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Full Length Research Paper

Assessment of the temporal change in groundwater quality when stored at different temperatures in household conditions, in the equatorial region of Central Africa

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The study carried out aimed at assessing the impact of groundwater storage temperature at household conditions on the temporal evolution of electrical conductivity and the future of heterotrophic aerobe bacteria (HAB). The storage duration was 7 days and the considered temperatures were 3, 10, 18 and 25°C. The electrical conductivity during storage reached 829 µS/cm at 3 - 18°C and 850 µS/cm at 25°C. The maximum HAB abundance was 9 x 10^3 cfu/ml at 3°C, 41 x 10^3 cfu/ml at 10°C, 44 x 10^3 cfu/ml at 18°C and 93 x 10^3 cfu/ml at 25°C. At the 3rd and 7th days storage at 3°C, changes in bacterial abundances values were significantly in the same direction as those of electrical conductivity ($P < 0.05$). The highest cell apparent growth rate at the 3rd day storage was 0.249 d^{-1} at 3°C, 0.559 d^{-1} at 10°C, 0.924 d^{-1} at 18°C and 1.233 d^{-1} at 25°C. However, at the 7th day storage, it was 0.362 d^{-1} at 3°C, 0.497 d^{-1} at 10°C, 0.690 d^{-1} at 18°C and 0.672 d^{-1} at 25°C. At the 3rd day storage, a decrease in cell abundance was noted in 90% of samples at 3°C and the cell apparent inhibitory rate varied from 0.012 to 0.989 d^{-1}. The storage of groundwater in households' conditions for a long period would alter its bacteriological quality.

Key words: Bacterial abundance's evolution, electrical conductivity, groundwater, storage temperature, duration.

INTRODUCTION

In most regions of the world, groundwater is one of the main drinking water supplies. Their dating using isotopic tracers and taking into account their flows and the soil depth at which it appeared showed that they are sometimes many decades old (Portniaguine and Solomon, 1998; Cook and Herczeg, 2000). Their origins are sometimes explained by several theories such as the infiltration theory which shows the impact of the soils porosity and permeability, that of water vapour condensation implying the water vapour contained in the air which penetrates in the ground and the youthful theory showing the implication of gas emanations from magma in the depths of the ground (Banton and Bangoy, 1997).

The origin of bacteria in underground waters is often discussed. According to some authors, although the origin of other micro-organisms is uncertain, most of the

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Abbreviation: HAB, Heterotrophic aerobe bacteria; **CAGR,** cells apparent growth rate; **CAIR,** cell apparent inhibition rate.

underground bacteria are derived from infiltrated bacteria through propagation, due to pollution of underground water by runoffs (Mayer et al., 1997; Dzeda et al., 1998). For other authors, the ancestors of natives in the underground ecosystem originated from anoxigenic microorganisms which likely were mutated (Fenchel, 2001). In natural environments, bacterial survival is supported by various parameters. The systematic changes in microbial community composition are related to the salinity (Jiang et al., 2007). According to Lozupone and Knight (2007), the major environmental determinant of microbial community composition is salinity rather than extremes pH or other physical and chemical factors. Furthermore and depending on the mineralogy, compatible solutes in water sample can be used as sources of nutrients for bacterial populations and therefore be responsible for the marked enhancement of bacterial growth (Krammer et al., 2008). Water storage conditions sometimes affect some of the bacterial properties such as culturability and cell activity, but would not affect others such as structural and genomic integrity (Caro et al., 1999).

Water from springs and wells are often stored in various conditions for many days drinking in households. This is often due to the long distances between groundwater points and households related to drought in the region. Many studies have been carried out on groundwater quality in the equatorial area of Central Africa. They show that bacterial distribution undergoes fluctuations in space and time and is influenced by physicochemical and weather factors (Nola et al., 2001, 2002). However, little is known about the bacteriological quality of this water at the moment of their consumption. Little data is available on the change of this microbial quality during household storage conditions. This study aims was to determine the impact of the storage temperature of groundwater on the temporal evolution of its bacterial microflora and electrical conductivity.

MATERIALS AND METHODS

Description of study sites

The Yaounde region (Cameroon) is located at latitude 3°52'N and longitude 11°32'E, with average altitude of 760 m. The climate is of typical equatorial type, with 4 seasons (Succhel, 1988); a mild rainy season from April to June, a mild dry season from July to August, a peak rainy season from September to November and a peak dry season from December to March of the next year. Its soil is ferro-lateritic and acidic, the pH values in general is lower than 6 (Bachelier, 1959). Two well water points coded W_1 and W_2 were chosen, based on their highest importance as a drinking water supply for the population, the higher density of these neighboured populations and the permanent presence of water in these wells during all seasons of the year.

Water samples collection

Samples were collected once every 15 days during peak dry season from mid-November 2008 to mid-April 2009. This period was chosen because it corresponds to the peak dry season during which ground waters are least affected by precipitation. Ten study campaigns were carried out. At each site, water samples were first collected in a 100 ml sterile glass bottle coded A_{d0} and in a 100 ml clean polyethylene bottle coded B_{d0}. Second samples were collected in 4 series of 7 sterile glass bottles of 100 ml each coded A^1_{d1}, A^1_{d2}, A^1_{d3},..., A^1_{d7}; A^2_{d1}, A^2_{d2}, A^2_{d3}..., A^2_{d7}; A^3_{d1}, A^3_{d2}, A^3_{d3}, ..., A^3_{d7}; A^4_{d1}, A^4_{d2}, A^4_{d3}, ..., A^4_{d7}, respectively and in 4 other series of 7 clean polyethylene bottles of 100 ml coded B^1_{d1}, B^1_{d2}, B^1_{d3},..., B^1_{d7}; B^2_{d1}, B^2_{d2}, B^2_{d3}..., B^2_{d7}; B^3_{d1}, B^3_{d2}, B^3_{d3}, ..., B^3_{d7} and B^4_{d1}, B^4_{d2}, B^4_{d3}, ..., B^4_{d7} respectively. Each sample series was done in triplicate. All samples were then transported to the laboratory in cool conditions (6 ± 1°C) after 45 min following their collection. Samples in glass bottles were used for bacteriological analysis and those in polyethylene bottles were used for chemical analysis.

Storage and sample analyses

In the laboratory, couple of samples A_{d0} and B_{d0} were immediately analyzed. Couples of samples A^1 and B^1, A^2 and B^2 and A^3 and B^3 were stored respectively in the refrigerators named R1, R2 and R3, in which temperatures were respectively adjusted to 3, 10 and 18°C. Samples A^4 and B^4 were stored at room temperature (25 ± 1°C). The duration of sample storage varied according to their codes. Samples A^1_{d1}, A^2_{d1}, A^3_{d1}, A^4_{d1}, B^1_{d1}, B^2_{d1}, B^3_{d1} and B^4_{d1} were analyzed after 24 h (1 day storage). Samples A^1_{d2}, A^2_{d2}, A^3_{d2}, A^4_{d2}, B^1_{d2}, B^2_{d2}, B^3_{d2} and B^4_{d2} were analyzed after 48 h (2 days storage), ... and samples A^1_{d7}, A^2_{d7}, A^3_{d7}, A^4_{d7}, B^1_{d7}, B^2_{d7}, B^3_{d7}, B^4_{d7} were analyzed after 168 h (7 days storage).

The bacteriological parameter considered was the heterotrophic aerobe bacteria (HAB). Analyses were performed on standard agar medium (Bio-Rad), using plate count method and incubations were done at room temperature (25 ± 1°C) during the 7 days. Physico-chemical parameters considered were the pH, electrical conductivity and biochemical oxygen demand in 5 days (BOD5). The pH and BOD5 values were measured only on the sampling days (d0). All analyses were performed according to standard techniques (Rodier, 1996; APHA, 1998).

Data analysis

The daily mean values of each parameter were calculated. The relationship between temporal evolution of the HAB abundance and that of electrical conductivity at each storage temperature was assessed. The straight log (number of CFUs) lines against storage duration were plotted. The slope a of each regression line was considered as the apparent evolution rate of the HAB abundance at the 3rd and 7th day of storage in each condition. This slope was then assimilated as the cell apparent growth rate (CAGR) when it was positive, or to the cell apparent inhibition rate (CAIR) when it was negative.

RESULTS

Chemical and bacteriological characteristics of water samples of the days (d_0)

The abundances of HAB isolated from well W_1 collected water samples varied from 46 x 10^1 to 67 x 10^2 cfu/ml (Figure 1). The highest abundance was observed at campaign C_4 and the lowest at campaign C1 (Figure 1). In wells W_2, HAB abundances varied from 39 x 10^1 to 61 x 10^2 cfu/ml. The highest value was recorded during

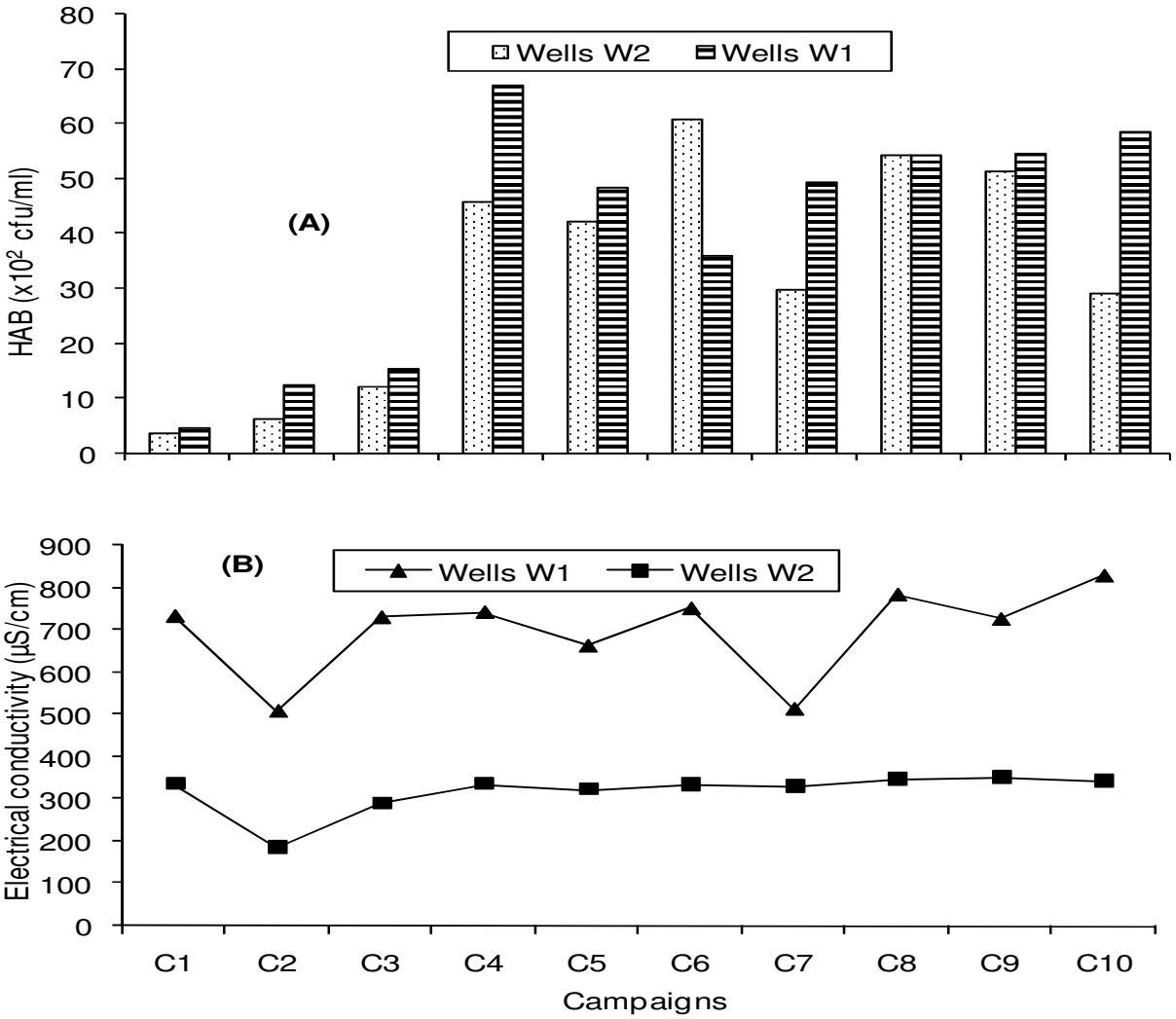

Figure 1. Abundances of HAB (A) and electrical conductivity values (B) in water samples during each campaign at each wells point, at the sampling day (d_0).

campaign C6 and the lowest during campaign C1 (Figure1). Abundances of HAB on the sampling days on the whole underwent spatial and temporal fluctuations (Figure 1).

Electrical conductivity values varied from 507 to 829 µS/cm in well W_1 and from 185 to 346 µS/cm in well W_2 (Figure 1). The values of this parameter also underwent spatio-temporal fluctuations. pH values in both wells varied between 4.9 and 5.4 (Figure 1). BOD_5 values remained 0.00 mg/l in samples over the campaigns in wells W_1 and W_2.

Chemical characteristic of water samples during storage

In samples from well W_1, mean electrical conductivity values for all campaigns varied from 279 to 829 µS/cm in samples stored at 3°C, from 301 to 829 µS/cm in those stored at 10°C, from 433 to 829 µS/cm at 18°C and from 507 to 850 µS/cm in samples stored at 25°C. In samples from well W_2, mean values of this parameter in water stored at 3, 10, 18 and 25°C varied from 159 to 352 µS/cm, from 148 to 378 µS/cm, from 149 to 352 µS/cm and from 185 to 376 µS/cm, respectively. The variation rate seemed to vary from one sampling campaign to another. During water sample storage at all incubation temperatures, the electrical conductivity values underwent temporal fluctuations. The variations of the mean values are presented in Figures 2 and 3. Because most of the curves were superposed, the standard deviations were not mentioned on the graphs. Their scale values are presented in Table 1. It was noted, in most cases and almost at all incubation temperatures, that there was a decrease in electrical conductivity values after 24 h of storage (Figures 2 and 3). After this period, the evolution

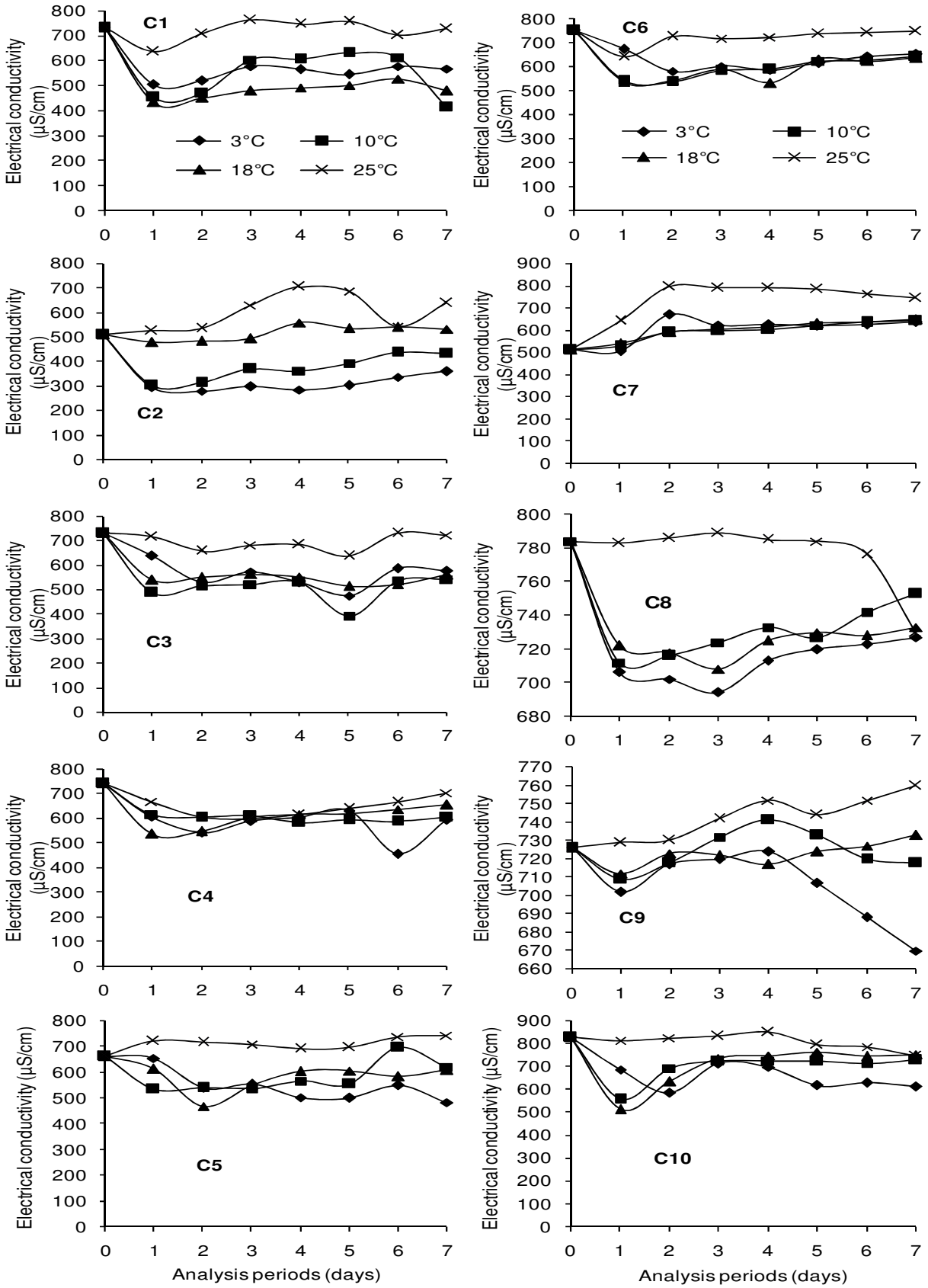

Figure 2. Temporal variation of the mean electrical conductivity values in samples stored at 3, 10, 18 and 25°C, collected from each of campaign (C1 to C10) from well W_1.

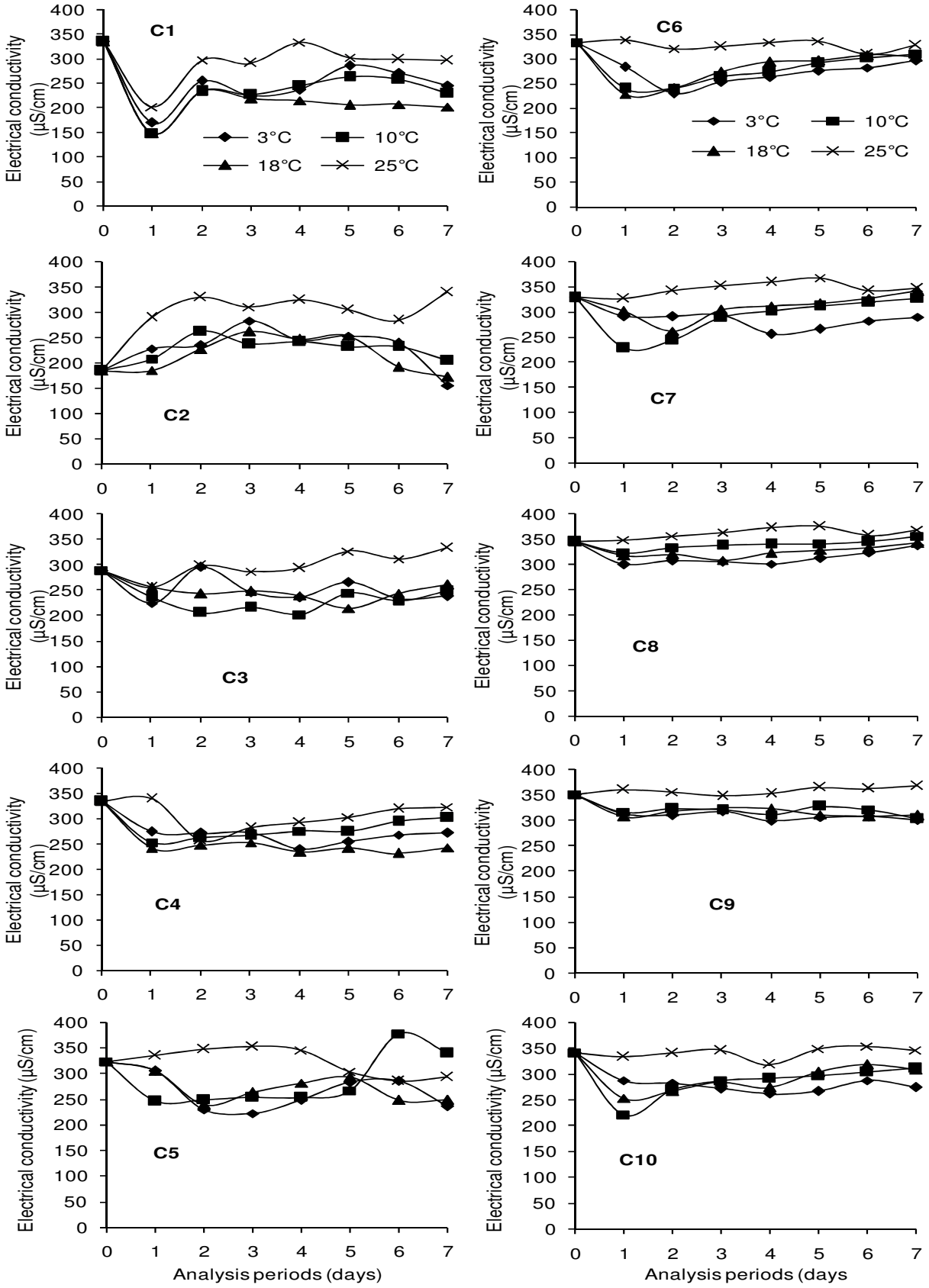

Figure 3. Temporal variation of the mean electrical conductivity values in samples stored at 3, 10, 18 and 25°C, collected from each of campaign (C1 to C10) from well W_2.

Table 1. Variation of standard deviation values of electrical conductivity and HAB abundances in water samples from each wells, stored at each temperature.

Storage temperature (°C)	Electrical conductivity (µS/cm)		HAB abundance (x 10^2 cfu/ml)	
	W_1	W_2	W_1	W_2
3	5.6 - 12.06	0.9 - 3.89	1 - 3	1 – 3
10	4.55 - 11.7	0.71 - 4.75	1 - 3	1 – 4
18	1.15 - 7.75	1.2 - 4.77	1 - 4	1 – 5
25	1.01 - 7.5	0.86 - 3.45	1 - 6	1 – 6

of this factor varied with the sample (Figures 2 and 3). During some rare cases as it was observed in samples from campaign C7 in well W_1 and in samples from campaign C2 in well W_2, electrical conductivity values during the first 5 days storage at all temperatures were higher than the recorded values on the sampling day (d_0) (Figures 1 to 3).

Bacteriological characteristics of water samples during storage

In water samples collected from wells W_1 for the whole investigation campaigns, mean values of HAB abundance during storage varied from 50 to 9 x 10^3 cfu/ml at 3°C, from 1.2 x 10^2 to 27 x 10^3 cfu/ml at 10°C, from 4.1 x 10^2 to 41 x 10^3 cfu/ml at 18°C and from 3.4 x 10^2 to 93 x 10^3 cfu/ml at 25°C. In samples from wells W_2, HAB abundance during storage varied from 30 to 26 x 10^3 cfu/ml at 3°C, from 1.3 x 10^2 to 41 x 10^3 cfu/ml at 10°C, from 2.1 x 10^2 to 44 x 10^3 cfu/ml at 18°C and from 13 x 10^2 to 69 x 10^3 cfu/ml at 25°C. During water sample storage at all incubation temperatures, the HAB abundance values underwent temporal fluctuations. The variations of the mean values are presented in Figures 4 and 5. The standard deviations were not mentioned on the graphs because most of the curves were superposed. Their scale values are presented in Table 1. In some cases for both wells, a decrease of HAB abundance was observed after one or two day's storage, mainly when storage temperatures were lower than 25°C. This was followed by the increase in HAB amount. In other cases, a relative decrease in HAB abundance was observed for more than 4 days storage (Figures 4 and 5). For the whole in both wells, abundances of HAB in water samples stored at 25°C were relatively higher than those in samples stored under other temperatures, at least during the first 5 days incubation. HAB abundance in water samples stored at 3°C seemed relatively lower (Figures 4 and 5).

The Spearman correlation test was performed between the average values of bacterial abundances and those of electrical conductivities recorded after 3 and 7 days and at each storage temperature for the 10 campaigns. It appeared that after 3 days of storage of water samples from wells W_1, changes in bacterial abundances values were significantly in the same direction as those of electrical conductivity ($P < 0.05$) at 3°C (Table 2). In samples from well W_2, this relationship was highly significant ($P < 0.005$) at 3 and 10°C (Table 2). After 7 days of storage, modifications of the 2 parameters were significant in the same way ($P < 0.05$) in water samples from wells W_1 stored at 3°C and very significant ($P < 0.01$) in those from W_2 stored at 10°C. No significant relationship was found between variations in the two parameters in water samples stored at 18 and 25°C (Table 2).

The HAB abundance's apparent evolution rates were estimated for each storage temperature, per investigation campaign and for each well point. The straight log (number of HAB counted) lines against storage duration was plotted. The slope *a* of each regression line was considered as the apparent evolution rate of the HAB abundance. This slope was assimilated to the cells apparent growth rate (CAGR) if it was positive or to the cells apparent inhibition rate (CAIR) if it was negative. The values of these evolution rates are given in Table 3. It was noted that in water samples from wells W_1 stored at 3°C, the HAB abundance's apparent evolution rates were negative at the 3rd day of storage, resulting in cell apparent inhibition. The CAIRs varied from 0.012 d^{-1} (campaign C8) to 0.738 d^{-1} (campaign C10) (Table 3). In water samples from wells W_2 and stored at the same temperature, most of the cell abundance's apparent evolution rates were also negative and the CAIRs varied from 0.042 d^{-1} (campaign C4) to 0.989 d^{-1} (campaign C10) (Table 3). CAGR was observed in samples from wells W_2 during the campaigns C2 (0.249 d^{-1}) and C8 (0.087 d^{-1}). At the 7th day of storage of samples from both wells at this temperature, either a decrease of CAIR or an increase of CAGR was noted. The CAGRs at the 7th day of storage ranged from 0.012 d^{-1} (campaign C8) to 0.362 d^{-1} (campaign C1) in samples from wells W_1 and from 0.041 d^{-1} (campaign C9) to 0.311 d^{-1} (campaign C10) in those from W_2 (Table 3).

In the water samples stored at 10°C, some CAIRs were observed at the 3rd day storage and their values ranged from 0.110 d^{-1} (campaign C5) to 0.994 d^{-1} (campaign C9) in samples from well W_1 and from 0.011 d^{-1} (campaign C4) to 0.708 d^{-1} (campaign C9) in those from the well W_2. The CAGRs ranged from 0.003 d^{-1} (campaign C7) to 0.559 d^{-1} (campaign C1) in samples from wells W_1 and

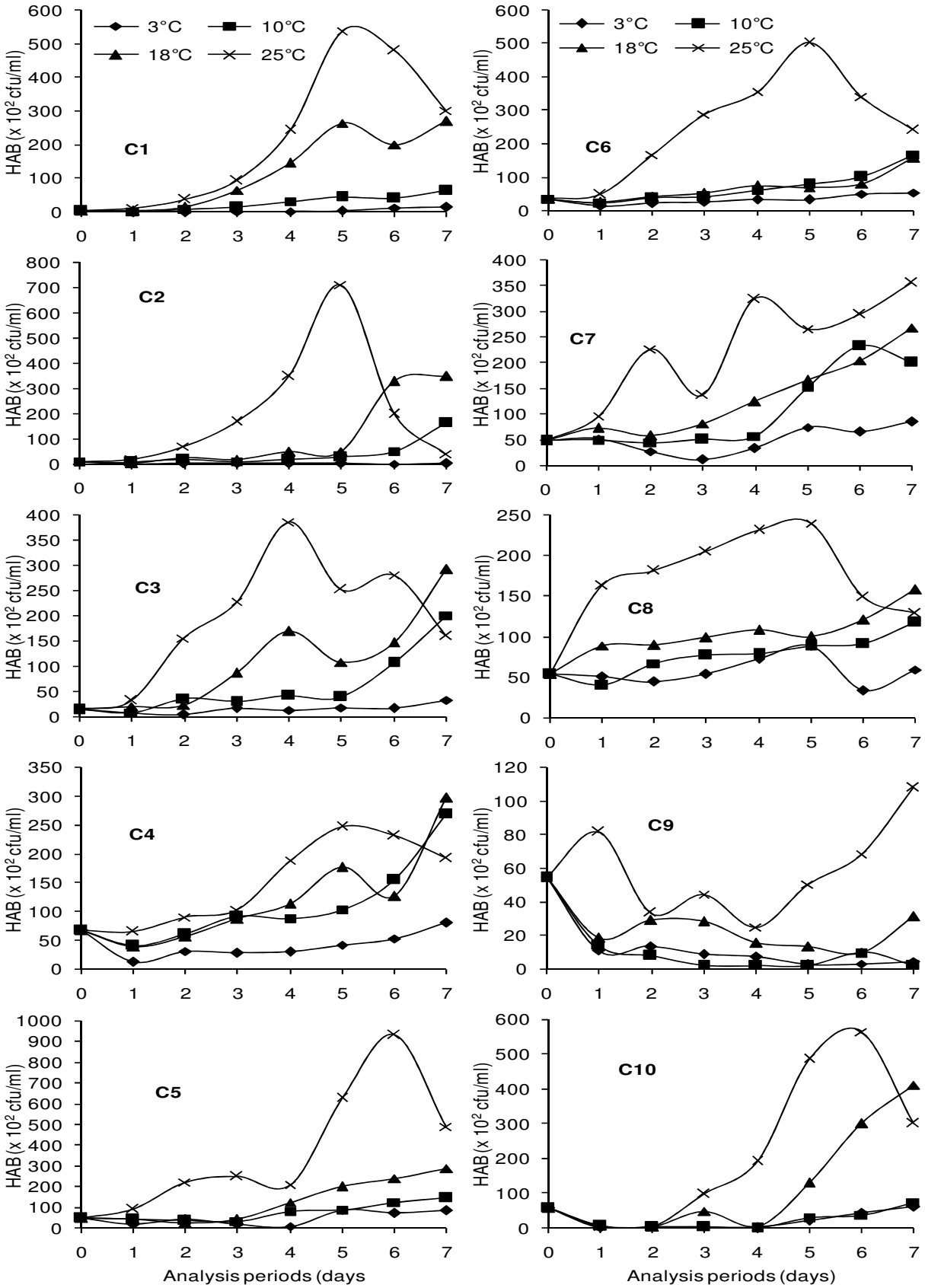

Figure 4. Temporal variation of the mean abundances of HAB in samples stored at 3, 10, 18 and 25°C, collected from each of campaign (C1 to C10) from well W_1.

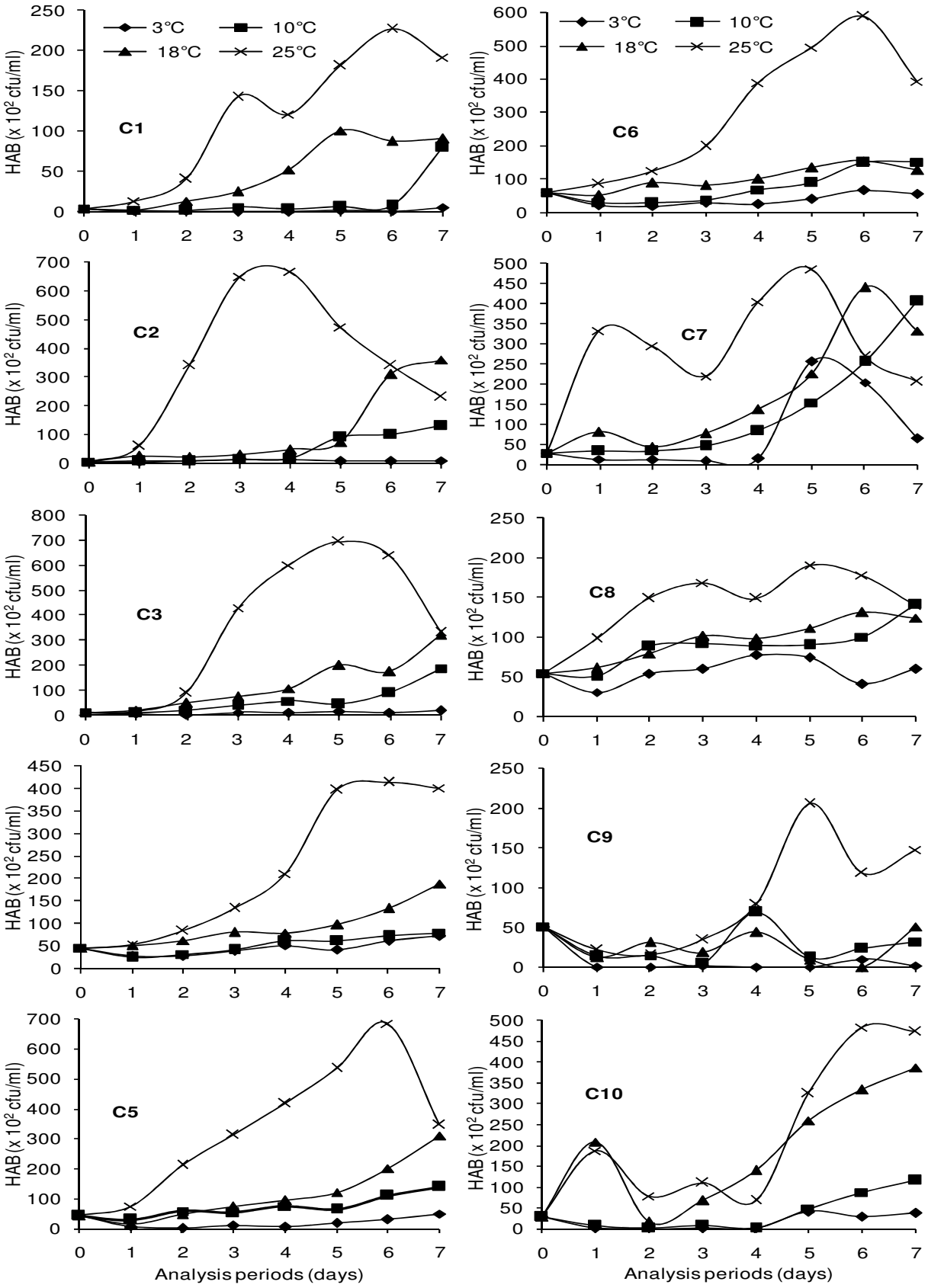

Figure 5. Temporal variation of the mean abundances of HAB in samples stored at 3, 10, 18 and 25°C, collected from each of campaign (C1 to C10) from well W_2.

Table 2. Spearman correlation coefficients between evolution rate values of cell abundance and mean values of electrical conductivity in water samples at the 3rd and 7th day of water sample storage, at each storage temperature.

Well	Correlation coefficient							
	3°C		10°C		18°C		25°C	
	3rd day	7th day	3rd day	7th day	3rd day	7th day	3rd day	7th day
W_1	0.413*	0.271*	0.309	0.047	0.313	-0.011	0.162	0.158
W_2	0.520***	0.201	0.468***	0.312**	0.089	0.008	0.126	0.004

3rd day of storage, n= 40 samples; 7th day of storage, n= 80 samples; *, P < 0.05; **, P < 0.01 ***; P < 0.005.

from 0.041 d^{-1} (campaign C1) to 0.409 d^{-1} (campaign C3) in those from well W_2 (Table 3). With the exception of the samples from well W_1 during the campaign C9; all HAB abundance's apparent evolution rates were positive at the 7th day storage at this temperature. The CAGRs ranged from 0.122 d^{-1} (campaign C8) to 0.0497 d^{-1} (campaign C1) in samples from well W_1 and from 0.017 d^{-1} (campaign C9) to 0.481 d^{-1} (campaign C2) in those from well W_2 (Table 3).

In water samples stored at 18°C, all HAB abundance's apparent evolution rates were positive with the exception of samples from well W_1 collected during the campaigns C5, C9 and C10. At the 3rd day storage, the CAGRs ranged from 0.117 d^{-1} (campaign C4) to 0.924 d^{-1} (campaign C1) in samples from well W_1 and from 0.005 d^{-1} (campaign C10) to 0.740 d^{-1} (campaign C1) in those from W_2. At the 7th day storage, it ranged from 0.112 d^{-1} (campaign C8) to 0.690 d^{-1} (campaign C1) in those from well W_1 and from 0.125 d^{-1} (campaign C8) to 0.565 d^{-1} (campaign C1) in those from well W_2 (Table 3).

In the samples stored at 25°C, negative HAB abundance's apparent evolution rates were observed at the 3rd day of storage in water samples collected from the well W_2 during the campaigns C2, C6 and C9 and from W_1 during the campaign C9 (Table 3). The CAGR values at the 3rd day storage varied from 0.123 d^{-1} (campaign C10) to 0.1012 d^{-1} (campaign C1) in samples from W^1 and from 0.312 d^{-1} (campaign C10) to 1.233 d^{-1} (campaign C3) in those from W_2. At the 7th day storage, the CAGRs ranged from 0.054 d^{-1} (campaign C9) to 0.672 d^{-1} (campaign C1) in samples from W_1 and from 0.121 d^{-1} (campaign C8) to 0.547 d^{-1} (campaign C1) in those from W_2 (Table 3).

It was noted that in the water samples collected from W_1 during the campaign C5 and from well W_2 during the campaign C9, the CAIR decreased gradually with the increase in storage temperature after 3 days of storage. However, in samples collected from W_1 during the campaigns C9 and C10, the CAIR values registered at 3rd storage at 10°C were greater than those recorded at 3°C (Table 3). In majority of samples from both wells which were stored at 3 - 18°C, the CAGR values increased from the 3rd to the 7th day of storage. However, in those stored at 25°C, the CAGR values decreased from the 3rd to 7th day of storage in most cases. On the whole, the negative bacterial abundance's evolution rates registered in some cases at 3rd day storage were followed by the positive values at the 7th day. No CAIR changed to any CAGR from the 3rd to 7th day of samples storage (Table 3). It was also noted that bacterial abundance's evolution rates in the stored water samples varied from one campaign to another, from one storage temperature to another and underwent temporal fluctuations at the same storage temperature (Table 3).

DISCUSSION

The investigations showed that water sample storage can result in an increase in HAB abundances (Figures 4 - 5, Table 3). This suggests the presence of biodegradable energetic substances. These waters might have contained biodegradable organic matter in microgram/l or nanogram/l level (the minimum concentration of the analysis method used could record 0.01 mg/l), or inorganic compounds that may have been used as an energy source through chemolithotrophic metabolism (Gounot, 1994; Holt et al., 2000; Fenchel, 2001). These processes may have been responsible for temporal variations in electrical conductivity values in water samples during storage.

A decrease of CAGR and the increase of CAIR at the 3rd day of storage were noted in most cases at 3°C. The lower temperature may have resulted in large number of cells in stress. This state would have induced some cells in a viable non-cultivable state, or would have destroyed others and the surviving cells then made use of the substances released. This may have led to an increase of CAGR or the decrease of CAIR noted on the 7th day of storage. These processes could have included among others the degradation of some macromolecules and the conversion of electrically neutral molecules to electrically charged molecules, which led to the variability of electrical conductivity of the medium (Nola et al., 1998). This explains the significant correlations shown in Table 2. These processes can also affect other cell properties. Caro et al. (1999) investigating the genomic integrity of the stressed *Salmonella typhimurium* population by salinity (0.9 and 3.8%) at 23°C, noted that the evolution of culturability and cellular activities followed a pattern different from that of the structural and genomic integrities. A slight decrease in cell structural and

Table 3. Apparent evolution rate values of HAB abundance (and regression coefficient) at the 3rd and 7th day of water sample storage at each temperature, from each campaign.

Cp	Apparent evolution rate (day^{-1})															
	Well W_1								Well W_2							
	3°C		10°C		18°C		25°C		3°C		10°C		18°C		25°C	
	3rd day	7th day	3rd day	7th day	3rd day	7th day	3rd day	7th day	3rd day	7th day	3rd day	7th day	3rd day	7th day	3rd day	7th day
C1	-0.457	0.362	0.559	0.497	0.924	0.690	1.012	0.672	-0.891	0.111	0.041	0.375	0.740	0.565	1.192	0.547
	(0.319)	(0.442)	(0.432)	(0.800)	(0.836)	(0.880)	(0.999)	(0.864)	(0.788)	(0.052)	(0.007)	(0.567)	(0.719)	(0.855)	(0.999)	(0.807)
C2	-0.349	-0.068	0.069	0.343	0.346	0.570	0.897	0.324	0.249	0.069	0.225	0.481	0.467	0.535	-1.567	0.416
	(0.524)	(0.130)	(0.081)	(0.778)	(0.285)	(0.839)	(0.982)	(0.324)	(0.853)	(0.345)	(0.813)	(0.890)	(0.681)	(0.919)	(0.946)	(0.412)
C3	-0.023	0.157	0.341	0.370	0.528	0.423	0.965	0.348	-0.077	0.115	0.409	0.383	0.625	0.451	1.233	0.568
	(0.002)	(0.423)	(0.461)	(0.835)	(0.795)	(0.880)	(0.949)	(0.558)	(0.020)	(0.239)	(0.832)	(0.926)	(0.989)	(0.956)	(0.938)	(0.703)
C4	-0.171	0.109	0.133	0.212	0.117	0.238	0.164	0.206	-0.042	0.102	-0.011	0.136	0.192	0.190	0.365	0.361
	(0.114)	(0.220)	(0.273)	(0.812)	(0.199)	(0.800)	(0.851)	(0.834)	(0.046)	(0.544)	(0.003)	(0.684)	(0.971)	(0.955)	(0.964)	(0.947)
C5	-0.207	0.143	-0.110	0.193	-0.094	0.338	0.585	0.366	-0.588	0.176	0.137	0.186	0.269	0.352	0.705	0.343
	(0.292)	(0.192)	(0.992)	(0.743)	(0.174)	(0.774)	(0.933)	(0.829)	(0.320)	(0.139)	(0.339)	(0.832)	(0.305)	(0.854)	(0.971)	(0.744)
C6	-0.060	0.123	0.083	0.243	0.158	0.214	0.747	0.316	-0.255	0.083	-0.144	0.216	0.137	0.144	-0.389	0.326
	(0.042)	(0.503)	(0.217)	(0.889)	(0.462)	(0.863)	(0.948)	(0.641)	(0.387)	(0.167)	(0.311)	(0.659)	(0.491)	(0.827)	(0.995)	(0.872)
C7	-0.486	0.106	0.003	0.252	0.130	0.244	0.390	0.246	-0.358	0.134	0.131	0.398	0.226	0.367	0.589	0.173
	(0.840)	(0.164)	(0.005)	(0.775)	(0.536)	(0.943)	(0.619)	(0.754)	(0.864)	(0.408)	(0.824)	(0.934)	(0.360)	(0.876)	(0.451)	(0.237)
C8	-0.012	0.012	0.155	0.122	0.183	0.112	0.408	0.078	0.087	0.041	0.209	0.119	0.213	0.125	0.381	0.121
	(0.029)	(0.011)	(0.519)	(0.831)	(0.750)	(0.813)	(0.742)	(0.161)	(0.134)	(0.110)	(0.781)	(0.805)	(0.981)	(0.912)	(0.929)	(0.517)
C9	-0.513	-0.331	-0.994	-0.331	-0.152	-0.118	-0.150	0.054	-0.907	-0.124	-0.708	0.017	-0.211	-0.238	-0.153	0.291
	(0.665)	(0.747)	(0.977)	(0.457)	(0.194)	(0.276)	(0.270)	(0.079)	(0.363)	(0.038)	(0.891)	(0.002)	(0.202)	(0.131)	(0.137)	(0.585)
C10	-0.738	0.262	-0.948	0.181	-0.088	0.483	0.123	0.607	-0.989	0.311	-0.504	0.356	0.005	0.349	0.312	0.333
	(0.383)	(0.203)	(0.814)	(0.084)	(0.007)	(0.433)	(0.008	(0.557)	(0.667)	(0.207)	(0.430)	(0.318)	(0.001)	(0.540)	(0.265)	(0.652)

Cp = Campaign.

genomic integrity was observed after 24 h incubation, but this increased quickly at the 4th day whereas, the culturability and the cellular activities decreased after 7 days of starvation; the percentages of structural and genomic integrity were still very high. In other respects, cell growth noted in samples stored at 3 and 10°C (Figures 4 - 5, Table 3), reflected the presence of psychrophilic tolerant microorganisms. Psychrophilic bacteria possess great concentration of unsaturated fats in their cytoplasm. Some of them contain polyunsaturated fats, the degree of unsaturation being related to the thermic transition point T (temperature at which fat melts or solidifies). Unsaturated fats remain liquid at low temperature, but are denatured at moderate temperature. Saturated fats are solid at ambient temperature meanwhile unsaturated ones remain liquid even at temperatures lower than 15°C (Todar, 2007).

Furthermore, it has been indicated that some bacteria strains such as *Bacillus subtillis* have multiple transportation systems leading to cell osmoprotection and others such as *Corynebacterium* genera are equipped with several systems of chemical molecules disintegration systems in the environment

(Wood et al., 2001).

At relatively high storage temperatures, no significant relationship was noted between cell abundance evolution rates and the electrical conductivity values (Table 2). The highest temperatures considered in this investigation relatively sped up the bacterial enzyme kinetics. Cell activities then occurred through various pathways and various chemicals were released into the medium and some of them interacted. In the aquatic environment, multiple microorganisms coexist as communities, competing for resources and are often associated as biofilms. Bacterial growth rate and production of quorum-sensing inhibitors constituted an attempt to identify attributes and allowed bacteria to effectively interact and coexist in a drinking-water environment. Simoes et al. (2007) when studying interactions amongst most species of *Methylobacterium, Sphingomonas, Burkholderia, Staphylococcus* and *Acinetobacter* genera noted synergy /cooperation between some species and antagonism and neutral interaction between others. According to Krammer et al. (2008), compatible solutes can be a source of nutrients for bacterial populations and therefore be responsible for the observed marked enhancement of bacterial growth. The exhaustion of biodegradable organic or inorganic compounds initially present would lead to a modification of the chemical characteristics of the medium; this latter becomes favorable for the survival of some bacteria and unfavourable for the others. At relatively low temperatures, the moderate celerity of enzymatic activities leads to a low impoverishment in celerity of the medium with energetic compounds (Todar, 2007).

In the aquatic medium, chemical elements highly influence micro-organism survival. In return, micro-organisms influence water physico-chemical properties through release of diverse metabolic wastes, rather than in normal conditions, or after physiological changes resulting in response to variation of environmental conditions. Some of these metabolic wastes can be harmful to other living micro-organisms in the ecosystem. These processes often contribute to significant fluctuations of the electrical conductivity in the milieu (Grouhel et al., 1995). This would have been one of the origins of the relative temporal increases of electrical conductivity values in stored samples at various temperatures (Figures 2 - 3, Table 1). It then resulted in the production of diverse chemicals, some alkaline others acidic, in the medium. In addition, some authors have observed that systematic changes in microbial community composition are mainly linked to the salinity gradient (Jiang et al., 2007).

In this study, the pH was not daily recorded in storage samples. It has been indicated that groundwater harbors various bacterial groups and despite the pH of the environment, the vast majority of the populations are tolerant vis-à-vis this factor (Tiago et al., 2004).

Nevertheless, it is known that the indirect action of pH creates an unsuitable environment for the survival of micro-organisms and is not directly associated with H^+ or OH^- concentrations, but acts through modification of the assimilation coefficient of different compounds by bacteria, which depends on the degree of bacterial tolerance vis-à-vis of acidity and of alkalinity of the environment (Nola et al., 2002). This modification can lead certain chemical substances towards the intracellular medium and are responsible for bacteria stress (Todar, 2007). This can partly be one of the causes of the decrease in HAB abundance in water samples during storage.

Conclusion

Physiological adaptations to chemistry may be an integral part of the evolution, ecology and diversification of water organisms. Their versatile character allows them to adapt to numerous apparently hostile conditions as lower temperatures. Their activity even though lower in speed, can permit them to colonize and modify biotope properties. Storage of drinking waters originating from underground sources for a long period would increase the health risk to the consumers in the short term if it contains potentially pathogenic bacteria, due to their potential growth and activity.

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Full Length Research Paper

Bacillus pumilus, a new pathogen on potato tubers in storage in Mali

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Soft rot occurred severely in potato tubers stored in traditional and ameliored storehouses at Sikasso, Mali. 17 infective bacterial isolates were isolated from potato rot tissues (*Solanum tuberosum* L var. Odessa). Out of all, the isolate Od23 was found pathogenic and was characterized as rod-shaped, Gram positive, endo-spore formers and yellow pigment producers. This isolate which was found to be the principal organism responsible for potato rot in storage in Sikasso, grew at a temperature range from 5 to 45°C, with optimum temperature of 30 - 35°C. However, it showed strong pathogenicity to potato tubers at 30°C at 3 days. Furthermore, the 16S DNA analysis confirmed that the obtained isolate was *Bacillus pumilus.* All Potato varieties cultivated in Mali responded to infection with *B. pumilus*. Potato var. Sahel was the most susceptible, while Pamina appeared the most resistant potato variety from Mali. According to literature review, this is the first report on the occurrence of *B. pumilus* as a causal agent of potato soft rot in storage in the region of Sikasso, Mali.

Key words: Potato, storage, *Bacillus pumilus,* soft rot, Mali.

INTRODUCTION

Potato (*Solanum tuberosum* L.) has been widely cultivated in Sikasso (Mali) as an important vegetable crop. Since most potatoes are harvested intensively from mid May to late June, they are stored under high temperature condition of about 30 - 35°C for a long period. However, potato harvesting often coincides with the rainy season in Mali, thus, adequate ventilation to maintain adequate humidity in the improved storehouse is not readily achieved. In addition, potato tubers are easily wounded and spoiled by pathogenic and even by saprophytic fungi inhabiting the surface of the tubers because of their soft and weak tissues (Kasmire and Cantwell, 1992). Potatoes stored under traditional and improved storage conditions have been known to be attacked mainly by *Penicillium* sp., and *Fusarium* sp. (Heilmann et al., 2006; Johnson et al., 1997; Salas et al., 2003). Unfortunately, potato suffers from several diseases at all stages of its life. All the parts of the plant particularly tubers are attacked by a number of, pathogens including fungi and bacteria. They cause several kinds of rot, anthracnose, scab, necrosis, spot and mildew (Messiha et al., 2007; Lee et al., 2002; Chuang et al., 1989). In Mali, the commercial viability of potato has been threatened by the frequent occurrence of bacterial soft rot in storage, a disease of potato which is generally reported to be caused by the phytopathogenic bacterium *Erwinia carotovora* var. *carotovora* (Bradbury, 1986; Beaulieu et al., 2008). Symptoms include water-soaking or yellowish-brown rot of tubers. It causes severe economic losses in traditional storehouses, where the incidence of this disease reached up to 30%. In Mali, we observed a resistance of this disease to all the treatments traditionally used against potato soft rot due to Erwinia species. Preliminary isolation trials indicated the presence of a pathogen, other than *E. carotovora* var. *carotovora*, associated with the disease.

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Thus, the aims of this work are to isolate and identify the causal agent(s), describe pathological properties and test the varietal sensitivity.

MATERIALS AND METHODS

Isolation of bacteria from soft rot potatoes

Bacteria were isolated from rot potato tubers collected at Sikasso, Mali. Potato samples were obtained from random storage chambers in the sites. A total of 323 rot potatoes were sampled. Potato tubers showing water-soaking or yellowish-brown rot under low temperature storage condition were used for isolation. These were washed with tap water and cut longitudinally. The diseased potato tissues were cut into 5 mm cubes by using sterilized surgical blade. 3 pieces of potato tissues were ground in 1 ml of distilled water using a mortar and pestle. The suspension was streaked onto potato dextrose agar (PDA). Plates were incubated at 28°C for 48 h. Bacterial colonies were picked at random from the plates, checked for purity and grouped according to colony colour and morphology, cell shape, growth rate and Gram reaction.

Culture and maintenance of isolates

Bacteria were cultured on Tryptic soy agar. Gram-negative and Gram-positive isolates were subsequently studied considering that soft-rot symptoms of potato were generally associated to Gram negative bacteria. The bacterial isolates were cultured in Tryptic soy broth (TSB) and for maintenance, in nutrient broth–glycerol (8: 2 (v/v) and frozen at –70°C) and French agar (nutrient broth, 8 g l^{-1}; thiamine, 80 mg l^{-1} and agar, 7.6 g l^{-1}).

Characterisation of the pathogen

Bacteriological characteristics of the isolates were examined by using the methods described by Lelliott and Stead (1987) and Palleroni (1984) (Bergy's Manual). Briefly, bacterial cultures (18 h old) were streaked on Tryptic soy agar (TSA) medium and incubated for 24 h at 28°C for colony characterisation, cell morphology, gram stain, LOPAT test (levan type colonies), oxidase reaction, potato rot, arginine dihydrolase, and fluorescent pigment on King's medium B (proteose peptone #3, 20 g; $K_2HPO_4 \cdot 3H_2O$, 2.5 g; MgSO4·7H2O, 6 g; glycerol, 15 ml; agar, 15 g; and distilled water, 1 L). Potato rot and pectate degradation tests were conducted according to the method described by Lelliott and Stead (1987). Biochemical characteristics of the isolates such as oxidation reaction, arginine hydrolysis activity and nitrate reduction were tested by using the methods described by Goto and Takikawa (1984a, 1984b). Utilization of carbohydrates was also investigated by using the Microlog GN2 microplates system (Lee et al., 1997; Wilson et al., 1999; Duncan et al., 2002; Gilho et al., 2002). The bacterial isolates were grown on Biolog universal growth medium (BUGM) (Biolog Inc., Hayward, CA) and incubated at 28°C for 18 h.

Antibiotic resistance

To screen for antibiotic resistance to differentiate between closely related micro-organisms, isolates were spread over tryptic soy agar (TSA) medium for 18 – 20 h at room temperature as described previously (Maniatis et al., 1989). The antibiotics tested were penicillin, enoxacin, ceftriaxone, erythromycin, cephalotin, chloramphenicol, Kanamycin and gentamycin.

Pathogenicity test

Healthy potato tubers were used for the pathogenicity test. With the use of a toothpick, wounds were made on a potato tuber slice to inoculate the causal agent. The bacterial suspension obtained from 48 h old cultures on PDA agar was collected in distilled water and adjusted to 1.0 x 10^7 cfu/ml. The causal agent was inoculated at the level of 100 µl (10^7 cfu/ml) on the wounds. Potato tubers inoculated were incubated at 25°C in a moist chamber for 3 days. Control samples were similarly tested with sterile distilled water only and kept at the same conditions. The inoculated tubers were examined for potato soft rot. In order to confirm pathogenicity of the casual agent in storage conditions in improved storehouses, potato tubers inoculated with the bacterial suspension as described above were incubated at 5°C for 2 months. Pathogenicity test against potato was similarly conducted using sliced samples.

To investigate growth pattern of Od23 isolate which showed the strongest pathogenicity, growth rates of the pathogen at different temperatures (5, 15, 20, 25, 30, 35, 40, and 45°C) were investigated by measuring turbidity.

Ribosomal sequences (16S rDNA)

DNA was extracted from the pathogenic isolates (Maniatis et al., 1989). Polymerase chain reaction (PCR) amplification of 16S rDNA was made using universal primers (Widmer et al., 1998). The fragment amplified was subsequently cloned into the PCR 2.1 TOPO vector (Invitrogen Corporation Carlsbad, CA, USA) for sequencing. DNA sequencing was done in the laboratory of Microbiology, department of Biology, at the University of Sherbrooke, Canada.

Response of potato varieties to infection: tuber slices of different varieties of potato cultivated in Mali were inoculated with 10 µl of bacterial suspension placed in a hole dug in the centre of each slice. Control potato slices were treated similarly with 10 µl of bacterial-free solution. Inoculated slices were placed in a plastic bag for 48 h at 30 - 35°C in the laboratory and daily examined. Rot severity was assayed using an arbitrary 0 - 5 scale where 0= no symptoms, 1 = 1 - 25% of rotten potato cells, 2 = 26 - 50% of rotten potato cells, 3 = 51 - 75 % of rotten potato cells and 5 = 76% - completely rotted potato slice cells. Disease severity index (DSI) was calculated 15 days after inoculation, according to the Methods of Vakalounakis (1990) as follows: $DSI = \Sigma d/(d\ max \times n) \times 100$. Where, d is the disease rating possible and n is the total number of potato slices examined in each replicate.

RESULTS

Isolation of bacteria from soft rot potatoes and pathogenicity test

From sampled rot potatoes, 210 bacteria were isolated. Out of the isolated bacteria, 17 were potato pathogenic. (Table 1A) Isolates showed various virulence effects which depended on the inoculation methods (Table 1B), incubation conditions (temperature) and the isolates tested. Isolate Od23 gave the highest virulence effect (75% rot severity) under wound inoculation methods.

Table 1A. Potato pathogenic bacteria isolated from different potato cultivars.

Cultivars	Total bacteria	Potato athogenic bacteria
Claustar (Cl)	4	0 (0)*
Spunta (Sp)	4	1 (25)
Odessa (Od)	9	1 (11)
Total	17	2 (33)

*Percentage of potato soft rot pathogenic bacteria compared to the total number of potato pathogen Isolated

Table 1B. Soft-rot severity of potato var. Sahel induced by *B. pumilus* Od23 as influenced by different inoculation methods.

Bacterial isolate	Soft rot severity (%) incited		
	Wounding	Puncturing	Spraying
B.pumilus Od223	75	60	0

Under spray inoculation, Od23 isolate failed to incite symptoms.

Characterisation and identification of the pathogens

The morphological, physiological and biochemical characters of Od23, indicated that the isolate was rod–shaped, gram positive, endo-spore former and yellow pigment producer (Table 2). The catalase, methyl red reaction, aerobiosis and H_2S production tests were positive. The isolate utilized galactose, glucose, inositol, mannitol, trehalose, xylose andd weakly utilized glycerol and had a negative reaction for starch hydrolysis, nitrate reduction, V.P. test and indole formation. It did not utilize arabinose, lactose and maltose. It is also able to grow in the presence of 50 µg erythromycin but sensitive to 50 µg penicillin and streptomycin. The results suggest that Od23 is *Bacillus pumilus.* The 16S DNA sequencing results, indicate 99% similarity between the bacterial isolate Od23 and *B. pumilus* and confirmed the morphological, physiological and biochemical identification results.

Reaction of potato varieties to B. pumilus Od23

Significant variances were obtained between potato varieties in response to infection with *B. pumilus* Od23 isolate (Figure 1A and Table 3). Potato var. Sahel was the most susceptible to *B. pumilus* Od23 infection (50% severity) followed by var. Odessa (35%), Claustar (30%) and Pamina that showed (20%) soft rot severity. A moderate reaction to *B. pumilus* Od23 infection was recorded with Yukon Gold (15% soft rot severity) (Figure 1B and Table 3). Potato var. Russet and Norland showed a sufficient resistance against *B. pumilus* Od23 infection since only 9% soft rot severity was exhibited. Yukon Gold, Russet and Norland are potato varieties from Canada selected for their resistance to *Erwinia* species.

Table 2. Morphological, physiological and biochemical characters of the pathogenic bacterial isolate Od23 obtained from naturally infected potato tubers.

Character	Bacterial isolate Od23
Shape of cell	Rod
Motility	+
Gram Reaction	+
Spore forming	+
Catalase production	+
Yellow pigment	+
Starch hydrolysis	-
Nitrate reduction	-
V.P test	-
Methyl red reaction	+
Indole formation	-
Potato soft rot	+
Aerobiosis	+
H_2S production	±
Carbon source utilisation	
Arabinose	-
Galactose	+
Glucose	+
Glycerol	±
Lactose	-
Maltose	-
Mannitol	+
Sensitivity to antibiotics	
Erythromycin 50 µg	R
Penicillin 50 µg	S
Streptomycin 50 µg	S

(+) = Positive reaction; (-) = Negative reaction; (±) = Weekly reaction; (R) = resistant and (S) = Sensitive.

DISCUSSION

In West Africa, many soft-rot diseases have been associated with *E. carotovora.* This pathogen is characterised by the production of extracellular enzymes such as pectate lyases (Valenzuela-Zapata, 1994; Daniels et al., 1988). Potato soft-rot symptoms were reproducible under laboratory conditions and were similar to those observed in storehouse during storage. In order to make an accurate diagnosis, it was important to

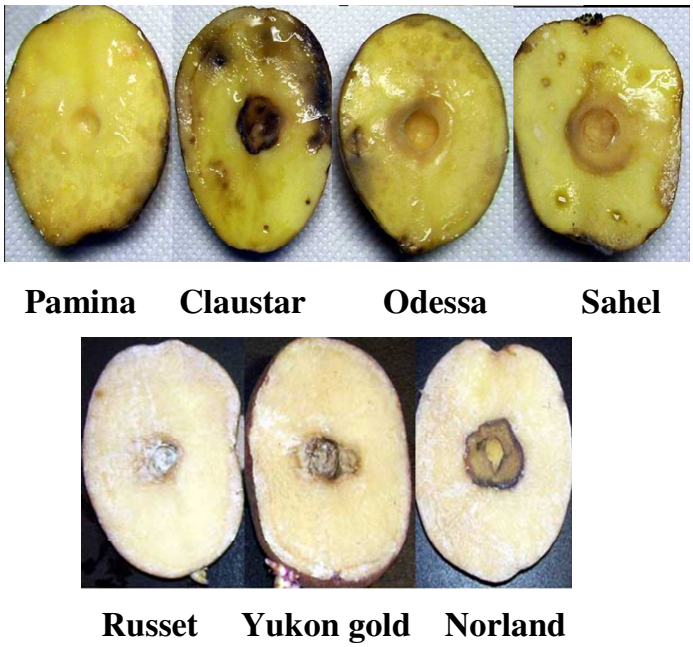

Figure 1. Soft rot severity of potato cultivars induced by *B. pumilus* Od23.

Table 3. Soft rot severity (%) to tubers of various potato varieties caused by inoculation with the isolate *B. pumilus* Od23.

Potato varieties	Provenance	Soft rot severity (%) caused by the bacterial isolate *Bacillus pumilus* Od23
Sahel	Mali	50
Odessa	Mali	35
Pamina	Mali	20
Claustar	Mali	30
Yukon Gold	Canada	15
Russet	Canada	9
Norland	Canada	9

identify and characterize the causal agents of the disease. The results show that Od23 is gram positive while *E. carotovora* is gram negative suggesting that the causal agent of patato soft-rot in storage in Mali is different from *E. carotovora*. Koch's postulates and extensive biochemical characterisation employing carbon source utilisation profiles, showed a 87% similarity between the bacterial isolate Od23 and *Cellulomonas hominis* CDC-A-3 (Bathily, 2007). Based on morphologic and physiologic properties, the isolate Od23 appeared to be different from *E. carotovora* (Bathily, 2007a, 2007b). The bacterial isolate Od23 appeared to be resistant to 50 µg erythromycin but sensitive to 50 µg penicillin and streptomycin, and did not utilize arabinose, lactose and maltose. These identification trials suggested that the isolate Od23 is *B. pumilus* as reported previously (Schaad, 1980; Gaber and Gazar, 1983). The last result was confirmed by the 16S DNA sequences showing a 99% similarity with Od23 and *B. pumilus*. Out of all the isolate bacteria, only the bacterial isolate Od23 appear to cause symptoms similar to sot-rot symptoms. Under spray inoculation, *B. pumilus* Od23 failed to incite soft-rot symptoms on potato tubers, indicating that this pathogen needs wound to infect potato tubers. These data are consistent with those reported by Gabr and Gazar (1983) and Saleh et al. (1997). *Bacillus* species are common in soil and some may be involved in post harvest diseases.

The bacterial isolate *B. pumilus* Od23, *Pseudomonas fluorescens, Corynobacterium, E. carotovora* subsp *atroseptica* and other *bacillus* spp. are known to cause bacterial soft rot to storage crops (Ciampi et al. 1976 and Ciampi and Huguelet, 1979). *B. pumilus, B. subtilis* and *B. polymyxa* are reported to cause post harvest soft rot of vegetables (Chiu et al., 1964). Gaber and Gazar

(1983) reported that the bacteria (*B. pumilus and B. polymyxa*) were causal pathogens of head rot of cabbage. *B. pumilus, B. subtilis, B. coagulans* and *B. polymyxa* have recently been reported to be the main causal agent of garlic cloves post-harvest decay (Saleh, 1995; Galal et al., 2002). Also, *B. pumilus* causes brown spots on fruit and leaves of Balady peach (Saleh et al., 1997).

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Full Length Research Paper

Dynamics of acid phosphatase production of the ectomycorrhizal mushroom *Cantharellus tropicalis*

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Ectomycorrhizal mushroom *Cantharellus tropicalis* was grown in axenic culture to study the effect of pH, temperature, nitrogen, carbon, phosphorus and heavy metals/trace elements on production of acid phosphatase and mycelial growth. The results of present study showed optimum mycelial growth with pH 4 at 15 and 35 ± 2°C. The ectomycorrhizal mushroom mycelia utilized lactose and yeast extract as best carbon and nitrogen source for biomass production. Ferrous sulphate supported the maximum mycelial growth when different trace and heavy metal were used. Among phosphorus sources, di-Sodium hydrogen phosphate supported maximum growth. The acid phosphatase production did not follow a uniform pattern as inferred from observations in this study. Moreover, results showed that high biomass did not mean more acid phosphatase production. However, pH 5 at temperature 15 and 35 ± 2°C supported high enzyme production. Fries Das medium supplemented with inositol and yeast extract produced maximum acid phosphatase at *in vitro* conditions. Ferric chloride produced considerably higher acid phosphatase among different trace elements tested. The present study demonstrates various factors affecting acid phosphatase production, an important feature for selecting ectomycorrhizal mushrooms for field inoculations.

Key words: *Dendrocalamus, Cantharellus*, acid phosphatase, ectomycorrhizal mushrooms.

INTRODUCTION

Ectomycorrhizal infection can increase the growth of host plant by increasing surface area and absorbing essential nutrients from soil which are otherwise unavailable to host. These fungi are mutualistic symbionts and increase nutrient uptake by production and secretion of surface bound extra cellular enzymes. They solubilize insoluble forms of nutrients not readily available to uninfected plant roots and have a significant role in carbon, nitrogen and phosphorus cycling in forested ecosystems (Cullings et al., 2008). The observed association of ectomycorrhiza with organic matter in forest soils has led to the suggestion that nutrients are obtained enzymatically from organic sources (Reddell and Malajczuk, 1984). Effect of ectomycorrhizal mushrooms on host plant growth or nutrient status depends on enzyme activities *viz.,* phosphatase, laccase, glucuronidase, cellobiohydrolase, *N*- acetyl- glucosamine, leucine aminopeptidase, xylosidase and β- glucosidase (Courty et al., 2007; Mosca et al., 2007), which provides an ecophysiological advantage for enhancing nutrient acquisition (Cameron et al., 2006).

Studies on acid and alkaline phosphatase have increased due to practical application of ectomycorrhizal mushrooms in field inoculations. There are several reports of acid phosphatase activity of *Amanita, Hebeloma, Tricholoma* etc (Antibus et al., 1986; Alvarez et al., 2005; Buee et al., 2005, 2007; Courty et al., 2005). These surface phosphatase activity are useful in selecting effective mycorrhizal symbiont for field inoculation of tree seedlings in reforestation of degraded land or mine sites. According to Antibus et al. (1986) and McLachlan (1980) external factors greatly affect production and activity of acid and alkaline phosphatase, thus affecting efficiency of potential ectomycorrhizal fungi. However, still the data on diversity and distribution of enzyme activities in native ectomycorrhizal

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Table 1. Different condition for experiment.

S. no	pH	Conditions				
		Temperation	Carbon source	Nitrogen source	Heavy metal and trace elements	Phosphorus source
1	1	5	Dextrose	Ammonium nitrate	Ferrous sulphate	Potassium di-hydrogen phosphate
2	2	10	Sucrose	Ammonium chloride	Magnesium sulphate	di-Sodium hydrogen phosphate
3	3	15	Maltose	Ammonium acetate	Manganese sulphate	di-Potassium hydrogen phosphate
4	4	20	Citric acid	Sodium nitrate	Cobalt sulphate	di-Ammonium hydrogen phosphate
5	5	25	Oxalic acid	Ammonium phosphate	Ferrous sulphate hepta hydrate	
6	6	30	Fructose	Potassium nitrate	Zinc sulphate	
7	7	35	Lactose	di-Ammonium sulphate	Copper sulphate	
8		40	Mannose	Ammonium oxalate	Iron citrate	
9			Inositol	Yeast extract	Ferric chloride	
10					Nickel sulphate	

communities are inadequate (Courty et al., 2005).

Cantharellus tropicalis Rahi, Rajak and Pandey is a delicious, edible, basidiomycetous fungus forming ectomycorrhiza with *Dendrocalamus strictus* Nees (Sharma, 2008; Sharma et al., 2008, 2009a,b ; Sharma et al., 2010a,b) in tropical region of Central India and also possesses medicinal properties. *C. tropicalis* uses a broad range of phosphorus sources under *in vitro* studies conducted. When it is grown in defined media, it releases phosphatase [acid phosphatase-(EC 3.1.3.2, orthophosphoric monoester phosphohydrolase)] as media has acidic pH (Sharma, 2008). Activity of acid phosphatase in *C. tropicalis* under controlled conditions has been studied (Baghel et al., 2009). However, there is no study on factors affecting acid phosphatase production by *C. tropicalis*. In present study, we assayed mycelia of *C. tropicalis* at different pH, temperature, nitrogen sources, carbon sources for growth and enzyme production and checked difference in enzyme production in response to various test factors.

MATERIALS AND METHODS

Growth conditions

Modified potato dextrose agar (PDA) plates were made for preparing inoculums [potato extract 200 g, dextrose 20 g (Hi-media, India), agar 20 g (Hi-media, India), malt extract 2 g (SRL, India). *C. tropicalis* culture from stock tube were transferred in 90 mm transparent plastic Petri dish (Hi-Media, India) and incubated for 10 - 15 days at 28 ± 2°C in BOD incubator (Caltron, India). When inoculum was ready, a 9 mm disc of 15 days old *Cantharellus* culture was cut with sterile cork borer and aseptically transferred to 150 ml Erlenmeyer flask with standard Fries Das Medium (FD) consisting- 5.0 g malt extract, 0.5 g KH_2PO_4, 0.5 g $MgSO_4$, 0.5 g NH_4Cl, 100 µm thymine HCL, pH adjusted to 5.6 which is best media for the growth of *C. tropicalis* mycelia in liquid media (Sharma, 2008). We used 50 ml of FD in 150 ml Erlenmeyer flask for growth and enzyme production of acid phosphatase. Mycelia were harvested after 15 days for biomass estimation and production of acid phosphatase. Three replicates were prepared for each experiment.

Experimental design

To examine the effect of pH on mycelial growth and production of acid phosphatase, liquid media was set at different pH levels (1-12 ± 0.2) adjusted with 1-5 M NaOH and 1N HCl and incubated at 28 ± 2°C. For studying the effect of temperature, inoculated flasks were kept at different temperature (5 - 40 ± 2°C) for 15 days before harvesting (Table 1).

To study the effect of different carbon source FD medium was replaced singly by various carbon compounds (Table 1). The quantity of different compounds was adjusted so as to obtain an amount of carbon equivalent to that present in 0.5 g of dextrose in the original FD medium except for starch and cellulose. For studying the effect of nitrogenous compounds, N source of FD medium was substituted by different nitrogen compounds (Table 1).

Different N source were incorporated separately in FD medium at the same nitrogen level as present in 0.5 g of ammonium chloride. A study was conducted to determine the effect of trace elements, heavy metals and phosphorus on growth and acid phosphatase enzyme production in *C. tropicalis* (Table 1).

Measurement of acid phosphatase production

Measurement of biomass and enzyme production was done according to Tibbett et al. (1998a) and Antibus et al. (1986) with slight modification according to the lab

Table 2. Effect of pH on growth and acid phosphatase production of *C. tropicalis*

S. no	Initial culture pH	Number of days (15)		
		Mycelial dry weight, mg (mean ± sem)	Enzyme production, mg p- NP liberated $h^{-1}g$ (mean ± sem)	Final culture pH (mean ± sem)
1	pH-1	-	-	-
2	pH-2	-	-	-
3	pH-3	-	-	-
4	pH-4	283 ± 0.068	2.975 ± 0.002	4.2 ± 0.317
5	pH-5	250 ± 0.015	4.300 ± 0.002	5.6 ± 0.850
6	pH-6	116 ± 3.333	3.537 ± 0.000	4.6 ± 0.100
7	pH-7	100 ± 0.020	4.068 ± 0.020	5.0 ± 0.066

Table 3. Effect of temperature on growth and acid phosphatase production of *C. tropicalis.*

S. no	Incubation Temperature (± 2°C)	Number of days (15)		
		Mycelial dry weight, mg (mean ± sem)	Enzyme production, mg p- NP liberated $h^{-1}g$ (mean ± sem)	Final culture pH[1)] (mean ± sem)
1	5	-	-	-
2	10	-	-	-
3	15	170 ± 0.020	3.512 ± 0.004	3.8 ± 0.00
4	20	160 ± 0.000	3.480 ± 0.010	4.5 ± 0.50
5	25	140 ± 0.035	3.425 ± 0.008	4.9 ± 0.25
6	30	140 ± 0.020	3.512 ± 0.008	4.6 ± 0.85
7	35	170 ± 0.013	3.512 ± 0.002	4.3 ± 0.41
8	40	120 ± 0.011	0.65 ± 0.003	5.0 ± 0.34

[1)] Initial culture pH for all treatments were 5.5.

requirements. Mycelia was separated from culture medium by gentle filtration through pre-weighed Whatman filter paper No.1 (Econ, India). Subsequently, it was washed (2-3 times) in modified universal buffer (MUB) (Skujins et al., 1962) prepared by titrating 120 ml of a stock buffer (7.26g tris- hydroxyl methyl amino methane buffer, 6.96 g maleic acid, 8.4 g citric acid, 3.7 g boric acid, 4 ml 0.5 M NaOH solution made up to 120 ml with d/w).

Mycelia was placed in 30 ml screw cap test tubes (Riviera, India), 4 ml of MUB (pH 5.5 for assay of acid phosphatase) and 2 ml of p-nitrophenol phosphate solution (made in MUB buffer) were added to it. The screw cap vials were closed with cap and incubated at 37 ± 2°C for 2 h in incubator. After incubation 4 ml 0.5 M NaOH was added to screw cap vials, mixed well for a few seconds and supernatant was filtered through Whatman No.1 filter paper. The yellow colour complex of p- nitrophenol (PNP) was measured using 1 cm glass cuvette (Optiglass Ltd, UK) in a spectrophotometer (Scigenics 118, India) at 410 nm. The amount of p-nitrophenol released was calculated by referring to a calibration graph and comparison with standard curve and represented as mg p-NP liberated $h^{-1}g^{-1}$.

Measurement of biomass

The biomass from each mycelial assay was required to calculate cleaved substrates on a mass basis. The assayed mycelia were re-filtered (as previously described) and together with the residual and assayed portion of mycelium were dried overnight at 70 ± 2°C and weighed (± 0.01 mg).

RESULTS

Optimum growth was obtained at pH 4 followed by pH 5 (Table 2). Mycelium of *Cantharellus* did not grow at pH lower than 4. At pH 7 mycelium growth was 35% of that obtained at pH 4. The effect of temperature on mycelial biomass showed that growth at 15 ± 2°C and 35 ± 2°C were considerably higher than other temperatures (Table 3). All carbon sources except oxalic acid (in which *Cantharellus* failed to grow) had a stimulatory effect on fungal growth. Lactose, sucrose, fructose, inositol supported good mycelial growth whereas media supplemented with citric acid and mannose showed least biomass (Table 4). All nine nitrogen sources supplemented in FD medium supported good mycelial biomass production. In general, organic sources supported best growth followed by ammonium (except NH_4Cl) and nitrate sources (Table 5). Media supplemented with trace elements *viz.,* Co, Zn, Cu and Ni showed no growth in flask, whereas ferrous sulphate and ferric chloride

Table 4. Effect of Carbon source on growth and acid phosphatase production of *C. tropicalis.*

S. no	Carbon source	Number of days (15)		
		Mycelial dry weight, mg (mean ± sem)	Enzyme production, mg p- NP liberated $h^{-1}g$ (mean ± sem)	Final culture pH[1)] (mean ± sem)
1	Dextrose	120 ± 0.010	0.468 ± 0.012	4.8 ± 0.033
2	Sucrose	310 ± 0.020	0.275 ± 0.002	3.1 ± 0.057
3	Maltose	260 ± 0.023	0.412 ± 0.028	3.5 ± 0.057
4	Citric acid	110 ± 0.016	2.281 ± 0.054	5.0 ± 0.033
5	Oxalic acid	-	-	-
6	Fructose	320 ± 0.023	0.375 ± 0.002	4.0 ± 0.033
7	Lactose	370 ± 0.051	0.330 ± 0.000	3.3 ± 0.088
8	Mannose	110 ± 0.020	0.55 ± 0.048	4.0 ± 0.033
9	Inositol	330 ± 0.092	0.675 ± 0.002	3.9 ± 0.033

[1)] Initial culture pH for all treatments were 5.5.

Table 5. Effect of nitrogen on growth and acid phosphatase production of *C. tropicalis.*

S. no	Nitrogen Source	Number of days (15)		
		Mycelial dry weight, mg (mean ± sem)	Enzyme production, mg p- NP liberated $h^{-1}g$ (mean ± sem)	Final culture pH[1)] (mean ± sem)
1	NH_4NO_3	340 ± 0.055	0.312 ± 0.011	3.7 ± 0.120
2	NH_4Cl	216 ± 0.019	1.100 ± 0.252	5.5 ± 0.066
3	$C_2H_3O_2NH_4$	278 ± 0.033	0.725 ± 0.008	3.2 ± 0.057
4	$NaNO_3$	235 ± 0.110	0.562 ± 0.023	3.5 ± 0.046
5	$(NH_4)_2HPO_4$	383 ± 0.102	0.588 ± 0.024	3.0 ± 0.120
6	KNO_3	227 ± 0.004	0.825 ± 0.002	6.1 ± 0.088
7	$(NH_4)_2SO_4$	286 ± 0.054	0.594 ± 0.009	2.9 ± 0.078
8	$(COONH_4)_2 H_2O$	333 ± 0.052	-	4.2 ± 0.115
9	Yeast extract	388 ± 0.044	3.175 ± 0.162	4.4 ± 0.67

[1)] Initial culture pH for all treatments were 5.5.

supported mycelial growth (Table 6). When FD medium was supplemented with different phosphorus sources it supported accumulation of mycelial biomass. However, mycelium growth on di-sodium hydrogen phosphate was significantly higher (370 ± 0.083 mg) (Table 7).

The pH had strong effect on production of wall bound acid phosphatase. Maximum production was observed at pH 5 followed by 7. The final pH

Table 6. Effect of Heavy metals and trace element on growth and acid phosphatase production of *C. tropicalis*.

S. no	Trace elements	Number of days (15)		
		Mycelial dry weight, mg (mean ± sem)	Enzyme production, mg p- NP liberated $h^{-1}g$ (mean ± sem)	Final culture pH[1)] (mean ± sem)
1	$FeSO_4$	236 ± 0.008	1.348 ± 0.042	3.4 ± 0.20
2	$MgSO_4$	135 ± 0.015	1.023 ± 0.085	4.0 ± 0.00
3	$MnSO_4$	186 ± 0.052	1.020 ± 1.415	3.7 ± 0.43
4	$CoSO_4$	-	-	-
5	$FeSO_4$. $7H_2O$	116 ± 0.039	3.60 ± 0.099	3.0 ± 0.11
6	$ZnSO_4$	-	-	-
7	$CuSO_4$	-	-	-
8	$C_6H_5FeO_7$	163 ± 0.020	0.803 ± 0.515	4.1 ± 0.20
9	$FeCl_3$	213 ± 0.012	1.742 ± 0.830	3.2 ± 0.11
10	$NiSO_4$	-	-	-

[1)] Initial culture pH for all treatments were 5.5.

Table 7. Effect of phosphorus on growth and acid phosphatase production of *C. tropicalis*.

S. no	Phosphorus source	Number of days (15)		
		Mycelial dry weight, mg (mean ± sem)	Enzyme production, mg p- NP liberated $h^{-1}g$ (mean ± sem)	Final culture pH[1)] (mean ± sem)
1	KH_2PO_4	280 ± 0.046	5.170 ± 7.513	3.8 ± 0.088
2	Na_2HPO_4	370 ± 0.083	3.400 ± 2.725	3.8 ± 0.218
3	K_2HPO_4	290 ± 0.018	3.950 ± 6.834	3.9 ± 0.185
4	$(NH_4)_2$ HPO_4	290 ± 0.040	5.375 ± 0.750	3.3 ± 0.0885

[1)] Initial culture pH for all treatments were 5.5.

of both treatments was approximately pH 5 which may be an important factor related to enzyme production (Table 2). It seems acid phosphatase production was not affected by various temperatures tested, except at 40 ± 2°C which showed marked reduction in enzyme production (Table 3). Of the nine carbon sources used, citric acid supported highest acid phosphatase production, while sucrose, inositol and lactose supported less enzyme production followed by inositol respectively. All tested nitrogen source showed enzyme production. However; yeast extract produced highest amount of enzyme. Among heavy metals and trace elements, ferric chloride produced highest amount of acid phosphatase (Table 6). All phosphorus sources produced large amount of acid phosphatase. However, potassium di- hydrogen phosphate and di- ammonium hydrogen phosphate produced significantly high enzyme when supplemented as only phosphorus source (Table 7).

DISCUSSION

Similar to many other ectomycorrhizal mushrooms *C. tropicalis* grow slowly (3-4 cm/wk) (Sharma, 2008). However, a fungus tries to adapt itself to conditions prevailing in soil environment. The optimum pH for growth was found to be pH 4 for *C. tropicalis* (Table 2). Temperature studies indicate maximum biomass production at 15 and 35 ± 2°C, which can be an adaptation to wide range of temperature prevailing in Central India during rainy season. However mycelium of *C. tropicalis* did not show any change in colour or growth morphology. Different temperature environments have considerable effect on physiological and ecological consequences of ectomycorrhizal associations (Tibbettt and Cairney, 2007). Hacskaylo et al. (1965) measured biomass after 24 days for *Suillus punctipes, Rhizopogon roseolus, Amanita rubescens,* and *Russula emetica* (all temperate species). Similar findings were reported for *Hebeloma* by Tibbett et al. (1998b). Like some secondary metabolites acid phosphatase production is directly related with mycelial growth of fungus.

Phosphatase production by ectomycorrhizal mushrooms is regulated directly or indirectly by several abiotic and biotic factors. Large increase in acid phosphatase production can be related with increase in mycelial biomass. Culture pH strongly influences extra cellular acid phosphatase production, as metabolic activities are

sensitive to external pH change whether in soil or *in vitro* studies (Baxter and Dighton, 2005). However culture pH affects extra cellular enzyme production by inhibiting enzyme stability after it is secreted into growth medium (North, 1982). In present study, culture pH showed a stimulatory effect on acid phosphatase production by *C. tropicalis* at pH 4 (Table 2). Generally ectomycorrhizal phosphatase has a pH optimum approaching that of native soil (Antibus et al., 1986). Moreover, it has been shown that increased ectomycorrhizal hyphal activity induces soil acidification and promotes greater production of acid phosphatase enzyme (Liu et al., 2005). Temperature 15, 30 and 35 ± 2°C showed highest enzyme production followed by 20 and 25 ± 2°C (Table 3). Although, differences in enzyme production were not pronounced, wall bound p–nitrophenyl phosphatase (p-NPPase) activities were detected for *C. tropicalis* at all growth temperature and tend to be high at higher temperature. The results of present study are different from other ectomycorrhizal fungi, which cannot be strictly compared due to difference in growth condition, species and culture media. *C. tropicalis* showed acid phosphatase production at 15°C which was also observed with arctic fungal strains by Tibbett et al. (1998b). Moreover, according to them, increased enzyme production at 15°C may be caused by cell plasma membrane confrontation and consequent leakage of intracellular p- NPPase. Based on results of present study, there is a need to work on ecological significance of extra cellular p- NPPase production at low temperature. It should be outlined that this is still an preliminary study and requires detail work such as the localization of enzyme in fungal cell as Bae and Barton (1989) reported that alkaline phosphatase are localized in cell wall of *Cenococcum graniforme.*

Citric acid appeared to be best for phosphatase production for *C. tropicalis,* even though it does not produce maximum biomass. Results suggest that, in general other carbon source represses acid phosphatase production in *C. tropicalis.* When different nitrogen sources were tested yeast extract produced highest amount of acid phosphatase. Other ammonium and nitrate sources which produced quite high amount of mycelia did not had any stimulatory effect on phosphatase production as observed by Kieliszewska-Rokicka (1992). However, *C. tropicalis* utilizes ammonium source much efficiently than nitrate sources, producing high mycelia biomass in pure liquid cultures (Sharma, 2008). This is true for other ectomycorrhizal mushrooms as observed with *Paxillus involutus* (Batsch) Fr. where ammonium source stimulated the mycelia growth and acid phosphatase (Kieliszewska-Rokicka, 1992). Trace elements reduce enzyme activity by interacting with enzyme-substrate complex, by denaturing the enzyme protein, or interacting with the protein active group (Nannipieri, 1995). Liquid media supplemented with ferrous sulphate, ferric chloride, magnesium sulphate and manganese sulphate had pronounced effect on regulation of enzyme production. Media supplemented with trace elements *viz.* Co, Zn, Cu and Ni showed no growth in flask, hence, no acid phosphatase enzyme production (Table 6). The change in final pH is shown in Table 6. Trace elements (metal ions) are assumed to inactivate enzymes by reacting with sulfihydril groups, a reaction analogous to the formation of a metal sulfide. It has been generally recognized that copper and cadmium are more toxic than other metals (Hattori, 1992). However, Gibson and Mitchell (2005) studying ericoid endomycorrhizal fungi showed that copper has no effect on wall-bound phosphatase activity up to 5 mM concentration. The production of extracellular p- NPPase appears to be enhanced when grown in a standard P medium (that is, with potassium di-hydrogen phosphate); however, effect of different concentration of phosphorus needs to be tested. Overall, results demonstrate no regulation of p-NPPase synthesis at different sources of P (Table 7). Dighton (1983) has also stated that, phosphatase production by basidiomycete fungi in liquid culture is independent of P in medium. Although, synthesis of p-NPPase is regulated by concentration of substrate, ability to restrict secretion of enzymes where product is plentiful and to increase production where product is limited suggests an economic regulation of phosphatase production attuned to environmental P concentration (Tibbett et al., 1998b). Baxter and Dighton (2005) found that phosphatase enzymes are differentially expressed under contrasting phosphorus conditions by different ectomycorrhizal fungi that may be due to difference in mineralization of organically bound phosphorus. *Piloderma* has also shown species specific substrate preferences in response to organic and inorganic sources of phosphorus (Rosling and Rosenstock, 2008). Calleja et al. (1980) have also suggested that wall- bound phosphatase are most important in cleavage and acquisition of P as intimate contact with substrate would guarantee uptake of liberated P molecules. However, ECM fungi differ greatly in their capacity to produce acid phosphatase due to differential potentiality to utilize phosphorus (Meyselle et al., 1991) which can also be affected by season and succession. Moreover, Courty et al. (2006) found less seasonal differences in ectomycorrhizal acid phosphatase activity while working with *Lactarius quietus, Cortinarius anomalus* and *Xerocomus chrysenteron.*

Although, the epifluorescent microscopy is an advance method to quantify acid phosphatase activity of ectomycorrhizal mushroom grown in soil or in liquid medium, studies on production is also important for ectomycorrhizal mushroom mycelia. Physiological conditions are an important factor in the ectomycorrhizal development and phosphatase production which forms a key factor of the symbiosis. Thus this study on phosphatase of *C. tropicalis* will help in understanding process of mycorrhization in *Dendrocalamus* and

selection of a potential strain for field inoculation with nursery seedlings. However, further studies are required on intra specific variation of acid phosphatase production would help us to screening potential strain/isolate for inoculation of host plant *Dendrocalamus* for forest plantation programs.

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Full length Research Paper

Analysis of compatibility relationships among some almond genotypes using fruit set and fluorescence microscopy

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Most of the Iranian almond cultivars are self-incompatible followed by pollination, fertilization, fruit set and lower yield problems. Therefore, selecting suitable cross-compatible cultivars for orchard establishment is necessary especially by new cultivars/genotypes obtained from breeding programs. In this study fruit set and pollen tube growth of ten late-bloom almond genotypes, obtained from a breeding program (D, E, F, I, G, L, K, O, P and Q) were investigated under filed and lab controlled pollination conditions. In order to study self-and cross-(in) compatibility they were pollinated by the pollens of overlap blooming-time genotypes in both conditions. Initial and final fruit set, fruit drop and some of the kernel traits were measured under filed condition. Measurements of pollen tubes at the style and in ovary were scored using fluorescence microscopy in lab, Results showed significant differences in some of the studied characters among crosses in both methods and Results confirmed each other in both methods. Fruit set percentage and pollen tube number in the ovary demonstrated that, all of the genotypes were self-incompatible but cross-(in) compatibility was not observed among them. In conclusion all of the genotypes could be used as a suitable polinizers for each others, regarding overlapping blooming-time of genotypes.

Key words: Almond, self-incompatibility, cross-incompatibility, fruit set, fluorescence microscopy.

INTRODUCTION

In fruit production industry self-incompatible cultivars are undesirable because, they cannot be grown in single-cultivar orchards; and their fruit set depends on the abundance of pollen transfer from other trees and finally produce lower yields. Self-incompatibility often occurs in fruit species of the genus *prunus* especially in sweet cherries and almonds (Milovic and Nicovic, 2007). However most of the commercial almond cultivars are gametophytical-self-incompatible therefore, successful pollination and fertilization are limiting factors for efficient almond production (Oukabli et al., 2000) because in this system self pollen tube growth stop in upper third of the styles and fertilization cannot take place successfully (Milovic and Nicovic., 2007). For this reason, high yields in almond need to plant at least 2 cross-compatible cultivars with overlapping blooming-time. Determination of self (cross)-compatibility relationships among cultivars/genotypes is one of the main objectives in almond breeding programs in the most almond producer countries such as Iran, in addition, determination of self (cross)-compatible cultivars/genotypes and their correctly plantation in the orchards, reduce orchard management costs and ensure pollination, fertilization, fruit set and producing consistent yields (Dicenta et al., 2002). Iranian

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almond cultivars are self-incompatible so, knowledge the self- and cross-(in) compatibility of them is necessary for the future breeding programs and orchard establishment. Traditional filed and laboratory controlled pollination, flore-scence microscopy studies and evaluation of pollen tub growth have been used in order to identify the self- and cross-(in) compatibility of cultivars/genotypes (Hajilou et al., 2006; Socias i company and Felipe, 1994a), obtaining the effective pollination period (EPP) of cultivars (Ortega and Dicenta, 2004) and studding the effects of pollen types on the fruit set, fruit quality and seed quality (Kodad and Socias i company, 2008; Oukabli et al., 2000, 2002; Socias i Company and Felipe, 1987; Vargas et al., 2005). Recently molecular methods have been used to identify the self- and cross-(in) compatibility of cultivars-/genotypes (L´opez et al., 2004, 2006). Therefore, most of the self-and cross-(in) compatible cultivars of fruit trees have been identified by field and lab controlled pollination methods (Lopez et al., 2006; Hajilou et al., 2006). 26 cross-incompatible groups (CIG) of almond cultivars having the same self-incompatibility genotypes, have been reported using described methods (Boskovic et al., 2003; L´opez et al., 2004). The first S genotypes and cross-incompatibility groups of almond were detected through cross pollination tests in the field (Kester et al., 1994) and later in the laboratory based on pollen tube growth (L´opez et al., 2004, 2006). Socias i Company and Felipe (1992) studied the self-compatibility and autogamy in 'Guara' almond cultivar and demonstrated its self-compatibility using pollen tube growth and fruit set. Hadjilou et al. (2006) studied self-and cross-(in)compatibility of 5 commercial apricot cultivars using field and lab controlled pollination and specific primers consequently, showed 3 self-incompatible cultivars and 2 cross-incompatible group. Furthermore, many researchers studied the effects of pollen type on fruit traits (L´opez et al., 2006). Socias i Company et al. (2004) studied the effects of pollen type on fruit set in some self-compatible almond cultivars and reported that some self-compatible almond selections had higher fruit set following cross-pollination than self pollination. Dicenta et al. (2002) studied several fruit characteristics after self- and cross-pollination in several self-compatible almond cultivars and showed no differences between both pollination types for any of the studied fruit traits. The objectives of this research were to study pollen tubes reaching the ovary, fruit set and fruit characteristics in ten improved Iranian almond genotypes, analysis of their self-and cross-(in) compatibility relationships and clarify cross-compatible groups with high quality and quantity yields.

MATERIALS AND METHODS

Plant material

This research was carried out on 10 improved genotypes (D, E, F, I, L, K, G, O, P and Q) obtained from a breeding program that, grown in Sahand horticultural research station (belong to Agriculture and Natural Research Center of East Azarbaijan, Iran). Genotypes divided to 3 groups based on overlapping bloom time, group 1: D, F and Q, group 2: I, L and O, group 3: E, G, K and P. Inter crosses program were QxD, FxD, FxQ (in group 1), LxI, OxL, OxI (in group 2), and KxG, ExG, ExK, PxE, PxG and PxK (in group 3. Pollens collected from the flower buds gathered in 'D' stage, from orchards dried and maintained in freezer until using in the field and lab pollination time. Pollen germination was carried out in an *in vitro* medium with 1.5% agar and 15% sucrose, pollens were incubated at 22°C for 24 h under dark conditions and then their growth protected with chloroform. 7 microscopic areas were counted randomly for evaluation of germinated pollens percentage and length of pollen tubes was measured using an ocular micrometer. Experimental model was completely randomized design with 4 replicates.

Field experiments: fruit, nut and kernel traits

In spring 2008, for each cross 4 repeats (each direction of the tree) were regarded and in each repeat at least 2 branches with 60 - 100 flower buds at 'D' stage were bagged to prevent the entrance of foreign pollens. Flowers were pollinated when the pistils were acceptable for pollens (only safe and complete ones). Branches on each tree were labeled and the percentages of initial and final fruit set were determined 4 and 8 weeks after pollination, respectively. In summer 2008, for each cross, samples of 40 fruits were collected from the branches, and then dried at room temperature. In order to select the suitable polinizer for genotypes, effects of pollens on main fruit, nut and kernel traits (usually evaluate in almond breeding programs) were studied as indicated by Ortega et al. (2006).

Fluorescence microscopy

Branches having at least 60 flowers in 'D' stage were selected for each cross and transmitted to the lab. Flowers of branches were emasculated and placed in trays with the 5% socrus, kept under controlled conditions (22 - 23°C and 75 - 80% relative humidity) in the growth chamber. After 24 h, the emasculated pistils were self (cross)-pollinated and kept again under the same conditions for 72 h, then pistils were collected and fixed in FAA solution and prepared for fluorescence microscopy observation as indicated in Ortega and Dicenta (2006). For each pistil the number of germinated pollen grains in the stigma, the number of pollen tubes in the first, second and third section and so, in the ovary were determined by a fluorescent microscope.

Experimental design and statistical analysis

Experimental design was completely randomized (CRD) for self-pollinations and pollen viability tests (10 treatment (genotypes) in 4 repeat) and completely randomized block design (CRBD) for cross-pollinations (different treatment (cross in each group), 4 repeat and 4 block (each direction of the tree). Differences between genotypes and crosses were analyzed following SAS software. Mean values were analyzed by Duncan's multiple range test in the each group crosses separately.

RESULTS

Pollen tube growth and germination

The analysis of variance indicated the significant

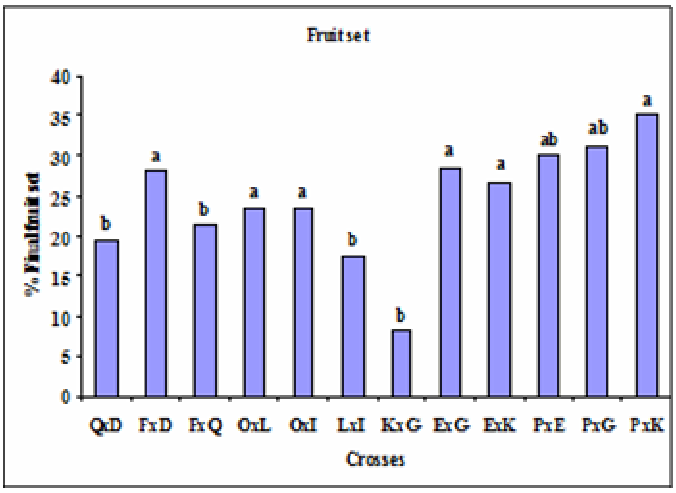

Figure 1. Means of the final fruit set percentage in the crosses (QxD, FxD,FxQ; group 1, LxI, OxL, OxI; group 2 and KxG, ExG, ExK, PxE, PxG and PxK group 3) (left genotypes pollinated by pollens of right ones and comparison of means carried out separately in each group crosses).

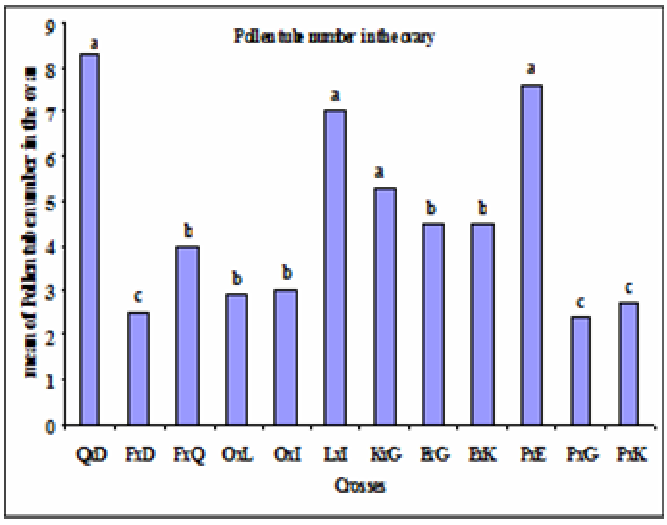

Figure 2. Means of the pollen tube number in the ovary of the crosses (QxD, FxD,FxQ; group 1, LxI, OxL, OxI; group 2 and KxG, ExG, ExK, PxE, PxG and PxK group 3). (left genotypes

differences in the percentage of pollen germination and pollen tube growth in all of ten studied genotypes at 5% level. Means of pollen germination percentage (Figures 1 and 2) were ranged among 26.5 - 78.9% in the *in vitro* medium. Respectively D: 78.9% (highest), E: 42.2%, F: 51.2%, G: 26.5% (lowest), I: 29.5%, L: 46.2%, K: 63.2%, O: 44.83%, P: 42.2% and Q: 65.86% (Tables 1 and 2). Also tables 1 and 2, showed the means of pollen tube length of the genotypes that, ranged among 155.2 - 737.2 µ, respectively D: 649.9 µ, E: 499.6 µ, F: 155.2 µ,

Table 1. Analysis of variances of the pollen germination percentage (PGP) and pollen tube length (PTL) in ten studied genotypes tested in the *in vitro* medium.

SOV	DF	PGP %	PTL(µ)
Genotypes	9	1066.9**	203035.6**
Experimental error	30	37	4734
CV		12.3	14.22

Table 2. Comparison of means of the pollen grain germination percentage (PGP) and pollen tube length (PTL) in 10 studied genotypes tested in the *in vitro* medium. (Means in each column with same letters are not significantly different at 5% level).

Genotype	Pgp %	Ptl (µ)
Q	65.86^{b}	737.2^{a}
D	78.9^{a}	649.9^{ab}
F	51.2^{c}	155.2^{de}
L	46.2^{c}	640.2^{ab}
I	29.5^{cd}	543.2^{bc}
O	44.83^{c}	116.4^{e}
E	42.73^{c}	499.6^{c}
K	63.2^{b}	685.8^{ab}
G	26.5^{d}	494.7^{c}
P	42.13^{c}	286.2^{d}

G: 494.7 µ, I: 543.2 µ, L: 640.2 µ, K: 685.8 µ, O: 116.5 µ (lowest), P: 286.2 µ and Q: 737.2 µ (highest). Pollen germination had not correlation with pollen tube length in the *in vitro* medium test (Tables 1 and 2). Means of germinated pollens percentage in the stigma of the self-pollinated crosses were 65 - 89.8%, respectively DxD: 79%, ExE: 81.8%, FxF: 74.5%, GxG: 89.8% (highest), IxI: 76.6%, LxL: 65.1% (lowest), KxK: 78.9%, OxO: 65.7%, PxP: 89.5% and QxQ: 72% (Tables 3 and 4). In addition, means of germinated pollens percentage in the stigma of cross-pollinated pistils were 51.9 - 89.5%, respectively QxD: 65%, LxI: 55.4%, KxG: 61.3% (lowest), ExK: 51.9%, ExG: 67.5%, FxD: 79.1%, FxQ: 81.4%, OxI: 89.5% (highest), OxL: 81.4%, PxE: 83%, PxG: 87.7% and PxK: 89.2% (Table 5). Means of the pollen tubes in the ovaries were 2.4-8.3 in the cross-pollinations, QxD: 8.3 (highest), LxI: 7, KxG: 4.5, ExK: 5.3, ExG: 4.5, F xD: 2.5, FxQ: 4, OxI: 2.9, OxL: 3, PxE: 7.6, PxG: 2.4 (lowest), and PxK: 2.7 respectively (Table 5). All of the crosses in each group, showed significantly differences in pollen grain germination percentage in the stigma, pollen tube number in the first, second and third section of the style and pollen tube number in the ovary (Table 5). The number of pollen tubes in the ovary had significant differences in the cross-pollination but, none of the pollen tubes reached to the ovaries in self-pollinated crosses. However, the pollen germination percentages in the

Table 3. Analysis of variances of pollen grain number in the stigma (PGN), pollen germination percentage in the stigma (PGP), number of pollen tubs in the first (style-1), second (style-2), third (style-3), section of style and number of pollen tubs in the ovary, in all of the genotypes pollinated by their own pollens. (**: significant in 1% level and *: significant in 5% level).

SOV	DF	PGN	PGP %	Style-1	Style-2	Style-3	ovary
Self-crosse	9	3636*	722**	756.6**	262.6**	12.5**	0
Experimental error	90	1441.5	84.4	224.5	73.8	1.9	0
CV		34	18	36	26	21	0

Table 4. Comparison of means of pollen grain number in the stigma (PGN), pollen germination percentage in the stigma (PGP), number of pollen tubs in the first (style-1), second (style-2), third (style-3), section of style and number of pollen tubs in the ovary, in all of the genotypes pollinated by their own pollens. (Comparison of means carried out separately in each group crosses).

Self-crosses	PGN	PGP	Style-1	Style-2	Style-3	Ovary
DXD	55.3^{ab}	79^{a}	17.5^{ab}	9^{ab}	0.5^{b}	0^{a}
QXQ	76.4^{a}	72^{b}	40.3^{a}	18.4^{a}	3.7^{a}	0^{a}
FXF	33.3^{b}	74.5^{a}	16.8^{b}	6^{ab}	0.1^{b}	0^{a}
OXO	76.1^{b}	65.7^{b}	22.5^{b}	1.7^{b}	0^{a}	0^{a}
LXL	101.2^{a}	65.1^{b}	38^{a}	4.7^{a}	0^{a}	0^{a}
IXI	80.8^{ab}	76.6^{a}	24^{ab}	5^{a}	0.7^{a}	0^{a}
GXG	47.2^{c}	89.8^{a}	16^{c}	2.7^{c}	0.7^{a}	0^{a}
KXK	73^{a}	78.9^{bc}	29^{ab}	12.2^{a}	0.7^{a}	0^{a}
EXE	70.5^{ab}	81.8^{ab}	32.4^{ab}	5.3^{b}	0^{a}	0^{a}
PXP	61.4^{b}	89.5^{a}	32.7^{a}	3^{c}	0^{a}	0^{a}

Table 5. Comparison of means of pollen grain number in the stigma (PGN), pollen germination percentage in the stigma (PGP), number of pollen tubs in the first (style-1), second (style-2), third (style-3), section of style and number of pollen tubs in the ovary; in the cross pollinations. (left genotypes pollinated by pollens of right ones and comparison of means carried out separately in each group crosses)

Crosses	PGN	PGP	Style-1	Style-2	Style-3	Ovary
QXD	95.9^{b}	65^{c}	48.8^{b}	29.6^{a}	14.2^{a}	8.3^{a}
FXD	106.3^{a}	79.1^{b}	55.6^{a}	20.6^{b}	9.1^{b}	2.5^{c}
FXQ	66^{ab}	81.4^{a}	35.3^{b}	15.8^{c}	7.8^{c}	4^{b}
OXI	39.1^{c}	89.5^{a}	25.4^{b}	13.7^{b}	6.5^{b}	2.9^{b}
OXL	68.2^{b}	81.4^{b}	25.6^{b}	13.7^{b}	6.5^{b}	3^{b}
LXI	88.3^{a}	55.4^{c}	35.5^{a}	26^{a}	15.6^{a}	7^{a}
EXK	129.2^{a}	51.9^{c}	53.9^{a}	29.3^{a}	16.6^{a}	5.3^{a}
EXG	107.3^{ab}	67.5^{a}	47.5^{ab}	27.4^{b}	11.8^{b}	4.5^{b}
KXG	78.5^{b}	61.3^{b}	35.3^{ab}	23,8^{c}	14.1^{a}	4.5^{b}
PXE	73.2^{c}	83^{ab}	43.1^{a}	25.3^{a}	15.8^{a}	7.6^{a}
PXG	93.3^{a}	87.7^{a}	44.8^{a}	18.2^{b}	5.1^{b}	2.4^{c}
PXK	59.1^{c}	89.2^{a}	27.6^{b}	11.7^{d}	6.1^{b}	2.7^{c}

stigma, in both of self and cross pollination had significant differences also, pollen germination in the stigma had not correlation with pollen tube number reaching the ovary, (Tables 3, 4 and 5). Data of Table 4 showed that highest pollen germination percentages in the stigma were observed in the stigmas witch received 40 - 60 pollen grains. Pollen germination and tube growth pattern showed in Figure 3 for some of the studied crosses.

Fruit and nut traits

Analysis of variances and comparison of the means were carried out in crosses of three groups separately

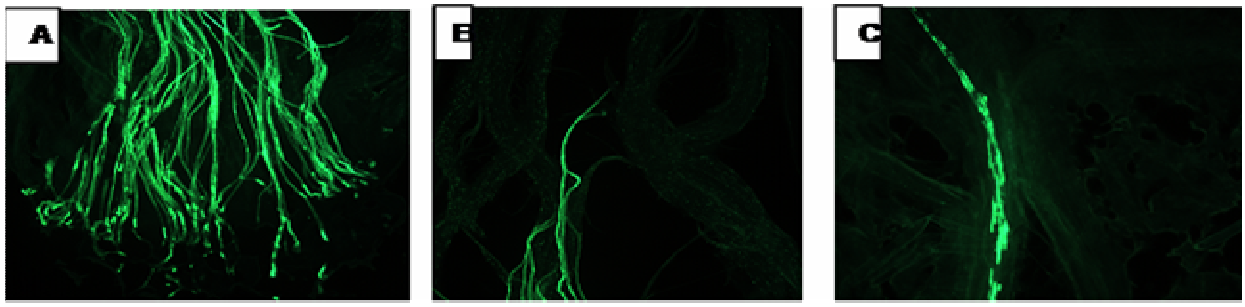

Figure 3. Pollen germination and tube growth pattern. (a) Pollens germinated in the stigma of the genotype P pollinated by itself pollens and tubs growth pattern in the style, (b) Deposition of pollen tube growth in the second section of the style in the self-pollinated G and(C) pollen tubes in the ovary of E pollinated by pollens of K.

Table 6. Comparison of means of the percentage of initial fruit set, final fruit set and fruit abscission. Means in each column with same letters are not significantly different at 5% level. (left genotypes pollinated by pollens of right ones and comparison of means carried out separately in each group crosses).

Crosses	Fruit set percentage		
	Initial	Final	Abscission
QXD	40^{b}	19.2^{b}	51.4^{ab}
FXD	48.7^{a}	28.12^{a}	40^{a}
FXQ	47.3^{a}	21.4^{b}	45.8^{ab}
OXL	62^{a}	23.4^{a}	62.2^{b}
OXI	62.3^{a}	23.4^{a}	61.6^{b}
LXI	47.8^{b}	17.6^{b}	62.4^{b}
KXG	67.5^{a}	8.2^{b}	88^{c}
EXG	55.3^{b}	28.6^{a}	54.3^{ab}
EXK	45.3^{c}	26.8^{a}	49.2^{a}
PXE	59.8^{b}	30.1^{ab}	47.6^{ab}
PXG	65.5^{a}	31.8^{ab}	49.7^{ab}
PXK	59^{b}	35^{a}	40^{a}

(genotypes that pollinated only with one type of pollens were not interfered). Analysis of variances showed differences at 5% level in some crosses for some of the studied fruit and kernel traits (data not shown). Initial and final fruit set percentage means were 40 - 67.52 and 8.2 - 34.96% in the crosses respectively, as well mean of fruit drop percentage was 40 - 88%. Final fruit set of crosses OxL, OxI, ExK and ExG were not shown significant difference among two pollen sources. Highest fruit set mean was observed in the crosses of group three, (Pxk; 35%, PxG; 31.8% and PxE; 30.1%) followed by lowest fruit absc-ission (Pxk; 40%, PxG; 49.78% and PxE; 47.6%), (Table 6). Crosses of group 2 were had highest fruit abscission (OxL; 62.2% and OxI; 61.5%). Initial fruit set percentage had not significant difference in group one crosses (FxD, FxQ and QxD), although their final fruit set and fruit abscission were have a few difference. Initial and final fruit set as so, fruit abscission were significantly different in the crosses of group two crosses (OxL, OxI and LxI) and group three crosses (PxE, PxK, PxG, ExK, ExG and KxG) (Table 6), although differences between crosses in each group were very little. Regarding the genotypes that pollinated at least by two types of pollens; nut and kernel weight, kernel size and kernel percentage were not significantly affected by pollen type in some crosses but affected very littlie in other some crosses (Table 7). For example, genotype E (pollinated by pollens of K and G) and F (pollinated by pollens of D and Q), showed not differences among 2 pollen type on nut and kernel weight and kernel size (Table 7). In the crosses of group 3, especially regarding PxE, PxK, PxG, kernel percentage, shape and weight were affected by pollen types but kernel size, thickness were not affected by different pollens (Table 7). Color of kernels was not affected by pollen type in none of the studied crosses (data not shown). Pollen type was not affected on nut shell hardi-ness in none of the studied crosses (Table 7) also blocks (each direction of the trees) had not showed significant effects on the studied traits of the fruit, nut and kernel (data not shown).

DISCUSSION

Results obtained from pollen tube growth pattern in the self-pollination in 10 studied genotypes demonstrated that all of the genotypes were self-incompatible because, in none of the self-pollinated crosses was stopped at the third upper section of the styles although, a few pollen tubes were received to the second or third section of the styles in some cases (Table 4). In the most case, pollen tube growth in the self-pollinated crosses were stopped at the third upper section of the styles although, a few pollen tubes were received to the second or third section of the styles in some cases (Table 4). Percentage of germinated pollens on the stigma was high in com-pared with the *in vitro* medium, this may caused by the ideal condition on the stigma versos to the *in vitro* conditions especially exist of proteins, amino acids and enzymes in the stigma. All of the crosses in each group, showed

Table 7. Comparison of means of the Pollen type effects on the kernel size, kernel thickness, kernel percentage (rate), kernel weight, nut weight, nut hardiness and kernel shape that evaluated by division the kernel length on its wight (L/W). (Left genotypes pollinated by pollens of right ones and comparison of means carried out separately in each group crosses).

Crosses	Kernel traits					Nut traits	
	Size	Thickness	Rate	Weight	L/W	Weight	Hardiness
QXD	4.6[b]	9.3[a]	0.7[a]	0.83[b]	1. 57[b]	1.18[b]	**9[a]**
FXD	7[a]	8.4[b]	0.67[b]	1.23[a]	1.8[a]	1.95[a]	**5[b]**
FXQ	8.7[a]	8.26[b]	0.63[c]	1.17[a]	1.75[a]	1.89[a]	**5[b]**
OXI	8.8[a]	8.12[b]	0.66[a]	1.2[b]	1.79[a]	1.89[b]	**7[a]**
OXL	8.3[a]	8.06[b]	0.64[b]	1.16[b]	1.72[a]	1.89[b]	**7[a]**
LXI	7[b]	9.9[a]	0.43[c]	1.36[a]	1.78[a]	3.35[a]	**3[b]**
EXK	4.6[b]	8.12[b]	0.7[a]	0.77[b]	1.54[a]	1.12[b]	**7[a]**
EXG	4.12[b]	8.8[a]	0.67[a]	0.77[b]	1.6[a]	1.16[b]	**7[a]**
KXG	6.5[a]	8.08[b]	0.49[b]	0.97[a]	1.53[a]	1.97[a]	**3[b]**
PXE	4.4[b]	7.58[a]	0.65[c]	.65[c]	2.18[c]	1[a]	**9[a]**

significantly differences in pollen grain germination percentage in the stigma, pollen tube number in the first, second and third section of the style and pollen tube number in the ovary (Table 5). Means of the pollen tubes in the ovaries were 2.4 - 8.3 in the cross-pollinations that, agreed with results obtained by Burgos et al. (1993) in different apricot cultivars. Pollen tube numbers were reduced from the stigma to the ovary in all of the self and cross-pollination systems (Tables 4 and 5).

In this study pollen tube number in the ovary and percentage of fruit set was not agree in the most crosses for example, regarding the crosses of group three (PxE, PxG and PxK) highest pollen tube number was observed in cross PxE (mean, 7.6) but highest fruit set was in the cross PxK (mean, 35%); this phenomenon represents variable environmental effects on different genotypes fruit set. High number of tubes in the ovaries and high fruit set, indicated the good compatibility of two genotypes for example, cross PxE had highest pollen tube number in the ovary and PxK had highest fruit set and could be introduce for orchard establishments (Figures 1 and 2). Pollen type in crosses of group 2 (OxL and OxI) had not significant effects on fruit set and pollen tube number in the ovary but crosses of group one (FxD and FxQ) showed very little differences (Figures 1 and 2). The main reason for the differences observed in fruit and kernel traits, may caused from the genetically differences among the genotypes or pollen types. In despite of our results, Socias i Company and Alonso (2004) detected cross-incompatibility of 'Ferragnès' and 'Ferralise' almond cultivars with controlled pollination and study of pollen tube growth by florescence microscopy. Many researchers studied the self-and cross-(in) compatibility of cultivars/genotypes using fruit set and fluorescence microscopy methods, and reported self-(in) compatible and cross-(in) compatible cultivars/genotypes in genus *prunus* species (Burgos et al., 1993; Hajilou et al., 2006; L´opez et al., 2004, 2006; Milatovic and Nicolic, 2007; Socias i Company and Felipe., 1992, 1994a). Burgos et al. (1993) studied self-and cross- (in) compatibility among 8 apricot cultivars using pollen tube growth in the laboratory and the percentage of fruit set in the orchard and finally resulted that five cultivars were self-incompatible but they were not observed cross-incompatibility among 25 cross-com-bination between cultivars. Those results agree with this work that, cross-incompatible groups were not observed among ten improved almond genotypes but all of them were self-incompatible.

Milatovic and Nicolic, 2007 studied self-(in) compatibility of 36 apricot cultivars using pollen tube growth and reported that, 22 cultivars were self-compatible and 14 cultivars were self-incompatible. Ortega and Dicenta (2006) studied the pollen tube growth pattern in the homozygous and heterozygous self-compatible almond individuals and showed that, in the heterozygous self-compatible almonds, rate of the pollen tube growth was high in compared with heterozygous ones and they related high fruit set of them for rapid pollen tube growth and high pollen tubes reaching to ovary. Ortega et al. (2004) following field studies showed that, although 'Marcona' cultivar and 'S_{5133}' genotype had similar pollen tubes in the style but, fruit set of 'Marcona' was higher than 'S_{5133}'. This phenomenon expresses interfering of other factors (etc of pollen tube number) in the fruit setting processes. Alonso and Socias i Company (2005), Socias i Company et al. (1976) and Dicenta et al. (2002c), following self-pollination and cross-pollination of self-compatible and self-incompatible almond genotypes found that, self-compatible almonds had a very low number of pollen tubes at the base of their styles after self-pollination and very slow growth rate (in despite of this work that, all of the genotypes were self-incompatible). Many researchers studied the effects of

pollen type on the fruit, nut and kernel traits and reported very inconsistent results; some of them reported significantly positive effects of pollen type on fruit traits and other some reported reverse results (Ortega et al., 2006). Vargas et al. (2005) indicated that fruits from open pollination in 34 self-compatible seedlings had higher weight in-shell, kernel weight, nut and kernel size in despite of self-pollination. Oukabli et al. (2002) observed a reduction of nut and kernel weight and kernel thickness in fruits from self-pollination of the self-compatible almond cultivar 'Tuono' in compared with cross-pollination with different cultivars. Furthermore, Socias i Company et al. (2004) demonstrated that some self-compatible almond selections had higher fruit sets following cross-pollination than after self-pollination and attributed the results to the different ability of set self-fruits instead of the influence of the pollination treatment. Consequently, in this work pollen tube number in the ovary, initial and final fruit set of cross-pollination groups showed that, all of the genotypes were cross-compatible and could pollinate each other regarding the overlapping time of blooming because, in none of the crosses final fruit set was 0% (0% final fruit set in a cross shows the cross-incompatibility of their pollens and pistils).

Conclusion

This research concluded that 10 studied almond genotypes were self-incompatible but cross-incommutability was not observed among genotypes and so, all of the genotypes could be used in breeding programs or orchard establishment for pollination each other based on the objectives. Moreover, based on the pollen tube number in the ovary, fruit set and fruit abscission percentages; group three genotypes (E, K, G and P) were constituted the best cross-compatible group for using as polinizers to each other especially, PxE and PxK compositions.

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Full Length Research Paper

Survival enhancement of probiotic *Lactobacillus plantarum* CMU-FP002 by granulation and encapsulation techniques

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Two processes of enclosure of probiotic *Lactobacillus plantarum* CMU-FP002, probiotic granules and calcium alginate beads, were studied. Sodium alginate solution at 1.0, 1.5 and 2.0 % (w/v) was used as a binder. The results showed that 20 log cfu/ml initial concentration of cells could be entrapped by the granules and beads with 12 to 13 log cfu/g and 16 cfu/g, respectively. The physical properties of granules and beads revealed that the strength increased when sodium alginate concentration was increased. On the other hand, the dissolution decreased. Probiotic granules completely released the cells within 60 min after being suspended in stimulate gastric fluid (SGF) pH 1.8 and had 2 to 3 log survival cells per gram. Calcium alginate beads, which were formulated from 1.0 and 1.5% (w/v) sodium alginate solution, gradually released bacterial cells and were completely released in SGF within 120 min. The beads formulated from 2.0 %(w/v) sodium alginate solution could not completely release the probiotics. The beads contained more survival cells than granules. Furthermore, the beads formulated from 1.5% (w/v) sodium alginate solution had the highest survival cells (9.30 log cfu/g). Probiotic cells in calcium alginate beads were still high (11 log cfu/g), although they were stored at 4˚C for 5 days alternating with room temperature for 5 days, for a total of 2 month. Further application in broilers will be studied.

Key words: Probiotic, survival enhancement, granulation, encapsulation, *Lactobacillus plantarum*.

INTRODUCTION

Intestinal coccidiosis, caused by various species of *Eimeria,* has become an economically important disease of poultry and livestock throughout the world. The disease can lead to reduced productivity and/or major losses of livestock (Girard et al., 1997). As the world's poultry industry continues to expand, so does the concern for the control of coccidiosis. Jeurissen et al. (1996) reported that the infection of chickens starts after the ingestion of oocysts causing sporozoites to penetrate the epithelium of the villi. After passing through the lamina propria, the sporozoites enter the crypt epithelial cells where they undergo several rounds of asexual and sexual proliferation, thus forming merozoites and later, gametocytes. When macrogametes are fertilized by microgametes, oocysts are formed that are shed in the faeces. For the 7 species of Eimeria, each parasitize inhabits different areas of the intestine. For example, *E. ucervulina* is restricted to the duodenum and *E. tenella* is located in the caeca (Girard et al., 1997). Currently, chemotherapy is used extensively to control the disease, but drug resistance among parasite strains has occurred (Chapman, 1998) and has created interest in the development of alternative control methods. The development and use of probiotics for poultry is based on the knowledge that the gut flora is involved in resistance to enteric infections.

Recently, Kasornpikul et al. (2009 succeeded in

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screening *Lactobacillus plantarum* CMU-FP002 from chicken intestines. This strain indicated probiotic properties such as tolerance to 0.3% bile, growth in widely pH range, utilization of starch, protein and fat. It also had antibacterial reactions to *Salmonella typhimurium* and *Escherichia coli*. *L. plantarum* CMU-FP002 exhibited an 85.63% reduction in the number of *E. tenella* oocysts shed in the faeces compared with the control group. Probiotics *L. plantarum* CMU-FP002 have also been shown to play an important role in protecting broiler from *E. tenella* by several mechanisms during infection, especially by local immunity mechanisms. However, the problems of probiotic application as a food additive in animal feed for livestock are the longevity of probiotic cells and the required properties of probiotics during storage and in the intestinal tract. According to the aforementioned reasons, several animal feed products with added probiotic as food additives have short shelf life. In order to impact the desired health benefits, probiotic bacteria should be contained in the product in higher viable count (7 to 9 log cfu/mL) during their whole product shelf life, which is required to successfully develop foods (Xinhuai et al., 2009).

One of the solutions to these problems is to granulate or encapsulate probiotic. Alginate is one of the materials for encapsulation. It is a natural biopolymer extracted from brown algae. It is composed of linear chains of the α-L-guluronic acid (G) and the β-D-mannuronic acid (M). Alginates form hydrogels in the presence of divalent cations like Ca^{2+} (Bajpai and Sharma, 2004; Pasparakis and Bouropoulos, 2006). Several reports revealed that *Lactobacillus* spp. cells in calcium alginate beads are entrapped by microencapsulation (Chandramouli et al., 2004; Gildas et al., 2009). Besides, some reports revealed that entrapped *L. plantarum* SP 1-3 cell (Ohmomo et al., 2007) and endospores of *Bacillus megaterium* (Chumthong et al., 2008) in granule forms could improve survival rates. Moreover, application of encapsulated probiotic in dry fermented sausages (Muthukumarasamy and Holley, 2006), yoghurt (Kailasapathy, 2006) and ice cream (Homayouni et al., 2008) could improve survival rates during storage and have no significant effect on their sensory properties. Thus, we are interested in enhancing probiotic *L. plantarum* CMU-FP002 living cells using granulation and encapsulation techniques performed by the probiotic granules and calcium alginate beads in this study.

MATERIALS AND METHODS

Materials

Sodium alginate, pepsin from porcine stomach mucosa, pancreatin, trypsin from hog pancreas and bile salts were purchased from Sigma (St. Louis, MO. USA). Calcium chloride and lactose was purchased from Ajax Finechem (New Zealand). Bromocresol purple was purchased from Fisher Scientific (UK). De Man Rogosa Sharpe (MRS) medium was purchased from Labscan (Spain). Bacteriological agar powder was purchased from Himedia (Mumbai, India). All other chemicals were reagent grade.

Bacteria, growth conditions and preparation of cell suspensions

The microorganism used in this study was *L. plantarum* CMU-FP002 from our previous research (Kasornpikul et al., 2009). The bacteria was cultured in MRS broth at 37˚C, checked for purity and maintained on MRS agar (1.5% (w/v) agar in MRS broth). Cells for survival experiments were propagated in MRS broth for 18 to 24 h (stationary phase) at 37˚C. Cells were harvested by centrifugation at 5,000 × g for 10 min at 4˚C, and washed twice with 0.9% (w/v) NaCl. Then, cells were harvested again by centrifugation at 5,000 × g for 10 min at 4˚C. Cell count was determined by pour plate on MRS agar incubate for 48 h at 37˚C. The cell pellet was resuspended in distilled water to 20 log cfu/ml as the initial cell concentration. Cell suspension was stored at 4˚C until formulating granule and calcium alginate beads.

Formulation and evaluation of probiotic *L. plantarum* CMU-FP002 granules

L. plantarum CMU-FP002 granules were prepared using the wet granulation technique. Cell suspension of *L. plantarum* CMU-FP002 with 20 log cfu/ml initial concentration were mixed with the diluent (lactose and corn starch, 1:3% (w/w)). Then, they were gradually added with various concentration of binder (1, 1.5 and 2% (w/v) sodium alginate solution) until the formation of coherent mass. Each step was homogenized by using mortar and pestle mixer. The obtained mass was then passed through a 12-mesh sieve (1.68 mm) and then dried at 40˚C for 24 h. The granules of *L. plantarum* CMU-FP002 were evaluated with regard to physical characteristic, percentage of moisture content and preliminary disintegration using the modified method of Chumthong et al. (2008). Moisture content was determined by moisture analyzer and MX-50 (Jay Instruments and Systems Pvt. Ltd., India). Physical characteristics were determined by using a digital camera (Canon IXUS 860 IS, Japan).

Formulation and evaluation of probiotic *L. plantarum* CMU-FP002 in calcium alginate beads

Sodium alginate was used as a wall material for the microencapsulation of *L. plantarum* CMU-FP002 by extrusion modified from Muthukumarasamy and Holley (2006). Cell suspension of *L. plantarum* CMU-FP002 (in distilled water) at initial concentration 20 log cfu/ml was added at a ratio of 1:5 (v/v) to alginate solution. Encapsule extrusion method was performed by expression of the wall material–culture mixture through a 27G syringe needle drop-wise into $CaCl_2$. The encapsulation parameters such as alginate concentration (1, 1.5, 2 and 2.5% w/v) and calcium chloride concentration (0.1 and 0.5 M) were studied. Hardening time of capsules in calcium chloride was fixed for 30 min at room temperature. Encapsules were held at room temperature for 30 min. Encapsules were separated by filtration through Whatman #4 filter paper. Microcapsules were dried at 40℃ for 24 h before storing at 4℃ until use. Physical characteristics were determined by inverted microscope (Olympus CK2, Japan).

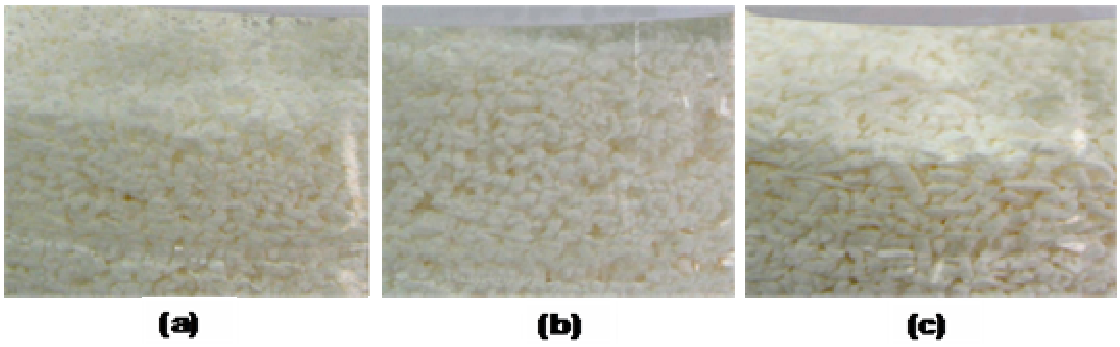

Figure 1. Physical characteristic of *L. plantarum* CMU-FP002 granule using: (a) 1% (w/v) sodium alginate, (b) 1.5% (w/v) sodium alginate and (c) 2% (w/v) sodium alginate. All were examined using digital camera.

Viability assay of cells in granules and calcium alginate beads

Granules and calcium alginate beads, both in dry and wet form, were dissolved in phosphate buffered saline (PBS, pH 7.4). A serial dilution of this suspension was made until a suitable cell density was obtained. The cell suspension was enumerated by pour plate on MRS agar. The plates were then incubated at 37˚C for 48 h. Colonies of bacteria were counted and converted to log cfu (colony forming units). The survival of probiotic cells reported as percentage survival was calculated according to the following equation:

$$\text{Survival (\%)} = \frac{\text{cfu of the dried granules or calcium alginate beads}}{\text{cfu of the wet granules or calcium alginate beads}} \times 100$$

Cell survival of *L. plantarum* CMU-FP002 granules and beads in gastrointestinal tract condition

The procedure for examination was modified from Gildas et al. (2009). Simulated gastric fluid (SGF), consisting of 9 g/L NaCl and 3 g/L pepsin from porcine stomach mucosa, was prepared. Then, pH was adjusted to 1.8 with 0.5 N HCl. The choice of pH 1.8 for SGF took into account the activity of pepsin, which was maximal in a pH range of 1.7 to 3.0 (Tobey et al., 2001). Simulated intestinal fluid (SIF), consisting of 9 g/L NaCl, 10 g/L pancreatin, 10 g/L trypsin from hog pancreas and 3 g/L of bile salts, was prepared. Then, pH was adjusted to 6.5 with 0.1N NaOH. Granules or beads were incubated in SGF for 120 min and subsequently, transferred to SIF for 180 min at 37℃. The control solution was sodium chloride solution (9 g/L) and control samples were free cells, blank granules and blank beads. Granules and beads were dissolved in phosphate buffered saline (PBS) of pH 7.4. Cell counts were determined by serial dilutions in the same buffer and plate counting of MRS agar.

Stability of *L. plantarum* CMU-FP002-loaded alginate particles during storage

A sample of free flowing *L. plantarum* CMU-FP002-loaded alginate particles was tested for stability, which modified the condition from Heidebach et al. (2010). It was stored for 8 weeks, alternating between 4℃ for 5 days and room temperature for 5 days, until the end of the storage period. For predetermined times at week intervals, 1 g of samples was collected and then dissolved and made into serial dilutions in phosphate buffered saline (PBS) of pH 7.4. Cell count was determined by pour plates on MRS agar and incubated for 48 h at 37˚C to determine survival cells of *L. plantarum* CMU-FP002.

Statistical analysis

All data is expressed as means ± SD from at least three independent experiments. Differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA). The differences were considered significantly different when $p < 0.05$.

RESULTS

Formulation and evaluation of probiotic *L. plantarum* CMU-FP002 granules

Three concentrations of sodium alginate were used to perform granules. The physical appearance of granules is shown in Figure 1. The strength of granules is directly related to the sodium alginate concentration. When sodium alginate concentration was increased, the strength of the granules increased. After drying at 40˚C in hot air oven for 24 h, the granules made from 1 %(w/v) sodium alginate looked like a white short worm line and was easily friable by hand. The properties of formulated granules using 1.5% (w/v) sodium alginate were similar to those using 1% (w/v) except for the white worm lines, which had more length and strength as shown in Figure 1. Among these granulation, 2%(w/v) sodium alginate produced the longest and strongest granules.

Moisture percentages of dried granules, which were produced from 1, 1.5 and 2%(w/v) sodium alginate were 3.04, 3.34 and 3.50%, respectively. The disintegration of obtained granules in distilled water at 37˚C was studied. The granules produced from 1 and 1.5%(w/v) sodium alginate could disintegrate in distilled water at 37˚C, better than granules produced from 2%(w/v).

Disintegration decreased when increasing sodium alginate concentration.

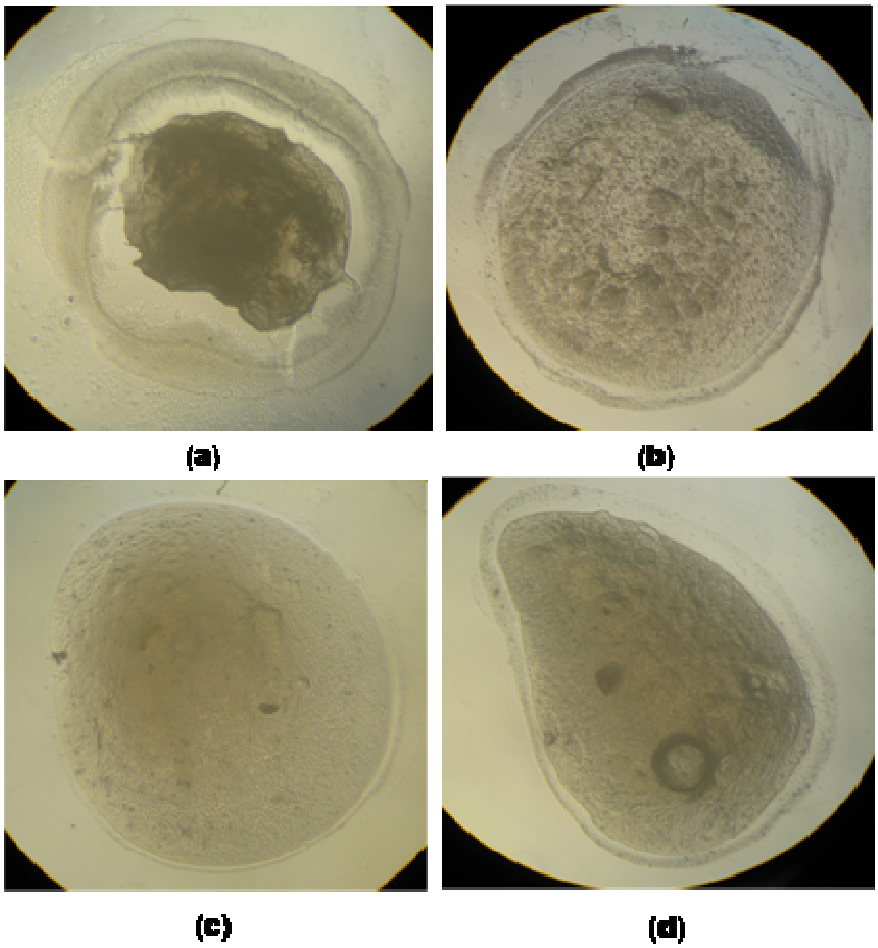

Figure 2. Physical characteristic of *L. plantarum* CMU-FP002 calcium alginate beads, (a)-(d) were prepared by using 1, 1.5, 2 and 2.5% sodium alginate, respectively. All were examined under 40X inverted microscope.

Formulation and evaluation of probiotic *L. plantarum* CMU-FP002 in calcium alginate beads

In this study, the influence of encapsulation parameters such as sodium alginate concentration and calcium chloride concentration were determined and optimized. The physical characteristics of calcium alginate beads were shown in Figure 2. The results showed that increasing sodium alginate concentration improved the beads shape and structural uniformity. Gel bead formation of 1% (w/v) sodium alginate presented a 2 to 3 mm gel bead size, cream-white color, non-spherical, non-smooth and revealed cracks on the surface. When sodium alginate of up to 1.5% (w/v) was used, 2 to 3 mm gel bead size, cream-white color, a nearly spherical shape and a nearly smooth surface ware observed. Cracks on the surface were less observed than using 1% (w/v) sodium alginate. The gel beads made from 2% (w/v) sodium alginate formed 2 to 3 mm gel bead size, cream-white color, spherical shape and smooth surface. Using 2.5% (w/v) sodium alginate, gel beads with a similar property to 2% (w/v) was observed except its spheroid shape. Gel beads, which performed in 0.5 M calcium chloride, had a higher strength than in 0.1 M calcium chloride.

For preliminary tests, dissolution of obtained blank beads in stimulated gastric juice (SGF) pH 1.8 was studied. The beads formed in 0.1 M calcium chloride could dissolve in SGF pH 1.8 better than blank beads formed in 0.5 M calcium chloride. In this experiment, low dissolution of blank beads made from 2.5% (w/v) sodium alginate and 0.5 M calcium chloride was observed. Thus, three sodium alginate concentrations, 1, 1.5 and 2% (w/v) with 0.1 M calcium chloride, were used to form the gel beads in the next experiment.

Survival of *L. plantarum* CMU-FP002 cell in granules and calcium alginate beads

Table 1 shows the viability and survival percentages of *L. plantarum* CMU-FP002 cells in granules and calcium

Table 1. Viable cell and survival percentages in wet granules, dried granules, wet calcium alginate beads and dried calcium alginate beads, which made from various concentration of sodium alginate (1, 1.5 and 2% w/v).

Sodium alginate (%w/v)	Viable cell (log cfu/g)				Survival (%)	
	Wet granules	Wet beads	Dried granules	Dried beads	Granules	Beads
1.0	18.74 ± 0.02^{b}	19.20 ± 0.02^{a}	12.95 ± 0.04^{h}	16.11 ± 0.03^{e}	69.10^{B}	83.91^{A}
1.5	18.36 ± 0.10^{c}	19.37 ± 0.01^{a}	13.27 ± 0.02^{g}	16.33 ± 0.06^{de}	72.28^{C}	84.31^{A}
2.0	18.46 ± 0.08^{bc}	19.41 ± 0.03^{a}	14.36 ± 0.02^{f}	16.37 ± 0.06^{d}	77.79^{D}	84.33^{A}

* Each value is the mean ± SD of three trials. The small alphabet indicate that the values are significantly different in viable cell amount among wet granules, wet beads, dried granules and dried beads treatment ($p < 0.05$, n = 3). The big alphabet indicate that the values are significantly different in survival percentages between granules and beads treatment ($p < 0.05$, n = 3).

alginate beads before and after being dried at 40˚C in a hot air oven for 24 h. Approximate 4 to 5 log cfu/g of *L. plantarum* CMU-FP002 cells in wet granules were significantly decreased after they were dried at 40˚C ($p < 0.05$). Similarly, cell survival in wet beads (~19 log cfu/g) were significantly higher than those in dried beads (~16 log cfu/g) ($p < 0.05$). Approximate 19 log cfu/g of *L. plantarum* CMU-FP002 cells in wet calcium alginate beads were achieved. Cell survival in wet calcium alginate beads was significantly higher in wet granules (~18 log cfu/g) ($p < 0.05$). After being dried at 40˚C, survival cells of dried beads were significantly higher than dried granules ($p < 0.05$). The results showed that live cells of calcium alginate beads, produced from 2% (w/v) sodium alginate, were the highest ($p < 0.05$). On the other hand, viable cells of dried granules, formed from 1% (w/v) sodium alginate, were the lowest ($p < 0.05$).

The results showed that increasing sodium alginate concentration, significantly increased the viability when it was in dried granules and beads ($p < 0.05$), but it was not achieved in wet granules and beads.

Survival percentages of dried granules and beads were calculated from viable cells in wet granules and dried granules and wet beads and dried beads, respectively. The results revealed that the survival percentages of the beads were higher than granules ($p < 0.05$). Furthermore, the result showed that survival percentages of both granules and beads increased with increased in sodium alginate concentration.

Cell survival of *L. plantarum* CMU-FP002 granules and beads in gastrointestinal tract condition

In order to evaluate the release and viability of *L. plantarum* CMU-FP002 granules and encapsules in gastrointestinal condition, *L. plantarum* CMU-FP002 granules and encapsules were tested in simulated gastric fluid (SGF) for 120 min and simulated intestinal fluid (SIF) for 180 min. The effect of sodium alginate concentrations on the viability of *L. plantarum* CMU-FP002 under SGF and SIF is shown in Figure 3. Three formulas of granules were disintegrated within 60 min in SGF (Line A-C). Moreover, all calcium alginate bead formulas were disintegrated with more difficulty than all granule formulas (Line D-F). Granules and gel beads produced from higher concentration of sodium alginate were more difficult to disintegrate. Gel beads made from 2% (w/v) sodium alginate did not completely dissolve (Line F). A viable count of *L. plantarum* CMU-FP002 in the granules decreased rapidly with increasing contact time in SGF (from ~ 12 - 14 to 8 - 9 log cfu/g) (Line A-C) and SIF (from ~ 8 - 9 to 2-3 log cfu/g) (Line A-C). On the other hand, the survival of cells in gel beads gradually declined with increasing contact time in SGF and SIF (from ~ 15 log to 6 - 9 log cfu/g) (Line D-F). However, in entrapped bacteria, both granules and beads survived well in gastrointestinal conditions compared to non-entrapped free bacterial cells. Moreover, the results revealed that the viability of encapsulated bacteria in gastrointestinal conditions increased with an increasing alginate gel concentration of 1 to 1.5% (w/v) as shown in line D and E. The higher the concentration of sodium alginate, the better the protection of bacterial cells was achieved. However, in 2% (w/v) of sodium alginate preparation, dissolution did not complete (Line F).

Stability of *L. plantarum* CMU-FP002-loaded alginate particles during storage

In this experiment, it was found that encapsulation technique could protect higher number of live cells. Therefore, probiotic encapsules, which were made from 1.5% sodium alginate were selected for storage study. The stability of *L. plantarum* CMU-FP002 entrapped in alginate gel beads during alternating storage between 4℃ for 5 days and room temperature for 5 days is shown in Figure 4. The survival of *L. plantarum* CMU-FP002 loaded in alginate beads gradually declined during storage. The survival was maintained at approximately ~11 log cfu/g after storage for 8 weeks.

DISCUSSION

Wet granulation, the process of adding a liquid solution to powders is one of the most common ways to granulate.

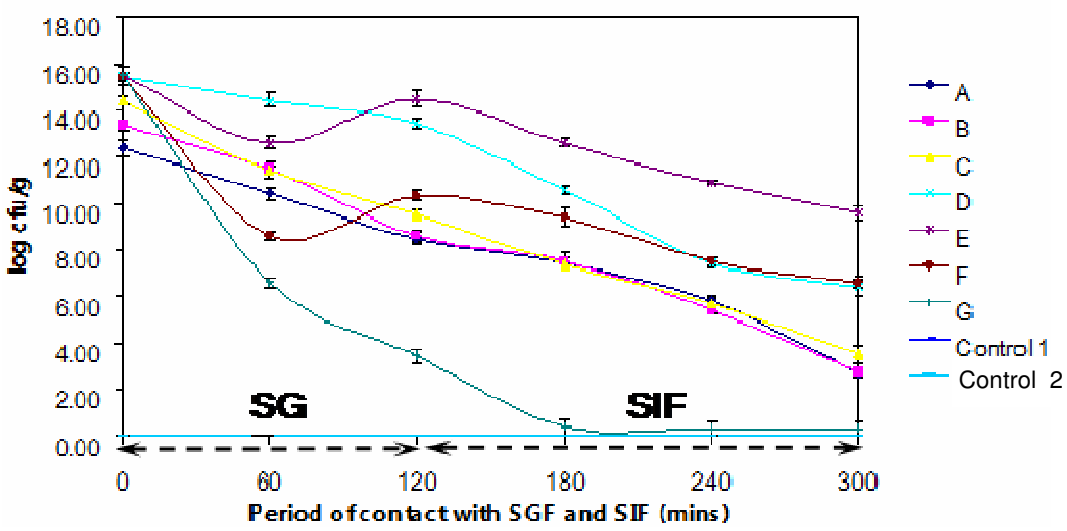

Figure 3. Viable cell of granules and calcium alginate beads formula in contact with SGF for 120 min and SIF for 180 min. *L. plantarum* CMU-FP002 granules, A-C were prepared by using 1, 1.5 and 2% sodium alginate, respectively. *L. plantarum* CMU-FP002 calcium alginate beads, D-F were prepared by using 1, 1.5 and 2% sodium alginate, respectively. G : free *L. plantarum* CMU-FP002 cells, Control 1 : blank granules, Control 2 : blank beads.

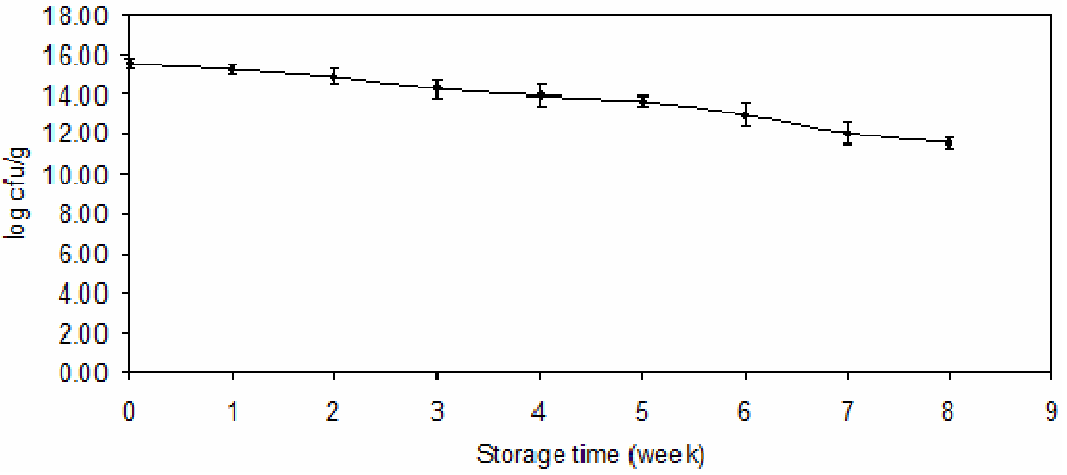

Figure 4. Viability of *L. plantarum* CMU-FP002 in calcium alginate beads during storage for 8 week. Each value is the mean ± SD of three trials.

In this study, the diluents were lactose and corn starch, 1:3% (w/w), with sodium alginate as a binder. Lindberg and Jönsson (1985) reported that lactose 100 mesh was granulated faster than the 350 mesh quality. Corn starch required large volumes of granulating solution. Consequently, a suitable ratio for the end-point determination when granulating a mixture of lactose 100 mesh and corn starch was 2:1 ratio, with povidone as a binder. Sodium alginate solution mixed into the powders can form bonds between powder particles that are strong enough to lock them together (Michael, 2002). The density of each granule increased by increasing the amount of binding solution. Therefore, controlling the quantity of binder was one factor to control the density and strength of the granules (Michael, 2002). In this study, results revealed that increasing the strength and length of granules depended on more viscosity of solutions that was caused by increase in the sodium alginate concentration. Similarly, Chumthong et al. (2008) reported that sodium alginate acted as a viscosifier in the granules formulation, which may be attributed to the bio-adhesive property. Moisture content in dried granules trends to increase with increase in alginate concentration. It may be caused by more granule strength not allowing

the water to evaporate into the atmosphere.

The formation of calcium alginate beads may be related to several factors. In this study, calcium alginate beads were formulated from 1, 1.5, 2 and 2.5% (w/v) sodium alginate solution with 0.1 and 0.5M $CaCl_2$. In the solution, alginates behave like flexible coils. The gel formation of alginate is mainly achieved by the exchange of sodium ions with divalent cations such as Ca^{2+}, Mn^{2+}, Zn^{2+}, or Cu^{2+}. However, depending on the interaction with divalent metal ions (such as Ca^{2+}), they form an ordered structure (Ana et al., 1999). In this study, the beads, which were formed in 0.5M $CaCl_2$, had more strength than in 0.1M $CaCl_2$, it might be explained based on the number of Ca^{2+} ions. As a result, in the pre-dissolution, stronger blank beads could not dissolve in SGF solution. Structural and mechanical properties of calcium-alginate capsules can also be tuned by adjusting the concentration of sodium alginate or calcium chloride. Low solubility calcium salts reduce the gelation rate and increase both the structural uniformity and the mechanical strength of gels (Thu et al., 1996; Wang and Spencer, 1998). Our result showed that 2.5% (w/v) alginate concentration, both in 0.1 and 0.5M $CaCl_2$, had difficult in forming spherical shapes because of their high viscosity. Similarly, Ariel et al. (2006) reported that maximum encapsulation efficiency was achieved by using higher molecular weight alginate and increasing the alginate concentration. Furtheremore, Chandramouli et al. (2004) reported that 2% (w/v) alginate solution had difficulty in forming spherical shapes. From our results, the higher concentration of sodium alginate and the better protection of bacterial cells were achieved. Increasing the biopolymer (sodium alginate) concentration may increase the number of binding sites for Ca^{2+} ions. As a result, a more densely cross-linked gel structure was formed.

Survival percentage of *L. plantarum* CMU-FP002 in dried gel beads was higher than in dried granules. This might be attributed to the interaction of gel formation causing stronger and denser structures than the interaction of granulation. In addition, granule structure had non-smooth surfaces and many cracks on the surface. According to these reasons, during the drying process, heat could contact with the cells in granules more than the cells in gel beads. Therefore, cells, which were entrapped in calcium alginate bead forms, were protected from heat, better than cells in granule forms. However, live cells in dried granules (~ 13 to 14 log cfu/g) in our experiment were still high enough for health benefits.

For the viability of *L. plantarum* CMU-FP002 granules and beads in SGF and SIF, results of our study corresponded well with Chandramouli et al. (2004), Lee and Heo (2000) and Ross et al. (2006) who indicated that *L. acidophilus* CSCC 2400, *Bifidobacterium longum* and *Bifidobacterium infantis* in calcium alginate spheres survived when exposed to SGF. Furthermore, Chandramouli et al. (2004) reported that the viability of encapsulated *L. acidophilus* CSCC 2400 in simulated gastric conditions increased when increasing alginate gel concentration from 0.75% to 1.8% (w/v). Cui et al. (2000) indicated that when the bifidobacteria was immobilized with alginate or even poly-*l*-lysine treatment, the survival of bifidobacteria was highly enhanced in the low pH conditions. Ross et al. (2006) reported that microscopic examination of the microcapsules showed that the bacteria remained entrapped within the capsule material in SGF and were released when transferred to SIF. It seemed likely that in our experiment, gastric fluid entered the microparticles through the surface pinholes resulting in a loss of viability. Thus, the survival of *L. plantarum* CMU-FP002 declined as the incubation time increased due to the detrimental effects of low pH on their cells. However, the dense membrane was expected to create diffusion resistance through the beads, which resulted in lower diffusion of SGF and SIF. Consequently, cell survival increased with increased alginate gel concentration. The strengthening of the surface membrane and the size of microparticles offered other choices for further enhancement of gastric resistance (Cui et al., 2000). Granulation technique has few reports regarding the application of entrapped bacterial cells. This might be as a result of the less protection of granulation when contacting SGF. Similarly, Al-Mohizea et al. (2007) reported that the wet granulation technique was not favorable for producing the yeast tablets due to the problems of color darkening and the reduction of the fermentation power of the yeast as a result of the early start of the fermentation process due to the presence of moisture.

The stability of bacterial cells entrapped in calcium alginate particles improved after encapsulation with alginate when compared to free *L. plantarum* CMU-FP002 cultures. The effect of storage conditions on viability of *L. plantarum* CMU-FP002 in calcium alginate beads was similar to Cui et al. (2000) who found that the stability of free flowing bifidobacteria-loaded alginate poly-*l*-lysine microparticles was significantly improved during storage at 4°C in a refrigerator when compared to bifidobacteria cultures. Our results indicated that encapsulated *L. plantarum* CMU-FP002 cells in calcium alginate beads could improve the viability of bacterial cells, although it was kept in an alternating condition between 4˚C for 5 days and room temperature for 5 days.

Conclusions

In summary, both granulation and encapsulation techniques were successful for entrapping *L. plantarum* CMU-FP002 with high survival cells enough for the use as a feed additive in broilers. However, the improvement of granulation technique is required to enhance the

retention efficiency during contact with SGF. The optimum conditions of microencapsulation preparation in this paper were 1.5% sodium alginate with 0.1 M calcium chloride and 30 min hardening time at room temperature. This condition was proved to be efficient in increasing the viability of probiotic bacteria in SGF compared to non-encapsulated free cells. The number of viable probiotic bacteria in gel beads, made from 1.5% sodium alginate, was 11 log cfu/g at the end of 8 weeks of storage. The results of this study also indicated that alginate microcapsules could be used as a protective delivery vehicle for administering viable probiotic bacteria. Further studies are needed for practical application in broilers.

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Full Length Research Paper

Does *dermatophagoides* cause different allergic rhinitis clinic than pollens?

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The aim of this study was to compare allergic rhinitis clinics caused by two most common allergens: house dust mites (HDM) and pollens. Three hundred and fifty patients were evaluated. These patients were pure pollen or HDM allergic according to skin prick test (51 HDM+, 299 Pollen+). Mainly, HDM were allergens for perennial allergic rhinitis, while pollens were for seasonal allergic rhinitis ($p < 0.01$). Both groups were found with similar symptom frequency except palatal, ocular, throat itching and eye redness indicating mostly pollen allergy ($p < 0.05$). Seasonal exacerbations used for the differential diagnosis were found to be very significantly different. Spring and autumn were the seasons where pollen allergy symptom exacerbation was mainly seen ($p < 0.01$). HDM allergy was uniquely found with symptom exacerbations in winter ($p < 0.05$). Rural area visit was found dominating triggering factor for pollen allergy ($p < 0.05$). The most common triggering factor was house dust exposure in HDM+ group ($p < 0.05$). HDM allergy being mostly mimicking pollen allergy in allergic rhinitis, however, differs from it with some clinical features. This could be detected with detailed history taken from the allergic rhinitis patients. While doing definitive diagnosis, prick test may be helpful with a clear patient history in patients hard to diagnose.

Key words: Mite, pollen, allergy, rhinitis.

INTRODUCTION

Rhinitis consists of allergic and non-allergic subgroups. Allergic rhinitis (AR) is one of the most common airway diseases that results from the inflammatory reaction mediated by specific IgE antibodies and is manifested after the exposure of the nasal mucosa to allergens (Obtułowicz and Składzień, 2005). 10 to 25% of the population suffers from AR (Valero et al., 2009). Patients suffering from specific rhinitis symptoms (nasal obstruction, rhinorrhea, sneezing) with positive allergic findings by either specific IgE or SPT are diagnosed as AR. Nasal provocation test is another technique to confirm the diagnosis (Chusakul et al., 2010). Allergens are divided into two subgroups: indoor and outdoor allergens. The mold, house dust mites (HDM), pets and cockroaches are kinds of indoor and outdoor allergens including pollens especially (Ferguson, 2008; Huss et al., 2001; Rosenstreich et al., 1997). The most common sensitizing allergen in the United States is HDM (Ferguson, 2008). Pollen calendars differ from one geographic area to another while pollens usually increase in spring and decrease in autumn (D'Amato et al., 1998).

HDM have been shown to be important sources of indoor allergens associated with asthma and other allergic conditions, especially AR. HDM are very small creatures that live indoors in warm, moist places. These creatures are not a type of bugs that you can spot and crush, being invisible to the unaided eye. Their translucent bodies further hinder their visibility. HDM have no eyes or antennae, just eight legs, and a mouth-like appendage. The most common dust mite species around the world include *Dermatophagoides pteronyssinus*

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(Dp), *Dermatophagoides farinae* (Df), *Euroglyphus maynei* and *Blomia tropicalis*. The pyroglyphid mite Dp is distributed from temperate to tropical regions (mainly in Europe). Other important pyroglyphid mite is Df found in drier regions (mainly in North America) (John and Petri, 2006). These microscopic arachnids colonize beds, upholstered furniture and carpets (Arbes et al., 2003). Because mites do not drink and rely on absorption of humidity from the atmosphere, reducing humidity below 50% is recommended as the primary prevention method. HDM do not bite, but live from shed human skin. Because of the medical implications, house dust and the fauna of mites associated with house dust have been tested for the source of the house dust allergen. Mite allergens are mainly present in feces of house dust mites and may become airborne, inhaled by patients, giving rise to asthma, rhinitis or atopic dermatitis (van Bronswijk et al., 1990). HDM allergic patients are also shown to have poorer psychological functioning, indicating the close relationship between moderate-to-severe persistent AR and psychological functioning (Lv et al., 2010).

Allergy clinics' algorithms for the diagnosis of allergic rhinitis consist of two powerfull tools: patient history and skin prick tests. As the most common allergens are HDM and pollens, the large part of the patients need to be differentiated by these tools. Besides, some patients may be allergic to both groups of allergens. The dominating allergen could only be found according to the clinic of the patient, and then further correct therapy methods could be chosen. The prevention from the allergen and allergen-specific immunotherapy should be applied against the clinically dominating allergen. The aim of this study was to compare allergic rhinitis clinics caused by two most common allergens as HDM and pollens. Also the discrepancies between the demographic findings of HDM and pollen allergic patients were discussed.

MATERIALS AND METHODS

Three hundred and fifty patients were evaluated. These patients were pure pollen or HDM allergic according to skin prick test (SPT). Patients were diagnosed with both detailed allergic history (clinical symptoms, season of the year, triggers, family history) and the SPT. In the HDM allergic group, there were 51 patients (20 male, 31 female) aged 31.8 ± 12.4 years (9 - 59 years); whereas pollen allergic group consisted of 299 patients (127 male, 172 female) aged 28.8 ± 12.5 (6 - 65 years).

Nasal congestion/obstruction, rhinorrhoea, sneezing, headache, postnasal drip, loss of smell (hyposmia or anosmia), nasal, pharyngeal, palatal itching and ocular symptoms (itching, redness, tearing) were questioned at the first visit to the clinic. Patients with rhinorrhoea were also asked about their nasal discharge characteristics (viscosity and color). Nasal examination was done by both anterior rhinoscopy and nasal endoscopy.

Skin prick tests were performed with multi-test applicators (Multi-Test® II device, Lincoln Diagnostics, Inc Illinois, USA) and the standardized allergen extracts solutions with negative (physiologic saline/0.4% phenol) and positive (histamine 1+999 w/v/0.4% phenol) controls (Allergopharma, MERCK KGaA, Darmstadt, Germany). The allergy panel, complying with allergy profile in the south of Turkey, consisted of both indoor and outdoor allergens: Grasses and cereals (velvet grass, orchard grass, rye-grass, timothy grass, bluegrass, meadow fescue, barley, oat, wheat, rye), weeds (mugwort, stinging nettle, dandelion, English plantain), trees blossoming both early (alder, hazel, poplar, elm, sallow) and mid (birch, European birch, oak, plane), mites (*D. farinea* and *D. pteronyssinus*).

We defined a reliable SPT in which the median wheal diameter of the positive control is more than 3 mm. We included patients with positive SPT which the median wheal diameter of the skin reaction is ≥ 3 mm. Non-allergic and mixed allergic (pollen + HDM) rhinitis patients were excluded. Groups were defined as purely HDM allergic patients (HDM+) and pollen allergic patients (Pollen+).

Normal distribution of the groups and homogeneity of variances were analyzed by Kolmogorof-Simirnov and Levene tests, respectively. Student t-test in independent samples with equal variances, and multiple chi-square tests were used for the comparison of groups. Statistical significance was determined as $p < 0.05$.

RESULTS

There were 17 (33.3%) students, 6 (11.8%) housewives, 1 (2%) retired and 27 (53%) working individuals in HDM+ group. Pollen+ group included 115 (38.5%) students, 40 (13.4%) housewives, 7 (2.3%) retired, 3 (1%) unemployed and 134 (44.8%) employed individuals (Table 1). There was no significant difference between groups according to their way to continue their life. Three (5.9%) patients of HDM+ group were diagnosed as seasonal and 48 (94%) as perennial allergic rhinitis. Seven patients (2.3%) of the Pollen+ group were diagnosed as perennial allergic rhinitis and remaining 292 (97.7%) as seasonal allergic rhinitis. This distribution was significantly different as HDM allergy causes perennial allergic rhinitis; and pollen allergy causes the seasonal allergy ($p < 0.01$). Symptom distribution of subgroups was given in Table 2. Palatal, ocular and throat itching as well as eye redness were more prominent symptoms in Pollen+ group ($p < 0.05$).

Time period since the beginning of allergic symptoms ranged from 1 to 16 years (mean 6 ± 4.6 years) in HDM+ group and 1 to 25 years (mean 5 ± 4.2 years) in Pollen+ group. There was no statistically significant difference between two groups about the time of the disease history ($p > 0.05$).

Although 48 of 51 (94%) HDM allergic patients gave perennial rhinitis history, 20 (39.2%) had exacerbations in spring, 8 (15.7%) in winter and 5 (10%) in summer. 182 (60.9%) patients in pollen group had exacerbations in spring, 61 (20.4%) in summer, 34 (11.4%) in autumn and 18 (6%) in winter. This was statistically different (Table 3). There was no statistical difference in the exacerbations of the symptoms in the morning between the two groups ($p > 0.05$).

Environmental triggering factors were effective in 115

Table 1. Occupational distribution of subgroups.

	HDM+	Pollen+	P
Student	17 (33.3%)	115 (38.5%)	p > 0,05
Housewife	6 (11.8%)	40 (13.4%)	p > 0,05
Retired	1 (2%)	7 (2.3%)	p > 0,05
Unemployed	0 (0%)	3 (1%9	p > 0,05
Employed	27 (53%)	134 (44.8%)	p > 0,05

Table 2. The frequency of the symptoms in subgroups.

	HDM+		Pollen+	
	Number	%	Number	%
Nasal congestion	33	64.7	211	70.6
Nasal discharge	43	84	256	85.6
Sneezing	46	90.2	279	93.3
Headache	16	31.3	93	31.1
Postnasal drip	26	50.9	133	44.4
Smelling disorder	9	17.6	81	27
Nasal itching	35	68.6	207	69.2
Palatal itching*	11	21.5	128	42.8
Throat itching*	17	33.3	144	48.1
Ocular itching*	21	41.1	190	63.5
Eye redness*	16	31.3	151	50.5
Eye tearing	30	58.8	184	61.5

*p < 0.05.

Table 3. Seasonal exacerbations.

	HDM+		Pollen+		p
	Number	%	Number	%	
Spring	20	39.2	182	60.9	P < 0.01
Summer	5	9.8	61	20.4	P > 0.05
Autumn	0	0	34	11.4	P = 0.011
Winter	8	15.7	18	6	P < 0.05

(38.5%) patients in pollen group whereas there were only 8 (15.7%) patients in HDM+ group (p < 0.05). House dust exposure was found as triggering factor mostly in HDM+ group (p < 0.05). There was no statistical difference between two subgroups about other triggering factors like thermal changes, smell and detergents.

There was not a statistical difference between two subgroups about allergic family history (p > 0.05). Incidence of concomitant allergic diseases (skin allergy, allergic eye disease, allergic lung disease) was similar in both groups (p > 0.05).

Papule diameter of positive controls ranged from 3 to 8 mm (mean: 5.8 ± 1.3) in HDM group and 4 - 19 mm (mean: 6.2 ± 1.8) in pollen group (p > 0.05). Erythema diameters ranged from 5 to 30 mm (mean: 15.5 ± 7) in HDM group and 5 to 35 mm (mean: 16.4 ± 6.7) in pollen group (p > 0.05).

DISCUSSION

Both pollen and HDM are the most common causes of allergic rhinitis. Pollens as main allergens in allergic rhinitis were widely discussed in the literature. Whereas, HDM are the main allergens of indoor environment. These two groups are worth comparing both clinically and demographically. Using purified groups, we augmented the scientific value of this study to eliminate other contributing factors in the diagnosis. The clinical differences may help to distinguish these patients before the definitive allergy tests. This paper is a summary of the similar and different findings of two groups.

Demographic findings as age, sex distributions and occupational conditions were found similar in two subgroups. Family history and associated allergic disease were not different for two groups. These findings indicated that both allergens work on the same pathogenesis ways.

Mainly, HDM are the allergens for perennial allergic rhinitis, while pollens are for seasonal allergic rhinitis. Both groups were found with similar symptom frequency except palatal, ocular and throat itching and eye redness indicating mostly pollen allergy. Both pollens and HDM were thought to exacerbate rhinitis symptoms in the morning. Patients having close contacts with HDM during the night may wake up in the morning with running nose, sneezing and nasal blockage. Pollens were also at a higher level in the air at early morning possibly causing more symptoms at this interval. Thus, we could not find any difference for morning symptom exacerbations between the groups. However, seasonal exacerbations used for differential diagnosis were found to be very significantly different. Spring and autumn were the seasons where pollen allergy symptom exacerbation was mainly seen. Sahney et al. (2008) reported pollen calendar of their country. In their report, the most frequent pollination season was spring for trees and flowers and autumn for grasses. Atmospheric pollen concentrations are at the highest level in April, May and August in our country (Erkara, 2008; Guvensen and Ozturk, 2003). This may be contributed to air temperature, humidity, and wind changes. HDM allergy was uniquely found with symptom exacerbations in winter. The highest concentrations of HDM are seen in November and December (Sun and Lue, 2000). This can be associated with humidity and climate conditions of winter. Also, people spend time mostly in indoor areas at this season and exposure to HDM increases. There was no statistical difference about allergic exacerbations between subgroups in summer (p > 0.05).

Rural area visit was found dominating triggering factor for pollen allergy. The most common triggering factor was house dust exposure in HDM+ group. There was no statistical difference between two subgroups about other triggering factors like thermal changes, smell and detergents indicating that both groups have similar associated nasal hyperreactivity.

Mites shed an abundance of allergenic proteins. Particularly abundant in allergens are the extracts of mite faeces as well as the extracts of their purified bodies or culture substrate (Thomas et al., 2004). Some of the gut enzymes (notably proteases) produced by the house mite persist in their fecal matter, and can be strongly allergenic. Using different means of assessing exposure to house dust mites (Acarex-test, major allergen (Der p 1 + Der f 1, Der p 2 + Der f 2), content measured by ELISA) has allowed better identification of mite reservoirs and allergens in patients' homes (Pauli et al., 2001). In this study, HDM allergy was detected with purified allergens using skin prick test.

There is convincing evidence that avoidance of mite allergen can effectively reduce allergic symptoms. Patients can be moved to a mite allergen-free environment, or mite and mite allergen abatement can be performed to reduce exposure in existing residences. House dust mite control measures are based on the cognition of factors contributing to mite development, especially indoor relative humidity. Mite allergen avoidance strategies include 3 different methods: avoidance of mite producing allergens, elimination of mite reservoirs, especially textile reservoirs, dwellings designed so as to inhibit mite proliferation (Pauli et al., 2001; Sheikh and Hurwitz, 2003).

In recent years, greater attention has been given to the role of indoor allergens as a cause of sensitization and allergic respiratory diseases. Although indoor allergen control measures to reduce symptoms in individuals allergic to mites have produced controversial results. Exposure to high indoor aeroallergen levels, especially to house dust mite allergens, is an important environmental risk factor for allergic sensitization. Effective house dust mite allergen avoidance will never be achieved using a single control measure; various methods are required to affect the multiple factors that facilitate high indoor allergen levels. Education of the patients and their families is also an important component of environmental control strategies.

As a conclusion, HDM allergy being mostly mimicking pollen allergy in allergic rhinitis, however, differs from it, with some clinical features. This could be detected with detailed history taken from the AR patients. Recommendation of specific preventive methods to patients could be possible with correct differential diagnosis methods. Definitive diagnosis with prick test may be helped by a clear patient history in hard to diagnose patients.

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Full Length Research Paper

Bio-nanomodeling of active site in oxidized azurin using by computational methods

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A cluster model for active site of oxidized azurin was presented and investigated the geometric structure and thermochemical parameters. Quantum-mechanical calculations were performed at the HF and B3lYP/6-31G levels of theory in the gas phase and eight solvents at four temperatures. Also, nuclear shielding parameters of the active site of oxidized azurin have been taken into account using GIAO and CSGT methods at the HF and B3LYP/6-31G levels of theory in the gas phase and in different solvents such as water, DMSO, nitromethane, methanol, ethanol, acetone, dichloroethane and dichloromethane. The results were revealed that the NMR chemical shielding parameters are strongly affected by inducing different solvent media. According to these theoretical results of energy values, some important relationships have been found between the dielectric constant and structural stability of active site of oxidized azurin. Thus, it can be drastically concluded that the dielectric permittivity of the solvent is a key factor that determines the chemical behavior of active site azurin in solution.

Key words: Azurin, IR, nuclear magnetic resonance, blue copper proteins, solvent effect.

INTRODUCTION

For more than 100 years, scientists have reported that bacterial infections can sometimes elicit remission in certain forms of cancer. A series of studies they have developed has shown that an opportunistic bacterium, *Pseudomonas aeruginosa* that grows in the soil and marshes but is often found in the lungs of cystic fibrosis patients, produces a protein, azurin, which it uses as a weapon, possibly to defend itself against cancer cells that might end up harming the microbe.

A team of researchers has shown that *P. aeruginosa* preferentially enters human melanoma and breast cancer cells, triggering apoptotic cell death. They further discovered that azurin sets off this death sequence by forming a complex with the well-known tumor suppressor protein p53, stabilizing it and activating caspases that induces apoptosis in cancer cells. P53 normally stops that are damaged from reproducing and encourages them to commit apoptosis, but a majority of cancer cells have damaged or missing p53 (Yamada et al., 2004).

Azurin is one of blue copper proteins (cupredoxins) with function of the electron transfer (Cascella et al., 2006). The blue copper proteins are characterized by an intense blue colour, distinctive electron spin resonance spectra, and unusually high reduction potentials. The copper proteins have been classified according to three types:

1. Type 1 copper proteins contain one copper ion, exhibit an unusual EPR spectrum with a hyperfine splitting appreciably smaller than that found for simple copper complexes. They exhibit an intense blue color (azurin and plastocyanin).
2. Type 2 copper proteins exhibit EPR spectra similar to those of simple copper complexes.
3. Type 3 copper proteins contain a dinuclear copper site and usually, as isolated, are EPR silent, which means that the copper atoms are either in the reduced form, or antiferromagnetically coupled. There are no pronounced features in the optical spectrum visible. The copper protein under study is azurin, a type 1 copper protein that serves as an electron transfer protein. The protein

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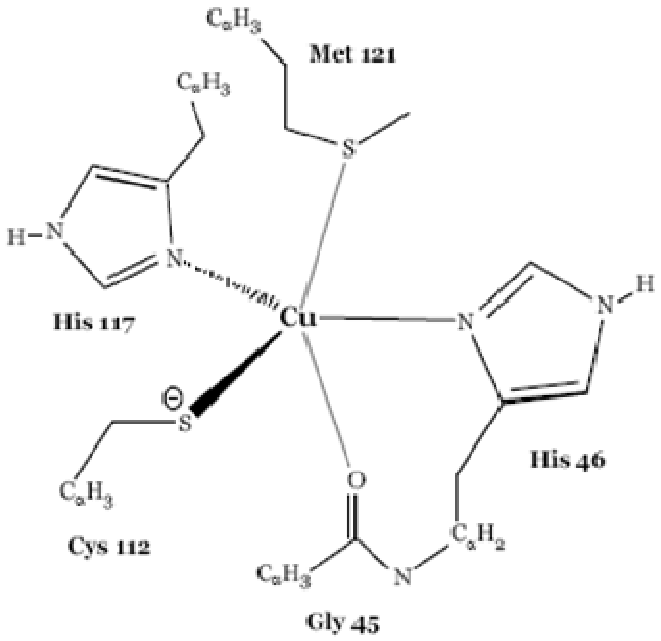

Figure 1. Cluster model for active site of oxidized azurin.

occurs in a variety of bacteria, the most commonly employed azurin are from *P. aeruginosa* (consisting of 128 amino acid) and Alcaligenes denitrificans (129 amino acid) (Olsson, 2000).

The active site of azurin consists of a copper ion and five residues, which are His 46, Cys112, His 117, Met 121 and Gly45. The copper ion is strongly connected to His 46, Cys112 and His117 with coordination bond and is weakly bonding to other residues and the structure around the copper ion is a distorted trigonal bipyramid (Figure 1) (Sugimori et al., 2005). Azurin(128 amino acids, 14 kD) consists of eight stranded β-strands, arranged in a β-sandwich, connected by random chains (turns) and of an a helical insertion.The copper site is at the top, or northern end, of protein, surrounded by an extensive hydrophobic patch that is the most striking surface feature of the protein (Arcangeli et al., 1999). The blue copper proteins serve as electron transfer agent. Their distorted trigonal geometry is intermediate between the tetrahedral coordination preferred by Cu(I) and the tetragonal geometry of most Cu(II) complexes(Rizzuti et al, 2007). As a results, the change in geometry when Cu(II) is reduced to Cu(I) is small, which gives a small reorganization energy and allows a high rate of electron transfer (Ryde et al., 2001). In the active site, electron transfer is with the redox reaction caused by charge transfer of S(Cys112)(π)→ Cu(dx^2 -y^2), which has been (investigated by resonance Raman (RR) spectra (Sugiyama et al., 2006).

Recently, the blue copper proteins including azurin have been investigated experimentally and theoretically by many groups (Lehmann, 2004; Thompson et al., 2000; Hansen et al., 2006). Experiments and computational studies are aimed at answering questions such as: To what extent are the local properties of the metal site determined by its protein and solvent environment? Which is the influence of protein internal dynamics on structural regions relevant for the protein functionality? How is protein conformation connected to solvent dynamics (Rienzo et al., 2004)?. The geometry of the active site of azurin has been estimated by density functional theory (DFT) with B3LYP method and quantum mechanics/ molecular mechanics (QM/MM) (Shuku et al., 2005)). The hyperfine coupling constants in the active site have been calculated by DFT with B3LYP method and also by QM/MM approach(2).The electronic structure and the g-tensor of the active site of azurin have been investigated by *ab initio* multireference determinantal configuration interaction (MRD-CI) calculations (Gastel et al., 2002). Solvent effects on electronic structure of molecules have been investigated by many chemists and physicists to understand molecular structure, mechanism of chemical reactions in solution, etc. by using quantum-chemical calculations and molecular dynamics simulations. Physical properties such as geometry of molecules and charge distribution in solution often vary from those in vacuum.

Ryde et al have also performed a geometry optimization of the active site of blue copper proteins including azurin by quantum-chemical calculations with polarizable continuum model (PCM). Their results suggest that weak axial bond of Cu-S (Met 121) in solution is more elongated than that in vacuum (Ryde et al., 2001). The ability to accurately calculate solvation energies of molecules using molecular simulation methods is an important development in computational chemistry. These methods have wide applicability not only in studies of free energies of salvation, but also in studies of free energies of binding and protein and nucleic acid stability. The solvent effect is taken into account using the self-consistent reaction field (SCRF) method. This method is based on the Onsager reaction field theory of electrostatic salvation. In this model, a solvent is treated as a uniform dielectric with a given dielectric constant. A solute is placed into a cavity within the solvent. Various SCRF approaches differ in how they treat the cavity and reaction field (Tomasi et al., 2005).

In this paper, we use non empirical calculation in one of the first detailed studies of active site, geometries, energies, enthalpies, free energies, entropies and other thermochemical properties with special emphasis on solvent effect on it. We optimized the geometries of the active site of oxidized azurin in various solvents using the Onsager model at the Hartree-Fock, B3LYP levels of theory and compared our results with those obtained for the gas phase in addition, the effect of the permittivity of solvents on the stability of this structure was explored and discussed .The calculations were performed using the 6-31G basis set. In our current research, we have also theoretically studied the effects of DMSO, nitromethane, methanol, ethanol, acetone, dichloroethane,

dichloromethane, water and gas phase on the chemical shielding parameters of C^{13}, N^{15}, ^{64}Cu, ^{32}S nuclei involving in active site and its structural stability. The gauge including atomic orbitals (GIAO) and continuous set of gauge transformations (CSGT) approaches within the SCF-Hartree-Fock and B3LYP approximation have been used in order to investigate the influence different solvent media on the magnetic shielding tensors through Hartree-Fock and B3LYP approximation using 6-31G basis set.

THEORETICAL METHODS

IR approach

The Onsager-SCRF code elaborated by Wiberg and co-workers for the Gaussian computational code has been fairy popular in the past years. The Onsager model is the simplest version of the MPE approach. Solvation is described in terms of a dipole moment drawn iteratively from QM calculations of the molecule. The appealing feature of the Onsager-SCRF method was the ability of directly exploiting almost all the computational facilities of the Gaussian package (Tomasi et al., 2005).
Following the Onsager model, the interaction energy of a dipole in a solvent is:

$$E^{solv}(t) = -m(t)R(t), \quad (1)$$

Where R(t) is the reaction field at time t caused by the surrounding solvent that acts on the dipole of the solute.
The Onsager model describes the system as a molecule with a multipole moment inside a spherical cavity surrounded by a continuous dielectric. In some programs, only the dipole moment is used, and calculations therefore, fail for molecules with zero dipole moment. The results obtained using the Onsager model and HF calculations as a rule is qualitatively correct. Accuracy increases significantly with the use of MP2 or hybrid DFT functional. This is not the most accurate method available, but it is stable and fast.This makes the Onsager model an attractive alternative when a PCM calculation fails (Monajjemi et al., 2008).

In this study, a theoretical analysis at the HF and B3LYP /6-31G level of theory was performed to characterize all the stationary points of the potential energy surface as minima and obtain thermodynamic corrections. Solvation effects were modeled by the Onsager method as implemented in the Gaussian98 program. The equilibrium free energy of salvation can be divided into smaller contributions corresponding to cavitations, universal salvation effect such as solute-solvent electrostatic, dispersion,
and repulsion interactions and non universal specific interaction, such as intermolecular hydrogen bonding or electron donor-electron acceptor interactions (Schleyer, 1998).
ΔG (solvation) =ΔG (electrostatic) + ΔG (dispersion) + ΔG (repulsion) + ΔG (cavitations) + ΔG (specific).
Electrostatic solute-solvent interactions are usually introduced into quantum-mechanical calculations by means of the self-consistent reaction field (SCRF) approach.

In the Onsager model, a solute is placed in a spherical cavity immersed in a continuous solvent and the full classical multipole expansion of the total solute charge distribution is truncated after the dipole term, that is, it only includes solute monopole and dipole
interactions with the continuum. Despite the simplicity of the Onsager approach, its applicability was proved for many systems.

The advantage of this model is analytic calculations of the solvent reaction field for spherical cavities, which speed up the process and allow geometric optimizations in solution to be performed for compact molecules at a modest computational cost. Although this method includes significant improvements of the Onsager approach, a new problem arises. For the definition of the density surface, an isodensity level that fixes the amount of electron density within the built cavity must be specified. However, a small percentage of charge density extends outside the cavity. This causes serious problems in the dielectric continuum treatment of solvents by affecting the charge inside the cavity.

Nuclear magnetic resonance approach

The calculation of nuclear magnetic resonance (NMR) parameters using semi-empirical, *ab initio* and DFT techniques has become a major and powerful tool in the investigation to look at how variations in the molecular structure occurs. The ability to quickly evaluate and correlate the magnitude and orientation of the chemical shielding anisotropy tensor with variations in bond length, bond angles and local coordination and nearest neighbor interactions has been a number of recent applications in the investigation of molecular structure. Nuclear magnetic resonance was shown that it is possible to calculate chemical shifts of individual amino acid residues of proteins without a detailed knowledge of the complete protein structure. The calculations also provide valuable information for exploring the experimental NMR chemical shifts with the molecular geometry and environment. Also NMR chemical shifts are quite sensitive to intermolecular interactions.

NMR is based on the quantum mechanical property of nuclei. The chemical shielding refers to the phenomenon which associated with the secondary magnetic field created by the induced motions of the electrons that surrounding the nuclei when in the presence of an applied magnetic field for chemical shielding (CS) tensors, which describes how the size of shielding varies with molecular orientation, we often use the following convention for the three principle component:

$$\sigma_{11} \leq \sigma_{22} \leq \sigma_{33} \quad (2)$$

The three values of the shielding tensor are frequently expressed as the isotropic value (σ_{iso}), the anisotropy shielding ($\Delta\sigma$) and the asymmetry parameter (η). In our current study, extensive quantum mechanical calculation of electronic structure of the active site of oxidized azurin and solvent effects on C^{13}, N^{15}, O^{17}, ^{64}Cu, ^{32}S-NMR parameters have been performed in different solvent media and in two available methods using GAUSSIAN 98 program.

A common feature among the blue copper proteins is the geometry around the Cu center. It has an approximately tetragonal symmetry, which is indeed distorted trigonal. The metal atom is coordinated by three strong atom ligands lying on a distorted trigonal plane, which are the S atom of a deprotonated cysteine and the N atoms of two histidines and by a weak axial atom ligand, namely the S atom of a methionine. In azurin from *P. aeruginosa*, these ligands belong, respectively, to Cys112, His46, His117, and Met121, and there is an additional weak axial ligand, that is, the O atoms of Gly45. After fully optimization of active site, we have calculated NMR parameters using the density functional B3LYP and HF method by Gauge Including Atomic Orbitals (GIAO) and continuous set of gauge transformations (CSGT).

RESULTS AND DISCUSSION

IR results

Quantum-chemical solvent effect theories give a self consistent description of the electronic structure of solutes,

which is closely related to the polarizable environment. Such calculations are indispensable for getting insight into the molecular properties and reactivity of condensed phases. This goal is usually achieved by means of a solute-solvent Schrödinger equation corresponding to some simplified representation of the solvent. In particular, the electronic structure of solute molecules can be closely related to the solvent structure and vice versa. This effect can be of key importance, for example, for the understanding of the microscopic mechanism of certain reactions in solutions. We should note that there is interaction energy between solutes and solvents. Because of this, solute properties that depend on energy and several other factors, such as geometry, vibrational frequencies, total energy and electronic spectrum, also depend on the solvent.

The presence of a solvent, particularly a polar solvent, can also stabilize charge separation within a molecule. This not only changes energy, but also causes electron density shifts and influences associated properties. In reality, this is a result of quantum mechanical interactions between the solute and solvent, which must be averaged over all possible arrangements of solvent molecules according to the statistical mechanics principles. The energy of solvation can further be divided into terms that describe the bulk solvent and terms that specify the first solvation shell. The bulk solvent contribution is primarily the result of dielectric shielding of electrostatic charge interactions. In the simplest form, it can be included in electrostatic interactions by means of the dielectric constant ε, as in the Coulomb interaction equation (Young, 2001).

$$\varepsilon = q_i q_j / \varepsilon r_{ij} \quad (3)$$

There are several effects in the region where a molecule meets its solvent shell. The first one is referred to as cavitations energy, which is the energy required to push aside solvent molecules to produce a cavity for the solute molecule. The second effect is related to forces attracting the solute molecule to the solvent. These are van der Waals, dispersion and hydrogen bonding interactions. Finally, the solvent molecules in the first shell can rearrange in order to maximize interactions with the solute. The largest amount of hydrogen bonding energy is usually related to solvent rearrangement to the preferred hydrogen bonding orientation. By solving the corresponding electrostatic equations inside and outside the sphere and applying proper boundary conditions, one can find the potential at any point inside the cavity and the total electrostatic energy of the interaction of molecular charge distribution with polarizable medium. In the quantum-chemical application of this theory, this interaction energy is represented by additional molecular Hamiltonian terms corresponding to nuclear-nuclear, nuclear-electronic, and electron-electron contributions. Because of the unavoidable deficiencies of the Onsager model, further insight into the nuclear polarization effects, that is, geometric optimization in solution would not yield better results. Therefore, the use of multipole expansions up to infinite order and a more realistic description of the solute cavity are necessary.

Solvent effects on relative stabilities

Active site of azurin was studied in the gas phase (ε = 1) and various solvent media with dielectric constants of water (ε = 80), dimethylsulfoxide (ε = 46.7), nitromethane (ε = 38.2), methanol (ε = 32.63), ethanol (ε = 24.55), acetone (ε = 20.7), dichloroethane (ε = 10.36) and dichloromethane (ε = 8.93) at 25, 27, 35 and 37°C. First, the active site was fully optimized by the HF and DFT (B3LYP) methods using the 6 - 31G basis set to obtain minima of the potential energy surfaces of the active site. The influence of the solvent on the relative stability of active site was studied by means of the Onsager approach. The values listed in Table 1 show that interactions between water molecules and active site reduce the energy of the whole system (ΔE). The only exception is nonbonding dispersion energy; this may imply that, in aqueous solutions, the import of polarized water molecules reduces the rate of active site polarization. The effect of solvents on the stabilization of the active site is of interest, it plays a major role in their activities.

The standard Onsager approach (the SCRF method) to active site with 6 - 31G basis set, as is used here, appears to be a good first step in theoretical investigations of solvent effects. In this paper, we studied the solvation of the active site of oxidized azurin. The influence of the dielectric constant on the standard geometry of active site in water, DMSO, nitromethane, methanol, ethanol, acetone, dichloroethane and dichloromethane was studied. We found that the relative energies (ΔE) of active site in solution are smaller than in the gas phase, because interactions in solution are stronger than in the gas phase. The interaction energies of the active site decrease as the dielectric constant of solvents increases according to HF and B3LYP calculations. The results obtained at the B3LYP/6-31G level are more negative than those of the HF/6 -31G calculations because these methods differently take correlation energy into account (Table 1). As regards the high dielectric constant of water molecules surrounding the hydrophilic part of amino acid chains, we optimized these parameters much better than for the other solvents. We found that there was no significant difference between thermochemical parameters at 25, 27, 32 and 37°C (Figure 2).

Solvent effects on thermochemical parameters

Dielectric constant values (ε) and thermochemical parameters have a great influence on the mechanism of

Table 1. Relative thermochemical parameters of active site of azurin obtained in water and other solvent using different methods.

Dielectric constant	Method	ΔE (Kcal/mol)		ΔH(Kcal/mol)		ΔG(Kcal/mol)		ΔS(Kcal/ molK)		SCF(Done) (Kcal/mol)
		25℃	27℃	25℃	27℃	25℃	27℃	25℃	27℃	
		32℃	37℃	32℃	37℃	32℃	37℃	32℃	37℃	
80	HF	0.0001	0.000088	0.000104	0.000088	0	0.000039	0.044915	0.045458	-183.67437
		0.000045	0	0.00004	0	0.000134	0.000229	0.04692	0.048377	
	B3LYP	0.513413	0.513396	0.513414	0.513396	0.513229	0.513263	0.042086	0.042664	-184.184521
		0.51335	0.513303	0.513349	0.513301	0.513356	0.51345	0.044223	0.045777	
46.7	HF	0.000161	0.00014	0.000162	0.000145	0.000044	0.000078	0.044305	0.044848	-183.67435
		0.000102	0	0.000101	0.000056	0.000172	0.000267	0.046311	0.047768	
		0.513409	0.513392	0.51341	0.513392	0.513213	0.513247	0.041663	0.042242	-184.18452
	B3LYP	0.513346	0.513295	0.513345	0.513293	0.51334	0.513429	0.043801	0.045355	
38.2	HF	0.00011	0.00009	0.00011	0.00009	0	0.00004	0.044769	0.045312	-183.67433
		0.00005	0	0.00005	0	0.00014	0.00023	0.046775	0.048231	
		0.51341	0.51339	0.51341	0.51339	0.51324	0.51327	0.042489	0.043067	-184.18452
	B3LYP	0.51334	0.5133	0.51334	0.51329	0.51336	0.51346	0.044627	0.046181	
32.63	HF	0.000114	0.0001	0.000117	0.0001	0.000011	0.000046	0.044737	0.04528	-183.67432
		0.000057	0.000013	0.000056	0	0.000101	0.000235	0.046743	0.0482	
		0.513409	0.513392	0.51341	0.513393	0.513178	0.513212	0.040485	0.041063	-184.18452
	B3LYP	0.513346	0.5133	0.513346	0.513298	0.513304	0.513397	0.042622	0.044174	
24.55	HF	0.00032	0.0003	0.00032	0.0003	0.00016	0.00019	0.043041	0.043585	-183.67429
		0.00025	0.00021	0.00025	0.00021	0.00028	0.00038	0.04505	0.046509	
		0.51341	0.51339	0.51341	0.51339	0.51324	0.51327	0.04251	0.043088	-184.18452
	B3LYP	0.51334	0.5133	0.51335	0.5133	0.51336	0.51346	0.044648	0.046202	
20.7	HF	0.000127	0.000111	0.000128	0.000112	0.000018	0.000053	0.044596	0.045139	-183.67428
		0.000068	0.000024	0.000067	0.000023	0.000147	0.000242	0.046602	0.048059	
		0.513412	0.513395	0.513413	0.513395	0.513253	0.513288	0.042928	0.043507	-184.184525
	B3LYP	0.513349	0.513302	0.513348	0.5133	0.513381	0.513475	0.045066	0.04662	
10.36	HF	0.00015	0.00013	0.00015	0.00013	0	0.00007	0.044345	0.044888	-183.67416
		0.00009	0.00006	0.00009	0.00004	0.00016	0.00026	0.046351	0.047808	
		0.51341	0.51339	0.51341	0.51339	0.51323	0.51326	0.042217	0.042796	-184.18456
	B3LYP	0.51335	0.5133	0.51334	0.5133	0.51336	0.51345	0.044355	0.045909	
8.93	HF	0.00016	0.00014	0.00016	0.00014	0.00004	0.00007	0.044305	0.044848	-183.67413
		0.0001	0	0.0001	0	0.00017	0.00026	0.046311	0.047768	
		0.51346	0.51344	0.51346	0.51344	0.51309	0.51312	0.035934	0.036501	-184.18458
	B3LYP	0.5134	0.51335	0.51339	0.51335	0.51321	0.5133	0.038027	0.039548	
1	HF	0.00034	0.00032	0.00034	0.00033	0.00018	0.00021	0.042699	0.043243	-183.67313
		0.00028	0.00024	0.00028	0.00024	0.0003	0.0004	0.046167	0.046167	
		0.5134	0.51266	0.51341	0.51266	0.51321	0.51125	0.041728	0	-184.18452
	B3LYP	0.51262	0.51259	0.51262	0.51259	0.51132	0.51139	0.001189	0.002378	

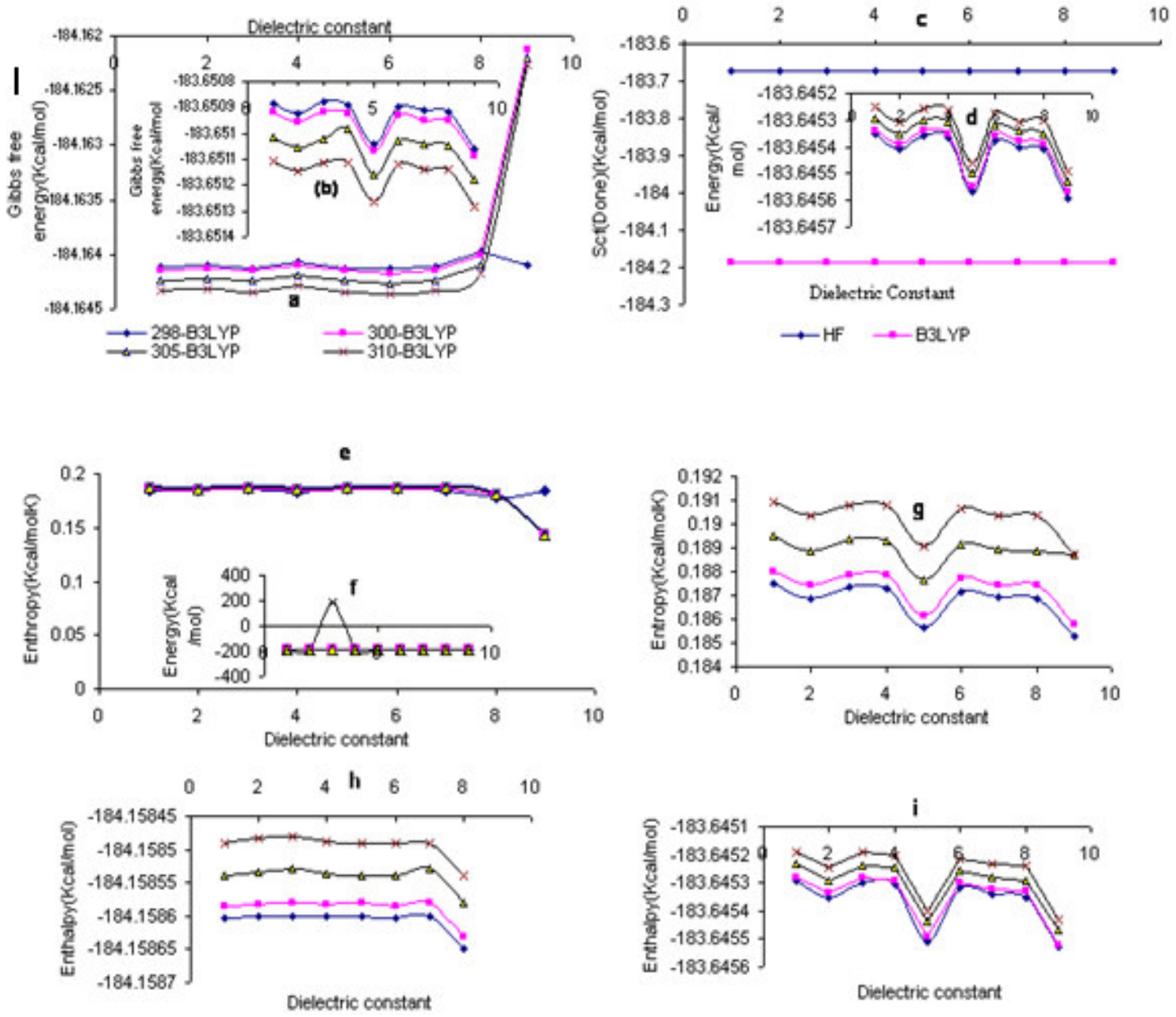

Figure 2. Comparison of energy(ΔE),enthalpy (ΔH),Gibbs free energy(ΔG) (Kcal/mol),and entropy (ΔS) (Kcal/ molK),of active site of oxidized azurin versus dielectric constants obtained using the HF and DFT methods at four temperatures (25,27,32,37˚C).(a) B3LYP- ΔG, (b) HF-ΔG, (c) SCF(Done)=E, (d)HF- ΔE ,(e) B3LYP- ΔS ,(f) B3LYP- ΔE ,(g)HF- ΔS, (h) B3LYP- ΔH, (i)HF- ΔH.

electron transfer in complexes and their studies could provide a deeper advance in quantitative treatment of charge transfer reactions in proteins (Chamorovsky et al., 2007). The standard enthalpies (Δ*H*), entropies (Δ*S*) and free energies (Δ*G*) of active site was obtained by theoretical methods using the GAUSSIAN 98 package (Table 1). We found that there was some difference between these functions obtained by the HF and B3LYP methods. A study of hydrogen bonding between the active site of oxidized azurin and water, DMSO, nitromethane, methanol, ethanol, acetone, dichloroethane and dichloromethane was performed for optimized structure in solution at 25, 27, 32 and 37℃ (Table 1).The best results in various solvent media were obtained at the B3LYP level of theory. Free energy variations in terms of dielectric constant at two levels of theory and four temperatures obtained using 6 - 31G basis set are plotted in Figure 2 in terms of the dielectric

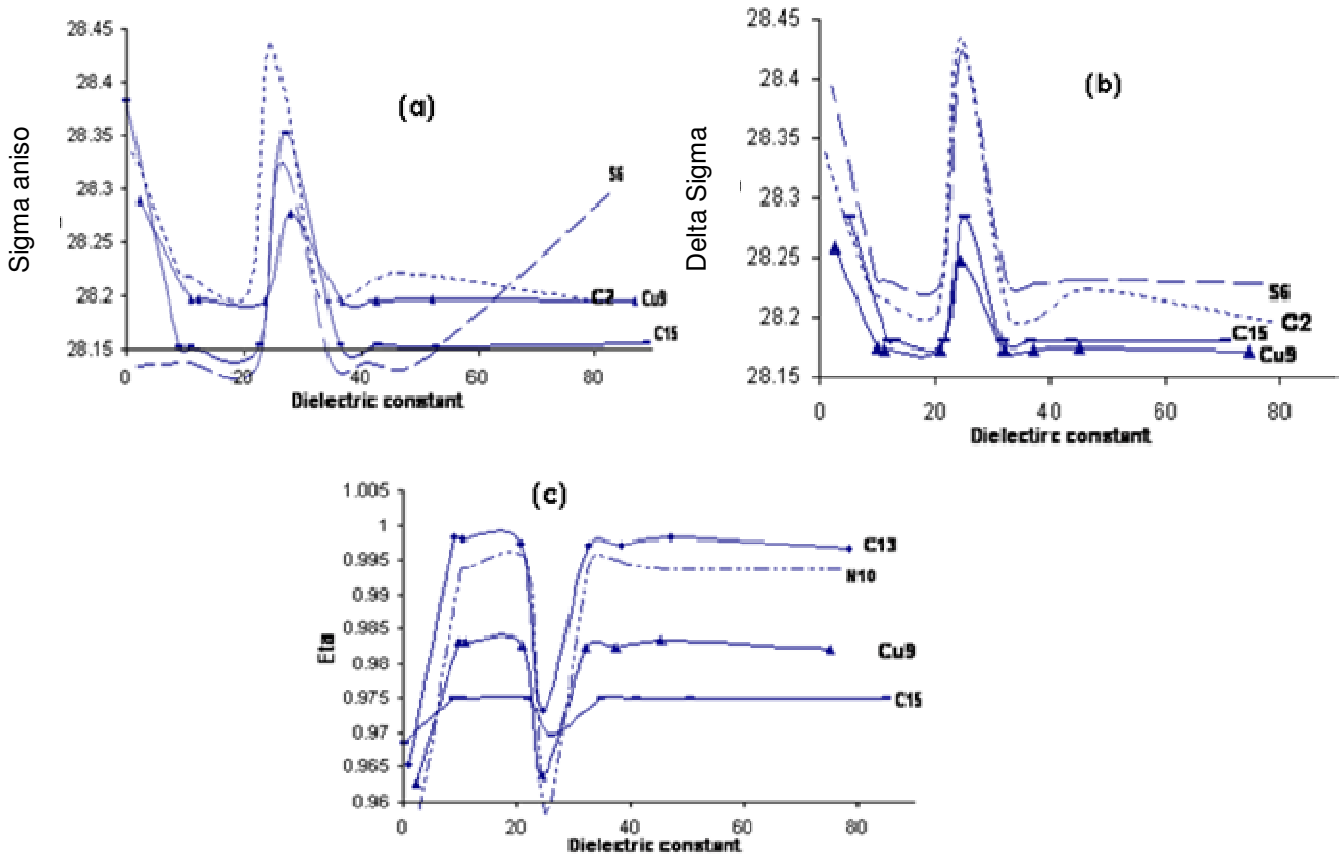

Figure 3. The graphs of (a) anisotropic shielding values(σaniso), (b) indirect shielding(Δσ), (c) asymmetry parameters (η) of propose atoms of active site azurin in different solvent media at the level of HF/6-31G theory in CSGT method.

constant. We see that, as the dielectric constant changes from dichloromethane (ε = 8.93) to polar water (ε = 80) at 25, 27, 32 and 37°C, the stabilization energy decreases at B3LYP levels (Figure 2). Table 1 and Figure 2 show that the free energies of interactions (Δ*G*) of active site in solution are more negative than in the gas phase, that is, interactions in solution are stronger than in the gas phase.

Solvent effects on nuclear magnetic resonance parameters

In this section we report and analyze the solvent effects on our NMR shielding tensors of ^{13}C, ^{15}N, ^{64}Cu, ^{32}S-NMR shielding of active site of oxidized azurin obtain at the HF and B3LYP levels. In our current research, we have presented the results of our extensive studies of solvent induced effects on the of ^{13}C, ^{15}N,^{64}Cu, ^{32}S-NMR shielding of active site of oxidized azurin site in a wide range of solvents encompassing a broad spectrum of polarity and hydrogen-bonding properties. According to our theoretical data, it is apparent that the solvent effects seems quite significant in either their diverse biological or physicochemical behavior. At first glance, the shielding variation seems to follow the polarity of solvent in the sense of enhanced deshielding with the increasing polarity, and this point is discussed in detail in conjunction with equations (Monajjemi et al., 2008). The ^{13}C, ^{15}N, ^{64}Cu, ^{32}S-NMR parameters of active site of oxidized azurin in various solvent media are given in Table 2. Also, several graph of NMR parameters of mentioned nuclei versus dielectric constant have been displayed in Figures 3 - 5. As expected, the NMR shielding tensors of ^{13}C, ^{15}N, ^{64}Cu, ^{32}S nuclei are drastically affected by what it is bonded to and the type of bond to its neighbor. Our obtained results yielded strong evidence that intermolecular effects such as electron transfer interactions play very important role in determining the ^{13}C, ^{15}N, ^{64}Cu, ^{32}S-NMR chemical shielding tensors of active site of oxidized azurin and some systematic trends appeared from the analysis of the calculated values.

Electron transfer between azurin and its proposed natural partners cytochrome C551 and nitrite reductase, as well as to some other redox proteins, was experimentally demonstrated. The need for specific but not too specific binding due to the fact that ET occurs

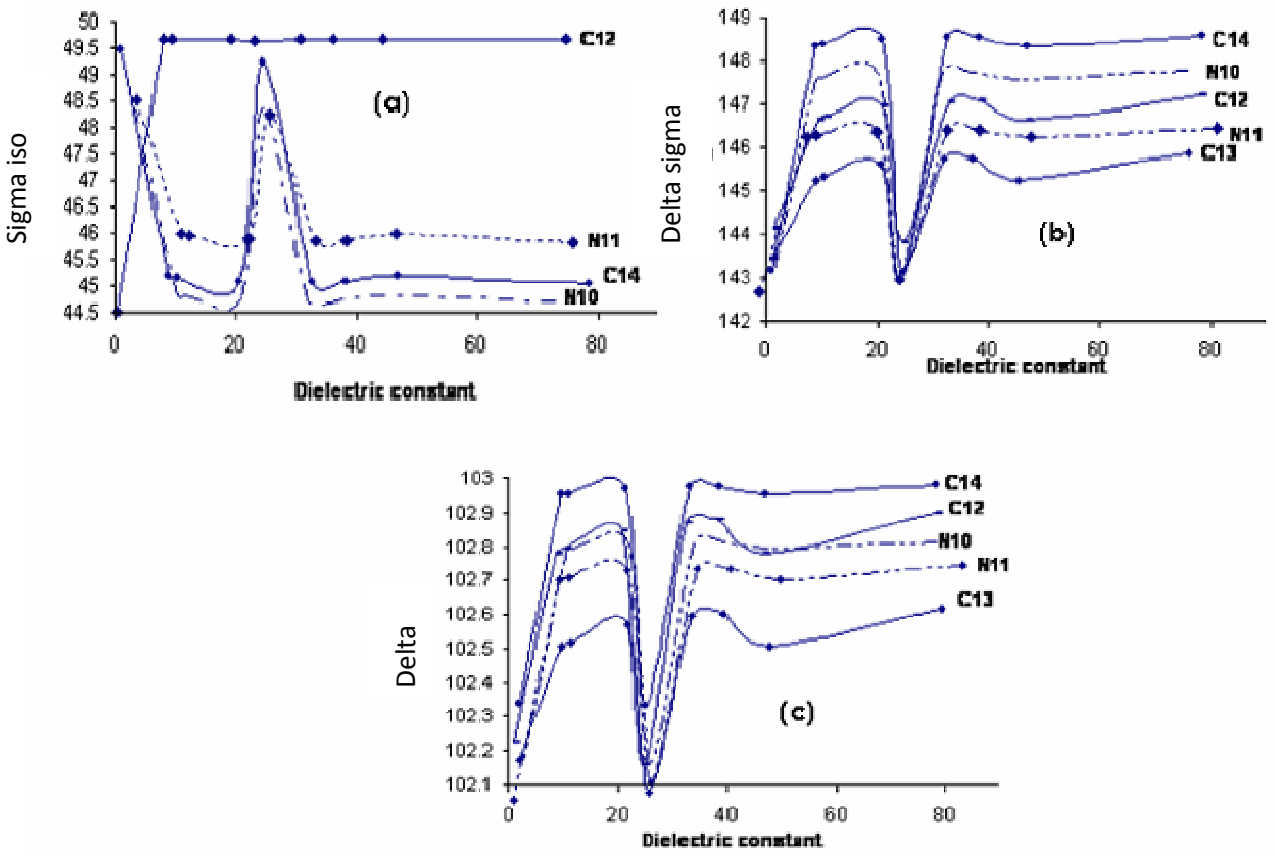

Figure 4. The graphs of (a) isotropic shielding values(σ iso), (b) indirect shielding (Δσ), (c)chemical shift tensor(δ) of propose atoms of active site azurin in different solvent media at the level of HF/6-31G theory CSGT method.

between azurin and various partners has also been addressed (Zhuravleva et al., 2004). On the basis of both geometrical positions of ^{13}C, ^{15}N, ^{64}Cu, ^{32}S existing in active site of oxidized azurin and computed results, for nuclei involved in electron transfer the obtained NMR parameters are not the same as those computed for other atoms. For Cu_9 atom which behaves as electron donor, the σiso component showed the largest intermolecular effects and it shows positive shielding values that are, the electron transfer interaction produced a deshielding in this position.

Comparison of σiso, σaniso, Δσ and δ values of Cu_9 atom with another shielding values in Table 2 and also analysis of graphs of σiso, σaniso, Δσ and δ 's versus dielectric constant exhibited in Figures 3 - 5 revealed that, the largest values observed for ethanol and water, whereas the smallest belongs to DMSO. It is interesting to note that on the contrary, the opposite trend has been observed for asymmetry parameter (η). This logical behavior may be readily understood in accord with biological conceptions.

The metal ion in the electron-transfer copper proteins such as the type I copper proteins has been proposed to exist in an entatic state. These studies as well as earlier reports suggest that the metal-ligand interactions in the blue copper proteins indeed play an important role in imparting extra stability to the metal binding site of the protein (Sujak et al., 2007). The holoprotein was more stable than the apo-protein, indicating that the metal ion plays an important role in stabilization of the protein. As expected, after Cu_9, S_6 shows positive shielding values. Cys 112 is among the ligand residues the one that more strongly hybridizes with the Cu orbitals. The covalency of the copper- ligand bonds is very anisotropic and it was suggested that this should favor hole super exchange pathways that couple to the Cu through the Cys112 ligand (Schleyer, 1998). The cysteine ligand decreases the reorganization energy. This decrease is caused by the transfer of charge from the negative charged thiolate group to CuII, which makes the oxidized and reduced structures quite similar (Ryde et al., 2001). As it can be seen in Table 2, an important piece of information can be derived from the analysis of the satisfactory correlation between the NMR parameters and dielectric constants of different solvents. So reported results in the presence of certain solvent molecules indicate that there is a significant cooperative effect that can strongly affect these parameters. For both N_{10} and N_{11} atoms which are

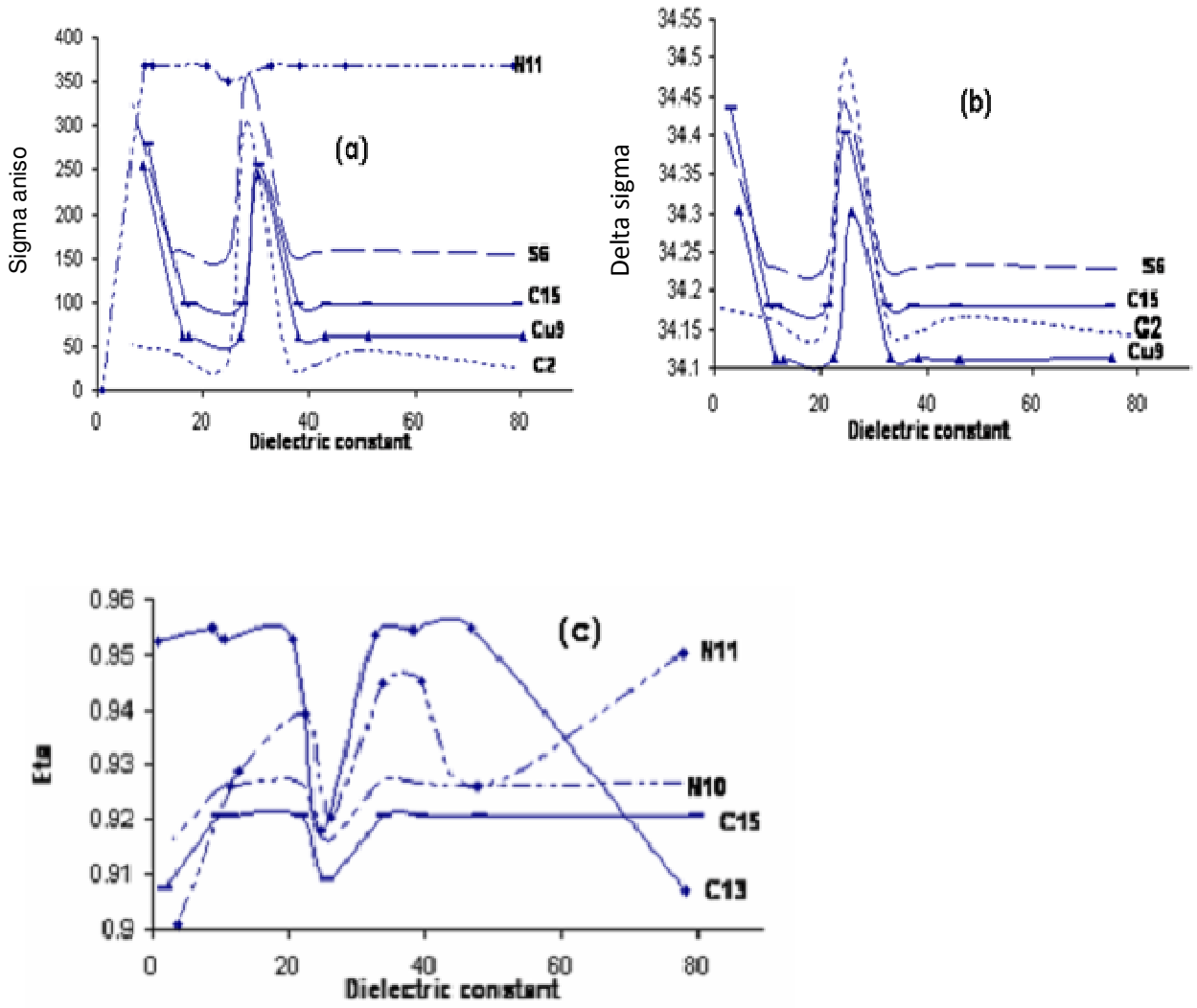

Figure 5. The graphs of (a) anisotropic shielding values(σaniso), (b)indirect shielding (Δσ),(c)asymmetry parameters (η), of propose atoms of active site azurin in different solvent media at the level of HF/6-31G theory in GIAO method.

fused in imidazole ring, shielding tensors are close to each other but according to our obtained results of Figure 4, as the dielectric constant in passing from the nitromethane to water, the σ_{aniso} and $\Delta\sigma$ of N_{11} and N_{10} increase and the η decrease in the ethanol which is expected to result in a significant shielding of this nitrogen nuclei at the Hartree-Fock level of theory with CSGT method. Nitrogen ligands give up appreciably lower reorganization energy than water, owing to the lower Cu-N force constant (19). Figure 5 shows that, as the dielectric constant of the solvent increases, the $\Delta\sigma$ and σ_{aniso} of C_{15} and C_{13} increase and the η of C_{15} and C_{13} decreased in the ethanol at the Hartree-Fock level of theory with GIAO method. Also, calculations at the HF in CSGT method (Figures 3 and 4) and the HF in GIAO method (Figure 5) have shown that molecular geometry and shielding properties are better than the other methods, B3LYP in GIAO and CSGT methods.

Conclusion

This article presents an *ab initio* DFT study on a model active site of oxidized azurin. All the residues directly interacting with the copper ion are considered in the model, including Gly45, which is specific for azurin (Corni et al., 2005). Two fundamental processes in which the BC proteins are involved have been analyzed and studied extensively and from different perspectives: the association of cupredoxins with their partners and their electron transfer. Deriving clues for understanding the details of ET processes in biology is a far more complicated matter to be approached computationally (Ryde et al., 2001). *Ab initio* QM methods are used to focus on the local properties of the region around the Cu site such as reorganization energy or stability. QM method is being increasingly applied and preferred to traditional methods due to their versatility and ability to

describe the same system at different levels of detail. Thus, the solvent (typically water) can be explicitly introduced in the simulations or described using implicit parameterization. The environment not only affects the protein structure but also protein functionality. In computational studies, it is important to take in to account modifications of solvent characteristics such as pH and ionic strength.

On the basis of the results of our calculations, we found that, among various modern quantum mechanical methods, the HF method was the most popular to date. System was optimized by the HF and B3LYP methods. In all cases, the steady-state nature (minimum of the potential energy surface) of the optimized complex has been confirmed through the investigation of theoretical levels. This will allow the most important reactions and the most important species to be identified for the compounds in question.

As a matter of fact, the energies and thermochemical parameters can provide valuable formation about binding stabilities of active site in oxidized azurin with possible electron transfer. The protein environment, which is often aqueous, affects the structure and folding dynamics and, therefore, the functionality of globular proteins. In fact, solvent-protein interactions, together with the interactions between residues in the protein matrix, facilitate the folding process and establishment of intermolecular interactions with other complex system. Furthermore, to be properly folded and fully functional, a protein requires a minimum level of hydration. Of course, the contrary is also true, that is, as far as water affects protein structure, protein can modify the structural and dynamic behavior of Water (Luise et al., 2000). The results reported in this paper indicates that it is possible to measure NMR tensors of various nuclei involving in biological compounds either in gas phase or in the presence of different solvent molecules theoretically. Several conclusions can be made on the basis of the observed results of the present study. Such amount of theoretical data can provide us important insights into the nature of molecular structures in biological systems. Our main findings from the point of view of solvent effects can be summarized as follows:

1. Optimization at the HF level of theory provides a suitable computational model in terms of calculated NMR parameters and relative energies.
2. NMR parameters are very sensitive to small changes in molecular geometry and chemical environment exhibited significant sensitivity to the intramolecular interactions. So, our obtained theoretical results emphasized on the influence of the environment factors.

The largest σ_{iso} value of mentioned nuclei of active site azurin observed for ethanol and water, whereas the smallest one belongs to DMSO. It is interesting to note that the opposite trend have been observed for asymmetry parameters (η). This usual behavior may be readily understood in accord with biotechnological conceptions. The calculation of NMR parameters using *ab initio* techniques seems to be a major and a remarkable tool for investigation of how variations of biological systems and provides information on the local environment of selected species and their next nearest neighbors. However, combination of NMR study embedded in solvent medium reveals a logical interpretation of the observed results.

In conclusion, we have shown that theoretical calculations can be used to successfully solve biochemical problems. In similarly with experimental methods, they involve assumptions and interpretation, and they have their limitations, but there are many problems that are best studied by theory. Thus, theoretical methods have become a competitive alternative to experiments for biochemical investigations.

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Full Length Research Paper

Campylobacteriosis in sheep in farm settlements in the Vhembe district of South Africa

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A total of 300 freshly voided sheep faeces were collected and screened for the presence of *Campylobacter* spp. using standard microbiological techniques. The samples were obtained randomly from 3 farm settlements in the Venda Region, South Africa in 2008 and 2009. The recovery rate was 30.0% (90 of 300) for all faeces. Of these, 65 (72.2%) were from diarrheic and 25 (27.8%) were from non-diarrheic faeces. Out of the 90 *Campylobacter* spp. isolated, 41(45.6%) were *Campylobacter jejuni* and 49 (54.4%) were *C. coli*. Sixty-three (70%) of the isolates were β-haemolytic, while 17 (18.9%) were α-haemolytic and 10 (11.1%) were non-haemolytic on 5% sheep red blood cells. The antibiotic resistance patterns of the 90 *Campylobacter* isolates were examined by the disc diffusion method. All *Campylobacter* isolates from the farms were resistant to at least one of the 12 antibiotics tested. The prevalence rate of *C. coli* resistance to ciprofloxacin was 20.4% compared with *C. jejuni,* 17.1%. Similar rates were noted for tetracycline for the two species. *C. jejuni* showed a higher rate of resistance to erythromycin (22.0%) compared with *Campylobacter coli* (10.2%). Significantly higher frequency of kanamycin resistance was recorded for *C. jejuni* compared to *C. coli* ($p < 0.005$). However, for ciprofloxacin, tetracycline, erythromycin, imipenem, gentamycin and ampicillin comparable resistant profiles were recorded for *C. jejuni* and *C. coli* isolates from the farms. The high prevalence of *Campylobacter* spp. in sheep is of public significance in the Venda Region. The observed multi-drug resistance and especially resistance to macrolides and fluoroquinolones in this study pose a threat of transfer of antibiotic resistance to human pathogens because of the close contact between sheep and humans.

Key words: Antibiotics, *Campylobacter*, haemolytic, macrolides, pathogens, resistance.

INTRODUCTION

The incidence of campylobacteriosis has increased during the last decade, and it is the leading cause of bacterial enteritis in developing and developed countries (Allos, 2001; Coker, 2002; Wingstand et al., 2006; EFSA, 2007).

Several epidemiological studies in different countries have identified sources of *Campylobacter* enteritis in man to include animals, food, water, and milk products (Oporto et al., 2007; Esteban et al., 2008). Reports of *Campylobacter* enteritis in developing countries (Padungton et al., 2005; Uaboi-Egbenni et al., 2008), point to an urgent need to explore prevalence rates, antibiograms and haemolytic activities in animals because of the zoonotic nature of infections and for proper planning of effective prevention and control measures (Raji et al., 1997; Oporto et al., 2009).

Studies on campylobacteriosis in sheep are scanty (Koides, 1991; Stanley et al., 2003; Oporto et al., 2009) despite the array of clinical manifestations. In sheep, campylobacteriosis is characterized by abortion, still births, and birth of weak lambs during late pregnancy (Kimberly, 1988; Dennis, 1990; Raji et al., 2000). *Campylobacter jejuni* and *Campylobacter fetus* have been identified as the most common causative agents of this disease. The infection is highly contagious and may cause up to 79% of ewes to abort when the organisms

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are newly introduced into the flock (Kimberly, 1990; Raji et al., 2000). Few reports suggest that sheep shed *Campylobacter* organisms but at a lesser rate than other animals (Stanley and Jones, 2003). Susceptible ewes may acquire infection through ingestion of contaminated *Campylobacter* organisms with fecal material or uterine discharge. Other sources of infection may include faeces of carrier sheep and other mammals (Raji et al., 2000).

Antimicrobial treatment is indicated for systemic *Campylobacter* infections in immune-suppressed patients and for severe or long-lasting infections (Allos, 2001). Erythromycin is considered the drug of choice for treating *Campylobacter* gastroenteritis, and ciprofloxacin and tetracycline are used as alternative drugs (Nachamkin et al., 2000) but several species are resistant to these antibiotics (Unicomb et al., 2008; Pezzotti et al., 2003; Oporto et al., 2009).

Although, the majority of *Campylobacter* infections are self-limiting, complicated cases may warrant antimicrobial therapy. Antimicrobial susceptibility data show an increase in the number of fluoroquinolone-resistant and, to a lesser extent, macrolide-resistant *Campylobacter* strains causing human infections (Pezzotti et al., 2003; Gibreel and Taylor, 2006; Anderson et al., 2006). Antibiotic resistance in human medicine is mainly linked to human misuse of antimicrobial agents, but there is accumulating evidence that antimicrobial resistance originating from the use of antimicrobials in food animals might complicate therapy of human infections (Oporto et al., 2009). Antimicrobials used therapeutically or prophylactically in animals husbandry can also exert selective pressure on bacteria that infect food animals and reach humans via food products (van den Bogaard et al., 2000; Oporto et al., 2007; Krutkiewicz et al., 2009). The extensive development of resistance to tetracycline and ciprofloxacin in various countries has led to a decrease in their clinical use (Trieber and Taylor, 2000). In addition, the increasing emergence of erythromycin resistance among isolates of *C. jejuni* and *C. coli* has prompted a search among newer macrolide derivatives for those useful against *Campylobacter* isolates. Local and international committees have highlighted the need for better control of antibiotics usage in human medicine and veterinary husbandry (EFSA, 2007).

In this sense, systematic monitoring of the occurrence of antimicrobial resistance in *C. jejuni* and *C. coli* originating from animals can serve as an indicator of the selective pressure these bacteria are undergoing and could assist in monitoring development of resistance. Isolates of *C. jejuni* and *C. coli* with resistance to various antimicrobial agents have been reported in both developed and developing countries (Hart and Kariuki, 1998; Valenza et al., 2010). Since the 1990s, a significant increase in the prevalence of resistance to macrolides among *Campylobacter* spp. has been reported, and this is recognized as an emerging public health problem (Engberg et al., 2001). It has been suggested by some investigators that resistance to macrolides is mainly found in isolates of animal origin, especially *C. coli* from pigs and also *C. jejuni* from chickens (Van Looveren et al., 2001).

A prevalence study recently carried out in the Basque Country (Northern Spain) identified 28.3% (34/120) of ovine and 18.0% (37/206) of bovine farms positive for *C. jejuni* (Oporto et al., 2007), and even higher values (38.2%, 13/34) in free-range poultry farms (Esteban et al., 2008). Bacterial antibiotic resistance genes are commonly carried on plasmids and could also be chromosomally borne as well as on extrusion factors (Anderson et al., 2006; Moore et al., 2005; Oporto et al., 2009; Chaban et al., 2010).

The application of PCR may provide a more accurate description of the prevalence of *Campylobacter* spp. associated with livestock. Marshall et al. (1999) and Samie et al. (2006) used the primer sequences designed to amplify a 1004 bp fragment within the coding region of the 16S rRNA gene in *Campylobacter* species. The design was based on an alignment of the full 16S rRNA sequences of *Campylobacter* species, which demonstrated common conserved regions that served as targets for the primers.

The common association between *Campylobacter* spp., animals and human diseases make them a potential source of antibiotic resistance genes, although information on the susceptibility of *Campylobacter* spp. from animals is scanty. The most important aspects of this study are the novel findings of campylobacteriosis among sheep in farm settlements in local communities, their multiple antibiotic resistant profiles and haemolytic activities and the untoward public health significance.

MATERIALS AND METHODS

Collection of faeces

Samples were collected from three farms (designated A, B and C for confidentiality). Briefly, one hundred (100) freshly voided faeces were collected at random from apparently healthy sheep from each of the farms with the aid of oven-sterilized spatula in sterile 50 ml plastic containers and transported to the laboratory within 2 h for initial processing. A total of 300 samples consisting of diarrheic and non-diarrheic faeces were collected. Once in the laboratory, the faeces were immediately processed. About 2 gm of each sample was transferred to 6 ml of sterile brain heart infusion broth and left to emulsify at room temperature for 10 to 20 min to release the bacteria. The suspension was used directly for detection of *Campylobacter*.

Isolation and identification of *Campylobacter* by conventional culture methods

Twenty microlitres of faecal suspension was spread on the surface of charcoal cefoperazone deoxycholate agar plates (CM 739 [Oxoid] with cefoperazone supplement SR 155E). The plates were incubated in a 2 L anaerobic jar under microaerophilic conditions employing the Campygen gas generating kit (Oxoid CM025) at 42°C for 48 h. Colonies suspected to be *Campylobacter* were further purified on blood agar plates (Blood Agar Base No.2 (Oxoid)

supplemented with 5% sterile laked horse blood). All isolates were characterized by their catalase, oxidase reactions, and susceptibility to nalidixic acid by standard procedures (Baker et al., 2008; Chaban et al., 2010). The resulting isolates were subsequently stored at -80°C in brain heart infusion broth with 15% glycerol until further investigation.

Confirmation of positive *Campylobacter* isolates

Identification of *Campylobacter* isolates was done using Dryspot *Campylobacter* test kit (Oxoid Basingstoke, Hampshire, England). The test is specific for pathogenic *Campylobacter* strains belonging to *C. jejuni, C. coli, C. upsaliensis* and *C. lari*. The Oxoid agglutination test was done according to the manufacturer's instruction. Agglutination under normal lighting conditions indicated that the test organism was *Campylobacter* and belonged to any of the four species mentioned earlier (Baker et al., 2008; Chaban et al., 2010).

Discrimination of *Campylobacter* species

The dryspot-positive campylobacters were further subjected to Mast diagnostic *Campylobacter* kits consisting of urease, indoxyl acetate and hippurate test (ampoules) and/or indoxyl acetate, urease and hippurate strips. Briefly, 24 h cultures of the *Campylobacter* were inoculated into the urease, indoxyl acetate and hippurate test solutions according to the manufacturer's instructions. These were then incubated for 4 h for colour development. For urease, development of pink colour was indicative of urease enzyme production (*C. lari*), development of pink colour in hippurate solution indicated production of hippuricase enzyme (*C. jejuni*). In the case of indoxyl acetate solution, change of colour from colourless to blue/purple was indicative of the presence of *C. jejuni/C. coli*. A reaction positive for Indoxyl acetate reaction but negative for hippurate test solution, confirmed C*. coli*. A positive for both reactions was indicative of *C. jejuni* (Baker et al., 2008; Chaban et al., 2010).

Alternatively, the indoxyl acetate strips and hippurate strips were impregnated with wet cultures and allowed to stay for 3 to 5 min. Development of blue/purple colour in the case of indoxyl acetate strips and development of pink colour in the case of hippurate strips within this period was indicative of positive reaction (*C. jejuni* and *C. coli*) and *C. jejuni* respectively.

Isolation of bacterial genomic DNA

Genomic DNA was obtained by the whole-cell lysate method as described by Marshall *et al*. (1999). Briefly, cells from a 24 to 48 h culture grown on Columbia blood agar were re-suspended in sterile distilled water to an equivalent of 2.5 McFarland value. The suspensions were boiled to 100°C for 20 min in an Eppendorff tube. The resulting templates were either used immediately for PCR or were kept at 4°C for up to 1 month.

PCR Confirmation of *Campylobacter*

In order to ascertain if the dryspot/Mast diagnostic kit-positive *Campylobacter* isolates were actually campylobacters, they were subjected to PCR identification using the general primers for the identification of campylobacteria. These primers are also specific for other members of the Campylobacteriaceae (*Helicobacter* and *Arcobacter*).

However, *Arcobacter* and *Helicobacter* spp. show negative reaction to the *Campylobacter* dryspot kit. Hence, any amplification of the primer sequences at the 1,004-bp fragment within the coding region of 16S rRNA confirms that such isolates are *Campylobacter* spp. and not *Helicobacter* or *Arcobacter* spp. The PCR-RFLP method used in this study was as previously described by Marshall et al. (1999). Briefly, amplification was done in 50 µl reaction volume containing 5 µl of whole-cell lysate, 1µl each primer, 10x buffer (invitrogen), 1.5 mM $MgCl_2$, 200 µM each deoxynucleotide (invitrogen) and 5U Taq DNA polymerase (invitrogen). The PCR amplification was performed with a - thermocycler (ESCO Swift Mini Thermal Cycler Version 1.0, ESCO Technologies, Philadelphia U.S.A). The samples were subjected to an initial denaturation for 2 min at 95°C, followed by 30 amplification cycles, each consisting of 94°C for 30 s, 52°C for 30 s, and 72°C for 90s. A final primer extension at 72°C for 10min. was included. Oligonucleotide primers employed in this study are CAH16S 1a (5' – AAT ACA TCA AAG TCG AAC GA – 3') and CAH16S 1b (TTA ACC CAA CAT CTG ACG AC – 3'), respectively. The oligonucleotides used in this study were synthesized by Inqaba Biotechnologies (Pretoria, South Africa).

Blood haemolysis

To ascertain the pathogenic status of the isolates, the *Campylobacter* spp were subjected to haemolytic test according to the procedure of Samie et al. (2006). Briefly, a 24 h broth culture of *Campylobacter* spp. were placed onto Columbia agar base supplemented with sheep blood. Plates were incubated at 35°C for 24 h. Thereafter, plates were observed for complete, partial and no haemolysis.

Antimicrobial agents

The antibiotics tested in this study were: Trimethoprim (2.5 µg), Nalidixic acid (30 µg), ciprofloxacin (5 µg), gentamycin (10 µg), tetracycline (30 µg), ampicillin (10 µg), erythromycin (15 µg), streptomycin (10 µg), methicillin (µg), cefexime (30 µg), imipenem (µg), kanamycin (30 µg) and vancomycin (30 µg) (Oxoid, Unipath Ltd, Basingstoke, England).

Antimicrobial susceptibility testing

The method of Gaudreau and Gilbert (1997) was used. Briefly, the confirmed *Campylobacter* isolates were inoculated onto Mueller-Hinton agar plates carrying a maximum of six (6) discs. All plates were incubated at 35°C under a microaerophilic atmosphere obtained with a Campy gas generator envelope (Oxoid), for 24 h. The resulting zone diameters were measured with a graduated metre rule. Analysis of diameter was done according to the procedures of NCCLS (2002) now known as ICLS for enterobacteriaceae.

Statistical analysis

χ^2 test was used to evaluate the results, using the SPSS version 17.1. The results obtained were used to compare the antimicrobial resistance amongst *C. jejuni* and *C. coli* isolates as well as comparison of the prevalence of resistance to the various antibiotics used in human campylobacteriosis.

RESULTS

Three hundred freshly voided sheep faeces were

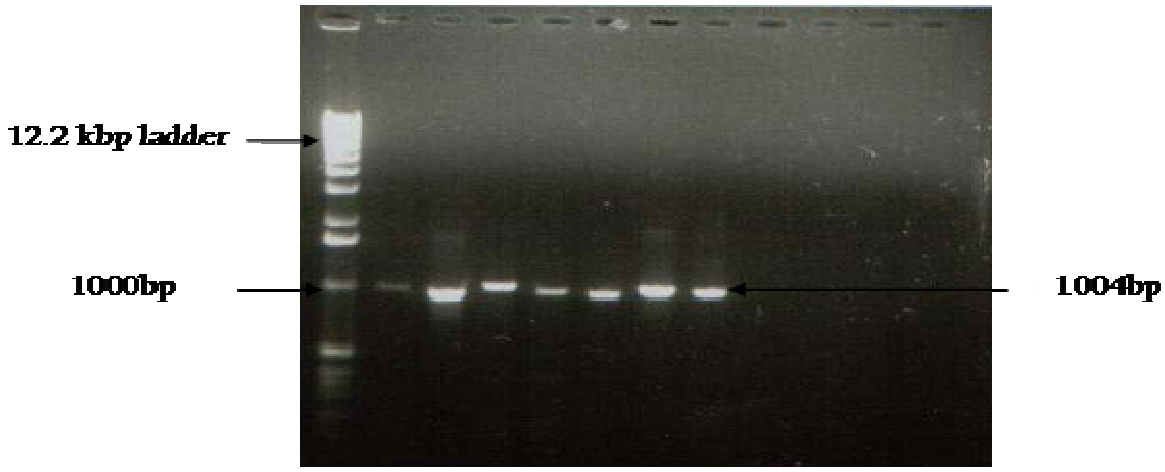

Figure 1. PCR products of amplified DNA from sheep *Campylobacter* isolates aligning at the 1004bp of a 12.2kbp ladder Lane 1= 1.9kb ladder; lane 2,3,4,5,6,7, 8 amplified bands of DNA from sheep *Campylobacter* strains.

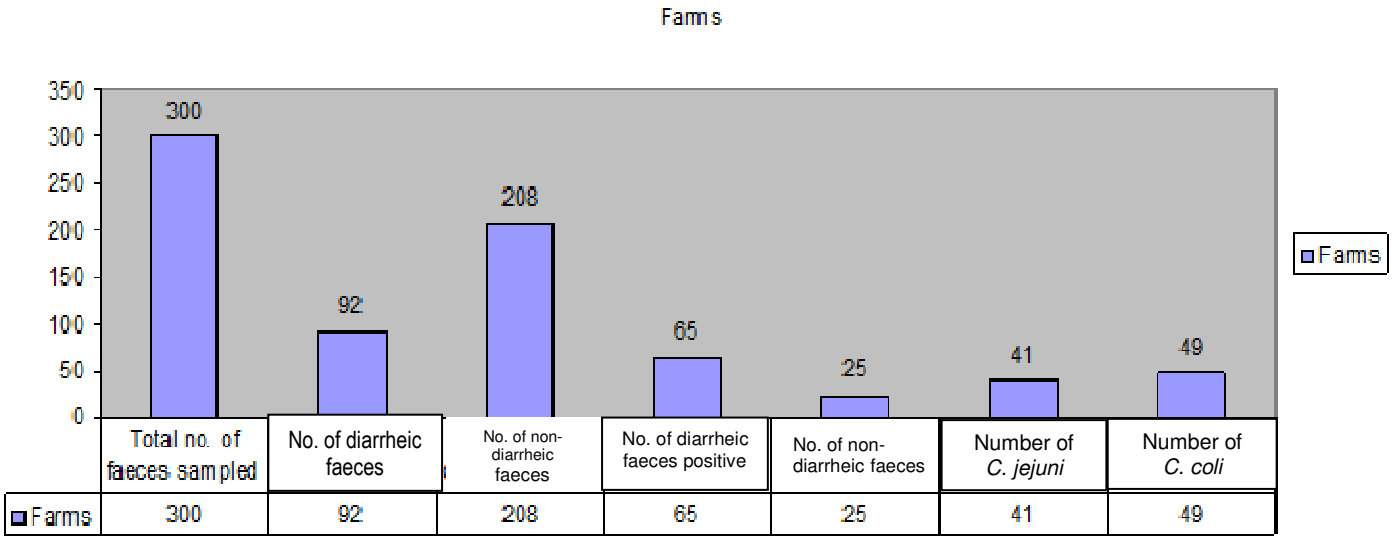

Figure 2. Distribution of *C. jejuni* and *C. coli* in diarreic and non-diarrheic faeces samples.

analyzed in the study. The recovery rate was 30.0% (90 of 300) for all cases. Ninety faecal samples were positive for *Campylobacter*. Of these, 65 (72.2%) were from diarrheic and 25(27.8%) were from non-diarrheic samples. Of the ninety (90) *Campylobacter* isolates obtained from the faeces, 41(45.6%) were *C. jejuni* while 49(54.4%) were *C. coli* (Figure 1). Of the 65 diarrheic positive faeces, 38 were *C. jejuni* and 27 *C. coli* and of the 25 non-diarrheic positive faeces, 3 were *C. jejuni* and 22 were *C. coli*. There was a higher prevalence rate of *C. jejuni* in diarrheic faeces than *C. coli* and vice versa. The high rate of incidence of *C. jejuni* in diarrheic faeces is an indication that the campylobacteriosis witnessed among sheep in these farms has its origin from *C. jejuni* infection.

Haemolysis of sheep red cells

Of the 90 *Campylobacter* strains isolated from the farms 63 (70%) were β-haemolytic, while 17(18.9%) were α-haemolytic and 10(11.1%) were non-haemolytic (Figure 2).

PCR study of *Campylobacter* isolates

The PCR micrographs of the DNA from *Campylobacter* strains from sheep are as indicated in Figure 3. The purified DNA from the *Campylobacter* strains amplified at the 1004 bp, which is the specific region for the conserved 16S rRNA for members of the genus

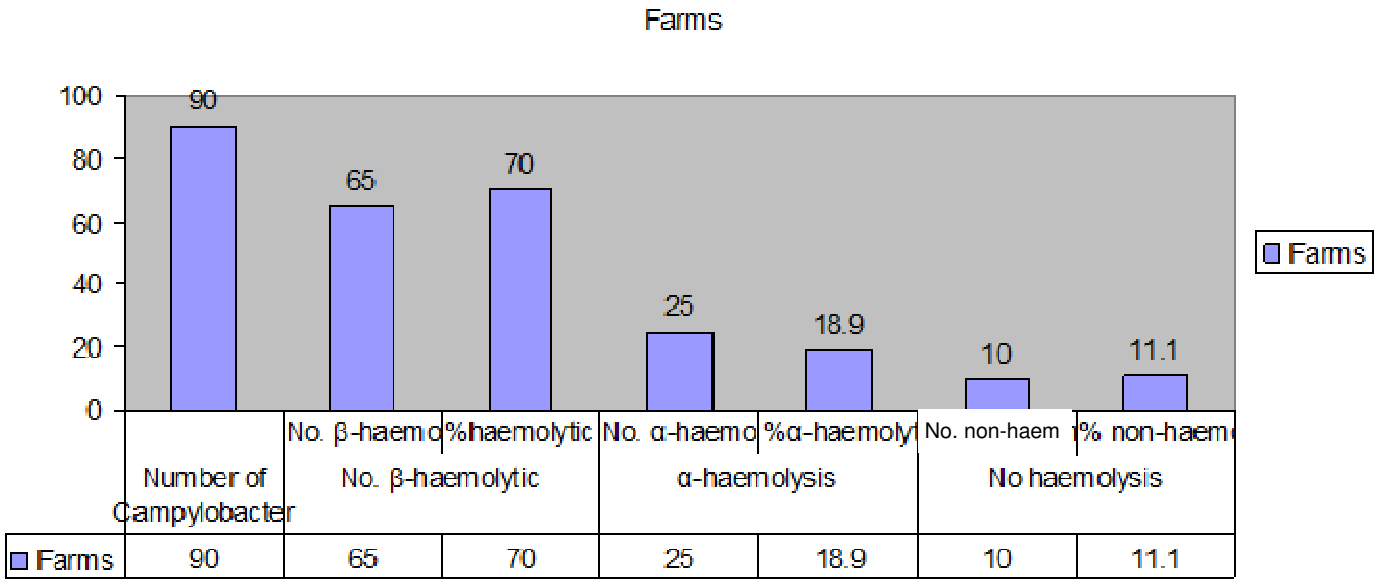

Figure 3. Haemolytic profile of campylobacters isolated from sheep faeces in South Africa

Campylobacter. The bands formed were confirmed as those for *Campylobacter* since the Oxoid *Campylobacter* dryspot agglutination test kit is specific for thermotolerant pathogenic members of the genus *Campylobacter* (*C. jejuni, C. coli and C. lari*). Furthermore, biochemical reactions involving catalase, cytochrome oxidase reaction, nalidixic acid sensitivity as well as motility test aided in the appropriate identification of the *Campylobacter* strains. However, since culture environments were done under 42°C, which is specific for the thermotolerant and pathogenic species, these *Campylobacter* strains could belong to any of *C. jejuni*, *C. coli* and *C. lari.* Specific identification by the Mast diagnostic kits differentiated the isolates into *C. jejuni* and *C. coli.*

Antimicrobial susceptibility

Ninety *Campylobacter* strains were exposed to 12 antibiotics. Of these, 73 were susceptible to ciprofloxacin with zone of inhibition diameters ≥28.3 mm, while 17 had zones ≤15.2 mm and were regarded as resistant. Seventy-two *Campylobacter* strains were susceptible to tetracycline with zone diameters ≥28.2 mm, while 10 resistant strains had zones of ≤17 mm and 8 had no zones around the disc. Seventy-eight erythromycin-susceptible strains had zone diameters ≥20 mm, while 8 resistant strains had zones of <20 mm and 5 had no zones around the disc. The 37 ampicillin-susceptible strains of *Campylobacter* spp. had zones of ≥27 mm, while the 40 ampicillin-resistant strains had zones of ≤15.2 mm, while 13 had no zones around the disc. The sixty-six gentamycin-susceptible and 24 gentamycin-resistant *Campylobacter* spp. had zone diameter of ≥28 and ≤16 mm (Table 1). *C. coli* had a higher rate of resistance to ciprofloxacin (20.4%) than *C. jejuni* (17.1%). The same trend was noticed for tetracycline with values of 20.4% *C. coli* and 19.5%, *C. jejuni* (Table 2). However, rate of resistance of *C. jejuni* to erythromycin (22.0%) was higher than *C. coli* (10.2%). The rest analyses were as shown in Figure 2.

A comparison of the occurrence of antimicrobial resistance among *C. jejuni* strains and *C. coli* in all farms is presented in Table 2. A significantly higher frequency of kanamycin resistance was recorded among *C. jejuni* and *C. coli* isolates from the farms ($p < 0.005$). However, for ciprofloxacin, tetracycline, erythromycin, imipenem, gentamycin and ampicillin comparable occurrences of resistance were recorded among *C. jejuni* and *C. coli* isolates from the farms.

DISCUSSION

Overall, in this study we found a high frequency of *Campylobacter* spp. in sheep (30%). The prevalence of species distribution of campylobacters in this study was *C. jejuni* 41 (45.6%), *C. coli* 49 (54.5%). The previously reported prevalence of campylobacters among sheep in Africa (Ethiopia) were C. *coli* (40.7%) and *C. jejuni* (59.3%) (Kassa et al., 2005). In their study they did not isolate *C. lari* from sheep. Our findings on the prevalence of campylobacters in sheep are in line with their report. There was a high prevalence of *Campylobacter* isolates in diarrheic (72.2%) than non-diarrheic faeces (27.8%) and the difference was of statistical significance ($p < 0.005$). Padungton and Kareene (2003) reported that

Table 1. Susceptibility profiles of 90 strains of *C. jejuni* and *C. coli* by disc diffusion using six (6) popularly employed antibiotics for the treatment of campylobacteriosis in humans.

Ciprofloxacin	Tetracycline	Erythromycin	Ampicillin	Gentamycin
73S S(≥ 28.3 mm)	72S(≥ 29mm)	78S(≥ 20mm)	37S(≥ 27mm)	66S (≥28)
17R (≤15.2 mm)	18R (≤ 17mm)	12R (<20mm)	53 (≤15.2mm)	24R (≤16.2)
Nalidixic acid				
69S (≥28 mm)				
21R (≤16.2 mm)				

S = Susceptible; R = Resistance.

Table 2. Antibiotic sensitivity patterns of *C. jejuni* and *C. coli* isolated from sheep in 3 farms in Venda Region, South.

Antibiotic	*C. jejuni*			*C. coli*			Combined resistance	
	No. of isolates	No. of resistant	% resistance	No. Exposed	No. Resistant	% resistance	Combined % resistance	P value
Ciprofloxacin	41	7	17.1	49	10	20.4	18.8	NS
Tetracycline	41	8	19.5	49	10	20.4	20.0	NS
Erythromycin	41	7	22.0	49	5	10.2	16.7	NS
Gentamycin	41	13	31.7	49	11	22.5	26.7	NS
Ampicillin	41	25	70.0	49	28	57.1	58.9	NS
Kanamycin	41	36	87.8	49	28	57.1	71.1	0.005
Imipenem	41	10	24.4	49	10	20.4	22.2	NS
Cefexime	41	36	87.8	49	41	83.7	85.6	NS
Vancomycin	41	33	80.5	49	37	75.5	77.8	NS
Methicillin	41	41	100	49	49	100	100	
Trimethoprim	41	41	100	49	49	100	100	
Nalidixic acid	41	11	26.8	49	10	20.4	23.3	NS

NS = Not statistically significant.

both *C. jejuni* and *C. coli* could cause a mild self-limiting enteritis and bacteraemia when inoculated orally into newborn calves. The frequency of *Campylobacter* spp. isolation (30%) in sheep in this study was higher than those reported in studies conducted in Portugal (15%) (Cabrita et al., 1992), in Norway (8.1%) (Rosef et al., 1983) and Brazil (20%) (Aquino et al., 2002).

The main species of *Campylobacter* isolated from sheep faeces in this study were *C. coli* (54.5%) and *C. jejuni* (45.5%). This pattern of shedding is similar to the report from Preston, Lancashire in the United Kingdom by Padungton and Kaneene (2003). However, in terms of species distribution we observed lower prevalence of *C. jejuni* and higher prevalence of *C. coli* compared with the findings of Kassa et al. (2005). The contamination of sheep carcasses during slaughter processes could also represent a potential source of human infection. Reports have shown that the shedding of campylobacters in faeces by sheep varies considerably with the time of the year (Stanley et al., 1998). The shedding of campylobacters by sheep has the potential to contaminate pastures and surface waters. In this wise, contamination of surface and sub-surface waters may transmit *Campylobacter* spp. within herds and between farms and other livestock groups (Jones et al., 1999). This may have been responsible for the high campylobacteriosis observed in the farms investigated. Our results are in line with the report of Stanley and Jones (2003) who observed campylobacteriosis and heavy shedding of *C. jejuni* and *C. coli* in cattle and sheep farms.

Few studies have reported campylobacteriosis resulting in abortion among sheep but the actual rates of prevalence were not ascertained (Dennis, 1990; Koides, 1991; Kimberly, 1988; Raji et al., 2000). The high occurrences of campylobacters in diarrheic faeces obtained from the farms were indicative of campylobacteriosis, which could result in economic loss of infected sheep in the flock. Kimberly (1988) reported Jenson and Swiff's diseases of sheep in Philadelphia, LA, USA, resulting from campylobacteriosis (enteritis and/stillbirth as well as abortion) caused by *C. jejuni* and its pathogenic allies.

Of the 90 *Campylobacter* isolates, 65 (72.2%) were

β-haemolytic, while 25 (18.9%) were α-haemolytic. These findings are consistent with the report of Samie et al. (2006) that most thermophilic *Campylobacter* isolates were β-haemolytic. The occurrence of non-haemolytic pathogenic thermophilic *Campylobacter* spp. as observed in this study may have presented a new idea that these *Campylobacter* pathogens could also lose their pathogenic attributes.

Resistance to fluoroquinolones has increased over the past years (Saenz et al., 1997; Prats et al., 2000; Reina et al., 1994). Results from recent susceptibility studies of *C. jejuni* and *C. coli* from poultry meat performed in different countries indicated substantial variation between countries. Relatively high resistance rates in *C. jejuni* strains isolated from chicken meat were reported from Belgium (Van Looveren *et al*., 2001), the United States (Ge et al., 2003), and Italy (Pezzotti et al., 2003), moderate rates were reported from Switzerland (Ledergerber et al., 2003) and Northern Ireland (Wilson, 2003), whereas limited occurrence of antimicrobial resistance among *C. jejuni* was reported from Sweden (Lindmark et al., 2004). A possible explanation for these differences might be that occurrences of antimicrobial resistance are reflecting the different national and regional policies in relation to the use of antimicrobial agents for food animals.

In the present study, there was a significant difference in ciprofloxacin and tetracycline resistance among isolates from sheep. This observation fits the finding of Oporto et al. (2009) who observed a high rate of resistance among *Campylobacter* isolates to these antibiotics. Our data (Table 2) also indicated that 19.5 and 20.5% of *C. jejuni* and *C. coli* isolates respectively from sheep faeces were resistant to tetracycline. Gibreel and Taylor (2006) in their review reported high rate of resistance to macrolides of clinical isolates of *C. jejuni* and *C. coli*. Most of the *Campylobacter* isolates were resistant to ampicillin (58.9%). This resistant rate of ampicillin is higher than that reported by Oporto et al. (2009) (26.4%) in their study of resistance profiles of *Campylobacter* spp. in Spain.

It is therefore possible that production of β-lactamase by *Campylobacter* isolates could have been responsible for the high frequency of ampicillin-resistant campylobacters (Baserisalehi et al., 2005). The *Campylobacter* isolates were also resistant to ciprofloxacin at 18.8% rate. However, 22.0% of *C. jejuni* and 10.2% of *C. coli* were resistant to erythromycin. These results are in contrast to reports by Isenberger et al. (2002) that most of the *Campylobacter* isolates in Vietnam and Thailand were resistant to nalidixic acid (73 vs. 7%, respectively, $p < 0.05$) and ciprofloxacin (77 vs, 7% respectively, $p < 0.05$). The observation was also contrary to studies of Oporto et al. (2009) where all their isolates were susceptible to erythromycin. Most isolates in this study (≥75%) were resistant to vancomycin, cefexime, methicillin and trimethoprim (Table 2). The combined rate of resistance of the *Campylobacter* spp. from the farms to gentamycin was 26.7%. Individually, resistance of *Campylobacter* to this antibiotic was *C. jejuni* (31.7%) and *C. coli* (22.5%). This observation harmonizes with the report of Nonga and Muhairwa (2010) in Tanzania who reported between 20 to 50% resistances, which contrasted with the observation of Oporto et al. (2009) who reported total susceptibility of *Campylobacter* isolates to gentamycin.

In addition, the report of Hong *et al*. (2007) and Nonga and Muhairwa (2010) were in contrast to our findings as these workers observed resistant rates as high as ≥94% of *Campylobacter* spp. to ciprofloxacin (95.9%), tetracycline (94.6) and nalidixic acid (94.6%). However, their reports on the resistant rate of campylobacters to erythromycin were in line with our findings.

Resistance to fluoroquinolones has increased over the past years in many parts of the world (Allos, 2001; EFSA, 2007; Engberg et al., 2001). In Spain, fluoroquinolone-resistant *C. jejuni* isolated from humans was first reported in 1988 (Reina et al., 1989) and resistance increased to high levels (Saenz et al., 2000; Prats et al., 2000). The activity of erythromycin against *C. jejuni* human isolates, the antibiotic of choice for the treatment of diarrhea caused by *Campylobacter* strains (especially in infants), seems to remain stable at rates below 5% (Saenz et al., 2000; Prats et al., 2000). The report of these workers is in contrast to our observation among animal isolates as we noted a higher rate of resistance to the macrolide (erythromycin) (16.6%). However, this is the most stable antibiotic to campylobacters isolated from sheep compared with other tested antibiotics. This susceptibility may be reassuring, but active and more extensive antimicrobial surveillance in campylobacters from animals is needed to allow future informed decisions about how macrolide antibiotics could be used in food animals while still safeguarding human health. With the emergence of resistant strains of campylobacters isolated from sheep as observed in this study, increasing passage of diarrheal faeces and with the way sheep are reared in Africa, there is every likelihood that an epidemic situation may arise through the dissemination of highly pathogenic antibiotic-resistant strains into the environment. Contamination of other farm animals co-reared with sheep and flock members as well as pollution of surface water through run-offs is of public health significance. To this end, it is important that the veterinary section of the department of health, South Africa investigate the cause of campylobacteriosis among sheep in these farms in order to prevent an epidemic situation that could in little time spread not only to other animals but also to humans.

Conclusion

This study has shown a high prevalence of campylobacteriosis in sheep in farm settlements in the

Vhembe district of the Limpopo Province, South Africa. In addition, we have also noted a high rate of β-haemolytic activities, which may indicate the ability of these isolates to cause pathological effects or death of cells. The high rate of isolation and multiple antibiotic resistances of *Campylobacter* species from diarrheic faeces of sheep calls for more elaborate studies to unravel the cause, origin and extent of campylobacteriosis not only among sheep but among other food animals, including their phylogenetic relatedness and the various genes coding for resistance and virulence in the Venda Region of South Africa.

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Full Length Research Paper

Serological and bacteriological study of leptospirosis in slaughtered cattle in north of Iran (Rasht)

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A serological and bacteriological study was performed in Guilan industrial slaughter house, in Rasht, North of Iran in 2009. To investigate the seroprevalence of leptospirosis in slaughtered cattle in Guilan, 59 and 39 random serum samples were collected from cows and bulls, respectively. None of the cattles was vaccinated against leptospirosis. Also urine samples were collected from all of the blood-sampled cattle and cultured. All serum samples were serologically tested by microscopic agglutination test (MAT), as a standard method for serological diagnosis of leptospirosis. The serum samples were tested for antibodies against eight live antigens of *Leptospira interrogans* serovars: Australis, Autumnalis, Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, Pomona and Sejroe. The lowest dilution that each serum was considered positive was 1:100. The results of this study showed that 37 (37.8%) animals had a positive reaction against one or more serovars. The most prevalent *Leptospira* serovars was Pomona (49.0%). One leptospiral organism was isolated from 98 urine samples of cows and bulls. The results of this study indicates that leptospiral infection is magnified in cattle in Rasht, and cattle have a major role in maintaining Pomona serovar; indeed they are a potential zoonotic risk to slaughter house workers, meat inspectors, milkers and farmers.

Key words: Iran, Rasht, serology, bacteriology, cattle, leptospirosis.

INTRODUCTION

Leptospirosis is known as a global public health problem because of its increased mortality and morbidity in different countries (Ahmed et al., 2006; World Health Organization, 2001). Leptospirosis causes economic loss to the cattle industry from decreased milk production, abortion, stillbirth, infertility and mortality. Diagnosis of leptospirosis is based on laboratory confirmation because its clinical signs are nonspecific and may be mistaken with other febrile diseases (Vado-Solis et al., 2002).The culture of *Leptospira* from body fluids (blood or urine) is the most confirmative test. The carrier cows secrete leptospires in their urine without clinical signs of disease because of the tendency of bacteria to accumulate in kidneys. Therefore, they have an important role in the epidemiology of disease (Ellis et al., 1986; Waitkins, 1986). The earliest recognised report of leptospirosis in Iran is published by Rafyi and Magami (1968). Since then the most prevalent *Leptospira* serovars reported in Iran includes: Hardjo, Pomona, Grippotyphosa, Canicola and Icterohaemorrhagia (Abdollahpour, 2009). As North of Iran, especially Guilan province, has a humid temperate climate with plenty of annual rainfall, is a suitable environment for maintaining of leptospira (Abdollahpour et al., 2009). The objective of this study was to investigate the seroprevalence of leptospirosis and urinary shedding in cattle population in Guilan industrial slaughter house.

MATERIALS AND METHODS

Study population

For this study, a total of 98 samples were randomly collected from

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Table 1. Distribution of leptospiral infection in cattle stratified by sex.

Sex	MAT reaction results		Total
	Number of positive	Number of negative	
Cow	25 (42.4%)	34 (57.6%)	59 (100%)
Bull	12 (30.8%)	27 (69.2%)	39 (100%)
Total	37	61	98

non-vaccinated cows (n=59) and bulls (n=39) of Guilan industrial slaughter house in Rasht, North of Iran, in May and August, 2009.

Seruma samples

Blood samples were collected from the jugular vein and were transferred to the Leptospira Research Laboratory of the faculty of Veterinary Medicine, University of Tehran, in 10 ml evacuated glass tubes. Serum was separated by centrifugation and stored in 2 ml cryotubes at -20°C until using. Microscopic agglutination test (MAT) was implemented on all of the serum samples, according to the methods of WHO (2003). MAT was performed using live antigens, which were grown in liquid Ellinghausen McCullough Johnson Harris (EMJH) medium for 7 to 10 days. The following serovars were used in this study: *Leptospira interrogans* serovars: Australis, Autumnalis, Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, Pomona and Sejroe. Serial dilution of test serum were prepared ranging from 1:50 to 1:800 and 10 µl of diluted test sera added to an equal volume of antigen suspension on a microscope slide. Following incubation at 28 - 30°C for 1.5 h, the slide was examined under a dark-field microscope, using a long working distance objective at x100 or x200 magnification. Agglutination was noted by observing clumps of leptospires. The lowest dilution that each serum was considered significant was 1:50. The end point titre was the highest titre in which 50% agglutination occurred, so that the lowest titre that was considered as positive was 1:100.

Urine samples

Urine samples were obtained about ten minutes after slaughter. Urinary bladders were carefully removed from the carcasses and they were flamed on the surface using a red-hot blade, then approximately 5 ml aliquots of urine were collected from each animal by aspiration using sterile disposable syringes. Urine samples were transferred to a location near the slaughter room immediately and 20 µl of each urine sample inoculated into EMJH medium enriched with rabbit serum. 5-fluorouracil added to medium at a concentration of 200 µg/ml, to inhibit the growth of contaminants. Then, cultures were incubated at 30°C and examined at 7-day intervals by dark-field (DF) microscopy for 24 weeks as recommended by WHO (2003).

Data analysis

The results of the MAT and culture methods were analyzed using Statistical Package for Social Sciences, version 16. Chi-square and Fisher's exact tests were used to detect significant differences among sex, MAT and culture results. A p value ≤ 0.05 was considered statistically significant.

RESULTS

This study indicated that 25 (42.4%) cows and 12 (30.8%) bulls had a positive reaction against one or more serovars of *L. interrogans* (Table 1). There were positive seroreactions against all serovars except for serovar Icterohaemorrhagiae. The most prevalent Leptospira serovars were Pomona, Canicola and Grippotyphosa. The least prevalent Leptospira serovars were Australis, Autumnalis and Sejroe (Table 2). Ten samples (27.2%) showed serological reaction with more than one serovar. One sample (2.7%) showed serological reaction with four serovars: Canicola, Grippotyphosa, Hardjo and Pomona, and two samples (5.4%) showed reaction with three serovars: Canicola, Grippotyphosa and Pomona. Seven samples (18.9%) showed serological reaction with two serovars: Grippotyphosa and Pomona (two samples), Hardjo and Sejroe (one sample), Canicola and Pomona (two samples), Canicola and Hardjo (one sample), Canicola and Grippotyphosa (one sample) (Table 3). The majority of titre levels were 1:100 for all serovars except serovar Icterohaemorrhagiae and the frequency of 1:100, 1:200 and 1:400 were 74.5, 25.5 and 0.0%, respectively (Table 1). In the present investigation, there was no statistically significant difference between sex and MAT results (p = 0.246).

Leptospires were demonstrated in one out of 98 urine cultures (1%) in EMJH medium. The isolate was obtained from a bull that had clinical signs of leptospirosis such as icterus and hemoglobinuria therefore, the carcase was removed. In this study there was no statistically significant difference between sex and culture results (p = 0.216).

Table 2. Serum antibody titres for serovars of *L. interrogans* in 98 cattle sampled at the Guilan industrial slaughter house in Rasht, Iran by MAT* (Titres ≥ 1:100 were considered seropositive).

Serovar	Total		
	1:100	1:200	seropositive
Australis	1	0	1 (2.0%)
Autumnalis	1	0	1 (2.0%)
Canicola	5	4	9 (17.6%)
Grippotyphosa	8	1	9 (17.6%)
Hardjo	3	2	5 (9.8%)
Icterohaemorrhagiae	0	0	0
Pomona	19	6	25 (49.0%)
Sejroe	1	0	1 (2.0%)

*Microscopic agglutination test.

Table 3. Incidence of MAT* reaction with one or more antigens in 37 positive reactors.

Number of antigen	Number of positive
One	27 (73.0%)
Two	7 (18.9%)
Three	2 (5.4%)
Four	1 (2.7%)
Total	37 (100%)

*Microscopic agglutination test.

DISCUSSION

Leptospirosis is a zoonosis of worldwide distribution, caused by *L. interrogans*. It is a well known causes of bovine reproductive losses such as abortion, infertility, stillbirth, birth of weak calves, decreased milk production. North of Iran (Rasht) has a humid temperature climate with plenty of annual rainfall, where is suitable for maintaining of Leptospira spp. Therefore, this study was conducted to investigate the seroprevalence of leptospirosis and urinary shedding in cattle that referred to Guilan industrial Abattoir in Rasht during May and August, 2009. The results of this study showed that the seroprevalence of leptospirosis in cattle in North of Iran was 37.8%. The reported results of seroprevalence of leptospiral infection in cattle are different from country to country. In Portugal, 15.3% of cattle reacted to one or more serovar of *L. interrogans* (Rocha, 1988). According to the report of Ozdemir and Erol (2000), the prevalence of leptospiral infection in cattle and sheep in Turkey was 44.77 and 8%, respectively. In Malaysia, 40.5% of cattle reacted to one or more serovar of *L. interrogans* (Bahaman et al., 1987). In Turkey, 25.42% of cattle reacted to one or more serovar of *L. interrogans* (Gumussoy et al., 2009). The Results of this study also showed that Pomona was the most predominant serovar in cattle in North of Iran. On the other hand, in a previous study in North of Iran (Guilan province), the predominant serovar was Canicola in cattle (Abdollahpour et al., 2009). These results showed that the predominant serovar varied in a period of time and in different situations. In Ahvaz (Southwestern Iran) the predominant serovar in cattle was Grippotyphosa (Hajj et al., 2005). The predominant serovar in sheep in Ahvaz was Pomona (Hajj et al., 2007). In Tehran suburb, the predominant serovar in cattle was Icterohaemorrhagiae (Sakhaee et al., 2007). The reported results of the predominant serovar of leptospiral infection in cattle are variable in different parts of Iran. This confirms the need for regional study for leptospirosis, because host-parasite relationship may change depending on the ecology of the region. Pomona is a common serotype in cattle and pigs, and these animals are considered to be the main reservoir for the mentioned serotype.

In the present study, 27% of seropositive cattle had antibodies against more than one serovar. This may be related to mixed serovar infection or cross-reactivity among serovars. In this study, the high prevalence of infection and dominant titre of 1:100 indicate that leptospiral infection in cattle in North of Iran is endemic and occurs mostly in subclinical form. In this study, one leptospiral organism was isolated. It would be interesting to follow up the isolated leptospira, that is characterize the isolate both genetically and serologically, as well as performing serological studies with the cattle sera using this local strain as antigen in the MAT.

In conclusion, this study revealed that infection with *L. interrogans* is common in cattle of North of Iran and is threaten to the public health and there is a potential zoonotic risk to slaughter house workers, meat inspectors, milkers and farmers.

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Full Length Research Paper

Textile effluent treatment by *Bacillus* species isolated from processed food

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Colour removal of industrial effluent has been a major concern in waste water treatment, especially for the waste water that originates from textile and dye stuff plant with a continuous discharge of great quantity of remaining dyes to the environment. The efficient treatment of the effluent is an eco- friendly method for the treatment of textile effluent. *Bacillus* species was isolated from the processed food-pickles and were characterized by means of biochemical reactions. Spore formers, non spore formers and their supernatant were used for the treatment. Effluent collected from the textile was diluted to 10, 50 and 90%, were subjected to biological treatment. The level of degradation rate was increased to 87.7 to 93.3% on dilution at 50 and 90% respectively when spore formers were used. The culture supernatant was able to degrade the dye at a rate of 34% with crude effluent and 98 with 90% of diluted effluent. Probably the metabolites produced by the organism were involved in the dye degradation process.

Key words: Textile effluent, *Bacillus* species, declorization, aerobic degradation.

INTRODUCTION

Synthetic dyes, high in usage, produced world wide every year which has lead the ecosystem blended by the hazardous compounds release at various stages of the operation from the dyeing industry. Textile dyes improves the human lifestyle on a positive account. But on the same side at a negative point they are affecting the environment due to the pollutants given out by them.

Dyes include a broad spectrum of different chemical structure, primarily based on substituted aromatic and heterocyclic groups such as aromatic amine, which is a suspected carcinogen, phenyl and naphthyl. The only thing in common is their ability to absorb light in the visible region. The removal of colour from waste water is often important than the removal of soluble colourless organic substance.

Contributing the major fraction of the biological oxygen demand (BOD) colour is the first contaminant to be recognized in waste water and has to be removed before discharging into water bodies or on land. Presence of colour in dye effluent gives an indication of water being polluted which will damage the receiving water, when discharged.

The discharge of dyes into the environment impedes light penetration and then toxic to food chain organisms and to aquatic life. Degradation of azo dyes by means of physical and chemical methods in waste water are recently done which are not viable options for treating large waste streams and cost prohibitive (Do et al., 2002; Maier et al., 2004). Living system, especially micro-organism can catalyze the degradation of wastes without disruption of the environment is represented.

Bacteria offers a cheaper and environment friendlier alternative for colour removal in textile effluents (Olukanni et al., 2006). Biological treatment has been effective in reducing dye house effluents and when used properly has a lower operating cost than other remediation process. Anaerobic and aerobic reduction of azo dyes to simpler compounds have all demonstrated the ability of microbes and sludges to effectively reduce azo dyes to their intermediate structures, thus destroying the apparent colour (Chinwetkitvanich et al., 2000) and Razo-Flores et al., 1997). Reduction of dye compounds to their intermediates reduces the aesthetic pollution but a larger and more deleterious problem may be created (Wallace, 2001).

Aerobic treatment of azo dye wastes though effective,

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is often the typical method of treatment. Recent combination of chemical and biological, physical and biological treatment has also proven to be effective (Seshadri et al., 1994; Horning, 1977). This research explores the ability of bacteria-*Bacillus sp* isolated from pickles for the treatment of a textile effluent containing indigo blue dye. The present investigation was taken up to screen the microorganisms *Bacillus* sp for decolourisation from home made pickles.

METHODOLOGY

Sources of the organism

Pickles of home made was collected from the local area , homogenized , appropriate dilution was made, plated on nutrient agar media, incubated at 37°C for 24 h. All isolations were done on nutrient agar using enrichment culture techniques and the organisms identified to the generic level using the Cowan and Steel (1993) Scheme. Two different isolates were considered for our present study; (1). *Bacillus* sp*.,* a spore former and (2). *Bacillus* sp*.,* a non spore former.

Effluent treatment with isolated bacteria

Collection of effluent

The effluent was collected from the dying industry near Tirupur and was stored under appropriate conditions for further use.

Determination of BOD of effluent

The BOD bottle was filled with the sample (effluent from dying industry) up to the rim and 2 ml of $MgS0_4$ and alkaline iodide azide solution was added and closed, the solution was mixed by inverting the bottle, then it is checked for the formation of brown precipitate. 2 ml of sulphuric acid was added along the sides of the bottle to dissolve the precipitate formed. From this 100 ml was taken and titrated against sodium thiosulphate taken in the burette. The color change of the solution to yellow was noted after which 2 ml of starch solution was added. The titration was continued by adding thiosulphate solution in drops till the disappearance of blue colour.The experiment was repeated to obtain concordant value. The BOD bottle was filled with the sample and incubated for 5 days and the OD values were calculated to find BOD value.

$BOD = DO_b - DOa$

DO_{bbn} = Dissolved oxygen of sample before incubation, DO_a = Dissolved oxygen of sample after incubation.

BOD procedure was repeated for the sample which was inoculated with respective cultures. The values were noted and tabulated.

Determination of biodegrdation activity

Potential decolourization of the stimulated effluent by each isolate was investigated

The crude effluent and diluted effluent to 10, 50 and 90% with distilled water was taken as samples. Into 20 ml stimulated diluted effluent, 2×10^5 CFU of the isolate was added and poured into transparent bottles (200 mg/L starch and 250 mg/L yeast extract were added as co-substrates) and cocked with sterile cotton wool. After 14 and 90 days decolourization was measured. Decolourization was determined by measuring the absorbance of the stimulated effluent at the effluent pre-determined λ max (485 nm) and the absorbance of the treated stimulated effluent. The percentage decolourization was calculated as

$[(A_O-A_t)/A_O] \times 100\%$

A_o-absorbance of the stimulated effluent
A_t-absorbance of the treated stimulated effluent 14 days post microbial inoculation.

Effluent treatment with supernatant of bacteria

Nutrient broth was prepared in a conical flask sterilized. It was allowed to cool. A loop full of bacterial culture was added to nutrient broth. It was incubated respectively. The following day the broth was dispensed into sterile centrifuge tubes centrifuged at 10,000 rpm for 15 min. The pellet was discarded and the supernatant was collected in a sterile beaker. To the 100 ml sample (effluent) 5 ml of supernatant is added using sterile pipette for bacteria. These flaks were also incubated accordingly to note the color change.

Plasmid screening

About 0.1 ml of the bacterial culture was dispensed into in eppendorf tube and centrifuged at 10000 rpm for 15 - 20 min and the supernatant was discarded. To the pellet 350 µl of STET buffer was added and mixed well. Centrifuged at 10,000 rpm for 10 min, pellet was removed using sterile tooth pick then added 40 µl of 3 M sodium acetate and 420 µl of isopropanol. Centrifuge at 10,000 rpm for 10 min. Finally the pellet was suspended in 20 µl of distilled water or TE buffer and electrophoresis on agarose gel to find the presence of plasmid DNA.

RESULTS

A total of ten isolates were obtained belonging to the genus Bacillus from the source pickle and were named as HB1, HB2, and HB3 respectively. Gram reactions of the isolates were observed in which all the isolates were gram positive. They were found to be rod shaped and grew under aerobic conditions. Few were able to ferment carbohydrates and almost all strains hydrolyzed starch, catalase positive and were oxidase negative. The isolates HB1, HB5, HB8 and HB10 were able to produce intracellular structures like spores.

BOD of the effluent on the initial day and on the fifth day was observed for the crude effluent and treated effluent and calculated. The BOD value was found to be reduced to 5.6. Biodegradation with the isolates and with their supernatant showed colour removing activities in the diluted effluents between 40.74 and 47.73%. The colour of the crude effluent was reduced to 25 - 35% after 14 days incubation. But complete degradation of dye was not possible within 90 days of incubation in crude effluent as evident in the lack of colour change. When dilution of

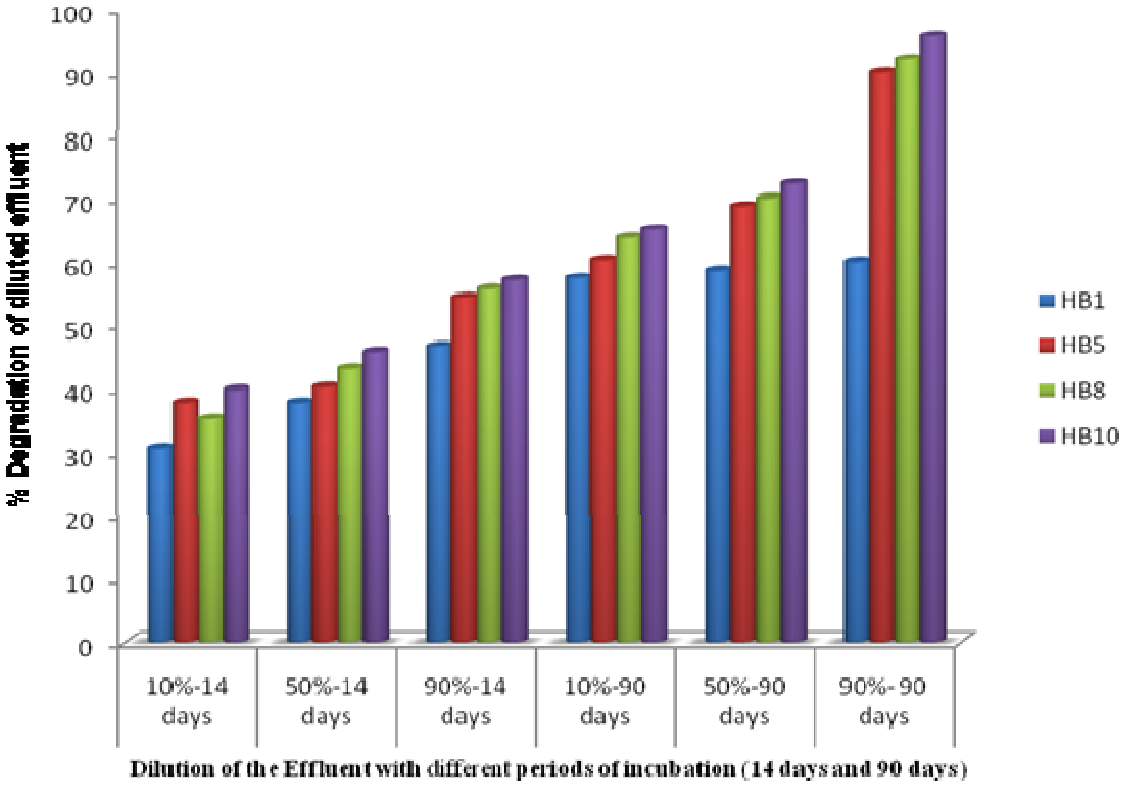

Figure 1. Endospore forming Bacillus treated effluent.

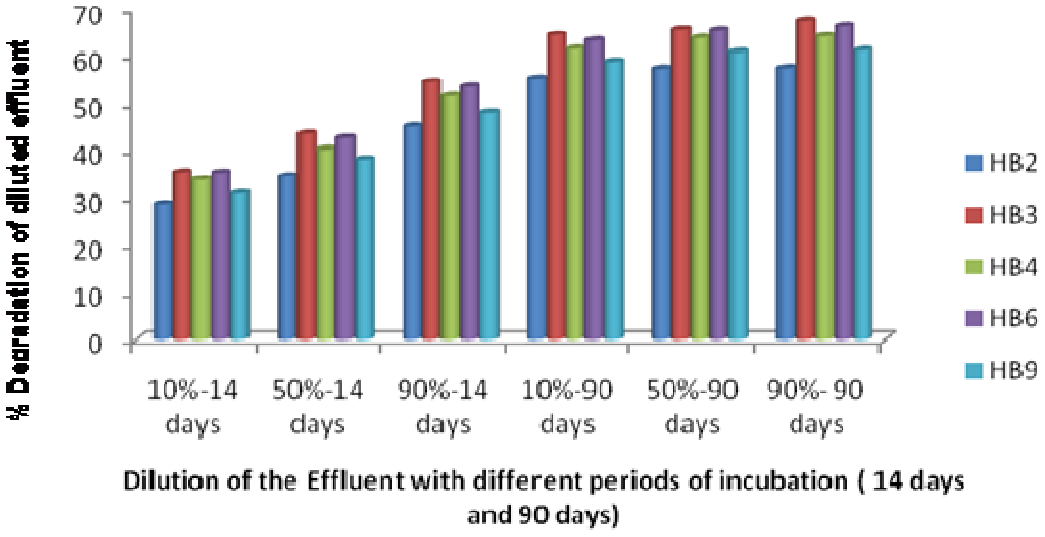

Figure 2. Non-endospore former Bacillus treated effluent.

the effluent was increased the decolourization of effluent was high by increase in activity of bacteria.

Degradation activity was compared with the spore formers and non spore formers. The latter showed better degradation level than the previous one (Figures 1 and 2). The level of degradation rate was increased to 72.3 to 95.63% on dilution at 50 and 90% respectively in case of spore forming isolates when compared to the non spore formers. HB10 spore formers showed the highest degradation level than any other spore formers (Figure 1). In case of non spore formers thought the level of degradation was very less. HB3 showed the highest

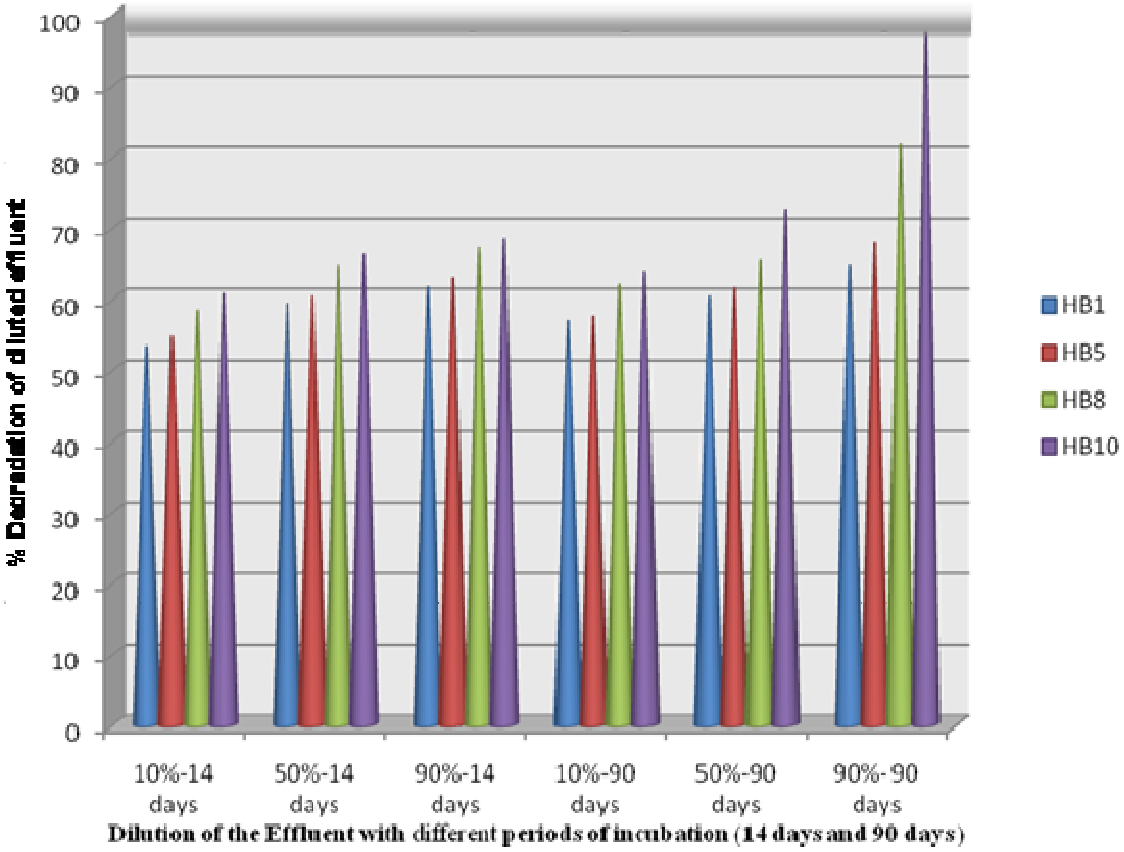

Figure 3. Effluent treatment using culture supernatant of endospore formers.

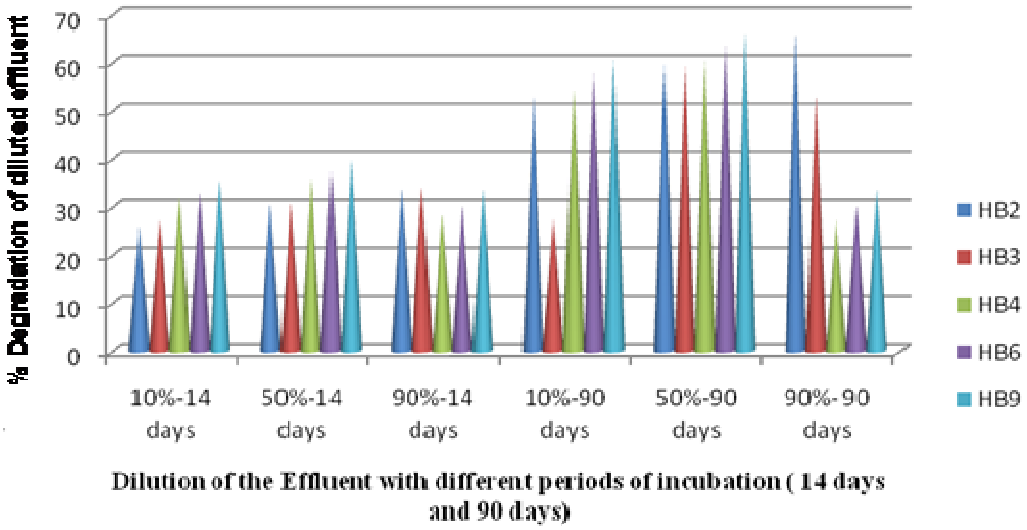

Figure 4. Effluent treatment using culture supernatant of non-endospore formers.

degradation level at 90% dilution after 90 days (Figure 2). The culture supernatants of non endospore formers and endoformers also shared the same results as above. The supernatants of endospore formers degraded the dye at very better level than compared to the non endosporers (Figure 3). Culture supernatants of non-endospore showed better degradation during initial days at 10 and 50% rather than at 90% dilution (Figure 4). When the

culture supernatant was used for the dye degradation, the crude extract reduction rate was found to be 34%. Probably the metabolites produced by the organism were involved in the dye degradation process. Plasmids were also detected from the isolates. Dye degrading genes may be of plasmid oriented or chromosomal oriented which can be our future work.

DISCUSSION

During the dying process a substantial amount of dyes and other chemicals are lost in waste water. Estimates put the dye loses between 10 - 15% (Vaidya and Datye, 1982). Dye is generally not toxic to the environment but the colour water bodies may hinder high penetration there by affecting the aquatic life and limiting the utilization (Ajaiyi and Osibanjo, 1980). Color removal of industrial effluent has been a major concern in waste water that originates from textile and dye stuff plant with a continuous discharge of great quantity of remaining dyes to the environment. The efficient treatment of the effluent is an eco-friendly method for treatment of textile effluent. The degradation of molecules of dyes in the environment by microorganisms is likely to be slow, which means that it is possible for high levels of dye to persist and potentially accumulate. Due to the low degradability of the dyes, conventional biological treatment process is inefficient in treating dye waste waters. Biological decolorization is employed under either aerobic or anaerobic environment. A number of reports discourage the azo dye decolorization by microorganism under anaerobic conditions as it leads to the formation of corresponding aromatic amines. The effectiveness of microbial decolorization depends on the adaptability and the activity of selected microorganisms. Over the past decades, many microorganisms are capable of degrading azo dyes, including bacteria, fungi and yeast.

In our present study the *Bacillus* isolates from home made pickle played a major role in degrading the effluent. Upon dilution and inoculation with isolated microorganisms, both spore and non-spore forming organisms were able to show the dye degradation at a maximum level within 90 days of incubation at a highest dilution when compared to 14 days of incubation under aerobic conditions. Ajibola et al. (2005) has stated that *Bacillus subtilis* reduced the colour of the crude effluent by 25%. Effluent when diluted, the activity of the bacteria increase the rate of decolourisation. Endospore forming isolates showed its high level of degradation both when whole cells were used and when the supernatant was used. Spore forming obligate aerobes under unfavorable conditions could form spores which might reduce its metabolic activities to a minimum (Ajibola et al., 2005). *Bacillus cereus* was not able to degrade crude effluent because of the environment conditions of the effluent sample. Increase in dilution of the effluent activated the bacteria more in declorization (Ajibola et al., 2005). In the current investigation, aerobic effluent treatment has involved in declorization of the dye though it has been stated that anaerobic effluent treatment has given a better reduction of dyes in previous research work. Effective treatment was obtained with spore forming *Bacillus* when compared to non- spore forming organisms.

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Full Length Research Paper

Identification of *Streptococcus salivarius* bacteriophage isolated from Persian Gulf as a potential agent for dental caries phage therapy

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The aim of this research was to detect oral *Streprtococci* bacteriophages from Persian Gulf. Dental plaque samples were collected using sterile explorer and cultured in brain heart infusion (BHI) Broth. The oral *Streptococci* were isolated in culture media. The Persian Gulf water sample was gathered using a sterile bottle from the depth of 50 cm under the inframarine surface at Boushehr Port, Boushehr state, Iran. The Persian Gulf water was centrifuged and its supernatant was filtered through a 0.45 micrometers membrane filter and with a sterile Millipore filtration system. The filtrates were added to activate oral *Streptococci* at their logarithmic phase and cultured in (BHI) Agar using overlay method. Bacteriophage plaque forming assay in (BHI) Agar and clearance of (BHI) Broth suggested the presence of specific bacteriophages in sample. Transmission electron microscopy revealed that the capsid of the isolated bacteriophage was hexagonal (diameter: ~ 83.33 nm) most probably related to *Cystoviridae* family. This is the first report of isolation and identification of oral *Streptococci* bacteriophages from Persian Gulf located in South of Iran. The applications of these lytic phages as a potential for phage therapy of dental plaque could be considered as the significance and impact of the present study.

Key words: Persian Gulf, *Streptococcus salivarius*, bacteriophages, phage therapy, dental plaque, pharmaceutical and medical biotechnology.

INTRODUCTION

The resident microorganisms of oral cavity especially those inhabit on tooth surfaces are responsible for dental plaque formation and conversion of dietary saccharides to organic acids. These acids decalcify the tooth enamel and lead to destruction of tooth hard tissue and consequently tooth decay (Loesch et al., 1986; Hitch et al., 2004; van der Ploeg, 2007). More than 500 species from 30 deferent genera reside in oral cavity (Schaechter et al., 2004). The most important species that play key roles in dental plaque formation are oral *Streptococci* (Tanzer et al., 2001). According to Bergey's mannual of systematic bacteriology, oral *Streptococci* are formed from 12 species including *Streptococcus salivarius, Streptococcus anginosus*, *Streptococcus constellatus*, *Streptococcus cristatus*, *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus parasanguis*, *Streptococcus pneumonia*, *Streptococcus sanguis* and *Streptococcus sobrinus* (Holt et al., 1994; Schaechter et al., 2004). These species are the first bacteria that attach to salivary glycoproteins on tooth surfaces through their specific surface capsular polymers such as glucan and fructan (Freedman et al., 1974; Tanzer et al., 1974; Tanzer et al., 2001). *S. salivarius* as well as mutans *Streptococci* and nonmutans *Streptococci* or sanguinis *Streptococci* are present at high levels in tooth and mucosal surfaces some of which are highly acidogenic and few are acid tolerant (Nyvad et al., 1990; Tanzer et al., 2001). *S. salivarius* along with *S. sanguis*, *S. oralis* and *S. gordonii* are the first tooth colonizers however, *S. sobrinus* and *S. mutans* are more dealt with dental

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diseases (Milnes et al., 1993; Smith et al., 1993; Jacques et al., 1998; van der Ploeg, 2008). The bacteriophages, viruses that attack their specific bacterial hosts, have a great impact on controlling bacterial population throughout the world as well as micro environmental niches in human body (Marks et al., 2000; Chanishvili et al., 2001). In recent decades bacteriophages have been studied as biotechnological tools for treatment and eradication of bacterial pathogens such as *Escherichia coli* in gastrointestinal infections (Smith et al., 1987a; Smith et al., 1987b; Smith et al., 1982; Smith et al., 1983; Drozdova et al., 1998; Marks et al., 2000), *Pseudomonas aeroginosa* and *Acinetobacter baumanii* in skin burns and grafts (Soothill, 1992; Soothill, 1994).

Several aspects of oral Streptococci and their influences on dental disorders and dentistry have been studied (Jacques, 1998; Tanzer et al., 2001; Okada et al., 2002; Franco and Franco, 2007) but there are few reports indicating the role of bacteriophages in ecology of oral cavity as a micro environment or the attitude toward phages as strong biotechnological and natural therapeutic agents for phage therapy of oral *Streptococci* (Bachrach et al., 2003; Hitch et al., 2004). Some reports have indicated the isolation and identification of lytic bacteriophages of *S. mutans* from human saliva (Delisle and Rostcowski, 1993; Armau et al., 1998) and recently the complete genome sequence of one of them, *S. mutans* lytic baceriophage M102, has been revealed (van der Ploeg, 2007). The main goals of this research were isolation and identification of oral *Streptococci* from dental plaques of healthy individuals as well as patients with mild gingivitis, isolation and identification of their specific bacteriophages as potential agents for phage therapy of dental caries.

MATERIALS AND METHODS

Culture media and chemicals

The culture media used were Brain Heart Infusion (BHI) Broth, (BHI) Agar. Mitis - Salivarius Agar (sucrose, 50 g/l; agar, 15 g/l; enzymatic digest of protein, 10 g/l; proteose peptone, 10 g/l; K_2HPO_4, 4 g/l; dextrose, 1 g/l; trypan blue, 0.08 g/l; crystal violet, 0.8 g/l; Na_2TeO_3 solution, 1 ml; distilled water, 1000 ml) (Atlas, 2004) and Blood Agar Base medium, all from Himedia, India, NaCl, glycine, $CaCl_2$, anaerobic class A gas pack, all from Merck, 0.45 µm membrane filter (Millipore, white gridded), api 20 Strep kit (bioMerieux, France) and H_2O_2 from Shimifan, Iran.

Dental plaque samples

The dental plaque samples were collected from healthy volunteers (10 students, aged 22 - 26, 8 females and 2 males at the Faculty of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran) and patients with mild gingivitis and periodontitis (aged 29 - 54, 6 females and 4 males). The dental plaque samples were obtained using sterile explorers from the upper right first molars in all individuals that had not applied antibacterial rinse and routine brushing 12 -14 h before sampling. The samples were taken in accordance with ethical guidelines and regulations prepared by Department of Operative Dentistry, Faculty of Dentistry, Isfahan University of Medical Sciences, which have been authorized by Iranian Ministry of Health and Medical Education.

Enrichment, isolation and primary identification of dental plaque *Stereptococci*

The dental plaque samples were cultured in BHI and then incubated at 37°C in 5% CO_2 for 24 h. After bacterial enrichment, the turbid broth media were cultured to Mitis – Salivarius Agar (MSA) using streak plate method and incubated at 37°C in 5% CO_2 for 48 h. The colonies were examined for catalase reaction using hydrogen peroxide.

Macroscopic, microscopic and biochemical characterizations

The individual colonies were examined for their macroscopic traits such as color, size, morphology, light reflection and hemolysis on 10% sheep blood agar. The microscopic morphology and arrangement of purified bacteria on selective media such as MSA were examined using gram staining method. For biotyping, the bioMerieux SA api 20 Strep kit, an identification system for Streptococcaceae was used. Before using api 20 Strep kit, a 48 h well isolated colony was picked from blood agar medium and suspended in 300 µl sterile distilled water, homogenized well and then swabbed aseptically the entire surface of blood agar culture media. Next procedures were followed using api 20 Strep instruction.

Bacteriophage resource sampling

The water sample was collected using a sterile 1000 ml bottle from the depth of 50 cm inframarine surface of the Persian Gulf at shorelines of the Boushehr harbor, Boushehr State, South of Iran. The sampling was taken for 5 min and the cap of the bottle was fitted below the water surface. Then the Persian Gulf water sample was transferred to our laboratory at 4°C.

Preparation of bacteriophage samples and bacterial treatment

Fifty milliliters of well shaked Persian Gulf water pipetted to sterile falcons and centrifuged at 1000 g for 15 min. The supernatants were filtered through 0.45 µm Millipore membrane filter using sterile Millipore filtration system and the filtrate was stored at 4°C. Then the identified bacterial isolate from dental plaque was cultured in 10 ml of BHI and incubated at 37°C for 24 h in order to be activated. After growth and obtaining appropriate turbidity, the bacterial inoculum was cultured to 250 ml Erlenmeyer flasks containing 100 ml BHI and incubated in a shaker incubator at 37°C and 120 rpm shaking speed for 16 -18 h. The shaking was then stopped at bacterial logarithmic phase and 10 ml of Persian Gulf filtrate were added to flasks aseptically. The shaking at 37°C was continued for another 45 - 60 min for attachment of probable bacteriophages to their specific bacterial hosts. Then 1 ml of BHI was added to 5 ml preheated 45°C BHA (0.7% agar), as top agar, vortexed and immediately over layed on BHA plates. The BHAs were incubated at 37°C for 24 - 48 h until lysis zones, bacteriophage plaques, were appeared.

Bacteriophage isolation and purification

After appearance of bacteriophage plaques, they were cut aseptically and washed with 1.25% glycine in sterile eppendorf tubes, vortexed well and the aforementioned procedures were repeated for 3 times. The overall BHI from the last trial after complete clearance, 24 h incubation at 37°C with 120 rpm shaking speed, were centrifuged at 1000 g for 15 min and supernatants were passed through 0.45 µm Millipore membrane filter. Ten milliliters of the filtrates were used for further purification using a previously described method as follows: NaCl 1M was added to phage solution, vortexed for 2 min and was kept on ice for

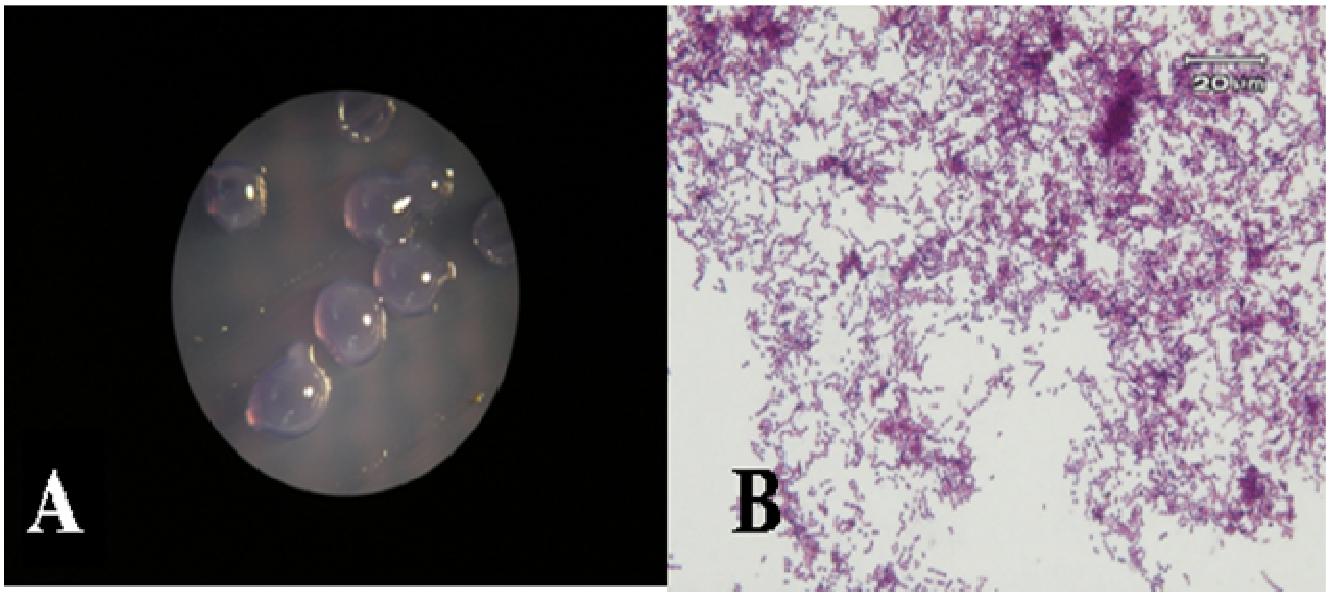

Figure 1. (A) Isolated oral *Streptococcus* from dental plaque in Mitis-Salivarius agar culture medium after 48 h incubation at 37°C and 5% CO_2. (B) The gram staining of *S. salivarius* on Mitis-Salivarius agar after 24 h at 37°C, isolated from dental plaque.

an hour. Then centrifuged at 11,000 g, 4°C for 10 min. The supernatant was transferred to a sterile tube and 10% (w/v) poly ethylene glycol 6000 was added to tube. The tube was kept on ice for another hour in order to phage precipitation. The suspension was centrifuged at 11,000 g, 4°C for 10 min. The supernatant was discarded and tube was placed in its reversal position for 5 min to be dried. Then SM buffer (NaCl, 5.8 g/l; MgSO4, 2 g/l; Tris 1 M, 50 ml; gelatin solution 2% (w/v), 5 ml; distilled water, 1000 ml) was added to pellet for soaking purposes and kept an hour in room temperature. The chloroform was added in equal volume of suspension, vortexed briefly for 30 s and centrifuged at 3,000 g, 4°C for 15 min. Organic phase discarded and aquatic phase, bacteriophage included, was kept at 4°C before TEM grid preparation (Sambrook et al., 1989).

Transmission electron microscopy

A drop of purified filtrated bacteriophage suspension transferred on a formvar coated grids (EM standard, 3.2 mm diameter). The additional suspension was removed by drying paper and grids were dried in front of light heat for 30 s. The grids were then negatively stained using 2% phosphotungstic acid (pH: 7.2 regulated with KCl, 0.5 molar) and dried following the same method and then observed through Transmission Electron Microscope (Philips, MC 10, Netherlands) at 78 K magnification.

RESULTS

The turbidity of BHI showed that the dental plaque bacteria were enriched after 48 h. The oral *Streptococci* were isolated on MSA at 37°C and 5% CO_2 after 48 h. The continual streak plate method on MSA at 37°C and 5% CO_2 confirmed the complete purification of isolates. The colonies were pale blue to bluish, smooth, mucoid with shiny reflection, convex and intermediate with 1 - 1.5 mm in diameter (Figure 1A). Microscopic observations showed large gram positive *Streptococci* and few diplococcic (Figure 1B). The negative catalase test indicated that the isolates were related to Streptococcacea family. The macroscopic and microscopic characterization of isolated oral *Streptococci* revealed that they were members of the genus *Streptococcus*.

The results of BioMerieux SA api 20 Strep kit after 4 and 24 h incubation periods at 37°C indicated two 7 – digit identification numbers, 5060045 and 5060445, respectively. Using the database (V7.0), analytical profile index and apiweb^{TM} identification software on the internet, it was confirmed that the isolated oral *Streptococcus* was *S. salivarius*. The main biochemical examinations that were used to identify the species of isolated oral *Streptococcus* as *S. salivarius* are shown in Table 1. This strain was isolated from patients with mild gingivitis. Using api 20 Strep kit, other Streptococci such as *Streptococcus ubris* and *Streptococcus thermophilus* were isolated from healthy volunteers (data not shown).

The addition of Persian Gulf filtrate to 16 - 18 h *S. salivarius* in BHI growth medium resulted in complete clearance of BHI after 12 h shaking incubation at 37°C. The cultivation of *S. salivarius*–Persian Gulf filtrate inoculums after 45 - 60 min shaking, on BHA plates showed the bacteriophage plaques after 24 h incubation at 37°C (Figure 2). The continuous contamination of new logarithmic growth of *S. salivarius* by bacteriophages obtained from individual plaques resulted in more purification of them. The results showed that these specific bacteriophages were lytic phages. Transmission Electron Microscopy of purified and concentrated bacteriohage suspension revealed that the capsid of the *S. salivarius* bacteriophage was hexagonal with

Table 1. The main biochemical characteristics of isolated *S. salivarius* from dental plaque on Mitis-Salivarius Agar by BioMerieux SA api 20 Strep kit after 4 and 24 h incubation periods at 37°C.

Biochemical tests	4 h	24 h
Voges Proskaur	+	+
Hipuric acid hydrolysis	-	-
Sculin hydrolysis	+	+
Pyrolidonyl arylamidase	-	-
α - Galactosidase	-	-
β – Glucuronidase	-	-
Alkaline phosphatase	+	+
Leucine amino peptidase	+	+
Arginine dihydrolase	-	-
Acidification of:		
D-Ribose	-	-
L-Arabinose	-	-
D-Mannitol	-	-
D-Sorbitol	-	-
D-Lactose	-	+
D-Trehalose	-	-
Inulin	-	-
D-Raffinose	+	+
Amidin	+	+
Glycogen	-	-
β-Hemolysis	+	+
Catalase reaction	-	-

approximately 83.33 nm diameter (Figure 3). These results suggested that the specific bacteriophage of *S. salivarius* isolated from Persian Gulf is most probably related to family *Cystoviridae* of bacteriophages.

DISCUSSION

The bacteriophages specific for *Enterococcus faecalis* have been isolated from human saliva but the efforts to detect bacteriophages for gram positive oral pathogens such as *S. sobrinus*, *S. mutans* and *S. salivarius* from human saliva was not successful (Bachrach et al., 2003). Hitch et al. (2004) isolated bacteriophages from oral cavity. Although, their aim was isolation of lytic bacteriophages of oral pathogens from human saliva, dental plaque and mature biofilms originated from salivary bacteria, they obtained phages specific for non-oral bacteria such as *Proteus mirabilis* but did not find any phage specific for oral pathogenic bacteria. They suggested that the bacteriophages do not play a key role in regulating the nature of micro environmental ecology of oral cavity that was against previous hypothesis that clarified bacteriophages are vital in modifying the bacterial ecosystems in most of milieus (Campbell et al., 2003). Nelson et al. (2003) reported the genomic sequence of C1 as the first *Streptococcal* phage. They showed that C1, a lytic phage that contaminating group C S*treptococci*, was a member of *podoviridae* family of phages that have been recognized by short and noncontractile tails. Three lytic bacteriophages of *S. mutans* known as M102, e10 and f1 have been characterized (Delisle et al., 1993). They reported each phage had an icosahedral head with 67 - 68 nm diameters and a noncontractile flexible tail that are characteristics of *Siphoviruses*. The complete genome sequence of *S. mutans* bacteriophage M102 has been resolved and reported that the ORFs responsible for structural proteins in M102 and *S. thermophilus* bacteriophages show similarity (Van der Ploeg, 2007). The characterization of prophage PH15 of *Streptococcus gordonii*, another oral *Streptococcus*, has been reported and the complete genome sequence of this lysogenic phage has been analyzed (Van der Ploeg, 2008).

A lysogenic bacteriophage of *S. mutans* PK1 (mucoid strain) has been identified as bacteriophage PK1. It has been revealed that the most PK1 phage particles had 95 nm hexagonal heads and 150 nm tails (Higuchi et al., 1976; Higuchi et al., 1977; Higuchi et al., 1981). While there is no report for lytic bacteriophages of oral *Streptococci* except for *S. mutans*, we isolated a lytic bacteriophage from Persian Gulf located at the South of Iran that attacked specifically to *S. salivarius*, a member of dental-caries producing *Streptoccoci*. The TEM micrograph of isolated *S. salivarius* bacteriophage showed that it had hexagonal head. Regarding the average diameter of phage particle, ~ 83.33 nm, as well as morphological characterizations it could be most probably related to *Cystoviridae* family of bacteriophages. So far there is no report indicating that *Cystoviruses*, the enveloped dsRNA phages with the size of 85 nm, could attack the gram positive bacteria e.g. *Streptococci*.

The isolation of a specific bacteriophage for an oral *streptococcus, S. salivarius*, from Persian Gulf water with a high salinity level, 23 g/l salt, is another significance of this study. While the investigation of lytic effects of this phage on other oral *Streptococci* is challenging, it could be applied as a potential for phage therapy of dental caries and other dental and periodontal disorders. The *S. salivarius* was isolated from dental plaque of patients with mild gingivitis. Other *Streptococci* such as *S. ubris* and *S. thermophilus* were isolated from healthy volunteers, but we did not examine lytic effects of the isolated bacteriophages on them because they were not classified as oral *Streptococci*.

In conclusion this is the first time that a *Cystovirus* is reported for *S. salivarius* as a gram positive bacterium. Although, there are a few documents in literature indicating the isolation of *S. mutans* lytic bacteriophages from salivary samples (Armau et al., 1988; Delisle et al., 1993) there has not been any report so far showing the isolation and identification of lytic bacteriophages of other eleven oral *Streptococci* species. We suggested that the

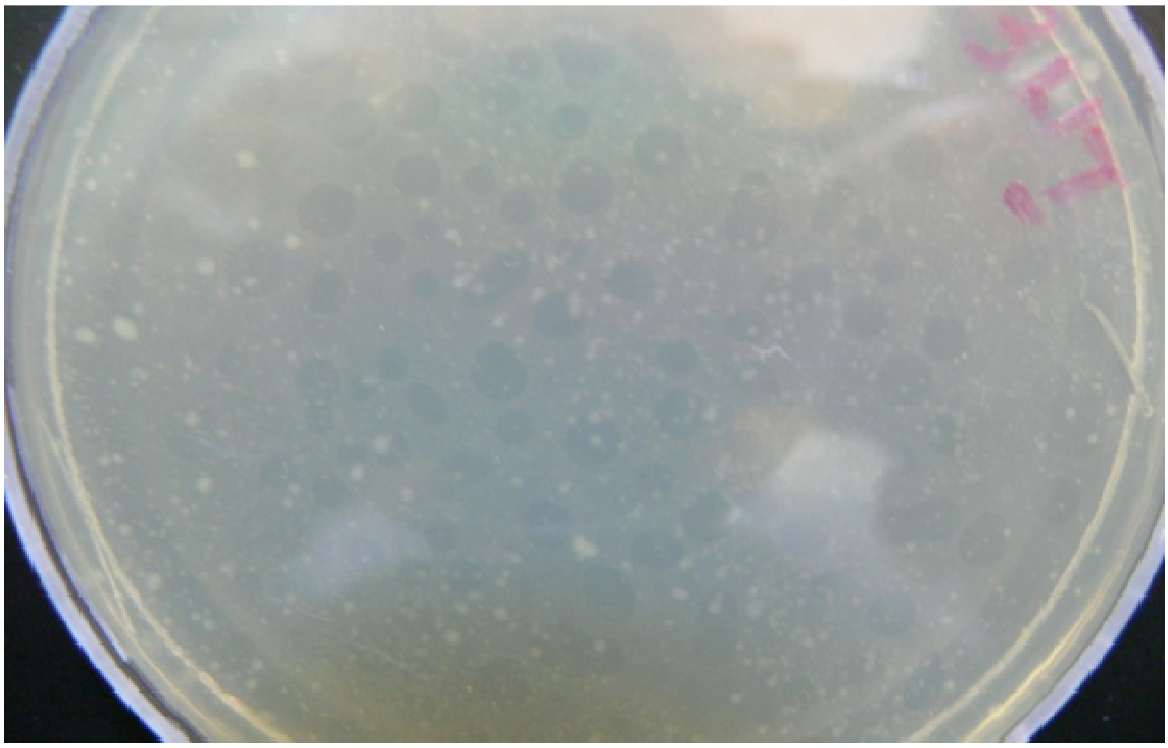

Figure 2. The plaques of *S. salivarius* bacteriophages isolated from Persian Gulf on Brain Heart Infusion Agar after 24 h incubation at 37°C.

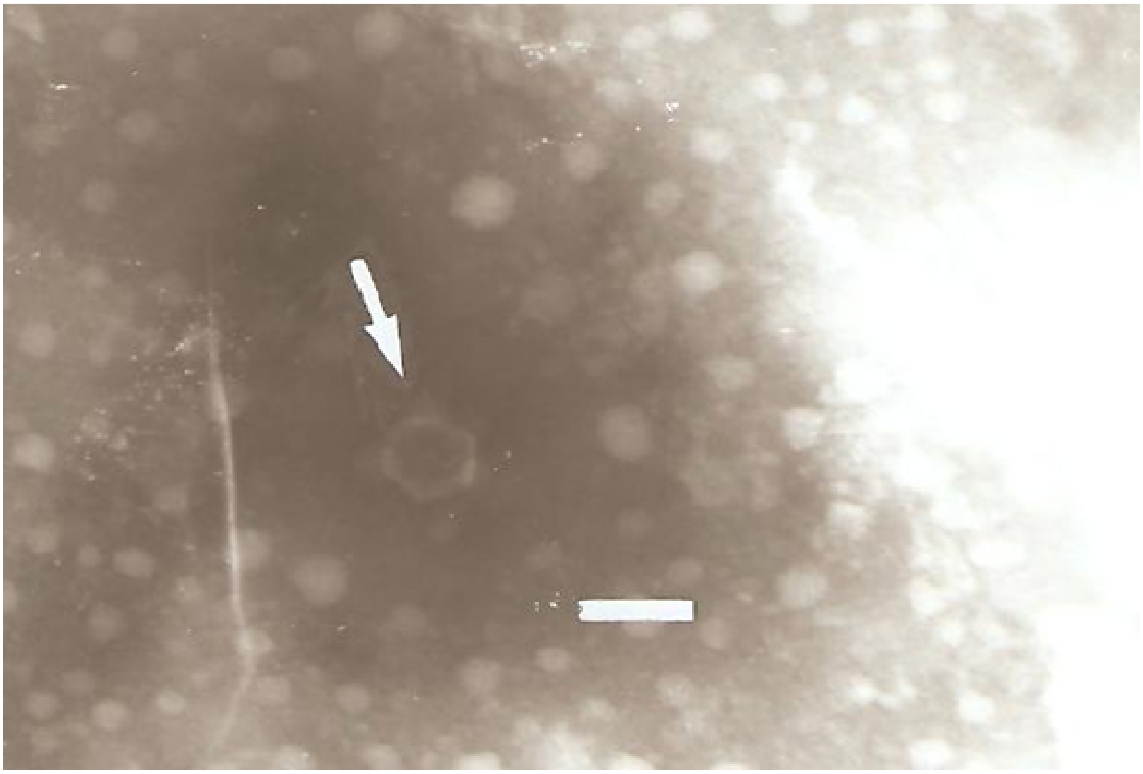

Figure 3. Transmission electron micrograph of the lytic hexagonal bacteriophage with approximately 83.33 nm diameter (arrow) isolated from Persian Gulf specific for *S. salivarius* isolated from dental plaque (Bar = 100 nm).

isolation and identification of new lytic baceriophages capable to eliminate oral *Streptococci*, starters of dental plaque formation, could be considered as a powerful approach for phage therapy of oral pathogenic bacteria in dentistry as well as modern medical and pharmaceutical biotechnology.

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Full Length Research Paper

In vitro antimicrobial activity of leaves of *Acalypha indica* Linn. (Euphorbiaceae)

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The antimicrobial activity of water, ethanol and chloroform extracts of *Acalypha indica* was tested against four bacterial and fungal strains using the disc diffusion method. The antibacterial activity against gram positive bacteria was more pronounced ($p < 0.05$) in water and ethanol extracts. Antifungal activity was more significant ($p < 0.05$) only in chloroform extract. This antimicrobial activity was compared to standard antibiotics (penicillin, enrofloxacin, ampicillin and chlorampenicol) and antifungal drugs (ketoconazole, itraconazole and fluconazole). Findings from current study support the use of *Acalypha indica* in traditional medicine for the treatment of various bacterial and fungal infections.

Key words: Antifungal, antibacterial, disc diffusion assay.

INTRODUCTION

Acalypha indica Linn. of the family Euphorbiaceae is a common weed in many parts of Asia including India, Pakistan, Yemen, Sri Langka and throughout Tropical Africa and South America (Ramachandran, 2008). It is an annual herb, about 80 cm high and commonly found in waste places or fields (Burkill, 1985). It is locally known as “kucing galak” or “rumput lis-lis”, “kuppaimeni” in India and “t’ie han tsai” in China (Kirtikar and Basu, 1975).

This plant is used as diuretic, anthelmintic and for respiratory problems such as bronchitis, asthma and pneumonia (Varier, 1996). The roots of *A. indica* is used as laxative and leaves for scabies and other cutaneous diseases (Perry, 1980). Major phytochemicals identified from *A. indica* are acalyphine, cyanogenic glycoside, inositol, resin, triacetomamine and volatile oils (Winter and Griffith, 1998). This plant has been used extensively in herbal medicine in many tropical and sub tropical countries (Kirtikar and Basu, 1975; Ramachandran, 2008).

Previous studies on *A. indica* revealed that this plant has antibacterial activity against several gram positive bacteria (Govindarajan et al., 2008; Krishnaraj et al., 2010). Others have shown that plants in the same genus has potential anti-microbial properties (Alade and Irobi, 1993). Recently, Rahman et al. (2010) reported *A. indica* having analgesic and anti-inflammatory effects. In Malaysia, *A. indica* is used for generations for the treatment of superficial fungal and several other bacterial infections (Abdul Rahman, 1996). Thus, the objective of this current study was to evaluate the antibacterial and antifungal activities of water, ethanol and chloroform extracts of *A. indica* and compare the anti-microbial activity with standard antibiotics and antifungal drugs.

MATERIALS AND METHODS

Leaves of mature *A. indica* plants (5 kg wet weight) were collected in the State of Selangor (Western Malaysia) and identified. A

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Table 1. Antibacterial activity of *A. indica* extracts and standard antibiotics.

Samples	Concentration (mg/ml)	Bacteria			
		E. coli	***S. enteriditis***	***S. aureus***	***B. subtilis***
Water	10	-	-	7.3 ± 0.4^{a}	8.9 ± 0.2^{a}
	20	-	-	14.2 ± 1.0^{b}	12.1 ± 2.1^{b}
	30	11.2 ± 0.7^{b}	10.1 ± 0.9^{a}	23.8 ± 2.1^{c}	20.7 ± 2.6^{c}
Ethanol	10	-	-	6.5 ± 0.3^{a}	-
	20	7.1 ± 0.1^{a}	-	10.7 ± 0.9^{a}	11.0 ± 1.3^{a}
	30	12.7 ± 0.3^{b}	9.3 ± 0.2^{a}	14.3 ± 0.2^{b}	12.3 ± 0.7^{ab}
Chloroform	10	-	-	9.2 ± 0.5^{a}	-
	20	-	-	-	-
	30	-	-	-	-
Penicillin G	10	-	15.0 ± 2.0^{b}	37.0 ± 4.2^{d}	8.8 ± 0.3^{a}
Chloramphenicol	30	20.3 ± 1.6^{c}	22.7 ± 1.6^{cd}	23.2 ± 1.6^{c}	22.3 ± 0.9^{c}
Enrofloxacin	5	26.0 ± 1.0^{c}	28.0 ± 1.2^{d}	25.4 ± 1.2^{c}	25.0 ± 1.3^{c}
Ampicilin	10	-	20.7 ± 0.6^{c}	40.3 ± 5.7^{e}	10.3 ± 0.6^{a}

Values are mean ± sd (mm) of 4 separate experiments. – No inhibition zone. $^{a-e}$ Means within a column with different superscripts differ significantly ($p \leq 0.05$) using ANOVA and Duncan multiple post test.

voucher specimen (Voucher number SK 1631/2007) has been deposited at the Phytomedicinal Herbarium, Institute of Bioscience, Universiti Putra Malaysia. Leaves of *A. indica* were washed, oven dried at 45°C overnight, then grounded into powder form and extracted using Soxhlet apparatus with either chloroform, ethanol or distilled water as solvent for 12 h. The solvent was concentrated under vacuum using a rotary evaporator. The yields were 2.57, 4.25 and 7.9% respectively. The solid residues were stored at -20°C prior to use.

Sterile 6.0 mm diameter blank discs (Oxoid, UK) were used to impregnate four different dilutions of the extracts as follows: 0, 10, 20 and 30 mg/mL extract (n = 4/extract). Discs were stored at –5°C prior to use. Tests were performed by the disc diffusion method (Somchit et al., 2004) and experiments were conducted four separate times.

Bacteria (*Escherichia coli*, *Salmonella enteritidis*, *Staphylococcus aureus*, *Bacillus subtilis*) and fungi (*Candida albicans*, *Candida tropicalis*, *Microsporum canis*, *Aspergillus fumigatus*) used in this study were from clinical isolates and identified at the Department of Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia. Detailed method was published previously (Somchit et al., 2003). These micro-organisms are commonly seen in both human and veterinary medicine in Malaysia.

Commercial antibiotics disc which consists of penicillin G (10 mg/ml), chloramphenicol (30 mg/ml), enrofloxacine (5 mg/ml) and ampicilin (10 mg/ml) were used as reference. Standard antifungal drugs of ketoconazole, itraconazole and fluconazole diluted in dimethyl sulfoxide were impregnated onto sterile blank discs with the concentration of 30 mg/ml respectively.

The results are presented as mean±standard deviation (SD). All data obtained were analyzed using One-way analysis of variance (ANOVA) with Duncan post hoc test using SPSS v. 17 and the result will be considered significant if $p < 0.05$.

RESULTS AND DISCUSSION

Antibacterial activity of *A. indica* is listed in Table 1 and Figure 1. All extracts of *A. indica* showed varying degrees of antibacterial activity against all microorganisms tested. The gram positive bacteria are more susceptible than the gram negative bacteria. These different antibacterial activities could be due to the nature and concentration of antibacterial compounds plus its/their mode of action (Tortora et al., 2001). Polar extract (water) and the semi-polar extract (ethanol) revealed more potent antibacterial activity than the non-polar extract chloroform. The antibacterial activity of water extract at 30 mg/mL against *S. aureus* and *B. subtilis* was statistically ($p > 0.05$) similar to the control antibiotics chloramphenicol and enrofloxacin. Interestingly, this activity was more potent than penicillin G and ampicillin (Table 1).

There are many reports of plants in the family Euphorbiaceae possessing anti-microbial activity (Perez et al., 1997; Awoyinka et al., 2007; Falodun et al., 2008). Interestingly, Irobi et al. (1994) reported that water and ethanol extracts of *Bridelia ferruginea* (Euphorbiaceae) produced *in vitro* antimicrobial activities mainly against bacteria against hospital strains similar to this current study. They concluded from their preliminary phytochemical analysis that phenols and tannins detected in the extracts may contribute to the antimicrobial effect. This may be the reason why *A. indica* also showed similar anti-microbial activity. Indeed, previous study on *A. indica* revealed this plant has anti-bacterial property against other bacteria (Govindarajan et al., 2008).

The antifungal activity of *A. indica* is shown in Table 2 and Figure 2. Only the non-polar extract showed antifungal action and at 30 mg/mL chloroform extract, the activity was statistically similar to the antifungal drug

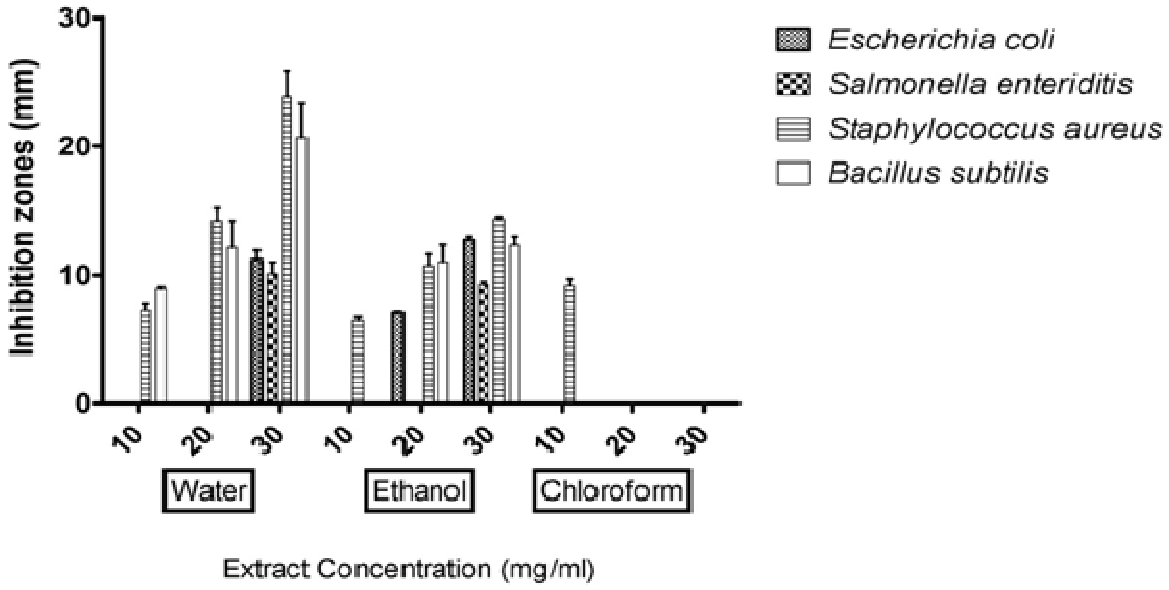

Figure 1. Antibacterial activity of *Acalypha indica* extracts. Values are mean ± S.d (mm) of 4 separate experiments.

Table 2. Antifungal activity of *A. indica* extracts and standard antifungal drugs.

Sample	Concentration (mg/ml)	Fungi			
		C. albicans	***C. tropicalis***	***M. canis***	***A. fumigatus***
Water	10	-	-	-	-
	20	-	-	-	-
	30	-	-	-	-
Ethanol	10	-	-	-	-
	20	-	-	-	-
	30	8.7 ± 0.6 [a]	-	9.3 ± 0.6 [a]	-
Chloroform	10	-	-	-	-
	20	8.3 ± 2.3[a]	-	9.3 ± 0.6 [a]	-
	30	12.7 ± 3.7 [b]	10.3 ± 1.1 [a]	13.0 ± 1.5 [b]	8.7 ± 1.4 [a]
Ketoconazole	30	13.3 ± 1.8 [b]	-	-	17.7 ± 2.6 [b]
Fluconazole	30	21.3 ± 0.7 [c]	15.7 ± 3.6 [b]	17.0 ± 1.9 [c]	-
Itraconazole	30	25.6 ± 1.7 [c]	17.0 ± 1.2 [b]	19.2 ± 3.0 [c]	22.0 ± 1.1 [c]

Values are mean±Sd (mm) of 4 separate experiments. – No inhibition zone. [a-c] Means within a column with different superscripts differ significantly ($p \leq 0.05$) using ANOVA and Duncan multiple post test.

ketoconazole. There is no previous study conducted evaluating the anti-fungal property of *A. indica*. Oksana et al. (2007) reported that flavonoids (quercetin, kaempferol, isorhamnetin, isoquercitrin), phenolic derivatives (gallicin, gallic, syringic, and caffeic acids), and coumarin (scopoletin) have potent anti-fungal activity against *Microsporum* spp. and *Trichophyton* spp. Interestingly, Ogunwenmo et al. (2007) stated that Euphorbiaceae showed high concentrations of flavonoids, phenols and alkaloids. These may be responsible for the potent anti-fungal activity of *A. indica* reported in this current study.

Results obtained revealed potent selective antimicrobial activity in all extracts of *A. indica*. The water and ethanol extracts exhibited better antibacterial activity against gram positive bacteria and this was as potent as several commercial antibiotics. The chloroform extract however, revealed antifungal activity mainly against *M. canis* and *C. albicans*. This antifungal activity was as

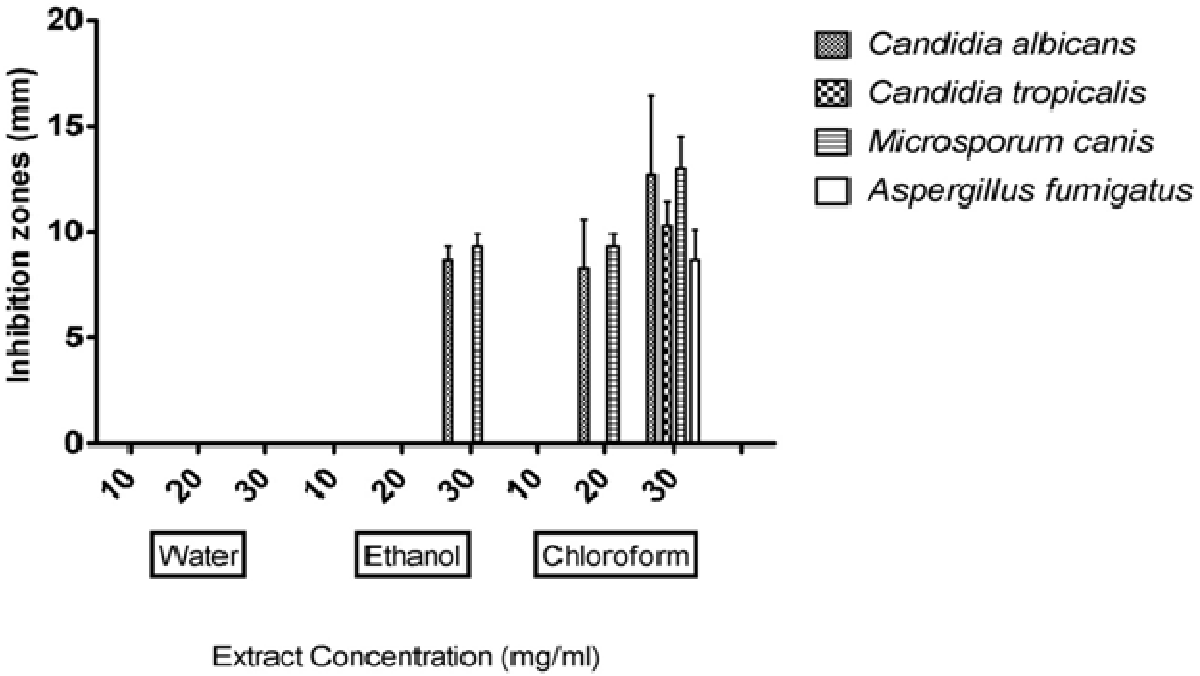

Figure 2. Antifungal activity of *Acalypha indica* extracts. Values are mean ± Sd (mm) of 4 separate experiments.

potent as ketoconazole and fluconazole. Hence, they can be used in treatment of infectious diseases caused by tested strains and potential antimicrobial agents may be developed. However, further studies must be performed to identify the specific principles responsible for the anti-microbial activity of *A. indica*.

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Full Length Research Paper

Molecular and biochemical characterization of surfactin producing *Bacillus* species antagonistic to *Colletotrichum falcatum* Went causing sugarcane red rot

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***Bacillus* species suppress phytopathogens by producing lipopeptide antibiotics, hydrolytic enzymes, siderophores and other secondary metabolites. Three bacterial strains *Bacillus subtilis* NH-100 (EU627167), *B. subtilis* NH-160 (EU627169) and *Bacillus* sp. NH-217 (EU627170) with proven ability to suppress red rot disease on sugarcane plants were further characterized to elucidate the multiple modes of action involved in their biocontrol activity. Plate assays pointed out the production of protease and antibiotics. Lipopeptide antibiotic surfactin was detected in the culture extract of *B. subtilis* NH-160 and *Bacillus* sp. NH-217 through LC-MS (Liquid chromatography - mass spectrometry). These results were further supported by identifying the presence of *sfp* and *srfAC* genes of surfactin biosynthetic operon using specific polymerase chain reaction (PCR) primers. Two strains *B. subtilis* NH-160 and *Bacillus* sp. NH-217 were further analyzed for their survival in compost which successfully retained consistency in their population 4.0 - 5.0 log $CFUg^{-1}$ after 14th day. Bacteria capable of suppressing pathogens and maintaining their population by competing with other microbes can be successfully utilized as biopesticide for sustainable organic farming.**

Key words: *Bacillus*, biocontrol, surfactin, sugarcane, red rot.

INTRODUCTION

The excessive use of chemical pesticides has caused soil pollution and detrimental effects on human beings. Accordingly, the use of eco-friendly biocontrol agents of plant pathogens has been greatly accentuated (Correa et al., 2009). These agents suppress pathogens by different mode of actions (Liu et al., 2009).

Bacillus species are outstanding biocontrol agents with proven excellent characteristics like effective root colonization, versatile activity against multiple pathogens and promising ability to sporulate (Kloepper et al., 2004; Romero et al., 2004). This assures their ubiquitous occurrence in the environment and use in the framework of integrated disease management (Correa et al., 2009). Cyclic lipopeptides of the surfactin, iturin and fengycin families are one of the important metabolites produced by *Bacillus* species and their involvement in disease control have been widely reported (Ongena and Jacques 2008). They impart successful biocontrol activity by direct suppression of phytopathogens and reinforcing of the potential host plant through stimulating induced systemic resistance phenomenon. These lipopeptides are the products of multimodular enzyme complexes called non-ribosomal peptide synthetases (NRPs). These molecules are usually synthesized as isoforms with variation in the fatty acid chain length and peptide part. Surfactin, exhibiting strong antibiotic activity, consists of heptapeptides containing a β-hydroxy fatty acid with 13 to

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15 carbon atoms. Genes involved in surfactin biosynthesis are encoded by the *srf* operon which consist of four open reading frames (ORFs) designated as *srf*A-A, *srf*A-B, *srf*A-C and *srf*A-D (Peypoux et al., 1999; Yakimov et al., 1998). These open reading frames contain seven modules organized in a linear array. Similarly, fengycin biosynthesis is encoded by an operon containing five ORFs viz *fen*A, *fen*B, *fen*C, *fen*D and *fen*E while the ORFs *itu*A, *itu*B, *itu*C and *itu*D are involved in iturin biosynthesis (Steller et al., 1999). In our previous work, we have reported that three *Bacillus* strains isolated in our laboratory suppressed red rot disease caused by *Colletotrichum falcatum* on sugarcane plants, and also showed good abilities to colonize sugarcane roots (Hassan et al., 2010). The aims of this study were to detect the production of various antifungal metabolites produced by these promising biocontrol agents to elucidate the underlying mechanisms responsible for their antagonistic activity towards *C. falcatum* and evaluate their survival in compost to formulate these agents as biopesticide.

MATERIALS AND METHODS

Bacillus strains and growth conditions

The antagonistic strains *Bacillus subtilis* NH-100 (EU627167), *B. subtilis* NH-160 (EU627169), *Bacillus* sp. NH-217 (EU627170) and red rot pathogen *C. falcatum* were isolated from the sugarcane plant and rhizospheric soil (Hassan et al., 2010). Isolates were grown at 37°C on Luria Bertani (LB) agar routinely and preserved in 20% glycerol at -80°C for a long time. *C. falcatum* was cultured on potato dextrose agar (PDA).

Production of extracellular metabolites, HCN, protease and antibiotics

Production of extracellular metabolites was determined as described by Montealegre et al. (2003) with certain modifications. The antagonistic isolates were grown in sterile nutrient broth (NB) for 6 days on a rotary shaker at 175 rpm at 28±2°C. Cell free supernatant of these strains obtained by centrifugation was mixed with PDA (Oxoid chemicals) at the rate of 5, 15 and 25% (v/v). LB instead of cell free supernatant was added in control PDA plates. A 5 mm mycelial disk of *C. falcatum* was kept at the centre of petri dish and incubated at 28±2°C. Production of diffusible and volatile antibiotics was determined as described by Montealegre et al. (2003). Mycelial growth of the fungus was observed after 5 - 6 days and percentage inhibition was determined using the following formula:

$$\% \text{ Inhibition} = [1 - (\text{Fungal growth/control growth})] \times 100$$

Production of protease and HCN was tested according to the method of Denizci et al. (2004) and Sun et al. (2006) respectively.

LC-MS analysis

The presence of lipopeptide antibiotics such as surfactin, fengycin and iturin etc in the supernatants of *B. subtilis* strain NH-160 and *Bacillus* sp. NH-217 were determined by liquid chromatography mass spectrometry (LC-MS) analysis. Bacterial strains were grown in Landy medium and incubated at 28 ±2°C for 96 h in an orbital shaker at 250 rpm. Cell free supernatant was obtained by centrifugation at 13000 rpm for 10 min passing through 2 µm syringe filter. The supernatants were extracted with methanol (Ahimou et al., 2000) while methanolic fractions were analyzed by LCT (Liquid Chromatograph Time of Flight Mass Spectrometer with Electrospray sample introduction; Waters Incorp).

Detection of genes involved in antibiotic synthesis

Genomic DNA was isolated from the *Bacillus* strains by standard protocols (Maniatis et al., 1982). Primers used for detecting genes involved in lipopeptide antibiotics (surfactin, fengycin, iturin) synthesis are described in Table 1. New primers were designed by retrieving the reported sequences from GenBank (http://www.ncbi.nih.gov) and aligning at clustal W (www.clustalw). PCR amplifications were carried out in 50-µL reaction mixtures containing PCR buffer (Qiagen Inc.), 1.5 mM $MgCl_2$, 1.5 U Taq DNA polymerase (Qiagen Inc.), 40 µg of each forward and reverse primer, 200 µM each of dATP, dGTP, dCTP, and dTTP and 2 µL of template DNA (approximately 100 ng of bacterial genomic DNA). The amplifications were performed using a thermocycler (Eppendorf) with the cycle conditions mentioned in Table 1. Amplified products were separated by electrophoresing on 1.2% agarose gel stained with ethidium bromide and visualized under ultraviolet light. Desired products were eluted from gels using the gel extraction kit (Qiagen Inc) and sequenced either directly or by cloning in TOPO T/A cloning vector (*Invitrogen*) on the sequencer ABI at the Central Genomics and Sequencing facility, University of Sheffield, UK. Nucleotide sequences were identified using the basic local alignment search tool (BLAST) and GenBank nucleotide data bank from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih. gov/).

Survival of antagonistic *Bacillus* strains in compost

Development of soil microcosms

The microcosm was developed by filling the 50-mL falcon tubes with 20 g non-sterilized soil-based compost obtained from the Department of Animal and Plant Sciences, University of Sheffield, UK. The falcon tubes were placed at temperature of 23±2°C.

Introduction of antagonistic strains to compost

Single colonies of *B. subtilis* NH-160 and *B.* Sp. NH-217 were cultured in LB for 24 h at 30°C on an orbital shaker at 200 rpm. The cells (10^9 CFU m/L) were mixed with 25% Ringer solution (2.25 g/LNaCl, 0.15 g/l KCl, 0.12 g/l $CaCl_2$, 0.05 g/l Na_2CO_3). The washed cells were mixed with compost in each falcon tube with a concentration of 10^8 CFU/g. The control treatment received ringer solution only without cells.

Enumeration of antagonistic Bacillus

The compost was sampled periodically on day 0th, 1st, 2nd, 4th, 7th, 14th and 21st after bacterial inoculation and *Bacillus* species were isolated according to the method of Hart et al. (1998). The inoculated strains were identified on the basis of their colony morphology, antagonistic activity and the presence of molecular marker that is, *sfp* gene.

Table 1. The primers used in this study and PCR profile.

Primer	Sequence	Antibiotics	Product size/ Amplification	PCR profile	Reference
Sfp-f	ATG AAG ATT TAC GGA ATT TA	Surfactin	675/Yes	95℃ - 5 min. 30 cycles (94℃ for 1 min, 48℃ for 1 min, 72℃ for 1 min) and 72℃ -5 min.	Hsieh et al. (2004)
Sfp-r	TTATAAAAGCTCTTCGTACG				
SrfA-f	GAT CAG GTT CAR GAY ATG TAT TA	Surfactin	3700/Yes	95℃ - 5 min. 30 cycles (94℃ for 1 min, 55℃ for 3 min,72℃ for 3 min) and 72℃ -15 min.	This study
SrfA-r	AGC ATT TCT GCG TGY GTK CC				
Fend-f	TCC TGC AGA AGG AGA AGT GA	Fengycin	281/No	95℃ - 5 min. 30 cycles (94℃ for 1 min, 54℃ for 1 min,72℃ for 1 min) and 72℃ -5 min.	This study
Fend-r	CGT CTT CCG TTT CTA AAA TGG T				
BmD-f	AAT CTT GCC TTT TTA TTT CCK G	Iturin	1200/No	95℃ - 5 min. 30 cycles (94℃ for 1 min, 48℃ for 1 min,72℃ for 1 min) and 72℃ -5 min.	This study
BmD-r	TTA TTT TAA AAT CCG CAA TTS TTC C				

* Degeneracy code: S= C or G, Y= A or T, K= G, T.

Table 2. Biocontrol characteristics of *Bacillus* strains isolated from sugarcane rhizosphere.

Strains	Inhibition of *C. falcatum* (%)					Survival in soil based compost							Production of antifungal metabolites				
	*Extracellular metabolites			**Antibiotics													
	5%	15%	25%	Dif	Volt	0th	1st	2nd	4th	7th	14th	21st	HCN	Prt	srf	Itr	Fen
B. subtilis NH-160	12.3 b	24.3 b	40b	43b	15.2 b	7.7 a	7.8a	7.6a	6.5a	5.6a	5a	5a	--	++	+	-	-
Bacillus sp. NH-217	18a	32a	48a	52a	27.5a	7.5a	7.5a	7.1a	5.5b	4.1b	4.2a	4.2a	--	--	+	-	-
B. subtilis NH-100	10 b	25 b	41b	39b	13.5c	ND	ND	ND	ND	ND	ND	ND	--	++	+	-	-
Control	0c	0c	0c	0c	0d	ND	ND	ND	ND	ND	ND	ND	++	+++	-	-	-
P.flourescence CHA0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-	-	-

HCN; SRF; ITR; FEN = + production; -- no production; PRT: ++ = zone = 5 mm; +++ = zone > 5 mm. Values are mean of three replicates and those bearing same letter are significantly same according to Duncan' s multiple range test at $p < 0.05$.
*LSD $_{0.05}$ (5%) = 4; LSD $_{0.05}$ (15%) = 3.9; LSD $_{0.05}$ (25%) = 1.9 ** LSD $_{0.05}$ (dif) = 7 and LSD $_{0.05}$ (vol) = 2.1.

Statistical analysis

All the data were subjected to analysis of variance (ANOVA) using computer statistical package MSTAT-C. Data values of bacterial colonies were log-transformed before analysis.

RESULTS

Detection of biocontrol determinants

Extracellular metabolites, diffusible and volatile antibiotics, HCN and hydrolytic enzymes are the major determinants of biocontrol activity. Only 2 out of 4 strains produced protease while all the strains were negative for HCN production (Table 2). The strain *Bacillus* sp. NH-217 showed maximum inhibition of fungus mycelium up to 53% by producing diffusible antibiotics and 28% through volatile antibiotic production followed by the strain *B. subtilis* NH-160 which caused 43 and 15% inhibition by producing diffusible and volatile antibiotics respectively. Inhibition of fungus by extracellular metabolites and antibiotics of the strains is shown in Table 2.

Detection of lipopeptide antibiotics produced by *Bacillus* strains

LC-MS analysis (Figure 1) of the crude lipopeptide extracts of strains *B. subtilis* NH-160 and *Bacillus* sp. NH-217 yielded a parent peak at m/z 542 along with the intense fragments at m/z 549 indicating the presence of surfactin antibiotic as detected by using KEGG (Kyoto Encyclopedia of

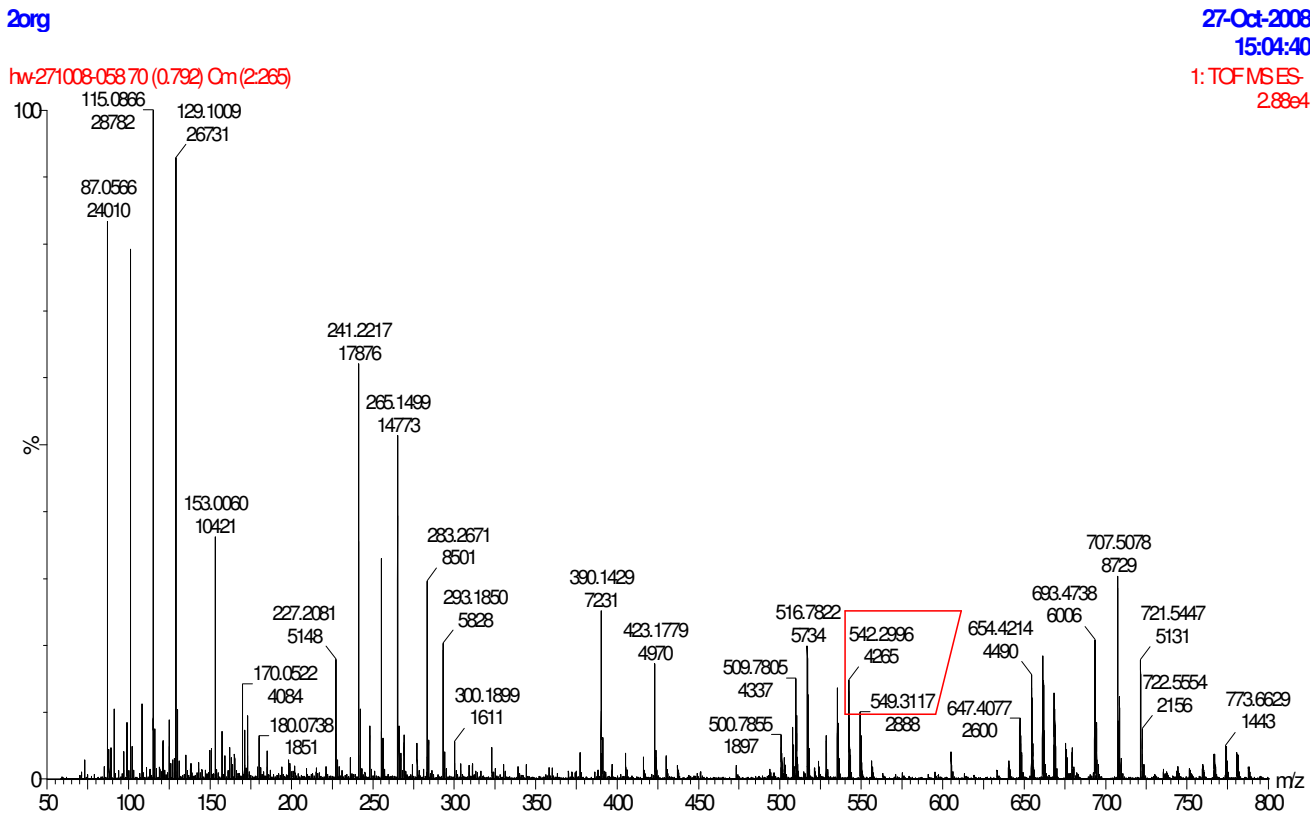

Figure 1. LC-MS analysis of the lipopeptide antibiotics produced by *Bacillus subtilis* NH-160. Peak at m/z 542 along with the intense fragments at m/z 549 indicating the presence of surfactin antibiotic as detected by using KEGG pathway database.

Genes and Genomes) pathway database.

Amplification of antibiotic related genes

Four primer pairs were used for the amplification of genes involved in the antibiotics biosynthesis from the *Bacillus* strains in this study. Primers *sfp*-f and *sfp*-r amplified a 629 bp of the *sfp* gene (Figure 2a) encoding 4'-phosphopantetheine transferase involved in surfactin biosynthesis from the *B. subtilis* NH-100, *B. subtilis* NH-160 and *Bacillus* sp. NH-217. A 3700 bp of *srf* AC gene (Figure 2b) involved in surfactin biosynthesis were also amplified from the *B. subtilis* NH-100 and *B. subtilis* NH-160 by using primers *srfA*-f, *srfA*-r. There was no amplification for *fenD* and *BmD* genes related to fengycin and Iturin antibiotic. Accession numbers of the partial nucleotide sequences of *sfp* and *srf* AC obtained from GenBank are FJ711067, FJ711068, FJ711069, FJ711070 and FJ711071.

Survival of *Bacillus* strains in un-sterilized compost

Population of *B. subtilis* NH-160 decreased until 14th day from 7.7 - 5 (log CFU/g) and then remained constant even at the 21st.day (Table 2) while *Bacillus* sp. NH-217 showed a decreased trend in the number of colonies from 7.5 - 4.1 (log CFU g^{-1}) during the initial seven days which afterwards persisted in this population level until the 21st day (Table 2).

DISCUSSION

Biocontrol activity of *Bacillus* strains against multiple plant pathogens have been widely reported and well documented (Correa et al., 2009; Kloepper et al., 2004). Their success as biocontrol agent is associated with the prominent property of producing lipopeptide antibiotics which exhibit wide spectrum antifungal activity, regulate attachment of microbes with various surfaces and enhance survival in the habitat (Sun et al., 2006). In our present study, the cell free supernatants of three *Bacillus* strains have effectively inhibited the mycelial growth of *C. falcatum*. Percentage inhibition of red rot pathogen was similar to the inhibition level of other pathogens by antagonistic *Bacillus* strains reported previously but our experiments were based on supernatants and sugarcane red rot pathogen rather than vegetative cells and other

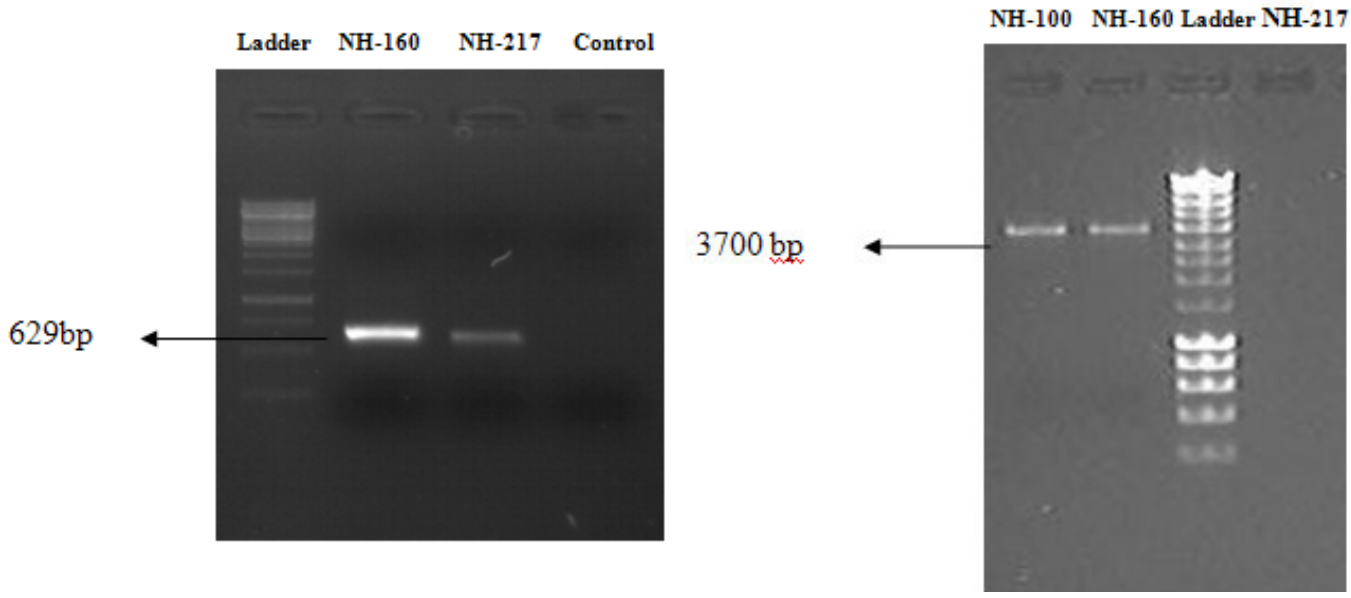

Figure 2. Amplification of (a) 629 bp *sfp* gene involved in surfactin synthesis (b) 3700 bp *srf*A gene involved in surfactin synthesis.

pathogenic fungi (Romero et al., 2004). Further plate assays indicated production of protease, diffusible and volatile antibiotics.

However, production of siderophores could not be assessed by plate assay due to inability of these strains to grow on the media described by Schwyn and Neilands., (1987). These findings supported the agreement with previous reports that antagonistic bacteria suppress pathogens by producing hydrolytic enzymes and antibiotics (Chen et al., 2008; Liu et al., 2009). Inability of strains to produce HCN will make them biocontrol agents of choice as HCN imposes negative effects on plant growth (Schippers et al., 1990). The LC-MS analysis of cell free supernatant attributed the presence of only surfactin- a lipopeptide antibiotic with proven antifungal activity against many pathogens (Yakimov et al., 1998). The *Bacillus* strains exhibiting pronounced antagonistic activity on sugarcane plants *in vivo* (Hassan et al., 2010) were found to be surfactin producer. These findings make the strains *B. subtilis* NH-100, *B. subtilis* NH-160 and *Bacillus* sp. NH-217 valuable and rare as most reported strains are co-producers of iturin and fengycin along with surfactin. Moreover, co-production of these lipopeptides results in purification problems. As surfactin is an industrially important compound, so a strain producing only surfactin would be really ideal.

Genes encoding surfactin synthesis are common to numerous antagonistic *Bacillus* sp used as commercial biopesticide and strains carrying such genes possess a pronounced capability to suppress the soil-borne pathogens (Joshi and Gardener, 2006). Comprehensive genetic analysis of vegetative cells also proved the occurrence of *sfp/ srfAC* genes and absence of *fend*, *bmd genes* involved in surfactin, fengycin and iturin synthesis respectively in *B. subtilis* NH-100, *B. subtilis* NH-160 and *Bacillus* sp. NH-217.

The coherence of results by chemical and genetic analysis has further strengthened the authenticity of LC-MS analysis coupled with identification of compounds using KEGG pathway (Hashimoto et al., 2006). Survival of the two antagonistic strains *B. subtilis* NH-160 and *Bacillus* sp. NH-217 was investigated in soil based compost for 21 days. Population was monitored by culture dependent assay by taking advantage of sporulation ability of these strains which were easily cultured on media after exposing the sample at high temperature. The inoculated strains were further confirmed by detecting *sfp* gene as a molecular marker. Both strains showed good survival until the 21st day. Population of strain NH-160 decreased from day 0 to day 14 but it remained constant from day 14 to day 21, while the population dynamics of NH-217 decreased over day 7 and remained constant afterwards (Figure 2). The stability in survival of *Bacillus* strains may be attributed to their ability to sporulate and produce surfactin; a characteristic which supports their persistence under extreme conditions (Kloepper et al., 2004; Romero et al., 2004). Initial decline in population may be due to competition with the micro flora present in un-sterilized compost. This indicates the strong competitive ability of surfactin producing strains *B. subtilis* NH-160 and *Bacillus* sp. NH-217 which has also been proved previously in root colonization experiments (Hassan et al., 2010). Moreover, it can be predicted that these strains will maintain a high level of population in sterilized formulations.

Results suggest that the strains *B. subtilis* NH-100, *B. subtilis* NH-160 and *Bacillus* sp. NH-217 are unique in their characteristics like being antagonistic to *C. falcatum,* deficient in HCN production and producer of only surfactin lipopeptide. Hence, these strains can be valuable candidates in context to develop biopesticide for sugarcane red rot. However, further studies are required to formulate these strains in suitable carrier material and explore their potential under field conditions.

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Full Length Research Paper

The role of *hp1165* gene on the efflux-mediated resistance to tetracycline in clinical isolates of *Helicobacter pylori*

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Tetracycline-resistant (Tet^R) *Helicobacter pylori* isolates have emerged in many parts of the world. We have previously demonstrated that among the mechanisms involved in the resistance of *H. Pylori* to Tet, its active efflux may be an important mechanism. This work, aimed to determine whether presence and/or expression of *hp1165* are associated with efflux-mediated resistance to Tet in clinical strains of *H. pylori*. Twenty five Tet^R strains including seven low level-resistant, eight intermediately-resistant, and ten high-level resistant strains/or mutants, of which 21 displayed the active efflux ability for Tet, were investigated. They were screened for the presence of mutation (s) in *16S rRNA* at 965-967 position and for the presence of *hp1165* gene. Detection of *hp1165* gene transcription /or gene expression was performed by RT-PCR Two low-level Tet^R strains displaying no efflux ability, that contained mutation (s) at 965-967 position of *16S rRNA*. Ten out of 21 Tet^R strains displaying active efflux abilities, that contained hp1165 gene. Their PCR product was similar to that of 26695 standard strains, susceptible to Tet. RT-PCR was positive for five out of them however; their product size was approximately 100 bps smaller than that of 26695 strains. Regarding to the results of PCR and RT-PCR, *hp1165* plays a role in the active efflux of Tet in resistant strains. A post-transcriptional regulation step may be involved in the expression of *hp1165* gene in Tet^R strains.

Key words: *Helicobacter pylori*, tetracycline, resistance, efflux, *hp1165* gene.

INTRODUCTION

Helicobacter pylori isolates resistant to Tet have been reported in many parts of the world especially in Asian countries (Dailidiene et al., 2002; Khan et al., 2008; Ribeiro, 2004; Trieber, 2002). Multiple mechanisms participate in the development of resistance to Tet in both gram negative and gram positive bacteria (Chopra et al., 2001; Aminove et al., 2001). In *H. pylori*, the most frequently reported mechanism have been mutations in the 16S rRNA tetracycline-binding site that corresponds to the triple base-pair substitution AGA (965–967) to TTC (Trieber and Taylor, 2002; Dailidiene et al., 2002; Gerrits et al., 2003; Ribeiro et al., 2004). Presence of such mutation affects the affinity of the drug-ribosome interaction and reduces the efficacy of tetracycline as a translational inhibitor. Other mechanism involved in the resistance of *H. pylori* to Tet may be defect in its uptake, and/or increase in its efflux (Gerrits et al., 2003; Glocker et al., 2005; Li and Dannelly, 2006; Wu et al., 2005; Bina et al., 2000). There are several classes of Tet efflux pumps including single subunits containing multiple transmembrane segments that are specific for the active transport of Tet (Chopra et al., 2001; Aminove et al., 2001; Nelson, 2002; Agerso and Guardabassi, 2005).

A putative gene for Tet efflux pump of this kind, hp1165, has been identified in the genome of *H. pylori* 26695. The *hp1165* gene displays 98% identity to the *jhp1092* gene in *H. pylori* J99 and 40 to 44% homology to the multidrug transporters in *Campylobacter jejuni* and 49.8% homology with tetracycline efflux gene *tetA (P)* in

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Clostridium perfringens (Kennan et al., 1997; Bannam et al., 2004). Furthermore, previous works have suggested that *hp1165* is prevalent in *Helicobacter* laboratory strains and clinical isolates (Li and Dannelly, 2006). However, the real role of HP1165 in tetracycline resistance of *H. pylori* remains unknown. Using clinical Tet^R isolates and high-level-resistant mutants, the authors have previously reported that proton motive force-dependent efflux plays an important role in the resistance of clinical isolates of *H. pylori* to Tet (Anoushiravani et al., 2009). In this work, they sought to determine whether the presence and/or expression of *hp1165* are associated with efflux-mediated resistance to Tet in low-, intermediate-, and high-level Tet^R clinical strains of *H. pylori.* In these strains presence of mutation (s) in the *16S rRNA* gene at AGA (965–967) position was also evaluated.

MATERIALS AND METHODS

H. pylori strains and growth conditions

Bacteria were routinely cultured on modified Campy-blood agar (Merck), as described previously (Falsafi et al., 2004; Falsafi et al., 2007). The clinical strains used in this work included 20 Tet^R strains (MIC above 2 mg L^{-1}), and two strains susceptible to Tet (MIC ≤ 2 mg L^{-1}). Five Tet^R mutants obtained in our previous work were also included in this study. The *H. Pylori* 26695 standard strain was used as control for detection of *hp1165* gene. Based on the results of MIC obtained by agar dilution method, these strains have been classified into three groups of: low-level (MIC 4 to 8 mg L^{-1}), intermediate-level (MIC 16 mg L^{-1}), and high-level (MIC 32 to 64 mg L^{-1}) Tet^R strains (Anoushiravani et al., 2009).

Measurement of antibiotic accumulation

Accumulation of Tet was measured in the presence and absence of carbonyl cyanide-m chlorophenyl hydrazone (CCCP) as described previously (Anoushiravani et al., 2009). Briefly, two days cultures were harvested and resuspended in sodium phosphate buffer (50 mM, pH 7.2) with a density approximating 1.2×10^9 by CFU. Antibiotic uptake assay was initiated by addition of TET to bacterial cell suspension at a final concentration of 20 µg/ml. At 10 min after antibiotic addition, CCCP (200 µM) was added to one–half of the reaction mixture and the other half was used as control. After 20 min, the collected samples were diluted in 1.5 ml ice–cold sodium phosphate buffer and centrifuged. The supernatants were discarded and the pellets were resuspended in 0.1 M glycine hydrochloride (pH 3.0), and shaken at 25°C to extract the accumulated antibiotics. After 16 h, it was centrifuged and the supernatant was used to measure the fluorescence of antibiotics with a Shimadzu RF 5000 spectrofluorometer (Shimadzu Scientific Instruments, Inc., Colombia, Md) at excitation and emission wavelengths of 400 and 450 nm. The concentration of Tet in the supernatant was obtained by comparison with a standard curve of Tet in 0.1 M glycine hydrochloride (pH 3.0). The results were expressed as nanograms of Tet per milligram (wet weight) of bacteria. An increase of ≥ 2 in the concentration of accumulated Tet in the presence of CCCP was interpreted as presence of active efflux ability.

DNA preparation and PCR

Bacterial DNA was prepared according to the previously described procedure (Falsafi et al., 2009). The detection of *16S RNA* gene mutation was performed by using the specific primers (Faza Biotech Inc, Iran) based on published sequences of *H. pylori* such that the 3´-end of the forward primer exactly matched with three nucleotides in 965-967 position of 16S rRNA gene. They were: 5´-GCA TGTGGTTTAATTCGAAGA -3´ (Forward) and 5´-CTTTGTGCACCCCATTGTA G-3´ (Reverse). The reaction was held at 95°C for 5 min for 1 cycle, followed by 40 cycles with parameters of 94°C for 30 s, 57°C for 1 min, 72°C for 1 min, and 1 cycle of 72°C for 10 min as final extension. The expected product size was 300 bp, and the absence of this product suggested that the 3´-end of forward primer could not match with the tetracycline binding site in *16S rRNA* gene due to a (the) mutation (s) in this position.

The detection of *hp1165*gene was performed by using the specific primers (Faza Biotech Inc, Iran) based on published sequences of *H. Pylori*. These primers were designed for detection of the middle sequences of the gene as described previously (Li and Dannelly, 2006). Their sequences were: 5´-AGGGAGTTCTTTGGGATCGT-3´ (Forward) and 5´-TCCAGACTGAGCGATAA-3´ (Reverse); the predicted product size was 624 bp. The PCR reaction was held at 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, 56°C for 1 min, 72°C for 1 min and 1 cycle in 72°C for 10 min.

Evaluation of *hp1165* gene expression by RT-PCR

To perform RT-PCR, the concentration of high-pure RNA prepared according to the manufacturer's recommendations (Roche, Germany), was measured at 260 nm and the cDNA was synthesized using 1st- strand cDNA synthesis kit for RT-PCR (Roche, Germany). The reaction for cDNA synthesis was held at 25°C for 10 min, 42°C for 60 min and 99°C for 5 min followed by PCR as described above. The PCR-products were visualized by electrophoresis on 1% agarose gel and 15% acrylamide gel with a 100 bp ladder size-marker (Cinnagene, Iran). RT-PCR analysis of *hp1165* was performed on tetracycline resistant, susceptible and 26695 *H. pylori* strains.

RESULTS AND DISCUSSION

PCR reaction for the detection of *16S rRNA* gene was positive for all of the strains except 10II, and 58II, two low-level Tet^R strains (Table 1 and Figure 1). This suggested that in these strains the 3´-end of forward primer could not match with the tetracycline binding site in the *16S rRNA* gene due to a (the) mutation (s) in this position which could affect efficacy of tetracycline binding. DNA prepared from the strains displaying active efflux ability for Tet, amplified normally for *16S rRNA* gene. This suggested that their resistance to Tet was most probably due to their active-efflux ability.

PCR reaction for the detection of *hp1165* gene was positive for seven Tet^R strains and three high-level resistant mutants. Association was observed between the presence of *hp1165* gene in these strains and the ability to pump-out actively Tet (Table 1 and Figure 2). The *hp1165* gene was not detected in the control susceptible strains (1 and 17B), and in strains with no-efflux ability. However, it was also absent in eleven Tet^R strains/or mutants capable to display active-efflux ability for Tet. This suggests that other determinant(s) than *hp1165* may

Table 1. Relationship between Tet-MIC, active efflux ability and *hp1165* in low-, intermediate-, high-level Tet^R and Tet-susceptible strains.

Strains	MIC to Tet	Active efflux [a]	PCR	RT-PCR
			Hp1165	Hp1165
12M	8	+	+	-
15M	8	-	-	-
27M	8	+	-	-
10II[b]	8	-	-	-
18B	8	+	-	-
58II[b]	8	-	-	-
23M	4	+	+	-
19M	16	+	-	-
20M	16	+	+	-
24M	16	+	+	+
25M	16	+	+	-
26M	16	+	+	+
28M	16	+	+	+
21D	16	+	-	-
2N	16	-	-	-
17M	32	+	-	-
18M	32	+	-	-
21M	32	+	-	-
1N	32	+	-	-
3N	32	+	-	-
25M-mut[c]	32	+	+	+
21M-mut[c]	64	+	-	-
23M-mut[c]	32	+	+	-
20M-mut[c]	64	+	+	+
18M-mut[c]	64	+	-	-
1B [d]	0.5	-	-	-
17B[d]	1	-	-	-
26695	2	-	+	+

a: Active efflux ability evaluated as ≥ 2 fold increase in amount of Tet in presence of CCCP. b: strains with mutation in 16S rRNA. c: high-level resistant mutants. d: negative controls.

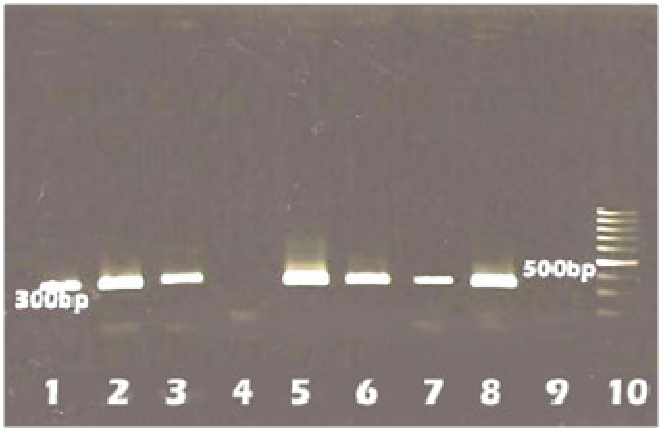

Figure 1. PCR detection of 16S-rRNA gene on 1% agarose gel. **Note:** 1. 12 M, 2. 15 M, 3. 17 M, 4. 17B (susceptible strain), 5. 18 M, 6. 19 M, 7. 20 M, 8. 26695 strain, 9. 10II, 10. Size marker.

be involved in the active-efflux of Tet in *H. pylori*. The *hp1165* gene was detected in low-level resistant, intermediately resistant and in high level-resistant strains/ mutants so, its presence may not be related to the level of resistance to Tet. Comparison of the PCR product between *hp1165*-positive Tet^R strains and 26695 Tet-susceptible strain, revealed that the same size, suggesting that similar DNA sequences were present in them (Figure 2).

RT-PCR was performed for all of the Tet^R strains and for 26695 Tet-susceptible *H. pylori* strain as control for presence of hp1165 gene. The reaction was positive for five out of ten Tet^R strains containing *hp1165* including low, intermediate and high-level resistant ones. This suggested that this gene may not be expressed in all of the strains containing *hp1165* gene, and its expression may be induced under certain conditions. Furthermore, the transcription/and or expression of *hp1165* gene may not be related to the level of resistance. The size of RT-PCR product for 26695 susceptible strains was 624 bps, similar to that of PCR whereas the size of RT- PCR product for the Tet^R strains was approximately 500 and 100 bps smaller than that of their PCR product in 15% acrylamide gel (Table 1 and Figure 3). Using 26695 laboratory strain and its mutant, Li and Dannelly (2006) have first studied the role of *hp1165* gene in the tetracycline resistance. They have proposed that *hp1165* gene is involved in the inducible tetracycline resistance in *H. pylori.*

Regulation of efflux-mediated resistance to Tet in Gram-negative bacteria is usually due to presence of *tetR*, which encodes a repressor protein and blocks *tetA/R* gene transcription in the absence of Tet (Speer et al., 1992; Chopra et al., 2001; Aminove et al., 2001). Several studies have confirmed non involvement of TetR kind of repressor in regulation of efflux genes in *H. pylori* (Nelson et al., 2002; Kutschke and Jonge, 2005; Liu et al., 2008; Morrison et al., 2003). Concerning the regulation mechanisms in *H. pylori*, a post- transcriptional regulation has been observed in the case of flagellin synthesis in *H. pylori* (Douillard et al., 2009), suggesting that this kind of regulation may be frequent in *H. pylori*.

Li and Dannelly (2006) have proposed that the expression of *hp1165* gene may be regulated at translational or posttranslational level. However, they could not observe a difference between the susceptible 26695 strain and its resistant-derivative concerning the result of RT-PCR. The results of this work may suggest the occurrence of a post-transcriptional regulation for the expression of *hp1165* efflux gene in Tet^R strains. As conclusion, the hp1165 gene exhibits a role in the active efflux of Tet in resistant *H. pylori* strains. A post-transcriptional regulation step may be involved in the expression of *hp1165* gene in Tet^R strains. An

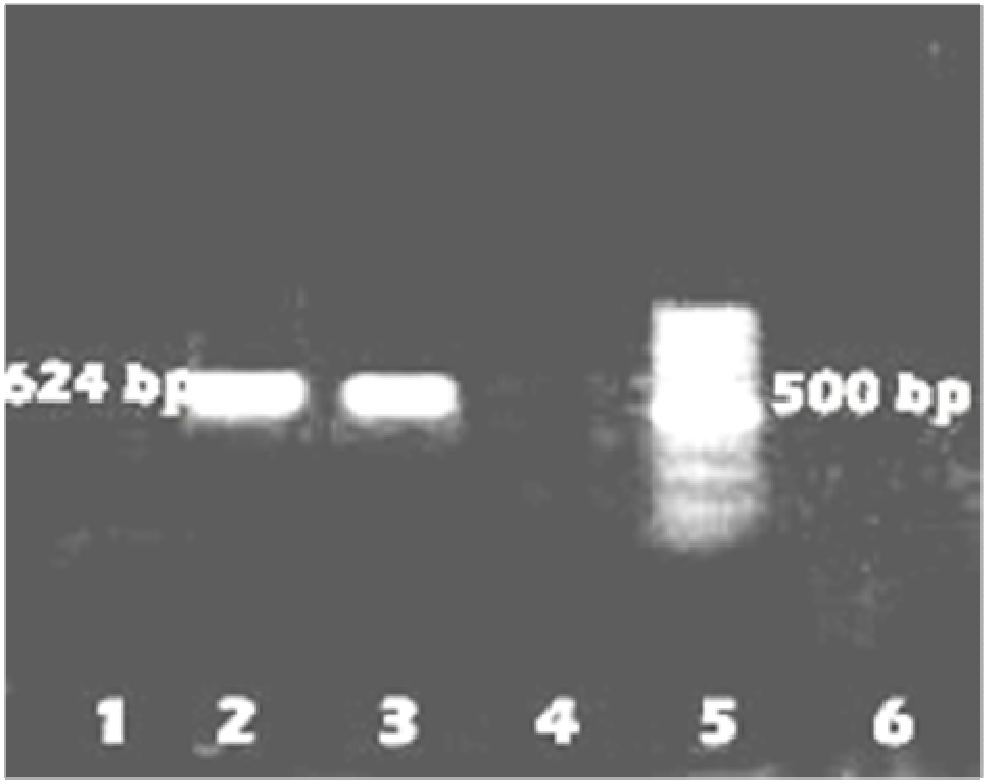

Figure 2. PCR detection of 1165 gene on 1% agarose gel. Note: 1.control, 2. 26695 strain, 3. 25M, 4. 17M, 5. size marker, 6. 10II strain.

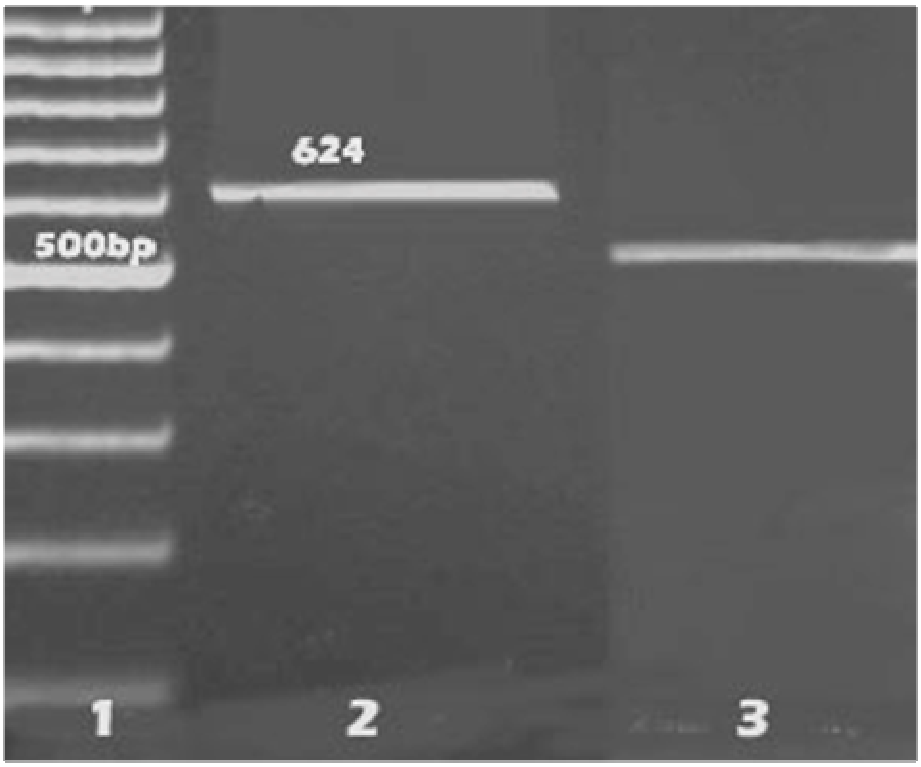

Figure 3. Presentation of the size difference of the RT-PCR products on 15% polyacrylamid gel. Note: 1. size marker, 2. 26695 standard strains, 3. 25M-mutant strain.

investigation of the HP1165 protein will help to understand its function and to identify the complete functional molecule involved in the active efflux of Tet in *H. pylori*.

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Full Length Research Paper

AdeABC efflux pump: Less important role in *Acinetobacter baumannii* against carbapenems

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AdeABC efflux pump in a total of 50 *Acinetobacter baumannii* strains were investigated and the role in contributing to hydrolysis carbapenems were further analyzed. All strains were divided into 3 groups according to their susceptibilities to Imipenem, Amikacin, Minocycline and Levofloxacin: Group A (22 imipenem-resistant strains), B (13 isolates which were imipenem-sensitive but resistant to at least one of the other three antibiotics) and C (15 isolates, sensitive to all the antimicrobials). Five gene types were observed according to REP-PCR and 39 isolates were included in the main one. Only one isolate (A2) was positive for efflux pump phenotype. All strains were positive for blaOXA51-like and AdeB gene and negative for blaOXA24, blaOXA58, VIM, IPM, and SIM-1. blaOXA23 were detectable merely in the 22 imipenem-resistant strains. 24 isolates from the same REP type representing for the 3 groups were selected for quantitative analysis of adeB expression. Compared with the mean level of Group B, only A2 expressed apparently higher (2.2 fold). The induction effects of imipenem and meropenem were analyzed as well. Compared with their freely grew isogenic counterparts, up regulated expression was observed only in 2 isolates under the pressure of imipenem and none in all under meropenem. These data indicate that AdeABC efflux pump play a less important role in *A. baumannii* against carbapenems.

Key words: *Acinetobacter baumannii*, Carbapenem resistance, AdeABC efflux pump, adeB gene.

INTRODUTION

Acinetobacter baumannii is increasingly an important opportunistic nosocomial pathogen, especially in the intensive care units (ICUs), and can cause various infections, including pneumonia, urinary tract infection and septicemia (Peleg et al., 2008; Wieczorek et al., 2008). Carbapenems were thought to be the last useful agents that could combat severe *A. baumannii* infections. But in the recent 20 years, carbapenem-resistant *Acinetobacter baumannii* isolates were reported with an ever faster frequency throughout the world (Peleg et al., 2008; Zhou et al., 2007; Coelho et al., 2006). OXA and MBL type carbapenemases were thought to be the main causes of the high resistance in *A. baumannii* (Walther-Rasmussen and Hoiby, 2006; Walsh et al., 2005). OXA-23-type carbapenemase, especially, is the most wide spread and powerful carbapenemase in *A. baumannii* against carbapenems (Peleg et al., 2008; Zhou et al., 2007; Walther-Rasmussen and Hoiby, 2006). It is thought that the AdeABC efflux pump play a significant role in *A. baumannii* against antimicrobial as well (Wieczorek et al., 2008; Magnet et al., 2001; Ruzin et al., 2007). AdeABC efflux pump, belonging to the resistance–nodulation-cell division family (RND) efflux pump, has a broad range of substrates, such as aminoglicosides, tetracyclines, erythromycin, chloramphenicol, trimethoprim, fluorquinolones, tigecycline and so on (Wieczorek et al., 2008; Ruzin et al., 2007). The pump has been divided into two types: inductive and constitutive, depending on whether inducible by the substrate or not (Wieczorek et al., 2008; Roberts et al., 1996). Although, meropenem resistance caused by the high expression of the efflux pump in *A. baumannii* has been reported by Huang and his colleagues (Huang et al., 2008), the role of AdeABC

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efflux pump in *A. baumannii* against carbapenems is still arguing (Bratu et al., 2008).

Thus, the appearance and expression of AdeABC efflux pump in a total of 50 *A. baumannii* strains, isolated from a tertiary medical center in China, were observed in this study. We describe here the role of AdeABC efflux pump in *A. baumannii* against carbapenems.

METHODS

Bacterial isolates

Fifty non-repetitive *A. baumannii* strains were isolated during March 2008 and March 2009 in a tertiary medical center in China. All isolates were assigned to Acinetobacter baumannii by Vitek GNI card (bioMe´rieux) and confirmed through the intrinsic $bla_{OXA-51-like}$ gene to the species (Turton et al., 2006).

Susceptibility testing

The susceptibility of Imipenem (Oxiod), Amikacin (Oxiod), Minocycline (Oxiod) and Levofloxacin (Oxiod) for all these isolates were determined by disk dilution method on M-H agar plates following CLSI guidelines. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls. Results were interpreted according to CLSI recommendations (CLSI, 2007).

REP-PCR

To characterize the 50 isolates genetically, Repetitive Extragenic Palindromic Sequence-Based PCR (REP-PCR) was performed with the primer pair REP1 5'-IIIGCGCCGICATCAGGC-3'and REP2 5'-ACGTCTTATCAGGCCTAC-3', as described previously (Bou et al., 2000). Strains belonging to the same clones showing identical or highly similar profiles (up to two bands variations). Discrepancy in the intensity of bands was not taken into account.

Detection of efflux pump phenotype

The minimum inhibition concentrations (MICs) of Imipenem of the 22 carbapenems-resistant *A. baumannii* isolates were obtained through broth dilution method following CLSI guidelines (CLSI, 2007). Meanwhile, additional CCCP (sigma) with the final concentration of 5 μg /ml was added to the broth in another group. Isolates with two-four fold MIC reduction of Imipenem with the addition of CCCP were considered as positive for AdeABC efflux pump. The interference of CCCP was examined by the growth in antibiotic free broth.

Detection of carbapenemases gene and AdeB gene

Genomic DNAs for PCR were prepared by boiling method. Carbapenemases genes: IPM, VIM, SIM-1, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-58}, $bla_{OXA-51-like}$ and AdeB were screened in all isolates. The regulators of AdeABC efflux pump of A2, AdeRS two-component system, were detected as well. PCRs were performed in a pre-mix PCR system purchased from SBS Gentech with the primers and parameters described previously (Zhou et al., 2007; Turton et al., 2006; Jeon et al., 2005; Lee et al., 2005; Marchand et al., 2004). PCR products were analyzed by agarose gel electrophoresis. All amplicons from A2 were sequenced by invitrogen (Shanghai) at the same time. Blasts were performed in NCBI. The positive control was generously provided by Professor YunSong Yu, first affiliate Hospital of Zhejiang University.

Real-time reverse transcript PCR

24 isolates with the same REP type, which represent for the 3 groups, were selected for the quantitative analysis of adeB expression. Total RNA was extracted from the bacterial cells grown on M-H agar plate over night with RNA isolation kit (SBS Genetech, Shanghai) following the manual. For inductive purpose, imipenem (Oxiod) and meropenem (Oxiod) contained disc were added in a parallel experiment and total RNA was isolated with cells growing within 5 mm from the edge of the discs. RNA concentration and purity were assessed spectrophotometrically at wavelengths of 260 and 280 nm. The purity was further confirmed by amplifying AdeB gene with the total RNA as template through polymerase chain reaction. Real-time RT-PCR was carried out with reverse transcription kit (TaKaRa Biotech, Dalian) according to the manufacturer's instructions on ABI7500 Real-Time PCR system. Each reaction mixture (20 μL) contained approximate 10 ng total RNA. RT-PCR condition was programmed as the kit recommended. The primers and probes (Table 1) used in this study were synthesized by SBS Genetech, Shanghai. 16S rRNA of *A. baumannii* was conducted in parallel as housekeeping. The samples and standards were determined in triplicate.

RESULTS

Antibiotic susceptibility

Among the 50 *A. baumannii* isolates, 22 were imipenem-resistant (inhibition zone<13 mm). Resistance to amikacin, minocycline and levofloxacin were 35, 33, and 35 isolates, respectively. Susceptibility with an intermediate result was assigned as resistant. According to these results, all the strains were separated into three groups. Group A consisted of the 22 imipenem-resistant strains. Group B contained 13 isolates which were imipenem-sensitive but resistant to at least one of the other three antibiotics. The left 15 isolates, susceptible to all of the antimicrobial, were sent into Group C.

REP-PCR typing

Five types were observed among the 50 strains through agarose gel electrophoresis with the amplified fragments. The main group containing 39 isolates consisted of the 22 imipenem-resistant isolates, 9 of B and 8 of C. Isolates contained in the left 4 types were no more than 5 each. The typing profiles of all the 39 strains showed identical bands with the variation only in the intensity.

Efflux pump phenotype

All isolates grew well in imipenem-free 5 μg /ml CCCP broth. The addition of CCCP led to a fourfold reduction of

Table 1. Primers and Taqman probes used in quantitative real-time RT-PCR

Primer	5'-3' sequence	Size(bp)	Reference
16sRNA	F: GTAGCGGTGAAATGCGTAGA	85	16
	R: CTTTCGTACCTCAGCGTCAG		
	Probe: CGAAGGCAGCCATCTGGCCT		
adeB	F: CCAGAGGAAGATCAAGGTTGGT	123	18
	R: TTTTACATCGGGATTGTCTTTCAA		
	Probe: CATGACTTCGTTCCAGCTACCTTCAGATGC		

the imipenem MIC in only one isolate (A2). No change in MIC was observed in the other 21 imipenem-resistant isolates.

Carbapenemases of *A. baumannii*

$bla_{OXA-51-like}$ gene was positive in all the 50 strains with an expected size as well as adeB. bla_{OXA-23} gene was detectable only in the 22 carbapenems-resistant strains with the size of 501 bp. IPM, VIM, SIM-1, bla_{OXA-24}, bla_{OXA-58} were negative in all the 50 isolates. Sequencing studies carried on amplicons of $bla_{OXA-51-like,}$ bla_{OXA-23}, adeB, adeR and adeS of A2 resulted in 100% identical with previously reported genes (GenBank NO.: AY750909, AJ132105, DQ294294, AF370885 and AF370885, respectively).

adeB expression

Relative adeB mRNA expression level to its own 16sRNA was 0.63 ± 0.015 (C.V = 2.38%) in Group B. Decreased expression was observed both in Group A and C compared with Group B, which were statistically significant ($P < 0.05$). Only one isolates (A2) showing increased expression which was 2.2 fold to the mean level of Group B. Increased expression of adeB were obtained in A1 and A3 under the pressure of imipenem, approximately 9.1 and 4.2 fold higher contrasted with their freely grew isogenic counterparts on M-H agar. No up-regulated expression was measured under the pressure of meropenem in all the 8 isolates. (Table 2)

DISCUSSION

Nosocomial outbreaks caused by carbapenems-resistant Acinetobacter baumannii have been reported worldwide. OXA-23, OXA-24, OXA-58 type carbapenemases and metal-β-lactamase play significant roles in *A. baumannii* against carbapenems (Peleg et al., 2008; Walther-Rasmussen and Hoiby, 2006; Walsh, et al., 2005). Meanwhile, AdeABC and AdeIJK, members of the resistance–nodulation-cell division family (RND) efflux pump, have been reported in *A. baumannii* (Wieczorek et al., 2008; Magnet et al., 2001; Damier-Piolle et al., 2008).

In this study, all the 50 strains were divided into 5 gene types according to the REP-PCR. As reported previously (Huang et al., 2008), most of the isolates were belonging to the same type, containing the 22 imipenem-resistant isolates, 9 of Group B and 8 of C. In accordance with previous findings (Zhou et al., 2007), bla_{OXA-23} was existed in all the 22 carbapenems-resistant *A. baumannii* isolates in this study. These isolates showed identical genetical profile and antibiotic pattern. $bla_{OXA-24,}$ bla_{OXA-58} and IPM, VIM, SIM-1 were undetectable in the all the 50 *A. baumannii* strains. The genes encoding the AdeABC efflux pump are located on the bacterial chromosome containing adeA, adeB and adeC. adeB captures its substrates either from within the phospholipids bilayer of the inner membrane or the cytoplasm in *A. baumannii* while adeA and adeC act as assistance (Magnet et al., 2001; Marchand et al., 2004). For all the *A. baumannii* strains studied here, adeB with the positive rate of 100%, not only in the 22 carbapenems-resistant *A. baumannii* isolates but also in the 28 carbapenems-sensitive ones. Sequencing was performed to confirm the gene. The high positive rate strongly indicate that AdeABC efflux pump is ubiquitous exists in A. baumannii, acting as a cryptic (Marchand et al., 2004) or even an intrinsic gene as $bla_{OXA-51-like}$. Given the fact that AdeABC efflux pump exists both in carbapenem-resistant and sensitive strains leave us the idea that it may not be an important contributor to carbapenems resistance.

Advanced study was carried out on the expression of adeB by Real-Time RT-PCR as result. The adeB mRNA of the 8 Group B isolates expressed steadily and identically with the C.V of 2.38% relative to their respectively 16sRNA. Compared with Group B, decreased expression was statistically significant ($P < 0.05$) both in Group A and C. The only exceptional was A2, giving 2.2 fold higher. It's in accordant to the phenotype analysis. This gives the further evidence that the AdeABC efflux pump act poor against carbapenem. Another study carried out by Simona Bratu and his colleagues have already supported the idea that the expression of adeB did not correlate with meropenem resistance (Bratu et al., 2008).

Table 2. Relative RNA expression level and efflux pump phenotype

Isolates	Relative expression ■	Inductive expression		phenotype
		IMP Pressure▲	MEM Pressure▲	
A1	0.2331	9.1	0.96	Negative
A2	1.386	0.22	0.41	Positive
A3	0.3969	4.9	0.5	Negative
A4	0.189	0.88	0.92	Negative
A5	0.4725	1.04	0.88	Negative
A6	0.5733	0.90	0.82	Negative
A7	0.693	1.02	0.95	Negative
A8	0.5985	0.91	0.89	Negative
B1	0.6489	N	N	N
B2	0.6363	N	N	N
B3	0.6111	N	N	N
B4	0.6237	N	N	N
B5	0.6048	N	N	N
B6	0.6615	N	N	N
B7	0.6174	N	N	N
B8	0.6363	N	N	N
C1	0.063	N	N	N
C2	0.1008	N	N	N
C3	0.1323	N	N	N
C4	0.015	N	N	N
C5	0.2205	N	N	N
C6	0.2646	N	N	N
C7	0.1386	N	N	N
C8	0.2331	N	N	N

■ Relative adeB mRNA expression to its own 16sRNA expression level. ▲ Relative expression level under the pressure of IMP and MEM to their isogenic counterparts growing on antibiotic-free M-H agar. N: Test has not been carried out.

The expression of the efflux pump has two types: inductive or constitutive, depending on whether induced by the substrate or independent of the environment (Wieczorek et al., 2008; Roberts et al., 1996). Thus, inductive effect of adeB mRNA expression was studied between strains grew under the pressure of imipenem and meropenem and their freely grew isogenic counterparts on M-H agar. Increased expression of adeB was observed only in A1 and A3 under the pressure of imipenem. They expressed 9.1 and 4.2 fold higher, respectively, while decreased expression appeared in all the eight isolates under the pressure of meropenem. Puzzlingly, the expression of adeB of A2 was even a little inhibited both by imipenem and meropenem, 0.22 and 0.41 fold decreased respectively. It's possible that A2 expressed constitutively and inhibited by CCCP, so that appeared with a positive phenotype. Although, the expression of A1 and A3 was inducible by imipenem, the failure of the phenotype detection may owe the high expression of bla_{OXA-23}. It's false negative in another word. As reported, adeB high expression isolates without the other carbapenemases background may turn out to be with a positive phenotype (Huang et al., 2008). Then, it's suggested that AdeABC efflux pump may have effect on the susceptibility of carbapenems in *A. baumannii* but far from been a primary.

The expression of the AdeABC efflux pump is stringently regulated by the AdeRS two-component system. Either variation in AdeR or AdeS resulted in up-regulated expression of AdeABC efflux pump, causing muti-drug resistance (Marchand et al., 2004). The response regulator- and sensor kinase-encoding gene, adeR and adeS, were amplified and sequenced. No mutations in the two regulative genes were observed in A1, A2 and A3 blasted with *A. baumannii* BM4454. No meaningful mutation points were found in the adeB over expression isolates by Huang and his colleagues, either (Huang et al., 2008). It is possible that promoters such as ISAba1 play a role in the expression of AdeABC efflux pump (Peleg et al., 2008).

Because of the co-existence of β-lactamases and efflux systems, assessment of the contribution of AdeABC

efflux pump is admittedly a difficult task. Gene knockout and restoration studies are still required to determine their precise roles in further studies.

Integrated with the evidence of its ubiquity both in carbapenem-resistant and sensitive strains, relatively low expression level and poor induction effect, we drawn the conclusion that AdeABC efflux pump play a less important role in *A. baumannii* against carbapenenms.

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Full Length Research Paper

Infections and aerobic bacterial pathogens in diabetic foot

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The aim of the study was to investigate the causative pathogens, profile of antimicrobial susceptibility of them and the extent of tissue lesions in diabetic foot infections. This is a prospective study in which infected diabetic foot presenting with Wagner grade 1 to 5 ulcers were investigated. 78 consecutive diabetic patients who were seen in the orthopaedic clinic were cultured during ordinary visits. Bacteriological diagnosis and antimicrobial susceptibility profiles were carried out and analyzed using standard procedures. Diabetic polyneuropathy was found to be a common finding (74.4%). 15 (19.2%) cultures revealed polymicrobial involvement. The most frequent organisms isolated were *Enterobacteriaceae* (36.5%), *Pseudomonas aeruginosa* (18.9%), *Enterococcus* spp. (14.9%), *and Staphylococcus aureus* (10.8%). While imipenem, meropenem, amikacin, piperacillin/tazobactam were found out to be the most effective agents against Gram-negative organisms, vancomycin, teicoplanin, chloramphenicol were the most effective agents against Gram-positives. The aetiologies of most of the ulcers were neuropathic and 81.6% of them were deep. Our study also revealed that Gram-negative bacteria were the most common pathogens in infected diabetic feet. The diabetic foot ulcer is the most important cause of non-traumatic foot amputations so it is important to know the causative pathogens of these ulcers, profile of antimicrobial susceptibility of them for their treatment.

Key words: Diabetes mellitus, foot ulcers, infection, pathogen.

INTRODUCTION

Diabetes Mellitus (DM) is a serious public health problem worldwide (Wild et al. 2004). This problem was redoubled with rising prevalence of DM ineluctable rise in foot ulcers. Contributory factors include peripheral neuropathy, vascular disease, foot deformities, local trauma and pressure. The situation is the most important cause of non-traumatic foot amputations (Wild et al., 2004; Vamos et al., 2010; Fosse et al., 2009). In Turkey prevalence of DM was 7.2% (Satman et al., 2002). Due to the increase in geriatric population, number of patients with DM is rising per year in Turkey. Therefore the number of diabetic foot and infections are also increasing.

Infection is a frequent complication of diabetic foot ulcers, and the presence of infection greatly enhances the risk of amputation (Fosse et al., 2009). Infections in diabetic foot are usually polymicrobial due to aerobic bacteria and fungi. Severe infections usually yield polymicrobial isolates, whereas mild infections are frequently monomicrobial. In cases of a severe diabetic foot infection, three to five organisms may be cultured (Lipsky, 2004).

The Wagner classification (Wagner, 1981) classifies the severity and depth of tissue injury into five grades. In the superficial grades (Wagner 1 and 2), aerobic bacteria (*Staphylococcus* spp., *Streptococcus* spp., and *Enterobacteriaceae*) are predominant pathogens while anaerobic bacteria add up in Wagner grade 3 to 5 ulcers (Pathare et al., 1998). Effective antimicrobial therapy for these infections should help to reduce morbidity.

The aim of this present study is to investigate the causative pathogens and the relation between them,

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profile of antimicrobial susceptibility and the depth of tissue injury in patients with DM.

MATERIALS AND METHODS

Participants

This is a prospective study in which 78 consecutive diabetic patients, who were admitted to the outpatient department of an orthopaedic clinic at a university hospital in a period of 18 months, were included.

The patients were included if they received no antibiotics (first episode) or if systemic antibiotic treatment was stopped at least 30 days before the time of onset of the current episode (recurrent wound).

The patients were classified at the time of their first assessment. Each patient was included only once in this study. Age, sex, clinical history, duration of DM, nephropathy, urinary incontinence, retinopathy, obesity, associated diseases (e.g., hypertension, ischemic heart disease, cerebrovascular accident), duration of foot ulcers, neuropathic or ischemic character, localization, any history of amputation were recorded, glycosylated haemoglobin was measured, and all wounds were graded according to the Wagner Classification System when they are admitted to the hospital. Grade 1 was defined as cellulitis or a superficial wound, grade 2 as subcutaneous infection, fasciitis, or tendonitis without osteomyelitis, grade 3 as osteomyelitis (Osteomyelitis was diagnosed on suggestive changes in the radiographs and bone scans), grade 4 as a localized gangrene, and grade 5 as widespread gangrene (Wagner 1981). The patients in grade 0 were not included into the study.

Microbiology and antibiotic susceptibility tests

Culture materials from all the wounds were obtained, either by washing the wound with sterile physiological saline and then making a puncture-aspiration from the base of the wound or by applying a sterile cotton swab to the wound (Shankar et al. 2005). Specimens were sent to the laboratory and processed for aerobic bacteria. To minimize bias, laboratory technicians were kept blind to the clinical data. Anaerobic cultures were disregarded because of the lack of technical and logistical support.

Bacteriological diagnosis was carried out and analyzed using standard medical microbiology laboratory procedures (Doern et al., 2003). Susceptibility testing of microorganisms was performed by the disk diffusion method and evaluated according to recommended National Committee for Clinical Laboratory Standards (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2010).

Statistical methods

Quantitative variables were expressed as means ±SD while qualitative variables were expressed as percentages. Comparison of mean values was performed using the Student's t test for continuous and chi-squared test for categorical variables. A p value≤0.05 was considered statistically significant. Analysis was performed with Statistical Package for Social Sciences (SPSS) version 13.0.

RESULTS

Demographic characteristics

78 patients were included in the study. 44 (56.4%) of them were male. Clinical features of the patients are shown in Table 1. The mean age of the patients was 59.72 ± 10.17 (min34 max79). Fifty eight patients (74.4%) had diabetic neuropathy, 25 patients (32.1%) had diabetic retinopathy, 22 patients (28.2%) had nephropathy and 11 (14.1%) had urinary incontinence. Their mean glycosylated haemoglobin was 9.7±3.5%. The aetiologies of ulcers were neuropathic in 42 (53.8%) patients, ischemic in 36 (46.2%) patients. The localization of ulcers was commonly on the distal phalanges (34.5%), with 28.2% on the sole, 20% on the heel and 17% on the interphalangeal area. 81.6% of these ulcers were deep and 71.4% of them were neuropathic in the patients with bacterial growth (Table 2). While 69.6% of the patients with bacterial growth had loss of protective sensation; all of them had deformity of foot; 75.7% of them had pain (Table 2).

The statistically significant relation was found between the bacterial growth and the deep ulcers, deformity of foot and size of cellulite (Table 2).

According to Wagner classification, the ulcers were found to be in grade 4, 3, 2, 5 and grade 1 in 26 (33.3%), 16 (20.5%), 14 (17.9%), 13 (16.7%), and 9 (11.5%) patients, respectively.

Microbiology

In 21 (26.9%) patients cultures were negative; while in 15 (19.2%) cultures revealed polymicrobial involvement and the most frequent organism isolated were *Enterobacteriaceae* (36.5%). Others were *Pseudomonas aeruginosa* (18.9%), *Enterococcus* spp. (14.9%), *Staphylococcus aureus* (10.8%), *Streptococcus* spp. (6.8%), Coagulase negative *staphylococci* (5.4%), *Candida* spp. (4%) and *Acinetobacter* spp. (2.7%). A total of 74 organisms were isolated. Thirty three of these organisms were isolated in polymicrobial cultures. *Enterobacteriaceae* (10/33), *Enterococcus* spp. (9/33) and *P. aeruginosa* (4/33) were mostly isolated in the polymicrobial cultures in predominantly grade 4 and 5 (Table 3). The majority of positive cultures were observed in grade 4 (39.4%), 5 (26.6%) and grade 3 (22.3%) (Table 4).

Imipenem, meropenem, amikacin, piperacillin/ tazobactam were the most effective agents against Gram-negative organisms while vancomycin, teicoplanin, chloramphenicol were the most effective agents against Gram-positives. The antimicrobial resistance to Gram-negative organisms and Gram-positive organisms are shown in Tables 5 and 6 respectively.

DISCUSSION

Diabetic foot ulcers are common and serious complications of chronic DM. In parallel with increased prevalence of DM, the prevalence of foot infection are increasing,

Table 1. Clinical features of the patients.

Feature	Number of patients (%)
Age (mean ± SD years)	59.72±10.17 (min34 max79)
Sex	
Male	44 (56.4)
Female	34 (43.6)
Diabetic medication	
Insulin	31 (39.7)
Oral antidiabetic	30 (38.5)
Oral antidiabetic+insulin	14 (17.9)
Associated diseases	
Hypertension	35 (44.9)
Ischemic heart disease	18 (23.1)
Cerebrovascular disease	7 (9)
Chronic heart failure	6 (7.7)
Chronic Obstructive Lung Disease	5 (6.4)
Chronic renal failure	4 (5.1)
Hyperlipidemia	3 (3.8)
Malignancy	2 (2.6)
Pregnancy	1 (1.3)
More than one disease	25 (32)
Duration of foot infection (mean±SD days)	79.0±108.7 (min 3. max 720)
>1 month	32 (41)
<1 month	46 (59)
Duration of diabetes mellitus (mean±SD months)	11.9±7.9 month
>1 year	72 (92.3)
<1 year	6 (7.7)
Glycosylated haemoglobin (mean±SD %)	9.7±3.5 (min 5.3. max 18.9)
Body mass index (mean±SD kg/m2)	27.6±4.3 (min 16.5. max 40.8)
Current or past history of smoking	19 (24.4)
Current or past history of alcohol use	7 (9)
Diabetes comorbidities	
Neuropathy	58 (74.4)
Nephropathy	22 (28.2)
Retinopathy	25 (32.1)
Urinary incontinance	11 (14.1)
History of previous amputation	13 (16.7)
History of previous diabetic foot ulcer	31 (39.7)
Insufficient foot care	37 (47.4)
Trauma	15 (19.2)
Foreign body	3 (3.8)

worldwide and also in Turkey (Wild et al., 2004; Satman et al., 2002; Kandemir et al., 2007; Ozkara et al., 2008).

This prospective study was performed to evaluate the diabetic foot infections, the causative pathogens, the antimicrobial susceptibility profiles of them and the dept of tissue injury in these patients with diabetic foot ulcers.

Table 2. The relationship between the clinical features and the culture results.

Findings		Positive growth n (%)	No growth n (%)	P
Fever		5 (100)	-	P>0.05
Location of ulcer	Deep	40 (81.6)	9 (18.4%)	P=0.027
	Superficial	17 (58.6)	12 (41.4%)	
Type of ulcer	Ischemic	27 (75.0)	9 (25)	P>0.05
	Neuropathic	30 (71.4)	12 (28.6)	
Loss of protective sensation		39 (69.6)	17 (30.4)	P>0.05
Deformity of foot		14 (100)	-	P=0.016
Pain		28 (75.7)	9 (24.3)	P>0.05
Heat of foot	Cold	11 (84.6)	2 (11.4)	P>0.05
	Hot	46 (70.8)	19 (29.2)	
Appearance of foot	Pink	51 (72.9)	19 (27.1)	P>0.05
	Pale	6 (75)	2 (25)	
Pulse positive		44 (73.3)	16 (26.7)	P>0.05
Leakage		36 (76.6)	11 (23.4)	P>0.05
Abse		21 (77.8)	6 (22.2)	P>0.05
Ecchymosis /petechia		19 (67.9)	9 (32.1)	P>0.05
Lymphedema		20 (74.1)	7 (25.9)	P>0.05
Necrosis		33 (71.7)	13 (28.3)	P>0.05
Crepitation		5 (83.3)	1 (16.7)	P>0.05
Cellulite		25 (71.4)	10 (28.6)	P>0.05
Spread of cellulite to the leg		12 (75)	4 (25)	P>0.05
Size of cellulite	≤2 cm	4 (40)	6 (60)	P=0.016
	≥2 cm	21 (84)	4 (16)	
Distance of cellulite and ulcer	Far	8 (80)	2 (20)	P>0.05
	Close	17 (70.8)	7 (29.2)	
Septicemia		2 (100)	-	P>0.05

In the patients included in this study the duration of DM and foot infection were found to be more than a year (92.3%) and less than a month (59%) respectively. The level of their mean glycosylated haemoglobin was high. Therefore it was confirmed that the complications of DM were seen in the patients who had irregular glucose levels. Hyperglycemias and other metabolic derangements cause impaired immunological (especially neutrophil) function and wound healing and excess collagen cross-linking (Boulton, 2008).

In this study diabetic neuropathy was found in 58 patients (74.4%). The most common serious complication of diabetic peripheral neuropathy that affects the foot is neuropathic ulcers. Loss of sensation results in failure to perceive damage caused by mechanical trauma-such as, friction from bad fitting shoes, penetration of pointed objects on the floor, or excessive heat from radiators or flames (Boulton, 2008).

However, as suggested by Lipsky et al. (2004) detection of neuropathy before its complications develop, is the best way to prevent diabetic foot infections.

In our study the ulcers were mostly on the distal phalanges. Infections of the lower extremities in diabetic patients commonly occur on the plantar surface of the forefoot, in particular the toes and metatarsal heads.

In this study majority of these ulcers were deep and neuropathic in the patients with positive growth. Also the significant relation was found between the bacterial

Table 3. Organisms in polymicrobial growth according to Wagner classification.

Organisms in polymicrobial growth	W1	W2	W3	W4	W5	Total
Staphylococcus aureus+Streptococcus spp.	-	-	1	-	1	2
Staphylococcus aureus+Pseudomonas aeruginosa	-	-	-	-	1	1
Escherichia coli+Enterococcus spp.	-	-	-	1	1	2
Pseudomonas aeruginosa+Enterococcus spp.	-	-	-	1	-	1
Klebsiella spp.*+Enterococcus* spp.	-	-	1	-	-	1
Klebsiella spp.*+Enterococcus s*pp.	1	-	-	-	-	1
Escherichia coli+Candida spp.	-	-	-	1	-	1
Enterococcus spp.*+Proteus* spp.	-	-	-	1	-	1
Enterococcus spp.*+Citrobacter* spp.	-	-	-	-	1	1
Enterococcus spp.*+Acinetobacter* spp.	-	-	-	-	1	1
Streptococcus spp.*+Escherichia coli+Candida spp.*	1	-	-	-	-	1
Streptococcus spp.*+Pseudomonas aeruginosa+Enterobacter cloacae*	-	1	-	-	-	1
Pseudomonas aeruginosa+Enterococcus spp.+Proteus spp.	-	-	-	1	-	1
Total	2	1	2	5	5	15

Table 4. Isolated organisms according to Wagner classification.

Microorganisms			W1	W2	W3	W4	W5	Total
No growth			3	7	2	6	3	21
ram negative bacteria	*Enterobacteriaceae*	*Escherichia coli*	2	1	3	3	2	1
		Klebsiella spp.	2	-	2	2	-	6
		Proteus spp.	-	-	-	3	2	5
		Morganella morganii	-	-	1	-	1	2
		Citrobacter spp.	-	1	-	-	1	2
		Enterobacter cloacae	-	1	-	-	-	1
	**Nonf.*	*Pseudomonas aeruginosa*	2	-	3	7	2	14
		Acinetobacter spp.	-	-	1	-	1	2
Gram-positive bacteria	*Enterococcus* spp.		1	-	2	5	3	11
	Staphylococcus aureus		1	3	1	1	2	8
	Streptococcus spp.		1	1	2	-	1	5
	Coagulase Negative Staphylococ		-	-	1	3	-	4
Yeast	*Candida* spp.		1	1	-	1	-	3
Polymicrobial			2	1	2	5	5	15

***** Nonf.; nonfermentative.

growth and the deep ulcers, deformity of foot, size of cellulite. There was bacterial growth at the cultures of the all patients who had foot deformity. These patients had friction from bad fitting shoes. This data was evaluated as the foot ulcer can be revealed out because of the mechanical trauma caused by friction from bad fitting shoes and can be infected easily.

The careful assessment is mandatory in the presence of severe diabetic foot infection. Ulcers and surrounding tissues must be evaluated for deep soft tissue involvement, presence of foreign bodies, and necrotic tissue. Categorization helps to determine the degree of risk to the patient and the limb and thus, the urgency and the method of management (Lipsky et al., 2004). As in previous studies (Candel et al., 2003), the ulcers were found to be mostly in grade 4 and 3 in this study. Severe infections usually yield polymicrobial isolates, whereas mild infections are frequently monomicrobial (Dhanasekaran et al., 2003). In cases of severe diabetic foot infection three to five organisms may be cultured

Table 5. Antimicrobial resistance among Gram-negative bacteria.

Antimicrobials	P. aeruginosa (n=14) No.	Acinetobacter spp. (n=2) No.	Enterobactericeae (n=27) No.
Mezlocillin	1	1	NT*
Ampicillin	NT	NT	22
Amoxicillin clavulanic acid	NT	NT	21
Ampicillin/sulbactam	NT	-	NT
Piperacillin/tazobactam	-	1	-
Ceftazidime	1	1	NT
Cefepime	NT	1	2
Cefuroxim	NT	NT	10
Cefazolin	NT	NT	19
Cefotaxime	NT	NT	4
Imipenem	-	-	-
Meropenem	-	-	-
Gentamicin	4	1	6
Amikacin	-	1	-
Tobramycin	1	-	NT
Netilmicin	NT	-	NT
Ciprofloxacin	-	1	5
Levofloxacin	-	1	5
Trimetoprim/sulphametoxazole	NT	NT	9

*NT; not tested.

Table 6. Antimicrobial resistance among Gram-positive bacteria.

Antimicrobials	*S. aureus* (n=8) No.	CNS* (n=4) No.	*Streptococcus* spp. (n=5) No.	*Enterococcus* spp. (n=11) No.
Ampicilin/Sulbactam	-	2	NT**	NT
Cefazolin	-	2	NT	NT
Vancomycin	-	-	-	-
Teicoplanin	-	-	NT	NT
Gentamicin	-	1	NT	NT
Erytromycin	2	2	1	NT
Ciprofloxacin	-	1	-	1
Ofloxacin	NT	1	NT	NT
Clindamycin	-	-	2	NT
Trimetoprim/sulphametaxasole	-	2	-	NT
Chloramphenicol	-	-	-	NT
Methicillin	-	3	1	NT
Tetracycline	NT	NT	2	11
Penicillin	NT	NT	1	3
Nitrofurantoin	NT	NT	NT	1
Cefotaxime	NT	NT	-	NT

* CNS; Coagulase Negative Stapylococci. **NT; not tested.

(Anandi et al., 2004; Abdulrazaka et al., 2005; Ozkara et al., 2008). The polymicrobial infection rate was low (19.2%) in this study. Similar with our findings Dhanasekaran et al. (2003) documented that 84% of diabetic foot ulcers are frequently monomicrobial. Several studies have previously described polymicrobial aetiology in diabetic foot infections (Anandi et al., 2004; Abdulrazak et al., 2005; Ozkara et al., 2008; Frykberg, 2003). Lipsky

et al. (2004) reported that polymicrobial etiology in diabetic foot ulcers may often be due to previous treatment history. However the aim of the present study is to investigate the aerobic pathogens in diabetic foot infections, disregarding anaerobic bacteria may be a limitation. Also this might be the reason of the lower polymicrobial infection rate.

Determining the causative organisms in diabetic foot infections and their antimicrobial susceptibility pattern is necessary for the antimicrobial therapy. Chronic wounds were developed more complex infections caused by Enterococci, various *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and sometimes, other nonfermentative Gram-negative rods (Anandi et al., 2004; Abdulrazak et al., 2005; Ozkara et al., 2008). Lipsky et al. (2004) reported a prospective evaluation of diabetic patients with non-limb-threatening lower extremity infections and who were not yet treated with antibiotics, and cultures revealed aerobic Gram-positive cocci as pathogens in 89% of patients.

Though previous studies (Frykberg, 2003; Ge et al., 2002) showed Gram-positive aerobes as predominant agents in diabetic foot infections, we frequently isolated Gram-negative bacteria (55.7%) compared to Gram-positive bacteria (40.5%). Similar to our findings, Shankar et al. (2005) and Gadepalli et al. (2006) showed predominant involvement of Gram-negative isolates.

Diabetic patients with foot ulcers have several factors that may be associated with a high risk of multidrug resistant microorganisms carriage, such as inappropriate antibiotic treatment, chronic course of the wound and frequent hospital admission (Kandemir et al., 2007). The causative pathogens and their antimicrobial susceptibility profiles should be considered when arranging the treatment of diabetic foot infections. In this study the most frequent bacteria isolates were Enterobacteriaceae. The majority of them were resistant to ampicillin, amoxicillin/clavulanic acid and cefazolin. Imipenem, meropenem, amikacin, piperacillin/tazobactam were the most effective agents against whole Gram-negative organisms included *P. aeruginosa* and *Acinetobacter* spp., while vancomycin, teicoplanin, chloramphenicol were the most effective agents against Gram-positives. Among the Gram-positive bacteria Enterococcus genus was isolated mostly and all of them were resistant to tetracycline, while no resistance to vancomycin was determined. Imipenem, meropenem and vancomycin were reported to be the most effective agents against to the bacteria isolated in diabetic foot infections in several studies similar with our study (Abdulrazak et al., 2005; Raja, 2007; Gadepalli et al., 2006).

Conclusions

We found that the aetiologies of the most of the ulcers were neuropathic in our study and 81.6% of them were deep. Detection of neuropathy before its complications develop is a strategic way to prevent diabetic foot infections. Our study showed that Gram-negative bacteria were the most common pathogens in diabetic foot infections. Imipenem, meropenem, were the most effective agents against Gram-negative organisms. Vancomycin was the most effective against Gram-positive organisms. Decisive therapy should be based on both the cultures and susceptibility data and the clinical response to the empirical regimen.

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Full Length Research Paper

Effect of 60Co γ radiation on mesenchymal stem cells (MSCs) proliferation and differentiation

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Mesenchymal stem cells (MSCs) are undifferentiated multipotent cells which reside in various human tissues and have the potential to differentiate into osteoblasts, chondrocytes, adipocytes, fibroblasts and other tissues of mesenchymal origin. In this study, we investigated the effect of 60Co γ radiation on the proliferation and differentiation of MSCs. MSCs were treated with increasing radiation doses to assess the effect on MSC. Results showed that 2 Gy of 60Co γ radiation did not significantly affect MSCs. When compared with the control group and 2Gy of 60Co γ radiation group, the MSCs viability after 4 Gy of 60Co γ radiation markedly decreased ($p < 0.05$). Two weeks of 8 and 12 Gy of 60Co γ radiation induced all cell death. After the 5-Aza treatment, the expression of myocardial-specific protein, C-TNT and β-MHC was not detected in the MSCs which were pretreated by 60Co γ radiation. In conclusion, middle dose (4 Gy) of irradiation induces MSCs morphological changes, as well as alteration in both proliferation and differentiation potentials of MSCs. 5-Aza treatment did not induce both proliferation and differentiation of MSCs because high dose (8 and 12 Gy) of irradiation killed all cells.

Key words: Mesenchymal stem cells, radiation, proliferation, differentiation.

INTRODUCTION

Mesenchymal stem cells (MSCs) are adult stem cells traditionally found in the bone marrow. However, mesenchymal stem cells can also be isolated from other tissues including cord blood, peripheral blood, fallopian tube, and fetal liver and lung. Multipotent stem cells, MSCs differentiate to form adipocytes, cartilage, bone, tendons, muscle and skin. Mesenchymal stem cells are a distinct entity to the mesenchyme, embryonic connective tissue which is derived from the mesoderm and differentiates to form hematopoietic stem cells (Wilkins et al., 2009; Rubio et al., 2008; Peter et al., 2010).

Mesenchymal stem cells are characterized morphologically by a small cell body with a few cell processes that are long and thin. The cell body contains a large, round nucleus with a prominent nucleolus, which is surrounded by finely dispersed chromatin particles, giving the nucleus a clear appearance. The remainder of the cell body contains a small amount of Golgi apparatus, rough endoplasmic reticulum, mitochondria, and polyribosomes. The cells, which are long and thin, are widely dispersed and the adjacent extracellular matrix is populated by a few reticular fibrils but is devoid of the other types of collagen fibrils (Wang et al., 2009; Vachiraroj et al., 2009; Yamada et al., 2007).

Mesenchymal stem or multipotent stromal cells (MSCs) are of importance for therapeutic use due to their ability to proliferate, moreover, differentiate into various cell phenotypes. Culturing or collecting the stem cells is usually necessary to obtain the adequate cell numbers for the clinical applications (Ghaedi et al., 2010).

We, therefore, investigated the effect of 60Co γ radiation on *in vitro* stability, viability and proliferation capacity of MSCs. Additionally, to date, little is known about the effects of 5-aza on MSCs proliferation, growth factors secretion and differentiation. Hence, the present

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Table 1. 60Co γ radiation.

Radiation dose (Gy)	2	4	8	12
Radiation time (s)	63	138	289	411

study was also designed to determine the effect of 5-aza on MSCs differentiation.

METHODS

Mesenchymal stem cell cultures

Mesenchymal stem cell cultures were prepared in Chinese PLA General Hospital and Postgraduate Medical School (BeiJing, China) according to the method described by Da Silva Meirelles and Nardi (2003); Nardi and Da Silva Meirelles (2006). Cells were dissociated using collagenase type 1 (0.5 mg/mL in DMEM 10 mM HEPES). After washing by centrifugation at 400 × g for 10 min and counting viable cells with trypan blue, the cells were resuspended in DMEM with 10% FBS and 10 mM HEPES in a final concentration of 5×10^6 viable cells per ml.

To initiate the cultures, cells were plated in 96-well tissue culture dishes at 3.5 ml/well (1.94×10^6 cells/cm^2) and kept in a humidified 5% CO_2 incubator at 37°C for 72 h, when non-adherent cells were removed by changing the medium. Confluent cultures from MSC were incubated with 0.25% trypsin solution containing 0.01% EDTA for detachment and maintained in culture by changing the medium every 3 - 4 days. For the experiments, cells between the 10 and 25th passages were seeded in 96-well plates in DMEM with 10% FBS and 10 mM HEPES in a density of 30,000 cells/well 2 days before the contact with the organotypic culture. Twenty-four hours before the contact, the medium was changed and conditioned during the next 24 h.

60Co γ radiation and MSCs cells viablity

60Co γ radiation

MSCs cells (3rd generation) in 96-well plates were exposed to 60Co γ radiation (dose rate, 165.44cGy/min) at 30°C for 90 s. The irradiated cells were incubated for continuous 4 weeks (Table 1).

Cell shape

After the removal of the culture medium, each well was washed once with Phosphate Buffered Saline (PBS) and the cells attached to the bottom of the plate were fixed and stained with 0.4% crystal violet solution in methanol for 30 min. After the microplate was washed with water and dried, cell shape of each well was observed using the microscope.

Cell viability

MSCs cell growth and viability was measured by adding 0.4% trypan blue in 0.9% saline to a 50% dilution, and cells were counted, using the hemocytometer according to standard procedure (Pienata and Lehr, 1993). Briefly, 0.5 ml of the trypan blue solution was transferred to a test tube and 0.3 ml of PBS plus 0.2 ml of the trypsinized cell suspension (dilution factor of 5) were added. The final solution was thoroughly and gently mixed and allowed standing for 5 min.

Then a drop of this dye-cell suspension was loaded onto both chambers of the hemocytometer. Cells were examined and counted in duplicates under light microscope at 200 × (Olympus BH2). Concentration and total number of cells were determined, and percentage cell viability was calculated by the formula:

$$\text{Cell iability (\%)} = \{[\text{Number of viable cells (unstained cells)}] \times 100 \over \text{Total number of cells}\}$$

MTT assay

Cell viability was checked using MTT assay. The MTT test is a colorimetric assay that measures the cell survival as percentage of cell survival compared to untreated controls (Williams et al., 2002). In brief, 100 µl of 0.45 g/l MTT solution was added to wells. Cells were incubated at 37°C for 45 - 60 min to allow colour development and thereafter, 100 µl of 20% SDS in DMF:H_2O 1:1 solution was added to the wells. Plates were incubated overnight at 37°C to solubilize the formazan products. Absorbances were measured at the wavelength of 570 nm. The levels of blue colour development in the control wells were designated as 100% viability, and all further comparisons were based on that reference level. Blank values, indicating the absorbance of MTT and 20% SDS in DMF:H_2O 1:1 solutions only, were subtracted from all samples.

Mesenchymal stem cells differentiation

Group and radiation

MSCs in 6-well plates were divided into A and B groups. Group A received no radiation; Group B was exposed to 60Co γ radiation (dose rate, 165.44cGy/min) for 138s. One day after radiation, culture medium was changed. Then, 10 µml/L 5-aza was added into the two groups (A and B). The total incubation time with 5-aza was 24 h. After that, culture medium was changed and MSCs were incubated for continuous 28 days.

RNA extraction

The RNA extraction method using guanidine thiocyanate, phenol and chloroform (all from Sigma) was as described (Verhofstedea et al., 1996). RNA extraction using the 'PURESCRIPTR' kit ('body fluid' protocol) was performed according to the instructions of the manufacturer (Gentra Systems, Minneapolis). Total RNA extracts were used directly for RT-PCR or first stored in small aliquots at -80°C for periods of up to 2.5 years (stock sample) and at -20°C for periods of up to 8 months (working sample). Samples were thawed and re-frozen no more than four times to minimize possible RNA degradation.

Real time-polymerase chain reaction (RT-PCR)

Subsequently, single-stranded cDNA was synthetized from 2.0 µg RNA with the use of 5 µmol/L random primers (Ambion, Austin, Texas) and 10 U AMV Reverse Transcriptase (Promega, Madison, Wis) in a buffer containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$ (Ambion), 40 U/µL ribonuclease inhibitor (Promega), and 2 mmol/L of dNTP mix (Ambion) in a total volume of 20 µL (final concentrations indicated). RT was carried out for 15 min at 25°C and for 90 min at 42°C, and the samples were subsequently heated for 10 min at 92°C as a means of terminating the RT reaction. Having obtained the cDNA, we carried out a PCR reaction using 6 µL of the RT reaction mix. To this, 2.5 U Taq

Table 2. Primers used for the analysis of gene expression on RT-PCR.

Gene	Serial number in gene bank	Sequence	Length (bp)
GAPDH		F : 5′- CGTATCGGACGCCTGGTT - 3′ R : 5′- CGTGGGTAGAGTCATACTGGAA - 3′	124
C-TNT	M26052	F : 5′- CAGCAGCGTATTCGCAATG - 3′ R : 5′- TTCTTCTTCCGGGCCTCA - 3′	119
B-MHC	AY701540	F : 5′- GTGTACCCTTCAAAGACGC- 3′ R : 5′- CACCTTGAGGAACCGTCT - 3′	202

polymerase (Promega), 0.6 µmol/L sense primer, and 0.6 µmol/L antisense primer (made by Roche Diagnostics GmbH, Mannheim, Germany) were added. The final reaction volume was 50 µL. The tubes were incubated in a Mastercycler personal thermocycler (Eppendorff-Netheler-Hinz GmbH, Hamburg, Germany) at 94℃for 2 min to denature the primers and cDNA. The cycling program consisted of 94℃ for 90 s, 55 to 57℃ for 1 min, 72℃ for 2 min (10 min in the last cycle) and comprised 30 to 35 cycles. For each primer set, an increasing number of PCR cycles with otherwise fixed conditions was performed to reveal the optimal number of cycles to be used. This point was determined to be halfway through the exponential phase. Human and rabbit primers that amplified the rabbit sequence for GAPDH, C-TNT, and β-MHC are provided in Table 2. PCR products and molecular-weight markers (Boehringer Mannheim GmbH, Mannheim, Germany) were subjected to electrophoresis on 1% agarose gel and visualized by means of ethidium bromide staining. The gels were analyzed by means of laser scanning densitometry with the use of a Gelprinter plus photodocumentation system (TDI, Madrid, Spain) and Zeiss KS-300 software (Imaging System, Münster, Germany).

Statistical analyses

All analyses were performed in duplicate. Data were analyzed by one-way ANOVA using the general linear model procedure of SAS (SAS Inst. Inc., Cary, NC, USA). Differences among means were tested using Duncan's multiple range tests. A significant level of 0.05 was used.

RESULT

Normal MSCs morphology

Most of first-generation MSCs exhibited a spindle shape. A few cells showed a triangular or round or polygonal shape. Third-generation MSCs still showed a spindle shape and displayed better permeability and light refraction.

MSCs morphology after radiation

MSCs morphology was not significantly affected with doses as high as 2 and 4 Gy of 60Co γ radiation. MSCs morphology started to markedly change with increasing dose of 60Co γ radiation (Figure 1). Most of the cell death was observed after 8 and 12 Gy of 60Co γ irradiation. All cells died after 2 weeks of 8 Gy of 60Co γ irradiation or after 1 weeks of 12 Gy of 60Co γ irradiation.

Effect of 60Co γ radiation on MSCs viability

After 2 Gy of 60Co γ radiation, the MSCs viability as assayed by trypan blue dye exclusive test were above 97%, and no significant difference in MSCs viability was found among the experiment groups ($P > 0.05$) (Figure 2). When compared with the control group and 2 Gy of 60Co γ radiation group, the MSCs viability after 4 Gy of 60Co γ radiation markedly decreased ($P < 0.05$). Two weeks of 8 and 12 Gy of 60Co γ radiation induced all cell death (Figure 3; Tables 3 and 4).

Effect of 60Co γ radiation on MSCs proliferation

The inhibitory effect induced by the 60Co γ radiation was related to the dose of radiation treatments, thus to the global radiative energy given to the cells. It should be noted that there was no significant difference ($P > 0.05$) in the number of dead cells between the control and 2 Gy radiation groups. After 4 Gy radiation treatments, the number of viable cells in the treated samples was significantly ($P < 0.05$) reduced with respect to the control sample. After 8 and 12 Gy radiation treatments, all cells were dead (Figure 4; Table 5).

5-aza-induced MSCs differentiation

Four weeks later, the cells were examined for the morphological changes and mRNA expression of myocardial-specific protein, such as C-TNT and β-MHC. In the group A, the MSCs cells showed various morphologies such as a long fusiform shape (Figure 5A). However, MSCs cells cultured in group B (Figure 5B) showed a spindle shape and cobblestone-like shape. Complete cell fusion and myotube formation were not detected in group A and B after 4 weeks culture.

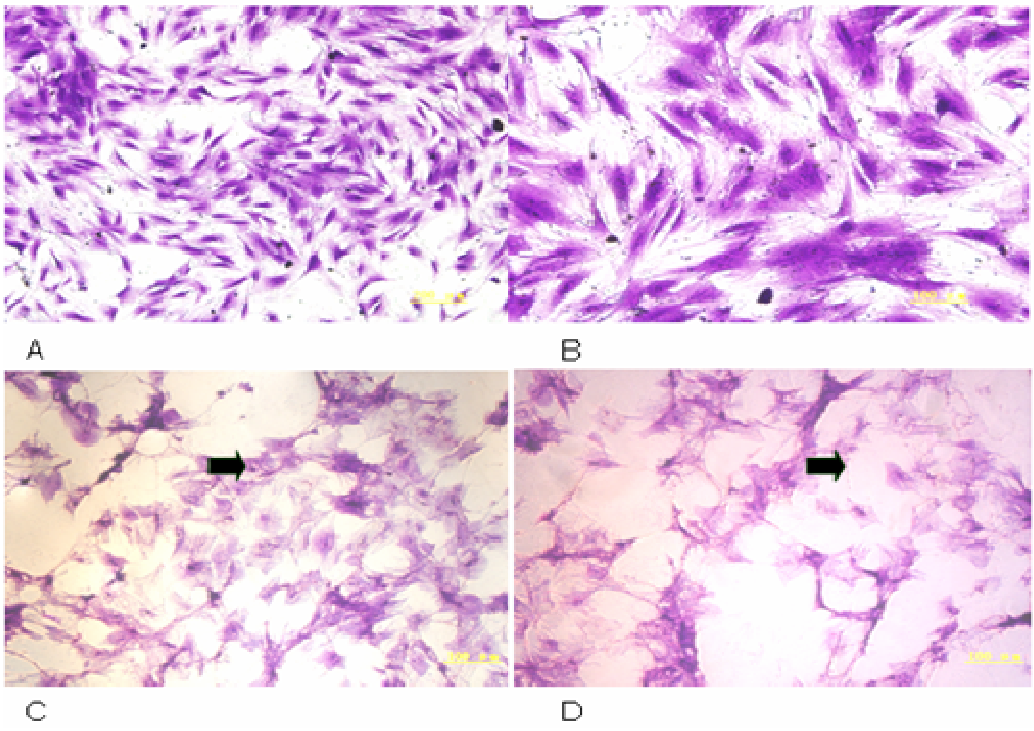

Figure 1. MSCs morphology after crystal iolet staining. (A) 2 Gy radiation (×100), (B) 4 Gy radiation (×200), (C) 8 Gy radiation (×200), (D) 12 Gy radiation (×200).

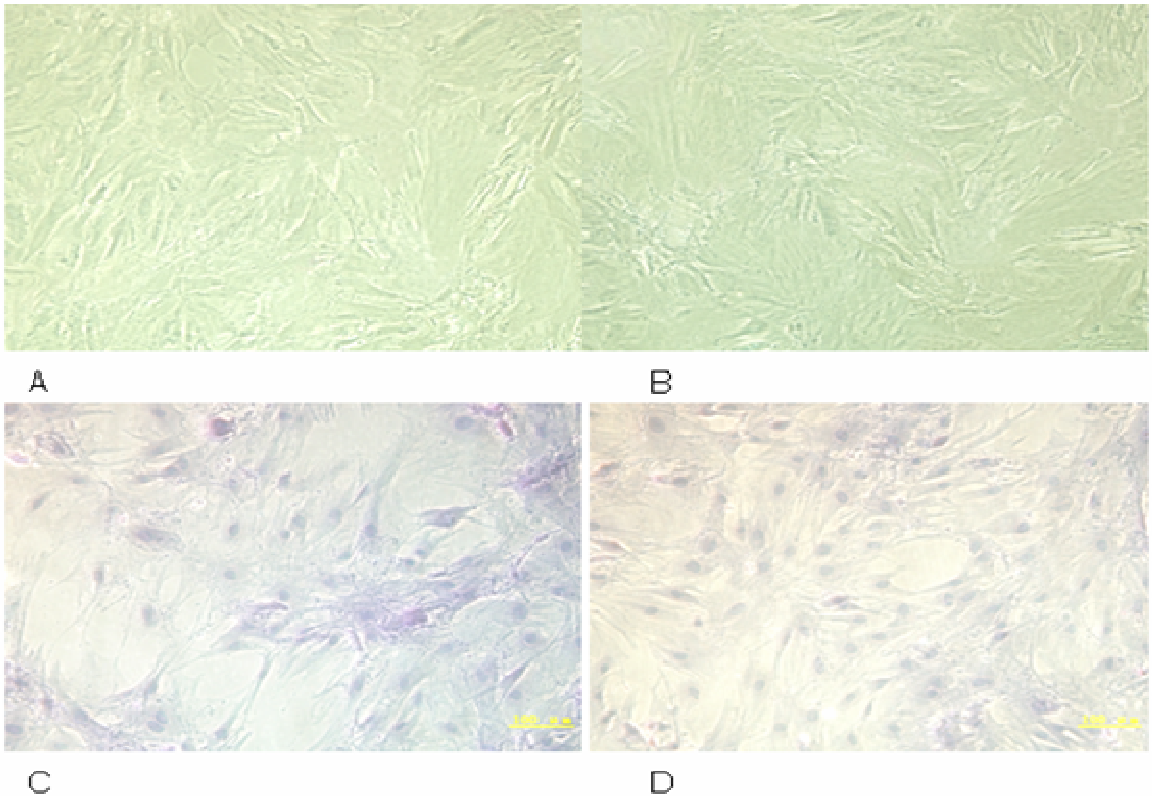

Figure 2. MSCs morphology after trypan blue staining. (A) 2 Gy radiation (×400), (B) 4 Gy radiation (×400), (C) 8 Gy radiation (×200), (D) 12 Gy radiation (×200).

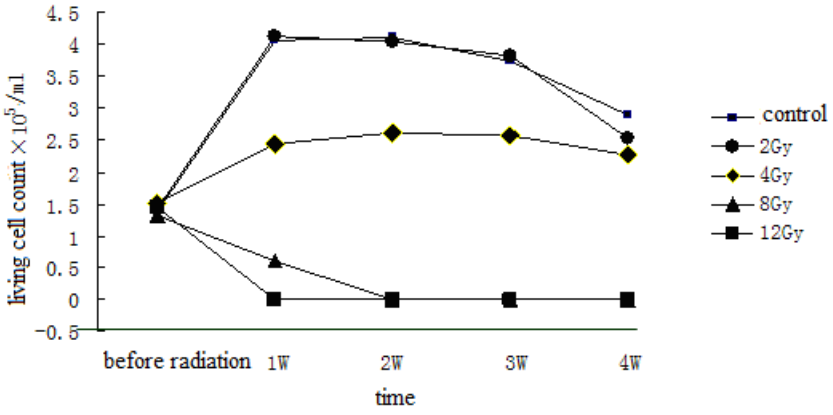

Figure 3. Trend in cell viability.

Table 3. Viable cell count (×10^5/ml, x ± s).

Group	Before radiation	Radiation (1W)	Radiation (2W)	Radiation (3W)	Radiation (4W)
Control	1.39 ± 0.27	4.07 ± 0.78	4.11 ± 0.74	3.74 ± 0.61	2.89 ± 0.35
2 Gy	1.42 ± 0.31	4.13 ± 0.81	4.05 ± 0.65	3.82 ± 0.63	2.54 ± 0.28
4 Gy	1.51 ± 0.36	2.45 ± 0.37#	2.61 ± 0.38#	2.58 ± 0.33#	2.27 ± 0.39●
8 Gy	1.32 ± 0.22	0.6 ± 0.11	0	-	-
12 Gy	1.44 ± 0.35	0	-	-	-

#p < 0.05， s. control and 2 Gy radiation groups, ●P<0.05， s. control group.

Table 4. MSCs iability (%, x ± s).

Group	Before radiation	Radiation (1W)	Radiation (2W)	Radiation (3W)	Radiation (4W)
Control	93 ± 3	293 ± 47	296 ± 49	269 ± 35	208 ± 33
2 Gy	92 ± 3	291 ± 39	285 ± 44	271 ± 39	179 ± 27
4 Gy	94 ± 2	162 ± 28#	173 ± 31#	170 ± 29#	150 ± 16●
8 Gy	93 ± 2	45 ± 7	0	-	-
12 Gy	91 ± 3	0	-	-	-

#p < 0.05， s. control and 2 Gy radiation groups, ● p < 0.05， s. control group.

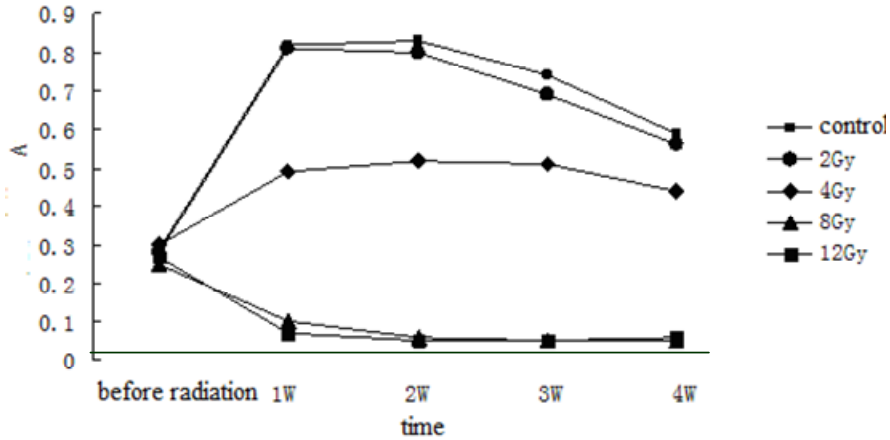

Figure 4. Trend in cell proliferation.

Table 5. Absorbance alue of different groups (x ± s).

Group	Before radiation	Radiation (1W)	Radiation (2W)	Radiation (3W)	Radiation (4W)
Control	0.29 ± 0.05	0.82 ± 0.13	0.83 ± 0.16	0.74 ± 0.12	0.59 ± 0.08
2Gy	0.28 ± 0.04	0.81 ± 0.15	0.80 ± 0.12	0.69 ± 0.11	0.56 ± 0.07
4Gy	0.30 ± 0.06	0.49 ± 0.09#	0.52 ± 0.08#	0.51 ± 0.07#	0.44 ± 0.06
8Gy	0.25 ± 0.03	0.10 ± 0.03	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
12Gy	0.27 ± 0.04	0.07 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01

#p < 0.05 s. control and 2 Gy radiation groups.

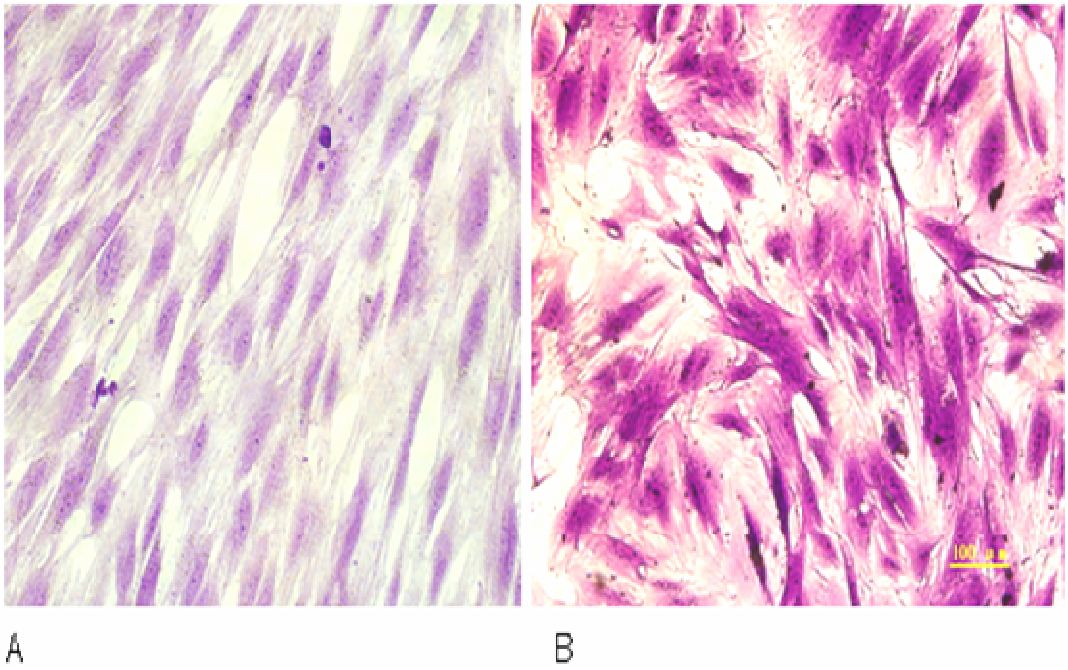

Figure 5. (A) Effect of 5-aza treatment on MSCs morphology in group A; (B) Effect of 5-aza treatment on MSCs morphology in group B.

The expression of myocardial-specific protein, C-TNT and β-MHC was not detected in the cells of group B (Figure 6). The expression of β-MHC (IOD, 0.36) and C-TNT (IOD, 0.21) in the cells of group A was detected after the 5-Aza treatment (Figure 6).

DISCUSSION

It is well documented that MSCs have the ability to differentiate osteoblasts, chondrocytes, myocytes, marrow stromal cells, tendon-ligament fibroblasts, adipocytes and other mesenchymal phenotypes. Because of their multiple differentiation or plasticity property, this is especially important for MSCs that could possibly repopulate other tissues than the bone marrow (Wang and Li, 2007).

In this study, we found that DNA damage was induced in a linear dose-dependent way by 60Co γ-rays. The data regarding viable cell counts showed a significant decrease in the MSCs viability index compared to control. These changes were dose and time dependent. This observation was confirmed by the MTT and trypan blue assays. Our results demonstrate that high dose (12 Gy) of radiation could accelerate cell dealth. In contrast, low dose (4 Gy) of radiation did not significantly affect MSCs. However, radiation may eventually affect cell growth, proliferation, viability and migration capacity of MSCs. In addition, radiation not only inhibited cell-cycle progression but also acted as a promoter of apoptosis.

MSCs have a large capacity for self-renewal while maintaining their multipotency. Like their hematopoietic counterparts, the differentiation of MSCs involves multi-step cell lineages controlled by bioactive factors existed in the local micro-environment or supplied in the culture environment of *ex vivo* cultivated cells. Nevertheless,

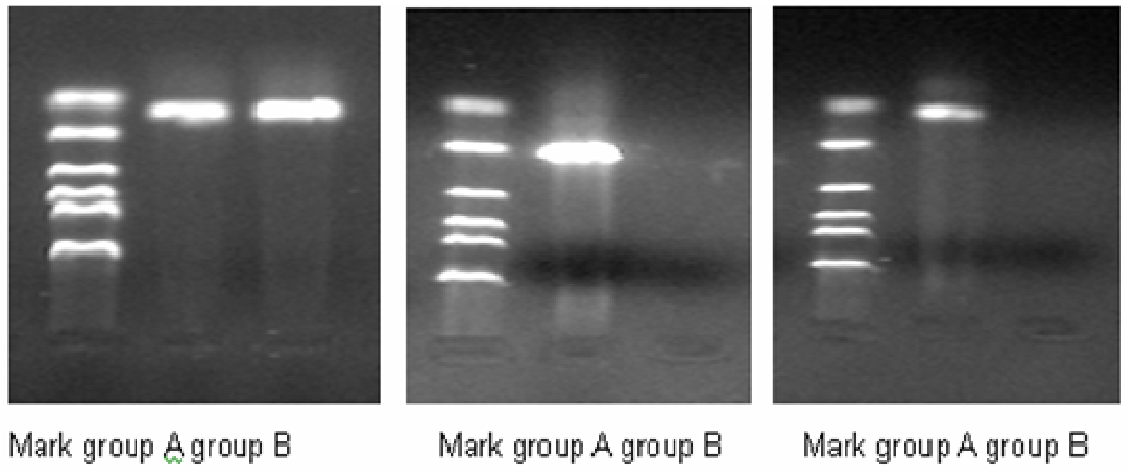

Figure 6. Effect of 5-aza treatment on expression of C-TNT and β-MHC mRNA in MSCs cells.

unlike their hematopoietic counterparts, MSCs can be ease of isolation and expanded highly in culture. Furthermore, MSCs display genetic stability, reproducible attributes from isolate to isolate, reproducible characteristics in widely dispersed laboratories and compatibility with tissue engineering principles (Pittenger et al., 1999; Lodie et al., 2002; Gronthos et al., 2003).

5-azacytidine (5-aza), sold under the trade name Vidaza, is a chemical analogue of cytidine, a nucleoside present in DNA and RNA. Cells in the presence of azacitidine incorporate it into DNA during replication and RNA during transcription. The incorporation of azacytidine into DNA or RNA inhibits methyltransferase thereby causing demethylation in that sequence, affecting the way that cell regulation proteins are able to bind to the DNA/RNA substrate. Inhibition of DNA methylation occurs through the formation of stable complexes between the molecule and with DNA methyltransferases, thereby saturating cell methylation machinery.

Cardiac troponin T (cTnT), a thin-filament contractile protein present in high concentrations in the myocardium but usually not in other tissues, is released rapidly after myocardial injury in direct proportion to the extent of injury. It persists in the serum for several days, probably as a result of ongoing release from the heart, but is not present in the serum following nonmyocardial muscle or other tissue damage (Gerhardt et al., 1991).

Expression of alpha- and beta-myosin heavy chain (MHC), the two functionally distinct cardiac MHC isoforms is species-dependent and tightly controlled by develop-mental and hormonal factors (Everett et al., 1984; Allen and Leinwand, 2001). Relative expression levels of these isoforms can be altered in disease states such as cardiac failure or hypertrophy (Nadal-Ginard and Mahdavi, 1989). For example, in failing adult mouse hearts, a shift from the normally predominant alpha-MHC toward beta-MHC is often observed (Harada et al., 1999; Jayakumar et al., 2010). Similarly, up-regulation of beta-MHC transcription can serve as an early and sensitive marker of cardiac hypertrophy (Jones et al., 1996).

The expression of myocardial-specific protein, C-TNT and β-MHC was not detected in the cells of group B (Figure 2B). The expression of β-MHC (IOD, 0.36) and C-TNT (IOD, 0.21) in the cells of group A was detected after the 5-Aza treatment (Figure 4B). A possible explanation was that 60Co γ radiation affected or inhibited the ability of MSCs to differentiate to myocardium.

In this study, we demonstrate that middle dose (4 Gy) of irradiation induces morphological changes, as well as alteration in both proliferation and differentiation potentials of MSCs. 5-azacytidine treatment did not successfully induce the differentiation of MSCs to myocardium, A possible explanation was that high-dose (8 and 12 Gy) of 60Co γ radiation affected or inhibited the ability of MSCs to differentiate to myocardium.

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Full Length Research Paper

Antagonistic activity of probiotic lactobacilli against *Staphylococcus aureus* isolated from bovine mastitis

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Staphylococcus aureus **is one of the major pathogens which cause Bovine Mastitis (BM). Probiotic lactobacilli have the great potential to produce antimicrobial compounds that inhibit and control pathogenic bacteria. Antagonistic activity of probiotic lactobacilli (*L. acidophilus* DSM 20079, *L. plantarum* ATCC 8014, *L. casei* ATCC 39392 and *L. reuteri* ATCC 23272) against *S. aureus* isolated from bovine mastitis (BM *S. aureus*) and standard *S. aureus* ATCC 25923 was the objective of this study. Antagonistic effect of probiotic lactobacilli was investigated by modified double layer method, well diffusion method, co-culturing assay and co-aggregation method. Among four lactobacilli, *L. plantarum* showed the greatest inhibitory activity. In modified double layer method the zone of inhibition of BM *S. aureus* and standard *S. aureus* ATCC 25923 by *L. plantarum* was 44 and 40 mm, respectively. Cell Free Supernatant (CFS) of probiotic lactobacilli in well diffusion method had inhibitory effect. Inhibition zone of BM *S. aureus* (13 mm) and standard *S. aureus* ATCC 25923 (9 mm) by *L. plantarum* was achieved. Co-culturing of *L. plantarum* with these two bacteria resulted in 87 and 77% inhibition growth of BM *S. aureus* and standard *S. ureus* ATCC 25923, respectively after 12 h. Co-aggregation between *L. plantarum* with two mentioned *S. aureus* was obtained 88.4 and 76%, respectively. According to these data, *L. plantarum* and its antimicrobial compounds can be one of the selective choices to control the BM *S. aureus.***

Key words: Antagonistic effect, probiotic lactobacilli, bovine mastitis, *Staphylococcus aureus.*

INTRODUCTION

In an economical point of view, bovine mastitis (inflammation of the mammary gland) is an important disease in dairy industry (Bradley, 2002; Viguier et al., 2009; DeRong et al., 2010). One of the major contagious pathogens which cause bovine mastitis is *Staphylococcus aureus*. This bacterium is adapted to survival within mammary gland and by establishment of infection, stimulates the inflammatory response (Bradley, 2002). It produces many virulence factors, such as alpha and beta toxins, protein A, coagulase, etc., which help it to colonize and damage mammary gland (Palma et al., 1999; Shana et al., 2009). Although antibiotic therapy to control bovine mastitis is effective, it can be detrimental too, because of the emergence of antibiotic resistant human pathogens (Craven 1987; Van vee and Margolles, 1999; Naheed et al., 2006). So an effective treatment by other substances than antibiotics becomes an urgent need (Pyrola, 2002).

Probiotic lactobacilli with a variety of applications are now the best choice to treat many infectious diseases of human and also have a great potential to control bovine mastitis (Green et al., 1991; Tagg and Dierksen 2003). These bacteria are well known as having many properties which make them beneficial to control pathogenic microorganisms. These include, the ability to adhere to cell, reduce pathogenic bacteria adherents, co-aggregate, produce organic acids, hydrogen peroxide,

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bacteriocin and etc., be safe and nonpathogenic, which antagonize pathogenic microorganisms (Gergor, 1999; Mami et al., 2008). The aim of this study is to assess antagonistic activity of the probiotic lactobacilli culture and their Cell Free Super-natant (CFS) against *S. aureus* from bovine mastitis (BM *S. aureus*) in comparison with standard *S. aureus* ATCC 25923.

MATERIALS AND METHODS

Bacteria strains and media

Probiotic lactobacilli (*L. acidophilus* DSM 20079, *L. plantarum* ATCC 8014, *L. casei* ATCC 39392 and *L. reuteri* ATCC 23272) and standard *S. aureus* ATCC 25923 were purchased from Persian Type Culture Collection. Isolated *S. aureus* from bovine mastitis was provided from Biotechnological Research Center, Azad University, Shahrekord branch. Deman Rgosa and sharp (MRS) broth and agar (Himadia), Brain Heart Infusion broth (BHI) (Himadia), Baird Parker agar (BP) (Himadia), Triptic Soy Broth (TSB) (Merck), Muller Hinton agar (MHA) (Merck) were used in this research.

Antimicrobial activity assays

Modified double layer method

Spot - on- lawn method (Tagg and Mc Given, 1971) which was called double layer method by (Maia, 2001) was used with somehow modification to evaluate the antagonistic activity. An overnight culture of each probiotic *Lactobacillus* in MRS broth at 37°C was prepared. 100 µl of each probiotic *Lactobacillus* culture (10^8 cfu ml-1) was spotted onto the surface of MRS agar and incubated for 24 h at 37°C. The plate of MRS agar containing lactobacilli spot was overlaid with melted Muller Hinton agar and allowed to solidify. 100 µl of (BM *S. aureus*) and Standard *S. aureus* ATCC 25923 (0.5 McFarland) individually inoculated by streaking the swab over the entire agar surface. The plates were incubated for 24 h at 37°C. The sensitivity of bacteria in the presence of each probiotic *Lactobacillus* spot was determined by measuring the clear zones around spot (Guessas et al., 2006).

Preparation of cell free supernatant

In order to prepare Cell Free Supernatant (CFS), each probiotic *Lactobacillus* was cultivated in MRS broth for 24 h at 37°C. CFS was obtained by centrifuging the culture (10000 rpm, 10 min) followed by filtration of the supernatant through a 0.2 µm pore size filter (Norroozi and Mirzaii 2004).

Well diffusion method

Inhibitory activity of CFS of probiotic lactobacilli was investigated by well diffusion method (Mami et al., 2008). An overnight culture of BM *S. aureus* and Standard *S. aureus* ATCC 25923 in TSB was prepared. These bacteria (10^8 cfu ml^{-1}) were inoculated by streaking the swab over the entire MHA surface. Wells sized (6 mm) were cut into the agar plate and 50 µl of each CFS was placed into each well. The plates were incubated for 24 h at 37°C and inhibition of growth was examined by clear zone surrounding each well.

Co-culture assay

Co-culture assay, another method for determination of antimicrobial effect of probiotic lactobacilli, was used (Lim, Sung-Me et al., 2009). BM *S. aureus* and standard *S. aureus* ATCC 25923 were grown in BHI broth for 24 h at 37°C. All lactobacilli were grown overnight in MRS broth at 37°C. All cultures were centrifuged at 10000 rpm for 10 min and washed twice with buffer phosphate saline (PBS). 1% of each *Lactobacillus* (10^5 cfu ml-1),BM *S. aureus* and standard *S. aureus* (10^7 cfu ml-1) were co-incubated in 50 ml BHI broth for 24 h at 37°C. Initial and then at predetermined intervals (after 12 h), 1 ml of cell culture serially diluted and plated on Baird Parker agar for *S. aureus*. The controls were the monoculture of each bacterium. The plates were incubated overnight at 37°C and the number of bacteria was evaluated. The inhibition was calculated using the following equation:

$$\% \text{ Inhibition} = \frac{(\text{CFU/ml in control}) - (\text{CFU/ml in co-incubation culture})}{(\text{CFU/ml in control})} \times 100$$

Co-aggregation method

Co-aggregation of each probiotic *Lactobacillus* with BM *S. aureus* and standard *S. aureus* ATCC 25923 was determined with somehow modified method of Svetoslav et al., 2009. Probiotic lactobacilli were grown in MRS broth for 24 h at 37°C. An overnight culture of BM *S. aureus* and standard *S. aureus* ATCC 25923 in TSB were prepared. Cells were harvested (10000 rpm, 5 min, 20°C) and washed with PBS. Then cells were suspended in sterile saline and adjusted on optical density (OD) of 0.4 measured at 660 nm. Equal volumes (500 µl) of each were mixed and incubated for 4 h at 37°C.Then centrifuged (1600 rpm, 5 min, 20 °C) and the OD of supernatants were measured at 600 nm. Co-aggregation was calculated using the following equation:

$$\% \text{ Co-aggregation} = \frac{\text{Atot} - \text{As}}{\text{As}} \times 100$$

Where Atot represents the OD of strains right after mixing and As refers to the OD of supernatants after 4 h.

RESULTS AND DISCUSSION

According to Ryan et al. (1999), using non-antibiotic formulations to prevent bovine mastitis can reduce the need of using antibiotics in treatment of this disease, so the problem of the emergence of antibiotic resistance pathogens can to a great extent be solved. In this study, the antimicrobial activity of non-antibiotic compounds, such as probiotic lactobacilli, against one of the major causes of bovine mastitis, *S. aureus* was investigated.

One of the important roles of probiotic lactobacilli is the production of inhibitory compounds that antagonize pathogenic bacteria (Nemcova et al., 1997; Jacobsen et al., 1999). The inhibitory activity of probiotic lactobacilli (*L. acidophilus* DSM20079, *L. plantarum* ATCC 8014, *L. casei* ATCC 39392 and *L. reuteri* ATCC 23272) against BM *S. aureus* and standard *S. aureus* ATCC 25923 by modified double layer method is shown in Table 1. The best zone of inhibition of BM *S. aureus* and standard *S. aureus* was achieved in the presence of *L. plantarum*, 44 and 40 mm, respectively. This result was supported

Table 1. The inhibitory effect of probiotic lactobacilli against BM *S. aureus* and standard *S. aureus* ATCC 25923.

Bacteria culture	Inhibition zone diameter(mm)	
***Lactobacillus* strains**	**BM *S. aureus***	***S. aureus* (ATCC25923)**
L. acidopillus DSM 20079	40	38
L. plantarum ATCC 8014	44	40
L. casei ATCC 3939	39	35
L. reuteri ATCC 2327	35	32

Table 2. The inhibition zone of BM *S. aureus* and standard *S. aureus* ATCC 25923 by CFS of probiotic lactobacilli.

Cell free supernatant	Inhibition zone diameter (mm)	
***Lactobacillus* strains**	**BM *S. aureus***	***S. aureus* (ATCC25923)**
L. acidopillus DMS 20079	11	8
L. plantarum ATCC 8014	13	9
L. casei ATCC 39392	10	7
L. reuteri ATCC 23272	10	7

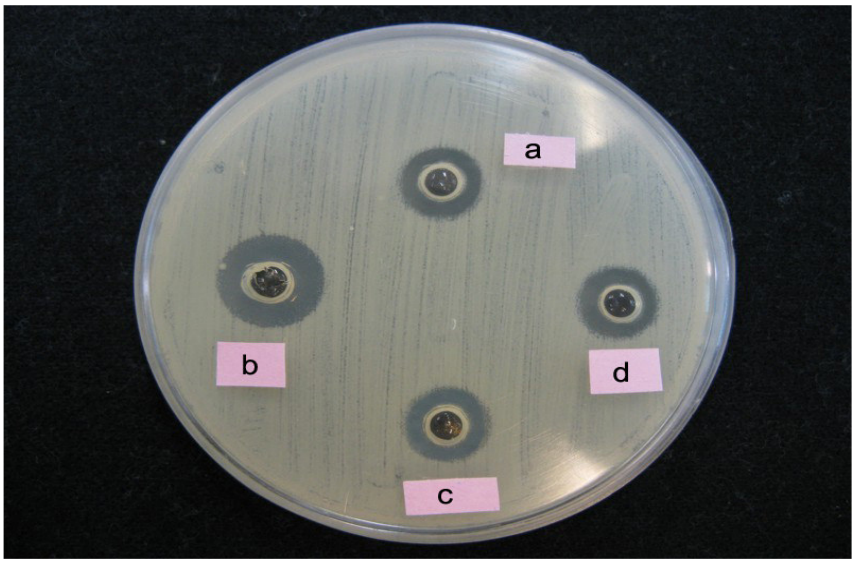

Figure 1. The inhibitory effect of CFS of probiotic lactobacilli against BM *S. aureus*. a) *L. reuteri*, b) *L. plantarum*, c) *L. acidophilus* and d) *L. casei*.

with the findings published by Mami et al. (2008) and in some extent was comparable with the findings of Bilg et al. (2005). According to Con and Gokalp (2000), this inhibitory effect was because of all or every metabolite such as lactic acid, acetic acid, diacetyl, bacteriocin, etc. which was produced during the assay period.

Antibacterial effect of cell free supernatant (CFS) of probiotic lactobacilli by well diffusion method is shown in Table 2 and Figure 1. A strong inhibition zone of BM *S. aureus* was obtained by *L. plantarum*, 13 mm. Standard *S. aureus* ATCC 25923 is nearly resistant to CFS of all lactobacilli, although *L. plantarum* had the most inhibitory effect against this bacterium, 9 mm. The findings of Paird et al. (1971) and Esaya et al. (2008) supported these results. According to Millettee (2006) antimicrobial effect of lacto-bacilli in co-culture with pathogenic bacteria is mainly due to production of organic acids which result in pH reduction, although they can produce some other substances as well. The inhibitory effect of probiotic lactobacilli in co-culture with BM *S. aureus* and standard

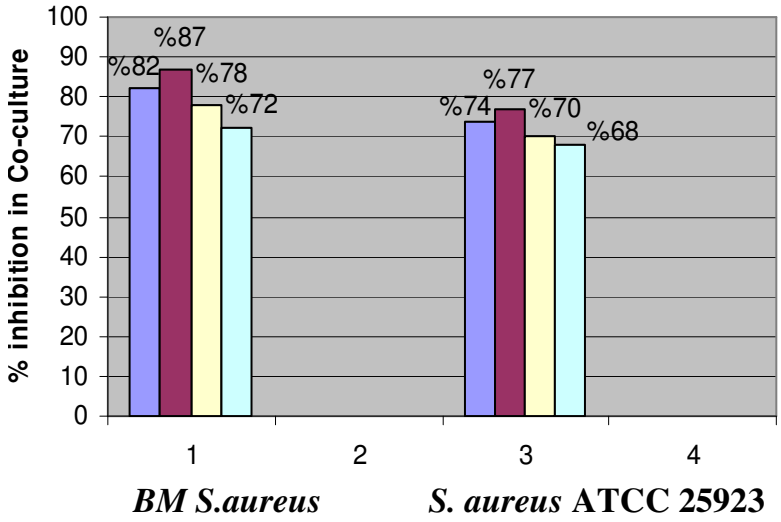

Chart 1. The inhibitory activity of co-culturing of probiotic lactobacilli with BM *S. aureus* and standard *S. aureus* ATCC 25923. ■ *L. acidophilus*, ■ *L. plantarum*, ■ *L. casei* and ■ *L. reuteri*.

Table 3. % Co-aggregation of probiotic lactobacilli with BM *S. aureus* and standard *S. aureus* ATCC 25923.

% Co-aggregation		
***Lactobacillus* strains**	**BM *S. aureus***	***S. aureus* (ATCC25923)**
L. acidopillus DSM 20079	81	72.4
L. plantarum ATCC 8014	88.4	76
L. casei ATCC 39392	80.1	68
L. reuteri ATCC 23272	79.9	65.4

S. aureus ATCC 25923 is shown in Chart 1. The best result was obtained when BM *S. aureus* and *S. aureus* ATCC 25923 co-incubated with *L. plantarum*, 87% and 77%, respectively. This result agrees with Bilge et al. (2005) who announced that antimicrobial sub-stances produced by *Lactobacillus* have a great potential for inhibiting the growth of pathogenic microorganisms. Co-aggregation assay is a reliable method to evaluate the close interaction between lactobacilli and pathogenic bacteria. Many surface proteins are found in lactobacilli which are predicted to promote binding to environmental surface like other bacteria surface. Co-aggregation may be beneficial to *Lactobacillus* that produces antimicrobial compounds, as it would force the cells into closer contact (Reid and McGroarty 1988). The result of co-aggregation of probiotic lactobacilli and BM *S. aureus* and standard *S. aureus* ATCC 25923 is shown in Table 3. Co-aggregation of *L. plantarum* with BM *S. aureus* and standard *S. aureus* 88.4% and 76% was the best result. As a conclusion, all used probiotic lactobacilli have an antagonistic activity against Staphylococcus aureus isolated from Bovine Mastitis and standard *S. aureus* ATCC 25923 but *L. plantarum* showed the great potential to inhibit mentioned pathogens. This bacterium can be one of the proper organisms to control bovine mastitis and a good choice for further investigations.

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Full Length Research Paper

Antimicrobial effect of slightly acidic electrolyzed water for inactivation of *Salmonella* spp. and *Escherichia coli* on fresh strawberries (*Fragaria* L.)

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Antimicrobial effect of slightly acidic electrolyzed water (SAEW: pH 5.6 ± 0.1, 20.5 ± 1.3 mg/L available chlorine concentration; ACC) against indigenous aerobic mesophiles and inoculated *Escherichia coli* and *Salmonella spp*. on fresh strawberry was assessed. The antimicrobial effect of SAEW was compared with that of strong acidic electrolyzed water (StAEW) and sodium hypochlorite (NaOCl) solution. SAEW effectively reduced total aerobic mesophilic bacteria from strawberries by 1.68 log_{10}CFU/g and was not significantly different from that of NaOCl solution ($p > 0.05$). Antimicrobial effect of SAEW against *Salmonella spp.* and *E. coli* was indicated by a more than 2 log_{10}CFU/g reduction of their population and the effect was not significantly different from that of NaOCl solution and StAEW at similar treatment conditions ($p > 0.05$). From these findings, SAEW with a near-neutral pH and low available chlorine concentration exhibits an equivalent bactericidal effectiveness to NaOCl solution and thus SAEW is a potential sanitizer that would be used as an alternative for StAEW and NaOCl solution in the fresh fruit and vegetables industry.

Key words: Slightly acidic electrolyzed water, strawberry, total aerobic mesophilic bacteria, *Escherichia coli*, *Salmonella spp*

INTRODUCTION

Fresh fruits are an important part of the human diet worldwide and consumers continue to eat more fruits partly because of reported health benefits (Beuchat, 1996). Strawberries are among the popular fruits and are mainly eaten raw as an important source of ascorbic acid (vitamin C). Fresh fruits can serve as a vehicle for many spoilage and food-borne pathogenic microorganisms with *Escherichia coli* O157:H7 and *Salmonella spp.* being the most frequent bacterial pathogens associated with fresh produce (Beuchat, 1996). Strawberries are reported to have a short postharvest life, mostly due to high metabolic bacterial activities and fungal decay. Studies have shown that *E. coli, Salmonella* spp. and *Listeria monocytogenes* are able to survive in fresh and frozen strawberries beyond the expected shelf-life of the fruit (Flessa et al., 2005) and therefore, their contamination with food-borne bacteria during harvesting or processing may pose a particular health hazard to consumers; this necessitate effective disinfection before they reach ultimate consumer. Washing produce with tap water cannot be relied upon to completely remove pathogenic and naturally occurring bacteria (Nguyen-The and Carlin, 1994; Yu et al., 2001; Koseki et al., 2004). Chemical compounds such as sodium hypochlorite (Adams et al., 1989), chlorine dioxide (Kim et al., 2009), sodium bisulfite (Krahn, 1977), sulfur dioxide (Bolin et al., 1977), organic acids (Adams et al., 1989), calcium chloride (Izumi and Watada, 1994, 1995), acidified sodium chlorite (Allende

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Abbreviations: **StAEW,** Strong acidic electrolyzed water; **SAEW,** slightly acidic electrolyzed water.

et al., 2009; Liao, 2009) and ozone (Nagashima and Kamoi, 1997) have been shown to reduce microbial populations on fresh produce. However, most of these sanitizers are made from the dilution of condensed solutions, which in handling involves some risk and is troublesome to the user and environment. A sanitizer that is not produced from the dilution of a hazardous condensed solution is required for practical use. For this reason, the use of electrolyzed water (EW) has been introduced as an alternative sanitizer in agriculture and food industry as it is safe for both environment and the user (Al-Haq et al., 2005). Strong acid electrolyzed water (StAEW), which is generated by the electrolysis of a dilute salt (NaCl) solution, has been proven to exhibit strong bactericidal activity for the inactivation of many pathogens (Venkitanarayanan et al., 1999; Park et al., 2004; Fabrizio and Cutter, 2005; Huang et al., 2008; Cao et al., 2009). However, the potential application of SAEW is limited because of its low pH values (≤ 2.7) and its corrosive characteristics. At this low pH, dissolved Cl_2 gas can be rapidly lost due to volatilization decreasing the bactericidal activity of the solution with time (Len et al., 2000) and adversely affecting human health and the environment. Moreover, the high acidity of SAEW may cause the corrosion of equipment and consequently limit its practical application (Abadias et al., 2008; Guentzel et al., 2008). Slightly acidic electrolyzed water (SAEW), a newly developed type of electrolyzed water with near-neutral pH value (5.0 - 6.5) is thought to be the best alternative for StAEW in disinfection of food and agricultural products. SAEW is produced by electrolysis of a dilute hydrochloric acid in a chamber without a membrane. At a pH of 5.0 - 6.5, the effective form of chlorine compounds in SAEW is almost (ca. 97%) the hypochlorous acid (HOCl) having strong antimicrobial activity (Honda, 2003; Cao et al., 2009). Therefore, the application of SAEW may improve the bactericidal activity with maximizing the use of hypochlorous acid, reduce corrosion of surfaces and minimize human health and safety issues from Cl_2 off-gassing (Guentzel et al., 2008). Despite these facts, the application of slightly acidic electrolyzed water (pH 5 - 6.5) as a food sanitizer has not been extensively studied in various types of fruits and vegetables as compared to strong acidic electrolyzed water (Koseki et al., 2001, 2004). The objectives of this study were to evaluate the antimicrobial effect of SAEW and compare its efficacy with that of StAEW and NaOCl solution in controlling the survival of indigenous bacteria as well as *Salmonella spp*. and *E. coli* inoculated onto strawberries at 20 ± 2°C.

MATERIALS AND METHODS

Strawberry sample preparation

Fresh strawberries of uniform size, color and maturity with a weight range of 25 - 30 g per fruit were purchased immediately after harvest from Katahira Kankou farm located in Kagoshima city and stored at 10 ± 2°C immediately after arrival at the laboratory. Strawberries were left whole, unhulled, or unwashed to evaluate bactericidal activity of SAEW against indigenous aerobic mesophilic bacteria associated with fresh strawberries. For simulated cross-contamination study, the calyx of the strawberries was removed.

Preparation of treatment solutions

SAEW was generated by electrolysis of a mixture of aqueous dilute solution of HCl (2%) and tap water using Apia60 generator (Apia60, HOKUTY Co., Kanagawa, JAPAN) at 5.0 V, 3.0 A and produced at a rate of 1.0 l/min. SAEW generator basically consists of an electrolytic cell with anode and cathode electrodes and no separating membrane between them (Figure 1a). StAEW was generated by electrolysis of 0.15% sodium chloride (NaCl) solution using a ROX-20TA generator (base model ROX-20TA, Hoshizaki Electric Co. Ltd., Japan) at 15.0 V, 14.5 A and at a rate of 1.5 l/min. The generator was left to run for 15 min before collecting water for the treatment. The StAEW generator consists of an electrolytic cell where the anode and cathode electrodes are separated by a diaphragm or membrane (Figure1b). With this type of apparatus, both StAEW and strong alkaline electrolyzed water are generated simultaneously. From the anode side of the generator, StAEW was produced and was collected to be used in this experiment. The cathode side produced strong alkaline electrolyzed water that was however, not collected. NaOCl solution was prepared by diluting 10% sodium hypochlorite solution (Wako Pure Chemicals Ind., Ltd., Osaka, Japan) using distilled water to obtain a final desired NaOCl solution. Tap water (TW) was used as control for this experiment.

Analytical measurements

The ORR, pH and ACC of treatment solutions were measured in duplicate immediately after preparation and before each bactericidal experiment. The pH was measured with a pH meter (HM-14P, TOA electronics Ltd., Tokyo, Japan) using a pH combination electrode (GST-2419C) and ORP was measured with ORP meter (RM-12P, TOA Electronics Ltd., Tokyo, Japan) using an ORP electrode (PST-2019C). The pH meter was calibrated using commercial standard buffers pH 4.01 and 6.86 (Nacalai Tesque, Inc., Kyoto, Japan). Available chlorine concentration of treatment solutions were determined by spectrophotometric method using a spectrophotometer (DR/4000V, HACH Co., Loveland, U.S.A). The detection limit is 0.2 mg/l Cl_2. Therefore samples were first diluted to desired lower levels of ACC using deionized water prior to measurement.

Preparation of inoculum and inoculation of strawberry samples

The pure L-dried culture of *E. coli* NBRC 3301 and salmonella spp. (NBRC 13245) were obtained from NITE Biological Resource Center (NBRC, Japan), revived soon after arrival according to L-dried culture reactivation procedures provided by NBRC and as described in details by Issa-Zacharia et al. (2010). The viable cell count of *E. coli* and *Salmonella* spp. cultures were verified by pour plate count methodology using standard method agar (NISSUI Pharmaceutical Co., Ltd, JAPAN). The colonies from plated pure culture were propagated once after every 4 days using a 4 by 4 looping out method on solidified standard method agar for preservation. Original *E. coli* and *Salmonella* spp. suspensions were prepared by transferring several colonies to a 10 ml of 0.1% peptone water using a sterile inoculation loop, vortexed using a thermal mixer (TM-100, Tokyo Thermonics Co. Ltd, JAPAN) and transferred to a 50 ml beaker that was filled up to a final volume of 50 ml by sterile 0.1% peptone water. The prepared original bacterial

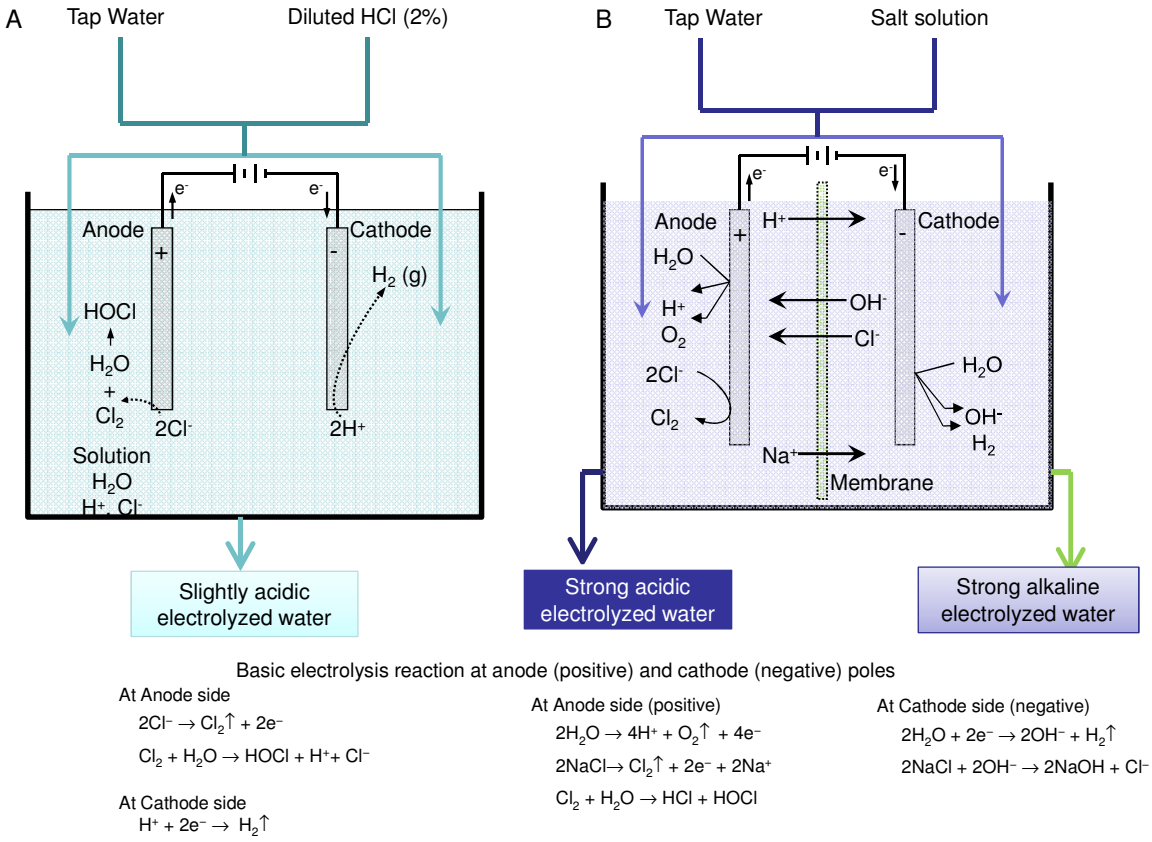

Figure 1. Schematic diagram of electrolyzed water generators resulting compounds during electrolysis. (A) is a SAEW generator and (B) is a StAEW generator. SAEW generator consists of an electrolytic cell with anode and cathode electrodes and no separating membrane between them (Figure 1A). StAEW generator consists of an electrolytic cell where the anode and cathode electrodes being separated by a diaphragm or membrane (Figure 1B). With this type of apparatus, both StAEW and strong alkaline electrolyzed water are generated simultaneously.

suspensions were continuously stirred using a magnetic stirrer (REXIM RS-6DR, ASONE Corporation, Osaka JAPAN) at 500 rpm to maintain the uniform distribution and applied to strawberries within 30 min of preparation. The final prepared bacterial suspension used for dip-inoculation onto the strawberry samples contained bacteria concentration of ca. 10 $\log_{10}$CFU/ml that was determined by plating 1 ml of portion of appropriately diluted *E. coli* and *Salmonella* spp. suspension on standard method agar plates and incubating plates at 37 ± 2°C for 24 ± 2 h. For a simulated cross-contamination study, strawberries were dip-inoculated into prepared *Salmonella spp.* and *E. coli* suspension at a fruit to bacterial suspension ratio (weight) of 1:3 with agitation on the rotary shaker at 150 rpm for 15 min to ensure uniform inoculation. The suspension was decanted and strawberries were placed on a sterile aluminum screen under a biosafety chamber and sterile air-dried for 30 min at room temperature (20 ± 1°C) before washing with different solutions.

Disinfection treatments of inoculated and un-inoculated samples

To evaluate the effectiveness of SAEW, StAEW and NaOCl solution on total aerobic mesophilic count and inoculated *E. coli* and *Salmonella* spp., both un-inoculated and inoculated strawberry samples received similar treatment. Four inoculated or un-inoculated strawberries (100 ± 20 g) were randomly selected and placed into a sterile 500 ml beaker with the aid of sterile forceps. SAEW, StAEW, NaOCl solution or TW (control) was added into the beaker at a fruit to treatment solution ratio of 1:3 by weight and treated for 5 min with agitation to facilitate the exposure between fruit and treatment solutions. The 5 min exposure time was selected to minimize product damage. Moreover, in the study by Udompijitkul et al. (2007) increase of exposure time from 5 to 15 min did not significantly increase the antibacterial effect of electrolyzed oxidizing water against *L. monocytogenes* and *E. coli* O157:H7 on strawberry, thus increase time would mean unnecessary loss of time. The antimicrobial effect of SAEW, StAEW and NaOCl solution against total aerobic bacteria, *E. coli* and *Salmonella spp.* was determined on the whole fruit tissue only.

Microbiological assay

Treated berries were put into sterile sampling bag with 99 ml Butterfield's phosphate buffer (BP) and homogenized in a stomacher (Model 22, TUL Instruments, Barcelona, Spain) at 230 rpm for 2 min. Fruit homogenate was serially diluted in BP, and 1 ml aliquots of appropriate serial dilution were pour-plated with plate standard plate count agar (NISSUI, Tokyo, JAPAN). Populations of mesophilic aerobic bacteria were counted after incubation at 35°C for 48 h and 37 ± 2°C for 24 h in case of *E. coli* and *Salmonella spp.*

Table 1. Physico-chemical properties of treatment solutions used for sanitization of strawberry.

	pH	ORP (mV)[a]	ACC (mg/L)[b]
TW[c]	6.8±0.4	632±6	<1
NaOCl[d]	9.7±0.1	655±7	125.3±1.7
SAEW[e]	5.6±0.1	940±7	20.5±1.3
StAEW[f]	2.5±0.1	1141±2	50.8±4.1

Values are the mean ± standard deviation of duplicate samples with n=10 for each solution; a: Oxidization reduction potential (mV) b: Available chlorine concentration (mg/L) c: Tap water. d: Sodium hypochlorite solution e: Slightly Acidic Electrolyzed water f: Strong Acidic electrolyzed water.

To obtain the initial population of *Salmonella spp.* or *E. coli* on strawberries, four inoculated but untreated strawberries were put into a sterile sampling bag with 99 ml of BP. Similarly, the baseline data for aerobic mesophilic bacteria were obtained by combining untreated samples with 99 ml BP and macerated in the stomacher for 2 min followed by plating procedures as previously described. Microbial counts were expressed as $\log_{10}$ CFU/g sample.

Statistical analysis

Mean of bacteria population reductions ($\log_{10}$ CFU/g) for each treatment was calculated from duplicate plates of each sample (n = 30 for each experiment). Data was expressed as mean ± standard deviation. The results were subjected to one way analysis of variance (ANOVA) and Tukey's HSD test was used to determine the differences at $p \leq 0.05$ using SPSS 13.0 (SPSS software for Windows, release 13.0, SPSS, Inc., USA).

RESULTS AND DISCUSSION

The physicochemical properties of treatment solutions used in current study are presented in Table 1. SAEW (pH 5.6; 20.5 mg/L ACC; ORP of 940 mV), StAEW (pH 2.5; 50.8 mg/L ACC; ORP of 1141 mV), NaOCl solution (pH 9.7; 125 mg/L ACC; ORP of 655 mV) were used to inactivate indigenous aerobic mesophilic bacteria, *Salmonella spp*. and *E. coli* on strawberry. In this experiment, tap water was used as control. The bactericidal effect of SAEW against indigenous aerobic mesophilic bacteria on strawberry was assessed and compared with other treatment solutions. The surviving populations of aerobic mesophilic bacteria in the macerate of strawberries treated with various sanitizers are summarized in Table 2. SAEW, NaOCl solution and StAEW treatment reduced levels of aerobic mesophilic bacteria in the macerate of the strawberry by 1.68, 1.71 and 2.07 $\log_{10}$CFU/g, respectively. These sanitizers resulted into a significantly higher reduction of indigenous aerobic mesophilic bacteria from strawberries ($p < 0.05$) than tap water (control) which achieved only 0.25 $\log_{10}$CFU/g. The difference in bactericidal effect against indigenous aerobic mesophilic bacteria between NaOCl solution and SAEW was not significant ($p > 0.05$). However, the bactericidal effect of StAEW was significantly higher than that of NaOCl solution and SAEW ($p < 0.05$). Similar reductions were reported by Udompijitkul et al. (2007) who reported a more than 2$\log_{10}$CFU/g of aerobic mesophiles by using electrolyzed oxidizing water (pH 2.27, ACC 68 ppm and 1137 mV of ORP) from strawberries.

The populations of *Salmonella spp.* and *E. coli* in the macerate of strawberries treated with various sanitizers are respectively summarized in Tables 3 and 4. When compared with the initial population (untreated), SAEW, NaOCl solution and StAEW significantly decreased the population of *Salmonella spp.* ($p < 0.05$) by 2.12, 2.15 and 2.22 $\log_{10}$CFU/g, respectively. Washing with tap water alone achieved insignificant *Salmonella spp.* reduction. The antimicrobial effect of SAEW, NaOCl solution and StAEW against *Salmonella spp.* (expressed as log reduction) was not significantly different ($p > 0.05$) as shown in Table 3 despite of their differences in available chlorine concentration. The available chlorine in NaOCl solution was > 6 times higher than that of SAEW, while the concentration in StAEW was more twice of SAEW (Table 1). The initial population of *E.coli* on inoculated strawberries was 8.13$\log_{10}$CFU/g. Washing with tap water resulted into insignificant ($p > 0.05$) reduction of *E. coli* population and 0.32$\log_{10}$CFU/g reduction was achieved. All the tested sanitizers significantly reduced the population of *E. coli* from strawberries and more than 2$\log_{10}$CFU/g of their population was evident. SAEW, NaOCl solution and StAEW respectively achieved a 2.21, 2.29 and 2.77 $\log_{10}$CFU/g reduction of *E. coli* population from strawberries. In the present study, SAEW (pH 5.6, 20.5 mg/L ACC) showed an equivalent antimicrobial effect against *E. coli* to that of NaOCl solution (pH 9.7, 125 mg/L ACC). StAEW (pH 2.5, 51 mg/L ACC) had a significantly higher antimicrobial effect against *E. coli* inoculated on strawberries than SAEW and NaOCl solution.

Strawberries are commonly eaten fresh without further heat treatment and therefore microbial control plays an important role in rendering them safe for human consumption. In the present study, SAEW and other tested sanitizers only achieved a 1.7- 2.0log reduction of indigenous aerobic mesophilic bacteria from strawberries. Higher log reduction in aerobic mesophilic bacteria was

Table 2. Efficacy of SAEW and other sanitizers against indigenous aerobic mesophilic bacteria associated with strawberries.

Treatments	Mean aerobic mesophilic bacteria in macerate (log $_{10}$CFU/g)		
	Before treatment	After treatment	log reduction
TW	7.82A	7.57A	0.25±0.02[a]
NaOCl	7.82A	6.11B	1.71±0.05[b]
SAEW	7.82A	6.17B	1.68±0.04[b]
StAEW	7.82A	5.75B	2.07±0.03[c]

Mean surviving population (log_{10}CFU/g) with different upper case letters (A, B) on the same row (before and after treatment) showed a significant difference at $p < 0.05$. Microbial reduction (log_{10}CFU/g) values are the means ± standard deviation and the mean log reduction (log_{10}CFU/g) with different lower case letters (a, b) on the same column for were significantly different at $p < 0.05$, Strawberries were dip-treated using NaOCl solution, SAEW and StAEW for 5 min to assess the antimicrobial effect of these sanitizers on the indigenous microorganism present on strawberries, TW: Tap water, NaOCl: Sodium hypochlorite solution, SAEW: Slightly Acidic Electrolyzed water and StAEW: Strong Acidic electrolyzed water.

Table 3. Efficacy of SAEW and other sanitizers against *Salmonella spp.* inoculated on strawberries

Treatments	Mean population of *Salmonella spp.* in macerate (log_{10}CFU/g)		
	Before treatment	After treatment	log reduction
TW	8.13A	7.64A	0.49±0.03[a]
NaOCl	8.13A	5.98B	2.15±0.03[b]
SAEW	8.13A	6.01B	2.12±0.03[b]
StAEW	8.13A	5.91B	2.22±0.02[b]

Mean surviving population of *Salmonella* spp. (log_{10}CFU/g) with different upper case letters (A, B) on the same row (before and after treatment) showed a significant difference at $p < 0.05$. Microbial reduction (log_{10}CFU/g) values are the means ± standard deviation and the mean log reduction (log_{10}CFU/g) with different lower case letters (a, b) on the same column for were significantly different at $p < 0.05$, Strawberry samples that were cross-contaminated by *Salmonella* spp. were dip-treated in NaOCl solution, SAEW and StAEW for 5 min. The antimicrobial effect was assessed by microbial log reduction (log_{10}CFU/g) as the result of a 5-min treatment and was calculated relative to un-treated samples, TW: Tap water, NaOCl: Sodium hypochlorite solution, SAEW: Slightly Acidic Electrolyzed water and StAEW: Strong Acidic electrolyzed water.

previously reported by Koseki et al. (2001) and Bari et al. (2003) when strong acidic electrolyzed water was used for decontamination of lettuce and tomato. This is due to the fact that lettuce and tomato have a relatively smooth surface, therefore strong acidic electrolyzed water was highly effective in killing or removing surface microorganisms (Koseki et al., 2004). A relatively lower reductions observed in current study could be attributed to the surface structure of the strawberry fruit. The strawberry has numerous achenes (seeds) that render its surface structure uneven and complex.

Results of current study have shown that SAEW and StAEW significantly reduced the population of *E.coli* and *Salmonella spp.* by more than $2log_{10}$ CFU/g. Udompijitkul et al. (2007) reported almost similar results in which washing with strong acidic electrolyzed water significantly decreased mean populations of *E. coli* O157:H7 and *L. monocytogenes* on fresh strawberry by $2log_{10}$ CFU/ml of solution, indicating that SAEW could equally be used instead of StAEW which is limited by its chlorine loss with time rendering loss of its antimicrobial activity. The observed efficacy of SAEW at low ACC could be due to its high content of hypochlorous acid (HOCl) and high ORP. At a pH of 5.0 - 6.5, the effective form of chlorine compounds in SAEW is almost the hypochlorous acid (HOCl) having strong antimicrobial activity (Yoshifumi, 2003; Cao et al., 2009). Current study showed that the effect of SAEW on the indigenous microbiota (total aerobic mesophilic count) of strawberries was smaller than that obtained with artificially inoculated *Salmonella spp.* and *E. coli.* This was probably because native microbiota on strawberries could have produced biofilms and could have therefore been more attached to, or become trapped in, the strawberry's tissue. Also, the total aerobic mesophilic count would have included many bacteria some of which might be resistant to SAEW and other tested sanitizers. In addition, Seo and Frank (1999) suggested that the effectiveness of disinfectants depends on the accessibility between the active sanitizing agent and the target microorganisms. Therefore, microorganisms that may be embedded in cracks, crevices and stomata or penetrate into interior structures can be protected from the action of disinfectants.

Current study demonstrated that SAEW at lower available chlorine concentration (20.5 mg/l ACC) exhibits a similar level of antimicrobial efficacy to NaOCl solution (125.3 mg/l ACC) against indigenous bacteria and other tested food pathogens. Unlike NaOCl solution, the

Table 4. Efficacy of SAEW and other sanitizers against *E. coli* inoculated on strawberries.

Treatment	Mean population of *E. coli* in macerate (log $_{10}$CFU/g)		
	Before treatment	After treatment	log reduction
TW	8.25A	7.93A	0.32±0.04[a]
NaOCl	8.25A	5.96B	2.29±0.07[b]
SAEW	8.25A	6.04B	2.21±0.05[b]
StAEW	8.25A	5.48B	2.77±0.02[c]

Mean surviving population of *E. coli* (log_{10}CFU/g) with different upper case letters (A, B) on the same row (before and after treatment) showed a significant difference at $p < 0.05$, Microbial reduction (log_{10}CFU/g) values are the means ± standard deviation and the mean log reduction (log_{10}CFU/g) with different lower case letters (a, b) on the same column for were significantly different at $p < 0.05$, Strawberry samples that were cross-contaminated by *E. coli* were dip-treated in NaOCl solution, SAEW and StAEW for 5 min. The antimicrobial effect was assessed by microbial log reduction (log_{10}CFU/g) as the result of a 5-min treatment and was calculated relative to un-treated samples, TW: Tap water, NaOCl: Sodium hypochlorite solution, SAEW: Slightly Acidic Electrolyzed water and StAEW: Strong Acidic electrolyzed water.

available chlorine in SAEW at pH 5.5 - 6.5 is predominantly (~97%) HOCl (Parish et al., 2003; Sapers and Gorny, 2006; Yoshifumi, 2003). The electrochemically activated HOCl in electrolyzed water is reported to be over 400% more effective than that formed chemically in for example, bleach (Schaik, 2009). It is widely believed that the bactericidal effect of slightly acidic electrolyzed water at a near neutral pH against various strains of bacteria is due to the combined action high oxidation-reduction-potential (ORP-reactions) and dissolved chlorine (HOCl). First, ORP-reactions at the cell membrane damage the outer and inner membrane and inactivate the microbes' defense mechanism. Then HOCl can penetrate the cell and oxidize it. SAEW has ORP of ≥ + 900 mV which directly and irreparably damages the microbial cell wall. In addition, the arrangement of water molecules is electrochemically altered which allows better penetrability and interaction of the microbicidal ions, a feature not found in conventional disinfectants (Schaik, 2009). This could further justify that observed antimicrobial efficacy of SAEW at low available chlorine concentration in current study could be due to its high content of hypochlorous acid (HOCl) and high ORP as previously stated. The ORP of ≥ + 900 mV for the SAEW used in current study would have contributed to its observed aerobic mesophilic bacteria reduction efficacy. Also, since *E. coli* and *Salmonella spp*. are facultative anaerobes, it is very likely that the high ORP of SAEW too played an influential role, in combination with its high proportion of HOCl for the effective inactivation of these pathogens from contaminated strawberry samples.

Due to the sanitizing equivalence of SAEW and NaOCl solution and increasing public health concerns about the possible formation of chlorinated organic compounds (Singh et al., 2002) and the safety concern that raised doubts in relation to the use of chlorine by the fresh fruit and vegetable industry, SAEW stands a promising chance as a novel disinfectant that could represent an alternative to sodium hypochlorite solution in fresh-cut industry. Moreover, the application of widely used StAEW might be replaced by SAEW, which may improve the antimicrobial activity while maximizing the use of hypochlorous acid, reducing the corrosion of surfaces and minimizing human health and safety issues from Cl_2 off-gassing (Guentzel et al., 2008). SAEW would thus be a potential environmentally friendly food sanitizer to be used towards the current green world advocacy era.

Conclusions

Populations of indigenous aerobic mesophilic bacteria were significantly reduced by SAEW, NaOCl solution and StAEW. Washing with tap water alone did not significantly reduce their population. SAEW at low available chlorine concentration demonstrated equivalent bactericidal effect to NaOCl solution against aerobic mesophilic bacteria and *E. coli* and *Salmonella spp.* inoculated on strawberries. SAEW (pH 5.6, 20.5 mg/L available chlorine) is promising and has potentials as sanitizer that would be used as an alternative for StAEW and NaOCl solution in the fresh fruit and vegetables industry.

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Short Communication

Inhibition of *Astragalus membranaceus* polysaccharides against liver cancer cell HepG2

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We have studied the inhibition of polysaccharides of *Astragalus membranaceus* against liver cancer cell HepG2. The polysaccharides at higher doses (25 mg/ml) have stronger antitumour effects, decreasing more than 40.5% (24 h) and 67.3% (48 h) of liver cancer HepG2 cell viability. A high percentage of apoptotic HepG2 cells was found at 25 mg/ml of *A. membranaceus* polysaccharides. 23.9 and 38.2% of cells experienced apoptosis when HepG2 cells were treated for 24 and 48 h with 25 mg/ml of *A. membranaceus* polysaccharides. Consequently, the results of the *in vitro* assays suggest that the *A. membranaceus* polysaccharides possesses strong antitumour activities, which is benefical to treatment of liver cancer.

Key words: Astragalus membranaceus polysaccharides, Antitumour, HepG2, MTT.

INTRODUCTION

Astragalus membranaceus (Fisch) Bunge (AM), Maxim of the Leguminosae family, is a traditional Chinese medicinal herb originated in Northern China. The dried root of AM, Huangqi, contains 2′4′-dihydroxy-5,6-dimethoxyisoflavone, kumatakenin, choline, betaine, polysaccharides, saponins, glucuronic acid, sucrose, amino acids, traces of folic acid and astraisoflavanin (Bensky and Gamble, 1993; Ma et al., 2002; Wu and Chen, 2004). Huangqi is the Chinese name for the root of AM. AM (root), also known as Huangqi in Chinese and Radix Astragali (RA) in Latin, is the dry root of *A. membranaceus* (Fisch) Bge. or *A. membranaceus* var. Mongholicus (Bge.) Hsiao of the Fabaceae family. AM grows mainly in Northern China, Mongolia and Siberia. Modern analytic techniques have identified more than 100 compounds that are contained in AM (root), such as flavonoids, polysaccharides (astragalan, APS), saponins (astragalosides), sucrose, amino acids and phenolic acids (Wu and Chen, 2004). The major bioactive constituents of AM (root) are flavonids, APS and saponins, and each has its own therapeutic properties.

Primary liver cancer is the fifth most common malignancy in the world, with a global annual incidence of about one million new patients. In 2004, the American Cancer Society estimated 18,920 new cases of Hepatocellular Carcinoma (HCC) and the estimated deaths were 14,720 (Shah and Bhowmick, 2006). The disease prevails in parts of Asia and Africa; yet appears rife in many European countries in recent years. Although, substantial advances have been made in chemotherapy regimen for HCC, the efficacy of drugs is often hampered by a range of adverse side-effects imposed on patients (Zein and Zein, 2002). Accordingly, it is urged to explore a new approach for development of an effective therapy against this disease.

In the present study, the polysaccharides of *A. membranaceus* were tested for its anti-tumour activity using liver cancer cell lines HepG2.

MATERIAL AND METHOD

Material

A. membranaceus was purchased from a local herb shop (Taizhou, China). The *A. membranaceus* was identified and authenticated by Institute of Botany, zhejiang Province, China. A voucher specimen (Number 20090876) was deposited in the herbarium of the Institute. Human liver cancer cell lines HepG2 was kindly provided from institute of pathology, our university.

Sample preparation

About 100 g of *A. membranaceus* sample was ground into powder

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Table 1. Inhibition rate (%) of *A. membranaceus* polysaccharides against HepG2 cell growth.

	Concentration					
	0 (Control)	**3 mg/ml**	**15 mg/ml**	**25 mg/ml**	**35 mg/ml**	**45 mg/ml**
Inhibition rate (%)	-	3.2±0.1	18.4±1.4	40.5±2.8	49.4±3.3	50.8±2.1
	-	4.2±0.2	29.1±1.5	67.3±4.9	76.2±6.1	78.1±5.3

Table 2. *A. membranaceus* polysaccharides-induced HepG2 cell death rate (%).

	Concentration					
	0 (Control)	**3 mg/ml**	**15 mg/ml**	**25 mg/ml**	**35 mg/ml**	**45 mg/ml**
Cell death rate (%)	1.3±0.1	2.3±0.1	10.6±1.4	23.9±1.5	40.9±3.3	47.8±2.3
	1.6±0.1	3.4±0.2	18.4±1.2	38.2±1.9	52.5±4.6	54.2±4.5

in a mortar, and was then extracted with 0.3 mol/L, pH 8.0, phosphate buffer containing a appropriate ratio of trypsinase at 40℃ for a given time. Then, protein in the extract was removed by Sevag method. The result material then was extracted with distilled water at 100℃. After filtration, the suspension was centrifuged for 10 min. The residues were extracted twice for 2 h with distilled water (100℃) and concentrated to dryness. The crude extract was then dialyzed against tap water, deionized with mixed ion exchange resins and dried under reduced pressure to give desire product. HPLC analysis showed that *A. membranaceus* polysaccharides contained glucose (34%), mannose (2.7%) and xylose (7.9%).

Gas chromatography–mass spectrometry (GC–MS)

Each of the *A. membranaceus* polysaccharides was dissolved and hydrolyzed in 200 µl 4.0 M trifluoroacteic acid (TFA) for 30 min in a sealed vial at 120℃. After hydrolysis reaction, excess TFA was removed by purging the sample with a stream of nitrogen at 2.0 ml/min. The hydrolyzed samples were derivatized using trimethylsilylation (TMS). The hydrolyzed samples were dissolved in 500 µl pyridine with vigorous shaking and/or sonication. 200 and 100 µl of trimethylchlorosilane (TMCS) was added. The mixtures were shaken vigorously for 1.0 min and were allowed to stand overnight to ensure complete derivatization of the sample. The final TMS-derivatized solutions were centrifuged for 1 min. 10.0 µl of the supernatant liquid was extracted and diluted with 990.0 µl pyridine. 1.0 µl of the sample solution was injected into the GC–MS system.

Treatment with antitumour agents and MTT colorimetric assay

Human liver cancer cell lines HepG2 (5×10^3 cells/0.33 cm^2) was plated in 96-well plates "Nunclon TM MicrowellTM" (Nunc) and was incubated at 37℃. After 24 h, cells were treated with *A. membranaceus* polysaccharides (final concentration 3-25 mg/ml). Untreated cells were used as controls. Microplates were incubated at 37℃ in humidified atmosphere of 5% CO_2, 95% air and then cytotoxicity was measured with colorimetric assay based on the use of tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). The results were read on a multiwell scanning spectrophotometer (Multiscan reader), using a wavelength of 570 nm. Each value was the average of 8 wells (standard deviations were less than 20%). Results are expressed as percent of cell proliferation inhibition calculated according to the following:% inhibition rate of tumour cells (%) = (Absorbance of control - Absorbance of test) / Absorbance of control × 100%

Flow cytometry

Apoptosis induction of human liver cancer cell lines HepG2 by *A. membranaceus* polysaccharides was verified by using a BD FACSCalibur flow cytometer (Becton–Dickinson) and staining with Annexin V-PE and 7-AAD. Cell samples (5×10^6 cells/ml) were induced with *A. membranaceus* polysaccharides (3-25 mg/ml). The cells were incubated for 24 and 48 h in medium. After incubation, an aliquot of cells was taken, washed, and resuspended in PBS prior to analysis. Cells were identified by forward and side scatters as well as the fluorescence Annexin V-PE and 7-AAD to discriminate apoptotic cells from live and dead cells.

RESULTS

Cell treatment with *A. membranaceus* polysaccharides at the concentration range of 3-45 mg/ml markedly affected HEPG2 cell growth. Cell exposure to aqueous polysaccharides of *A. membranaceus* induced an inhibition of cell proliferation and the effect increased in a dose-dependent manner. Compared with untreated control, the concentration of 45 mg/ml of *A. membranaceus* polysaccharides resulted in 50.8% growth inhibition HEPG2 after 24 h of exposure and in 78.1% growth inhibition after 48 h of exposure (Table 1).
Flow cytometric analyses (Table 2) indicated that 47.8% of cells experienced apoptosis when HEPG2 cells were treated for 24 h with 45 mg/ml of *A. membranaceus* polysaccharides. In the control cells, only a minor fraction of the cell population (1.3%) experienced apoptosis. When HEPG2 cells were treated for 48 h with 45 mg/ml of *A. membranaceus* polysaccharides, 54.2% of apoptotic cells were detected as compared to the untreated control with 1.6% apoptotic cells.

DISCUSSION

Despite the decrease in incidence, liver cancer remains the second leading cause of cancer related death worldwide. Prevention is likely to be the most effective means of not only reducing the incidence but also mortality from this disease. In recent years, attention has been focused on the anticancer properties of plant-derived dietary constituents of food, an important role in the prevention of disease (Block et al., 1992; Lambert and Yang, 2003; Wang et al., 2009; Wei et al., 2009; Xu et al., 2009; Yoon et al., 2009).

In this study, we identified the antitumor activity of *A. membranaceus* polysaccharides by the MTT method, and determined the high level (40.5% for 24 h and 67.3% for 48 h) of inhibition of liver cancer HEPG2 cell survival (at a dose range of 25 mg/ml).

Apoptosis is characterized by a number of well-defined features (Hengartner, 2000). In contrast to necrosis, apoptotic cell death is thought to be physiologically advantageous (Shacter et al., 2000; Anderson et al., 2002; Lauber et al., 2004). In this study, *A. membranaceus* polysaccharides induced apoptotic cell death in liver cancer HEPG2 cells in a dose-dependent manner. Our results showed that *A. membranaceus* polysaccharides, at doses higher than 25 mg/ml, reduced more than 23.9% (24 h) and 38.2% (48 h) of the HEPG2 cells viability, respectively.

Our results showed that *A. membranaceus* polysaccharides could inhibit HEPG2 cells proliferation, at least partly through apoptosis. The inhibition rate and the percentage of apoptotic cells were all dose-dependent. *A. membranaceus* polysaccharides at 25 mg/ml dosages had a prominent inhibition rate than the lower dosages.

Consequently, the results of the *in vitro* assays suggest that the *A. membranaceus* polysaccharides possesses strong antitumour activities, which is benefical to treatment of liver cancer.

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Full Length Research Paper

Identification and characterization of tenuazonic acid as the causative agent of *Alternaria alternata* toxicity towards groundnut

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***Alternaria alternata* (Fr.) Keissler, the causal agent of Alternariosis of groundnut seeds (*Arachis hypogaea* L.) was toxigenic when cultured on various laboratory media. The cell free extract or culture filtrate induced severe chlorosis and necrosis on leaves, inhibition of root and shoot growth of germinating seeds and wilting of seedlings. A phytotoxin was isolated both from cell free extract/culture filtrate and infected tissues, purified and identified as tenuazonic acid, by using thin layer chromatography, ultra violet and infra red spectral analysis. Toxicity was monitored with standard bioassay techniques. The results show patho-physiological significance of tenuazonic acid in disease syndrome.**

Key words: *Alternaria alternata*, groundnut, alternariosis, tenuazonic acid.

INTRODUCTION

Several phytopathogenic species of *Alternaria* have been reported to produce phytotoxic metabolites, many of which have been chemically characterized and play a significant role in pathogenesis. *Alternaria alternata*, an important pathogen of many plants, produces tenuazonic acid (TA) with bioactivity to microbes, plants and animals. TA is one of the main mycotoxin to humans and other organisms (Zhou and Qiang, 2008).

A. alternata (Fr.) Keissler, though a weak pathogen, is known to cause a large number of leaf spot and blight diseases in plants. The phytotoxic metabolites isolated from this fungus have been implicated in the disease syndrome.

On groundnut it has been reported to cause leaf spot and veinal necrosis or alternariosis leading to considerable damage to the crop in the South Indian States, where the crop is grown intensively as post-rainy season crop (Subramanyam et al., 1981). *A. alternata* species produce several mycotoxins such as alternariol (AOH), alternariol monomethyl ether (AME) or tenuazonic acid (TA). Natural occurrences of AOH, AME and TA have been reported in various fruits, including tomatoes, olives, mandarins, melons, peppers, apples and raspberries (Scott, 2001).

Tenuazonic acid, iso-tenuazonic acid, and their salts exhibit herbicidal activity with broad spectrum, quick killing, and high efficiency. The addition of adjuvants improves the herbicidal activity of these compounds

The symptoms of the disease indicate that the fungus may be producing some powerful toxic metabolite(s) during pathogenesis and preliminary studies indicated the same. The present study, reports both the *in vitro* and *in vivo* production of tenuazonic acid, by this pathogen, its isolation and characterization.

MATERIALS AND METHODS

In vitro production of toxic extract and assay for phytoxicity

A virulent strain of *A. alternata* isolated from infected groundnut leaves was used in the present study. The production of tenuazonic acid was carried out in the medium consisting following chemical

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composition (g/L) (Glucose-40; KH_2PO_4-1,$(NH_4)_2HPO_4$-2; $MgSo_4$-$7H_20$, 0.5; KCl, 0.5; yeast extract-1, were dissolved in 1000 ml distilled water, and the medium pH was adjused to 5.5. 200 ml medium aliquotes was transferred in 1 L Roux bottles. The fungus was grown for 3 weeks at 29 ± 2℃ as stationary culture and filtered through several layers of cheese cloth.

Half litre portion of the culture filtrate was autoclaved at 15 lbs pressure for 10 min, to inactivate any possible enzymatic activity before being used for phytotoxicity testing on the leaves of groundnut by leaf spot assay tests. Growth inhibiting activity of the culture filtrate was assayed on germinating groundnut seeds. One hundred germinating seeds were placed in Petri dishes containing filter papers moistened with culture filtrate and incubated at 30℃; root and shoot elongation was measured after the incubation period of 6 days in order to evaluate the activity. The phytotoxic activity was also assayed on one month old seedlings of groundnut by dipping the cut shoots in 100 ml of culture filtrate in 250 ml Erlenmeyer flasks. Uninoculated medium served as control in all the above experiments.

Isolation of toxin from culture filtrate

The toxin isolation was carried out by the method of Janardhanan and Hussain (1975). The fungal filtrate was collected by filtering through cotton wool. An equal volume of methanol was added to the fungal filtrate to precipitate proteins and kept in a refrigerator for 24h. The precipitate was filtered off and methanol recovered at 40 - 50℃ *in vaccuo*. The filtrate was then extracted with ethyl acetate 3 times and the extract concentrated with 40 - 50℃ *in vaccuo*. An orange red viscous substance obtained was dissolved in acetone, absorbed on activated charcoal and then eluted with the same solvent. The acetone eluate was concentrated *in vaccuo* for further purification and identification.

Identification of toxin

The concentrated eluate containing the toxic metabolite(s) isolated from the culture filtrate was chromatographed by thin layer chromatography (TLC) on silica gel G using chloroform : methanol (99:1), chloroform : methanol (80:20) and ethyl acetate and benzene (99:1) as solvent systems. Some of the known *Alternaria* toxins namely AOH, AME and TA were used as reference samples in order to identify the compound by comparison with its Rf value. The spots were detected by treatment with iodine vapours or spraying with ethanolic ferric chloride. The tenuazonic acid was confirmed by UV and IR spectral analyses. UV absorbance was measured in Spectrophotometer (Hitach) and IR spectrum was determined. UV and IR spectra of the reference sample taken under identical conditions were used for comparison.

Isolation of the toxin from infected leaves

The method followed was that of Janardhanan and Hussain (1983). Infected leaves (5 gms) showing early and advanced stages of infection were collected from inoculated plants and processed by homogenizing with 100 ml 80% aqueous acetone. The homogenate was filtered through Whatman No.1 filter paper and concentrated at low temperature. The aqueous extract thus obtained was acidified with dilute HCL to pH 2 and extracted thrice with 10 ml 5% $NaHCO_3$. The aqueous layer was separated, pH adjusted to 2 with dilute HCl and extracted with ethyl acetate. The ethyl acetate extract was dried over anhydrous Na_2SO_4 and the solvent evaporated at low temperature *in vaccuo* and the residue was chromatographed by TLC on silica gel G using chloroform: methanol (90:10) solvent system using a standard sample of tenuazonic acid as reference. The spots were developed by exposing the TLC plates to iodine vapours/ethanolic ferric chloride. Samples of leaves from uninoculated plants and processed in a similar manner were used as control.

Figure 1a. Effect of phytotoxin of crude culture filtrate on groundnut leaves applying 50 ul at 12, 24 and 36 h of incubation. (a) control (b) 12 h (c) 24 h (d) 36 h of incubation.

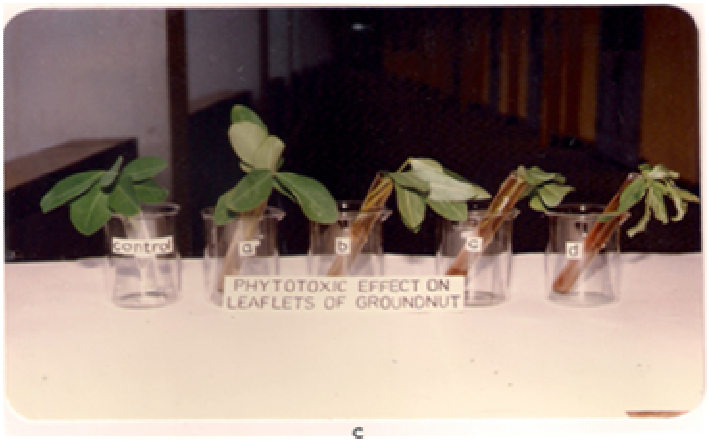

Figure 1b. Effect of phytotoxin on groundnut leaflets at different dilutions. Control, (a) 4:1 (b) 1:1 (c) 1:4 (d) phytotoxin (water/phytotoxin)

RESULTS

Phytotoxic activity of culture filtrates

Culture filtrate applied on leaves of groundnut produced chlorotic spots after 24 h of treatment, which enlarged and turned necrotic after 48 h. The necrotic spots were surrounded by chlorotic margin at later stages. The toxic symptoms elaborated were almost similar to natural infection. Leaflets of groundnut treated with culture filtrate exhibited irreversible wilting and dehydration within 24 h (Figure 1)

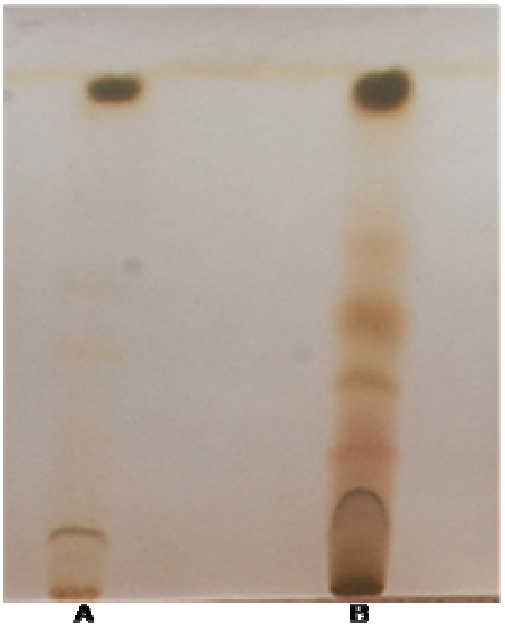

Figure 2a. Isolation of toxin from culture treating with iodine vapours, B. Authentic sample of TA, B. Isolated toxic metabolite from culture filtrate

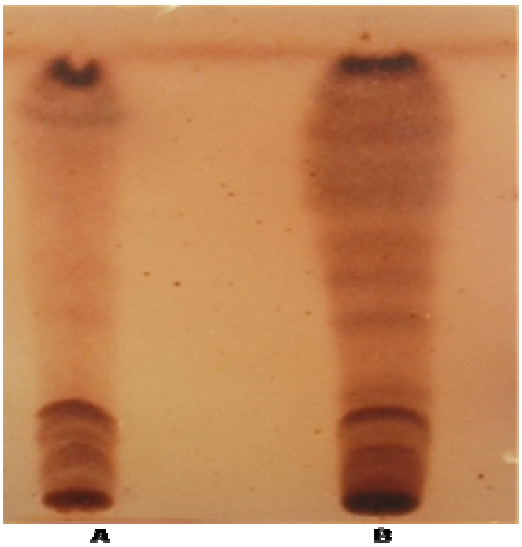

Figure 2b. Isolation of toxin from filtrate treating with Iodine vapours, A. Authentic sample of TA, B. Isolated toxic metabolite from culture filtrate

Isolation of tenuazonic acid from the culture filtrate

The analysis of the crude toxin by TLC, using chloroform: methanol (90:10), showed 6 spots on exposing the TLC plates to iodine vapours. Toxicity test of the spots eluted from unexposed TLC plates showed that the spot having Rf value 0.40 was the major phytotoxic principle. Other spots were either non toxic or weakly toxic (Figures 2a and b).

Identification of tenuazonic acid (TA)

Tenuazonic acid was identified based on the comparison of the Rf value of the toxic metabolite isolate from the culture filtrate with that of AOH, AME and TA showed agreement with that of tenuazonic acid. The toxin and the reference sample of tenuazonic acid produced brilliant orange colour spots on TLC plates by ethanolic $FeCl_3$ spray. Rf values of the toxic metabolite as compared to tenuazonic acid on TLC using various solvent systems was presented in Table 1.

The compound showed UV absorption peaks at 226 and 288 nm in spectrophotometry (Figure 3a). These were found to be identical with UV spectra of standard tenuazonic acid (Figure 3b). IR spectral analysis of tenuazonic acid isolated from culture filtrate showed absorption peaks at 3300, 2990, 2140, 1930, 1650, 1460, 1390, 1275, 1090, 1050 and 800 cm^{-1} (Figure 4a). Comparison of IR spectrum with that of standard TA revealed striking similarity (Figure 4b). Strong absorption was noticed in the region of 2990, 1650 cm^{-1}. Based on these observations the phytotoxin was identified as tenuazonic acid.

Detection of tenuazonic acid in infected plants

Tenuazonic acid in infected plants dectected by TLC analysis of toxin isolated from infected leaves indicated the presence of ethanolic $FeCl_3$ positive orange colored spot with identical Rf value and similar UV (Figure 3a) and IR (Figures 4a and b) spectral peaks as of standard tenuazonic acid. The spots were developed by exposing the plates to iodine vapours.

DISCUSSION

Experimental results indicate that *A. alternata* culture filtrate induced Alternariosis on leaves, as well as growth inhibition of germinating seeds. The observations of Fulton et al. (1965) support these conclusions, demonstrating that the pathogen produces several metabolites into culture filtrate, one of which was tenuazonic acid. Identification of the compound was done by TLC, besides UV and IR spectral analysis support this conclusion.

Tenuazonic acid was first isolated by Rosett et al. (1957), from *Alternaria tenuis* and its structure was established by Stickings (1959) as 3-acetyl-5-sec butyl-4-hydroxy-3pyrolin-one. Later, it was isolated from *Alternaria longiceps, Alternaria.kikuchiana, Alternaria mali* and *Pyricularia oryzae* as a phytotoxin and from

Table 1. Comparison of Rf value of the toxin isolated from *A. alternata* with tenuazonic acid by TLC.

Solvent	Rf value	
	Isolated phytotoxin	Standard tenuazonic acid
Chloroform : Methanol (90:10)	0.40	0.40
Chloroform : Methanol (80:10)	0.60	0.63
Ethyl acetate : Benzene (99:1)	0.21	0.21

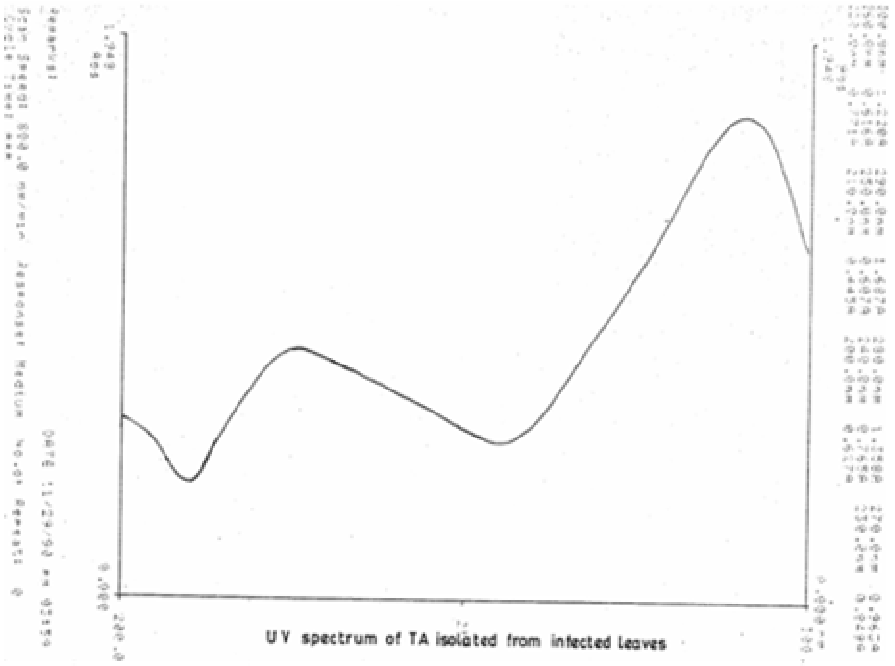

Figure 3a. UV absorption peaks of authentic TA at 226 nm.

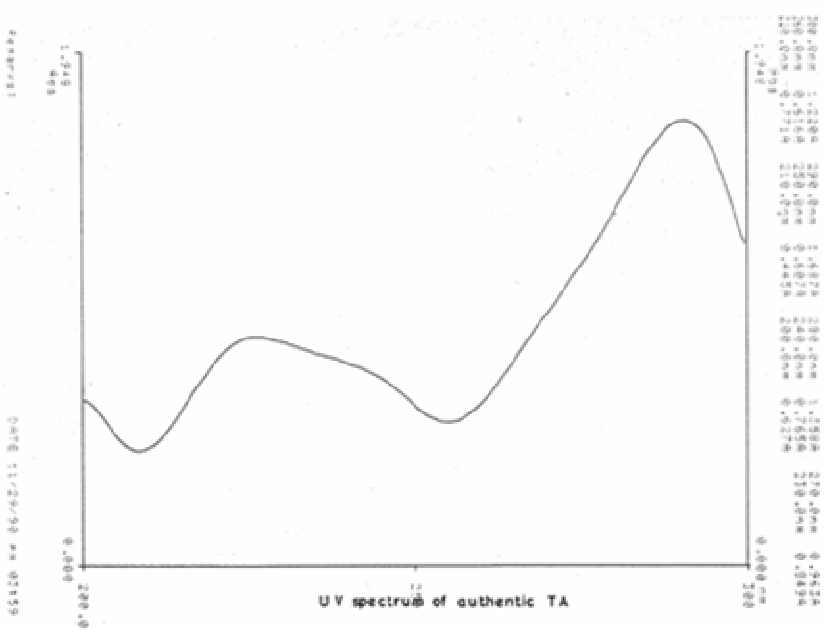

Figure 3b. UV absorption peaks of TA isolated from infected leaves at 226 nm.

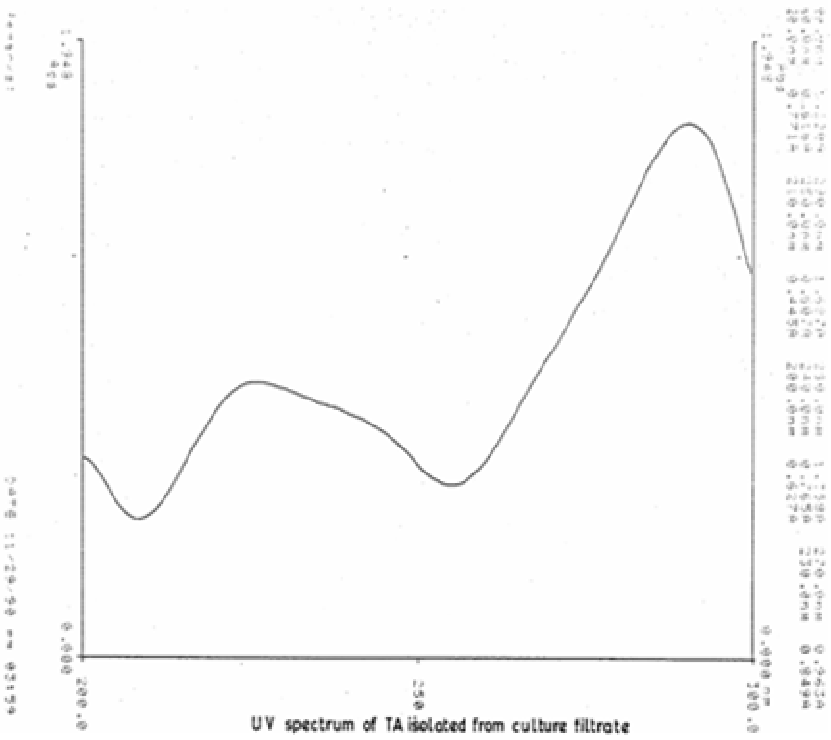

Figure 3c. UV absorption peaks of TA isolated from culture filtrate at 226 nm

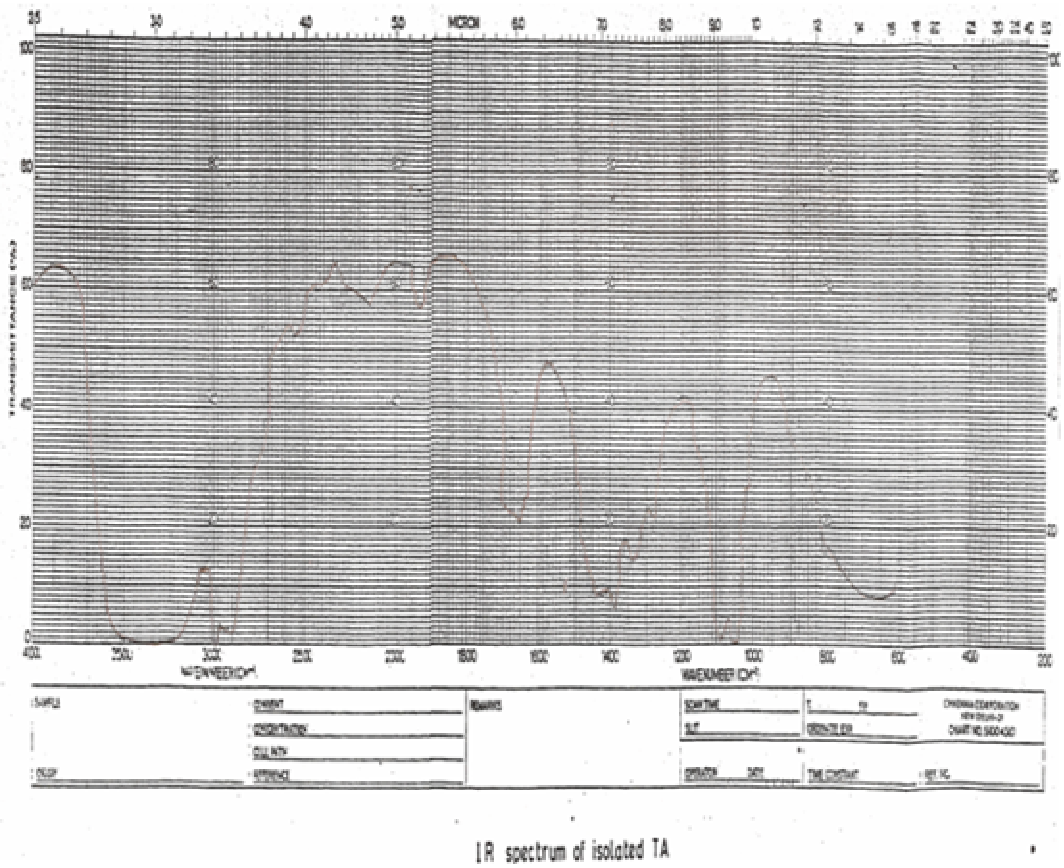

Figure 4a. IR Spectrum of Tenuazonic acid from isolated culture filtrate

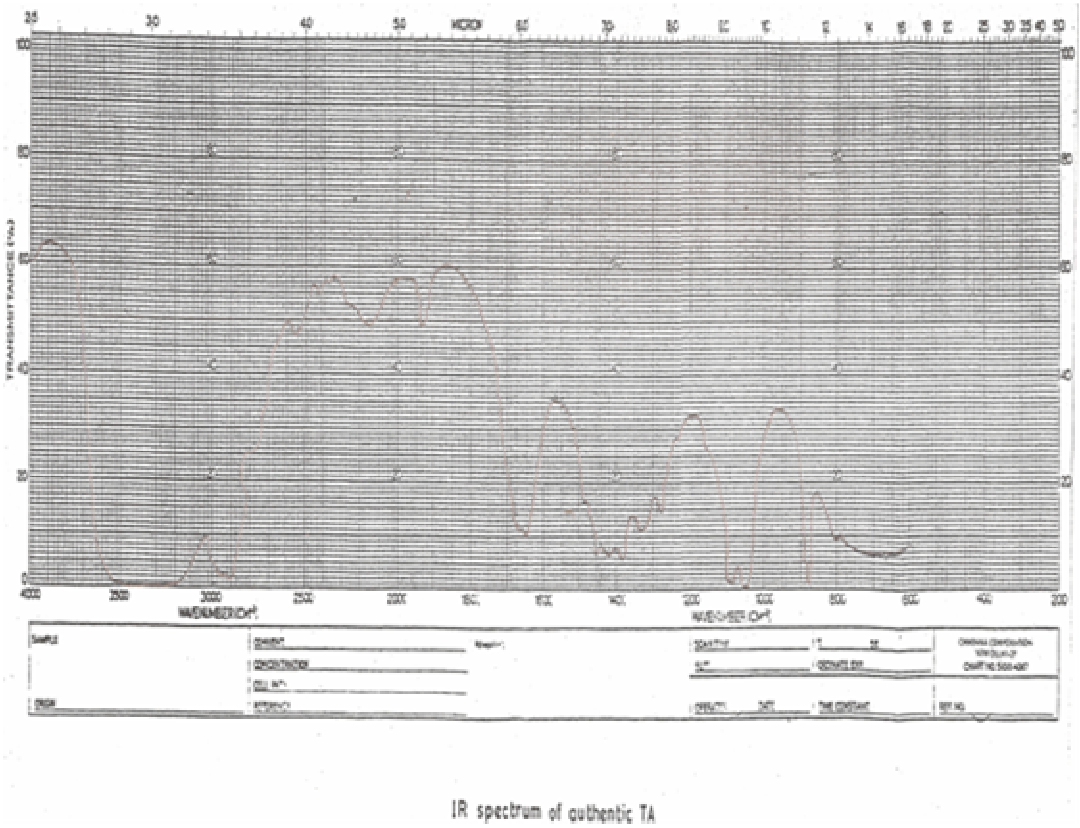

Figure 4b Figure 4a. IR Spectrum of authentic Tenuazonic acid

Alternaria alternata, *Alternaria.tenuissima* and *phoma sorghina* as a mycotoxin. Davis et al. (1976) and Kinoshita et al. (1972) screened 185 strains of *Alternaria* species and found the wide-spread occurrence of tenuazonic acid (TA). Thus, it was thought to be a characteristic metabolite of this genus and not a pathogen-specific toxin. Several other workers also observed the production of TA from *Alternaria* species isolated from different host plants (Chulze et al*.,* 1995; Hasan, 1996; Ozcelik and Ozcelik, 1997. Based on the present study finally we conclude that isolation of tenuazonic acid from infected, groundnut leaves indicate that the toxin was produced by the pathogen during pathogenesis. Thus tenuazonic acid can be considered as a vivotoxin in the case of Alternariosis of groundnut. The results also support the findings of Mikami et al. (1971) on leaf blight of *Datura innoxia*.

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