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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing *Escherichia coli* in the Calgary Health Region: emergence of CTX-M-15-producing isolates. *Antimicrob. Agents Chemother.* 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). *Microbiology: Concepts and Applications.* McGraw-Hill Inc., New York, pp. 591-603.

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The interactions between *esp*, *fsr*, *gelE* genes and biofilm formation and pfge analysis of clinical *Enterococcus faecium* strains

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Enterococcus faecium has become an increasingly important nosocomial pathogen due to formation of biofilms on several surfaces. Sixty one (61) *E. faecium* strains isolated from blood, urine and fecal were assessed for biofilm production, the effect of different glucose concentration on biofilm production and also the presence of *esp*, *fsr* and *gelE* genes. Pulsed field gel electrophoresis (PFGE) method was performed to show chromosomal similarities and also to determine correlation between biofilm formation ability and genetic identity of *E. faecium* strains. It was observed that glucose concentration of the medium and incubation period can affect biofilm formation of the bacteria. When tested strains were incubated in a medium containing 1% glucose for 48 h, 66.66% of urine isolates, 60.71% fecal isolates and 25% of blood isolates produced strong biofilm structures. *esp*-positive strains (80% of the all isolates) were also identified as strong biofilm producers compared to *esp*-negative isolates. As a result of PFGE analyses, isolates numbered 14 (isolated from fecal sample) and 81 (isolated from blood sample) were classified in minor group B at a level of 48% similarity. Out of these two isolates, all the isolates were included in major group A with 43% similarity level and this group was subdivided into six subgroups.

Key words: *Enterococcus faecium*, biofilm, Pulsed Field Gel Electrophoresis (PFGE), *esp*, *fsr*, *gelE*.

INTRODUCTION

Biofilm formation is a dynamic process involving the attachment of bacteria to a biotic or abiotic surface and encased in a hydrated matrix of exopolymeric substances, proteins, polysaccharides and nucleic acids (Tendolkar et al., 2006; Mohamed and Huang, 2007; Stepanovic et al., 2007). Biofilms are notoriously difficult to eradicate and are a source of many chronic infections. According to the National Institutes of Health, biofilms are medically important, accounting for over 80% of microbial infections in the body (Mohamed and Huang, 2007).

However, more than 30 species in the genus

Enterococcus have been described to date; the two most studied enterococcal species are *Enterococcus faecium* and *Enterococcus faecalis* (van Schaik et al., 2010). Contrary to most of lactic acid bacteria, enterococci are not considered "generally recognized as safe (GRAS)" because of they are considered to be pathogens with low virulence (Hallgren et al., 2003; Ogier and Serror, 2008). Inhabitants of the human gastrointestinal and genitor-urinary tracts enterococci (Zhu et al., 2010) are also known to cause serious infections such as bacteraemia and endocarditis (Hallgren et al., 2003).

Table 1. The strain numbers of *Enterococcus faecium* isolates.

Fecal isolates	Urine isolates	Blood isolates
1	3	2
7	5	4
13	8	6
14	24	11
15	25	12
17	26	21
18	29	28
19	30	46
36	32	69
38	33	71
39	35	77
41	37	81
42	43	
45	50	
55	52	
59	56	
63	60	
65	67	
68	72	
70	76	
84	94	
86		
87		
89		
90		
91		
93		
95		

Similarly, some researchers reported that enterococci have become increasingly important as nosocomial pathogens and have been found to form biofilms on several medical devices implanted in patients, such as central venous catheters, urinary catheters, intrauterine devices, and prosthetic heart valves (Cheng et al., 2002; Kristich et al., 2004; Laverde Gomez et al., 2011; Extremina et al., 2011). For biofilm formation of *Enterococcus* species, several genes have been found important such as *esp* (Shankar et al., 1999; van Schaik et al., 2010) and *fsr* via effect on gelatinase (Singh et al., 2007). Van Wamel et al. (2007) explained that *Esp* correlated with biofilm formation depending on growth conditions as well. The object of another study (Macovei et al., 2009) was to search relation between biofilm formation and gelatinase phenotype regulated by *fsr* operon in *E. faecalis* strains. As a result of that study, the researchers observed that *E. faecalis* with the complete *fsr* operon and the potential to form a biofilm were relatively common in the agricultural environment and might represent a source/reservoir of clinically relevant strains. Out of *esp* and *fsr* genes, several other factors have been

associated with biofilm development such as sugar-binding transcriptional regulator BopD, heterogeneity in surface charge, the *bee* locus and the secreted metalloprotease *gelE* (Van Wamel et al., 2007).

There are several factors used to identify differences between *Enterococcus* species, such as amplified rDNA restriction analysis (ARDRA), pulsed field gel electrophoresis (PFGE) of DNA macro-restriction patterns, randomly amplified polymorphic DNA (RAPD-PCR), amplified fragment length polymorphisms (AFLP). Among these methods, PFGE has been successfully used to introduce the differences between clinical and food isolates, and between isolates from poultry and hospitalized patients (Ogier and Serror, 2008).

The aim of this study was to exhibit biofilm formation of *E. faecium* strains isolated from human urine, blood and fecal samples. To present the role of the genes, *esp*, *fsr*, *gelE*, correspond biofilm formation was also aimed in this study. PFGE method was used for illustrating chromosomal similarities between the isolates in order to determine correlation between biofilm formation ability and genetic identity of strains.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A total of 61 previously isolated strains and identified as *E. faecium* strains (University of Ankara, Department of Biology, Prokaryotic Genetic Laboratory, Ankara-Turkey): 12 blood, 21 urine and 28 fecal originated human isolates (Table 1) were used in this research. *E. faecium* ATCC 6057 and *E. faecium* NCD0 942 were used as control strains. The stock cultures maintained in 40% glycerol solution at -80°C were activated in Tryptic Soy Broth (TSB, Merck®, Germany) for 24 h at 37°C prior to each trial.

Biofilm production and the effect of glucose concentration and incubation time on biofilm formation

A method described by Extremina et al. (2011) and Baldassari et al. (2001) was used to test the microorganisms for biofilm formation within the combination. Overnight cultures were diluted in fresh TSB until standardized the OD₅₉₅ to 0.07 (CFU 10⁷). Briefly, 200 µl of active cultures in TSB was inoculated into microtitre polystyrene plate wells. After 24 h growth at 37°C, the plates were gently washed three times with phosphate buffered saline (PBS, Sigma®, USA). The plates were allowed to dry for 1 h at 60°C and then fixed by using methanol (95%). For biofilm quantification, 200 µl of 1% crystal violet solution (Sigma®, USA) was added to each well, and the plates were allowed to stand for 20 min. The wells were subsequently washed thrice with sterile dH₂O to wash off the excess crystal violet. Crystal violet bound to the biofilm was extracted with 200 µl of ethanol-acetone (80/20%), and the absorbance of the extracted crystal violet was measured at 570 nm in ELISA Reader (Molecular Devices Spectra Max M2 Microplate Reader, USA). All biofilm assays were performed in triplicate.

The ability to form biofilm of the strains was scored as follows: OD < 0.120; non producers, 0.120 < OD < 0.240; weak producers, OD > 0.240; strong producers. Wells containing uninoculated served as negative controls (Tsirikionis et al., 2012).

It is known that glucose concentration of the medium have effect on biofilm formation of the microorganisms (Pillai et al., 2004;

Table 2. The *esp*, *fsr*, and *gelE* primers and their properties.

Primer	Oligonucleotide sequence	Product size (bp)	Reference
<i>esp</i>	5' TTGCTAATGCTAGTCCACGACC 3' 3' GCGTCAACACTTGCATTGCCGAA 5'	955	Eaton and Gasson, 2001
<i>fsr</i>	5' AACCAGAATCGACCAATGAAT 3' 3' GCCCCTCATAACTCAATACC 5'	3268	Pillai et al., 2002
<i>gelE</i>	5' AGTTCATGTCTATTTTCTTCAC 3' 3' CTCATTATTTACACGTTTG 5'	402	Mannu et al., 2003

Tendolkar et al., 2004). For this purpose, biofilm producing levels of standard strains (*E. faecium* ATCC 6057, *E. faecium* NCDO 942) and the strains determined as strong biofilm producers was assessed using TSB medium containing glucose at levels of 0.25, 0.50, 0.75, 1.00, 1.25%. After determining the optimum glucose concentration level, biofilm producing levels was identified at 24 and 48 h incubation periods as well. All experiments were performed in triplicate.

Isolation of *esp*, *fsr* and *gelE*

Enterococcal genomic DNA was used as the template for Polymerase Chain Reaction (PCR). Genomic DNAs of enterococcal strains were extracted by DNeasy Blood and Tissue Kit (Qiagen®, USA). Table 2 shows the specific primer pairs used for amplification of *esp*, *fsr*, *gelE* genes. PCR amplifications were performed in a ThermoCycler (Techne TC-512) in 0.2 ml reaction tubes each with 50 µl reaction mixtures composed of the 0.4 µM primer, 0.5 mM dNTP mix (Fermentas®, Finland), 1 X reaction buffer, 1.5 mM MgCl₂, 0.025u Taq polymerase (Promega®, USA) and 2.5 µl extracted enterococcal genomic DNA. PCR amplifications were performed for *esp*, *fsr* and *gelE* as previously described by Eaton and Gasson (2001), Pillai et al. (2002), and Mannu et al. (2003) respectively. According to this, after an initial denaturation procedure (94°C, 5 min) the reaction was subjected to 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, and a final elongation procedure of 72°C for 10 min for the amplification of *gelE*. It was applied by adjusting the Tm temperature to 55°C for PCR reaction of *fsr*. Initial denaturation procedure was performed at 94°C, and 5 min for *esp* amplification. Then, reaction was subjected to 1 cycle of 94°C for 2 min, 56°C for 2 min and 72°C for 2 min, another 30 cycles of 92°C for 15 s, 56°C for 15s and 72°C for 15 s and a final elongation procedure of 72°C for 10 min. The PCR products were analysed on 1% agarose gel electrophoresis, stained in ethidium bromide solution and visualised under UV light.

PFGE analysis

Isolation of genomic DNAs

CHEF-DR III applications guide protocol (1992) (Bio-Rad®,USA) was modified for isolation of intact genomic DNAs of isolates with the purpose of PFGE analysis. *E. faecium* cultures were passed twice into the TSB medium at 37°C prior to use. An overnight culture was diluted in fresh broth until standardization at OD₆₀₀ between 0.5 and 1.0. The culture was centrifuged (10,000 x rpm, 5 min, 4°C) and then washed twice with cell suspension buffer [10 mMTris (pH 7.0), 20 mM NaCl, 50 mM EDTA (pH:8.0)] and resuspended in 100 µl of cell suspension buffer. Equal volumes of cells and 2% low melting grade agarose (BioShop®, Canada) were mixed in a microcentrifuge tube. Then approximately 100 µl of this

mixture was pipetted into the disposable plug molds (10 mm x 5 mm x 1.5 mm, Bio-Rad Laboratories) before solidifying. In agarose, embedded cells were lysed *in situ* with lysis solution [30 mMTris (pH 8.0), 5 mM EDTA (pH 8.0), 50 mM NaCl, 10 mg/ml lysozyme] for 4 h at 37°C. Following this treatment agarose plugs were washed with 1x TE buffer [50 mM EDTA (pH 8.0), 20 mM Tris (pH 8.0)] so as to elimination lysis solution. Agarose plugs were added with proteinase K solution [100 mM EDTA (pH 8.0), 0.2% sodium deoxycholate, 1% sodium N-lauroylsarcosinate, 1 mg/ml proteinase K] and treated overnight without stirring at 50°C. Agarose plugs containing intact genomic DNA were washed ten times for half an hour at 50°C; four times with 1x TE supplemented by 1 mM NaCl, twice with 1x TE supplemented by 1 mM PMSF, twice with 1x TE and finally twice with 0.1x TE, respectively and then stored at 4°C in 0.1x TE.

Restriction enzyme digestion and electrophoresis

Each DNA embedded agarose plug was cut into about four slices. Slices of the plugs were digested for 16 h with 30 U of *Sma*I at 30°C in 80 µl of the 1x SE-Buffer Y (SibEnzyme®, Russia). Restriction enzyme mixture was removed on the slices of agarose plugs by washing with 0.5 x TBE prior to electrophoresis. Afterwards, DNA fragments were resolved in 1% (w/v) pulsed-field certified agarose (BioShop®, Canada) in 0.5 x TBE buffer by pulsed field gel electrophoresis (PFGE) using CHEF-DR III System (Bio-Rad®, USA). Lambda ladder PFG Marker (New England Biolabs®, UK) was used as a molecular size standard. Electrophoresis was performed for 14 h at 120°C included angle. Pulse times for a total running time of 17h ranged from 0.1 to 5 s for 5 h, from 5 to 35 s for 6 h and from 40 to 125 s for 6 h at a constant voltage of 6 V/cm. The agarose gels were stained with ethidium bromide (10 µg/ml) and visualized under UV light. A digital image was obtained with Gel Logic 200 Imaging System (Kodak Company). NTSYS-pc version 2.2 (Rohlf, 1993) computer software was used for the cluster analysis of the enterococcal isolates. The Dice coefficient of similarity was calculated and comparison of the banding patterns was performed by the unweighted pair group method with arithmetic averages (UPGMA) (Sneath and Sokal, 1973).

RESULTS AND DISCUSSION

Biofilm formation of the enterococcal strains and the effect of glucose concentration and incubation time

Biofilm assay was performed according to the method described by Extremina et al. (2011) and Baldassari et al. (2001). In this method, strains were grown in TSB medium without any supplementation and were incubated at

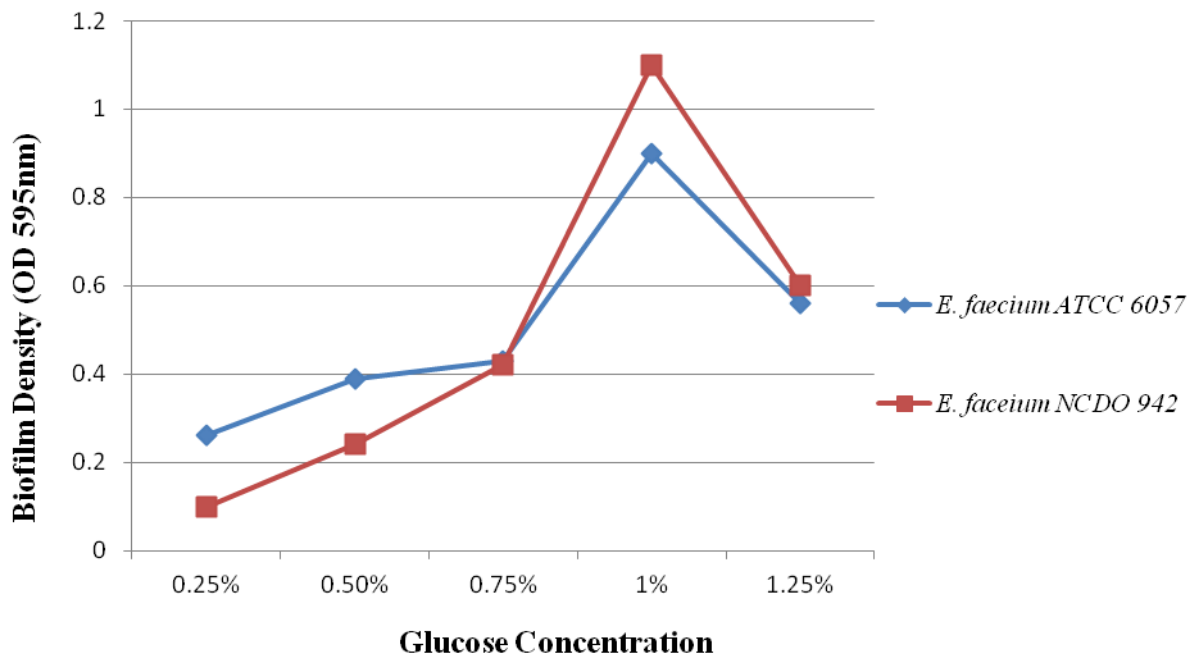


Figure 1. Effect of glucose concentration on biofilm formation of standard strains *E. faecium* ATCC 6057, *E. faecium* NCDO 942.

37°C for 24 h. According to results obtained in this experiment, 4 of 21 urine isolates, 9 of 12 blood isolates and 16 of 28 fecal isolates were able to produce biofilm. This means 48% of *E. faecium* strains could be defined as biofilm producers with the method comprising above. After this pre-trial, further experiments were performed.

Some researches indicated that factors such as nutrient concentration of the media can effect biofilm formation of the bacteria (Yoshida and Kuramitsu, 2002; Loo et al., 2000; Klausen et al., 2003). In addition to this knowledge, the enterococcal cell surface associated protein, Esp, may enhance biofilm formation by *E. faecalis* in a glucose-dependent manner (Tendolkar et al., 2005; van Wamel et al., 2007; Macovei et al., 2009). To demonstrate nutrient concentration effect on biofilm formation by *E. faecium*, we performed the microtiter plate assay to grow biofilms at the presence of different glucose concentration. Among the tested glucose concentrations (0.25, 0.50, 0.75, 1.00, 1.25%), 1.00% glucose concentration was used to detect an increased biofilm formation for standard and strong biofilm producer strains. Figure 1 shows the results for the standard strains. When the glucose concentration reached 1.25%, biofilm formation started to decline. Following this, all isolates were subjected to produce biofilm in TSB medium containing the mentioned glucose concentration. Out of our study, many researchers also reported on the effect of glucose concentration on biofilm formation. Whereas Pillai et al. (2004) detected 20-40% increased biofilm formation when they used 1.00% glucose added to TSB and Baldassarri et al. (2001) showed glucose supplementa-

tion may enhance biofilm formation. Kristich et al. (2004) demonstrated glucose-mediated inhibition of biofilm production among *Enterococcus* strains. In a study performed by Marinho et al. (2013), a synergistic effect on biofilm at 10, 28, 37 and 45°C and glucose was observed for *E. faecalis* and *E. faecium* as well. This thread can be taken in another angle; the glucose effect on biofilm formation can be considered important due to its ability to detect enterococcal biofilms in early stages or to select the biofilm producer bacteria in a more efficient way so that early detection of biofilm producing enterococci can be one of the essential steps towards prevention and management of nosocomial infections, as most of the hospital-acquired infections are biofilm related. Making changes in growth medium is one of the way to modify biofilm formation values output (Extremina et al., 2011).

In addition to glucose concentration assay, the effect of incubation time (24 and 48 h) on biofilm formation of *E. faecium* strains were investigated. As it is seen in Table 3, incubation for 48 h allowed strains to show more biofilm formation than 24 h incubation. Furthermore, the strains not producing biofilm after 24 h-incubation were able to produce biofilm by elongating incubation period to 48 h. Among all strains, 85.7% of urine and fecal isolates, 58.33% of blood isolates were determined as biofilm producers (Table 4). When comparing the isolates depending on origins, it can be reported that 66.66% of urine isolates, 60.71% fecal isolates and 25% of blood isolates were included in a group of strong biofilm producers (Table 4).

Table 3. Effect of incubation time and 1.00% glucose concentration on biofilm formation.

Parameter	Incubation time of the strains (h)	Non n (%)	Weak n (%)	Strong n (%)
Blood (12)	24	9 (75%)	2 (16.66%)	1 (8.33%)
	48	4 (33.33%)	5 (41.66%)	3 (25%)
Urine (21)	24	4 (19.04%)	6 (28.57%)	11 (52.38%)
	48	3 (14.28%)	4 (19.04%)	14 (66.66%)
Fecal (28)	24	16 (57.14%)	3 (10.71%)	9 (32.14%)
	48	4 (14.28%)	7 (25%)	17 (60.71%)

Table 4. *esp*, *fsr*, and *gelE* genes carriage, and biofilm formation of *Enterococcus faecium* isolates.

Origin of isolates (n)	<i>esp</i> gene n (%)	<i>fsr</i> gene n (%)	<i>gelE</i> gene n (%)	Biofilm formation (at 1.00 % glucose concentration and 48 h incubation)
Blood (12)	6 (46.15%)	0 (0%)	0 (0%)	7 (58.33%)
Urine (21)	8 (38.09%)	0 (0%)	0 (0%)	18 (85.7%)
Fecal (28)	6 (22.22%)	0 (0%)	0 (0%)	24 (85.7%)

Occurrence of *esp*, *fsr* and *gelE*

The presence of genes encoding the enterococcal surface protein (Esp), gelatinase enzymes (GelE), quorum-sensing locus *fsr*, were studied at 61 *E. faecium* isolates. The *esp* gene was detected for 46.15, 38.09 and 22.22% of blood, urine, and rectal isolates, respectively. *esp*-positive strains (80%) were significantly stronger biofilm producers than the *esp*-negative isolates (data not shown); this could indicate a role for *esp* in the formation of biofilm in clinical enterococci. The significantly higher incidence of *esp* gene in clinical isolates may reflect a role that Esp protein has in infection. Additionally to its role in adhesion, Esp is also thought to play a role in evasion of the host immune response, which is an important factor in the disease development (Shankar et al., 1999). There are conflicting results on the role of *esp* gene in biofilm formation but the opinions in general are in the line with positive effect of *esp* on biofilm production. Toledo-Arana et al. (2001) determined that *esp*-positive *E. faecalis* strains (93.5% in total) produce high level on abiotic surfaces whereas no biofilm formation was observed by *esp*-negative *E. faecalis* strains on the same surface. Another study performed by Tendolkar et al. (2005) aimed to localize the specific domain(s) of Esp that plays a role in Esp-mediated biofilm enhancement. It was reported by the researchers that an *E. faecalis* strain expressing only the N-terminal domain of Esp fused to a heterologous protein anchor formed biofilms that were quantitatively similar to those formed by a strain expressing full-length Esp. It is understood from this result that the minimal region contributing to Esp-mediated biofilm enhancement in *E. faecalis* was confined to the nonrepeat N-terminal domain. These results suggest that Esp may require interaction with an

additional *E. faecalis*-specific factor(s) to result in biofilm enhancement. In contrast to our findings, there are also some studies that report that there is no relationship between *esp* gene and biofilm formation (Dworniczek et al., 2005; Ramadhan and Hegedus, 2005). Dupre et al. (2003) could not detect any relationship between the *esp* gene and biofilm formation of 15 clinical *E. faecalis* and 32 *E. faecium* strains. In addition, any bond between the presence of *esp* gene and biofilm formation of 70 *E. faecalis* and 38 *E. faecium* strains isolated from the circulatory system has not been established (Sandoe et al., 2003).

The relationship between biofilm formation, the enterococcal surface protein (Esp) and gelatinase in clinical isolates of *E. faecalis* and *E. faecium* was searched by Di Rosa et al. (2006). In the study, neither *esp* nor gelatinase seemed to be required for biofilm formation: both *E. faecalis* and *E. faecium* but in *E. faecium* while *esp* was found in isolates the presence of both *esp* and biofilm together was only found in strains from clinical settings, suggesting that there exists a synergy between these factors which serves as an advantage for the process of infection.

In a study (Top et al., 2013) very recently published, it was reported that the *E. faecium* enterococcal biofilm regulator, EbrB, regulates the *esp* operon and is implicated in biofilm formation and intestinal colonization. The study also determined that *esp* is part of an operon of at least three genes putatively involved in biofilm formation. In a mouse intestinal colonization model, the *ebrB* mutant was less able to colonize the gut compared to wild-type strain, especially in the small intestine. These data indicate that EbrB positively regulates the *esp* operon and is implicated in biofilm formation and intestinal colonization.

gelE, the other gene thought to have an effect on biofilm formation, is an extracellular zinc metalloprotease which can hydrolyze gelatin, collagen and casein (Qin et al., 2001). Some researchers showed that *gelE* has a critical role on development of the biofilms (Hancock and Perego, 2004; Kristich et al., 2004; Mohamed et al., 2004). Except *gelE*, it was demonstrated that *fsr* gene has a significant effect on enterococcal biofilms (Hancock and Perego, 2004; Mohamed et al., 2004; Pillai et al., 2004). Unlike all these literature knowledge, in our study, it was determined that *fsr* locus and *gelE* gene were not present in any of tested *E. faecium* isolates. Further, any relationship between the genes (*fsr*, *gelE*) and biofilm producing capacity of the strains could not be detected. It suggests that the physiological factors may trigger the production of biofilm, instead of *fsr* locus and *gelE* gene.

In clinical enterococcal strains, gelatinase activity is generally associated with virulence factors (Pillai et al., 2002; Roberts et al., 2004). Because of no detection of gelatinase activity or presence of *fsr* and *gelE* genes in the tested clinical *E. faecium* isolates, no significant correlation was found between gelatinase production and *fsr*, *gelE* genes and biofilm formation. *fsr* locus (*fsrABDC*) of *E. faecalis* is determined as a global regulator and it acts as a signal transmission system which controls biofilm formation of *E. faecalis* for only different environmental conditions. Besides of regulation function of gelatinase and serin protease expression, many genes like *bopD* can be one of the parts of *fsr* regulon (Paganelli et al., 2012). Different from our findings, Qin et al. (2000) found that 69% of clinical isolates were positive for *fsr B* gene. In another study performed by Pillai et al. (2002), all the isolates obtained from patients with endocarditis and 53% of fecal isolates were declared as positive for *fsr B* gene. Based on this data, the researchers offered to identify the enterococcal strains which have *fsr* locus among clinical isolate.

Further analysis of the subgroup of *esp* negative human and animal isolates showed that the ability to produce gelatinase was positively associated with biofilm formation only in animal originated *E. faecalis* isolates. This could indicate that production of this protease may be a selection mechanism for animal *E. faecalis*, as it may enable the *esp*-lacking animal isolates to produce biofilm. However genetic manipulation studies have offered that gelatinase is essential for biofilm formation, epidemiological studies have not supported the link between gelatinase and biofilm production among clinical *E. faecalis* isolates (Tsirikonis et al., 2012).

PFGE assay results

PFGE has emerged as one of the most widely applicable, reproducible, and stable methods to examine strain identity in enterococci (Patterson and Kelly, 1998). In order to investigate relationship between genetic similari-

ties and biofilm formation abilities of clinical *E. faecium* isolates, the chromosomal DNA of the strains were cut with *SmaI* restriction endonuclease. Afterwards, macrorestriction fragment numbers and also sizes of all blood, urine and fecal strains were compared with PFGE. When the obtained data were analyzed, it was observed that there were high levels of heterogeneity between clinical isolates despite they all belong to same species. Cluster analysis of macrorestriction patterns generated by *SmaI* digestion of chromosomal DNAs revealed one major and a minor cluster for 61 *E. faecium* strains as shown in Figure 2. The fecal isolate numbered as 14 and the blood isolate numbered as 81 were classified in minor group B with around 48% similarity rate. Out of these two isolates, all the other isolates were included in a major cluster A at 43% similarity level and subdivided into six subgroups (Figure 2). Our PFGE results are similar from the point of low level homology among clinical *E. faecium* isolates with the study conducted by Weng et al. (2013). In another study performed previously by Bedendo and Pignatari (2000) were investigated genetic diversity among 20 clinical *E. faecium* isolates by using REP-PCR and PFGE, so PFGE had revealed easier interpreted band patterns in comparison with REP-PCR.

PFGE analysis of enterococci isolates from recreational and drinking water in Greece was searched by Grammenou et al. (2006). A collection of enterococci recovered from recreational and drinking water were applied to biotyping and DNA fingerprinting by PFGE, in order to identify possible genetic relationships. Even though genetic diversity was observed among the studied strains, common clonal types were also identified in different sources, suggesting a possible common origin of the enterococci. As a conclusion of that study, cluster analysis revealed a genetic relationship between certain environmental *E. faecium* and clinical strains.

The isolates numbered as 65, 68, 17, 18 and 41 constituted a subgroup A6 with their common traits, which are fecal isolates and also *esp* negative, although there was no found correlation in terms of biofilm formation abilities of them. On the contrary, isolates numbered as 42 and 45, which were previously isolated from the same medium (University of Ankara Department of Biology, Prokaryotic Genetic Laboratory, Ankara-Turkey) and exhibited the same *SmaI* band profile, were found also as the same with regard to all investigated properties in this study. Surprisingly, strain numbered 94, a urine isolate, showed strong biofilm formation and 70% chromosomal similarity with ATCC 6057 standard strain. However, we could not determine for all strains a correlation between all the tested strains biofilm formation and genetic similarity detected by PFGE. Increase in the ratio of differences between the strains indicates genetic distinctions, as well. It is possible to explain genetic differentiations among enterococcal strains with gaining linear plasmids, moderate prophages and transposones which can be integrated into chromosome via horizontal

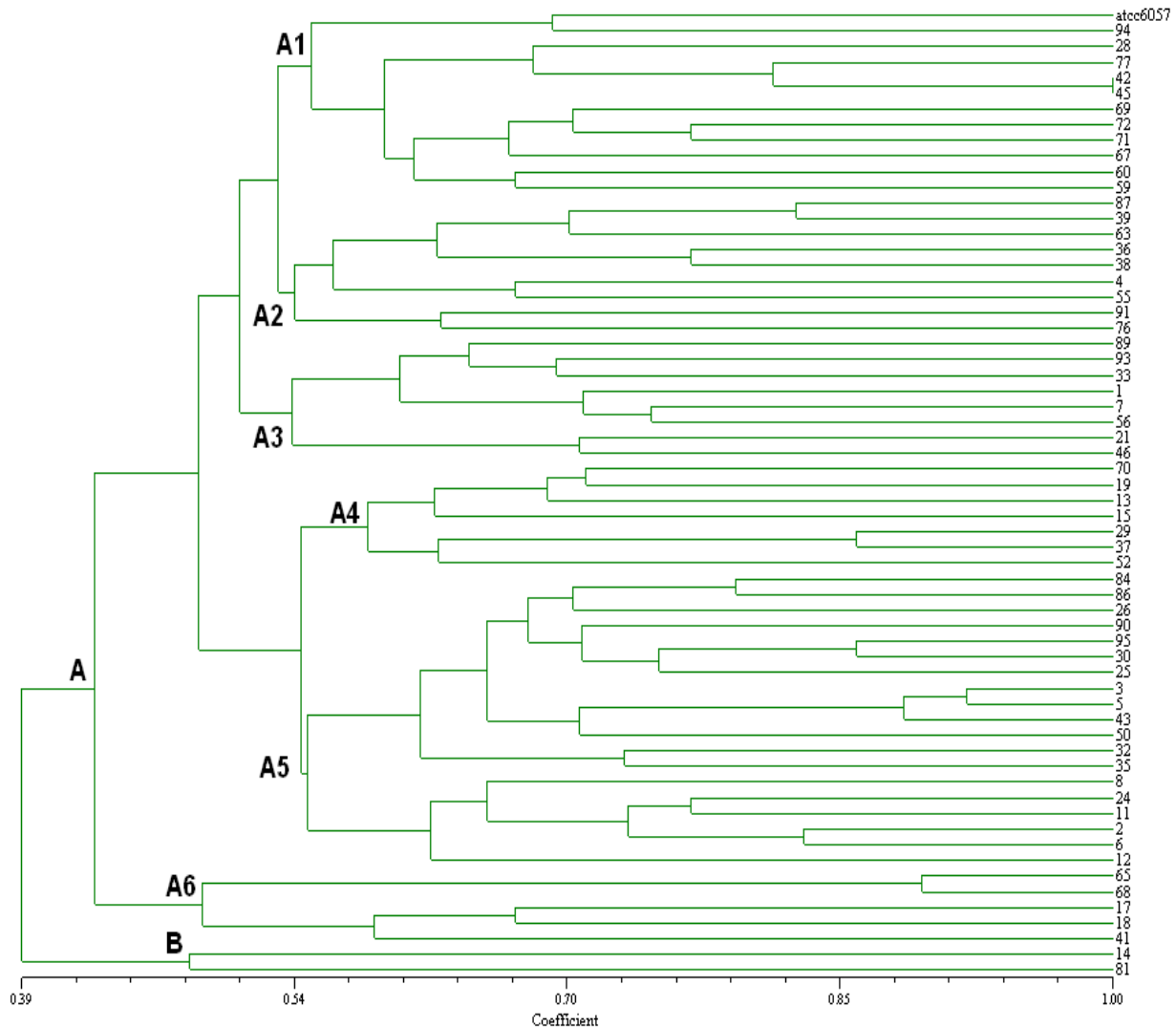


Figure 2. PFGE Dendrogram of *E. faecium* strains obtained by unweighted pair group method using average linkage of dice correlation coefficients

gene transfer between the isolates surviving in the same environment (Hacker et al., 2003; Paulsen et al., 2003). It is known that, this situation mentioned above can cause huge chromosomal differences between the strains belonging to *E. faecium* species and give a chance to them to show different phenotypic properties in evolutionary process, such as antibiotic resistance, virulence traits and also biofilm formation (Rice et al., 2005; Hegstad et al., 2010; Palmer et al., 2010).

As a result of PFGE assay, we can conclude that the chromosomal similarity detected around 60-70% between the clinical enterococcal isolates emerged due to gain or lose genes during evolutionary process. It was also found that biofilm production capacities of closely related isolates may vary. This assignation is another consequence of this study.

Conclusions

Enterococci are important pathogens and are one of the major causes of infection within hospitals. This study focused on the determination of phenotypic and molecular basis of biofilm formation traits in clinical *E. faecium* strains isolated from human origin blood, urine and rectal samples in Turkey. As a result of the present study, we can emphasize that glucose concentration has a significant effect on biofilm formation. The changes in incubation time may also introduce different biofilm formation cases. However, we focused on three genes, *esp*, *fsr*, *gelE*, thought as having relationship between biofilm formation of *E. faecium* strains; we could only detect a relation between *esp* gene and biofilm formation which was 80% of *esp*-positive strains and were strong biofilm

producers as well. A major and a minor group were determined by using PFGE analysis for *E. faecium* isolates. Surprisingly, it was observed that biofilm formation varied even among closely related species in PFGE assay.

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REFERENCES

- Baldassarri L, Cecchini R, Bertuccini L, Ammendolia MG, Losi F, Arciola CR, Montanaro L, Di Rosa R, Gherardi G, Dicuonzo G, Orefici G, Creti R (2001). *Enterococcus* spp. Produces slime and survives in rat peritoneal macrophages. *Med. Microbiol. Immunol.* 190:113-120.
- Bedendo J, Pignatari ACC (2000). Typing of *Enterococcus faecium* by polymerase chain reaction and pulsed field gel electrophoresis. *Braz. J. Med. Biol. Res.* 33:1269-1274.
- CHEF-DR III Pulsed Field Electrophoresis Systems, Instruction Manual and Applications Guide. (1992). Catalog Numbers 170-3690 through 170-3703, Bio-Rad, U.S.
- Cheng AF, Char TS, Ling M (2002). Are multiply resistant enterococci a common phenomenon in Hong Kong. *J. Antimicrob. Chemother.* 50:761-763.
- Di Rosa R, Creti R, Venditti M, D'Amelio R, Arciola CR, Montanaro L, Baldassarri L (2006). Relationship between biofilm formation, the enterococcal surface protein (Esp) and gelatinase in clinical isolates of *Enterococcus faecalis* and *Enterococcus faecium*. *FEMS Microbiol. Lett.* 256:145-150.
- Dupre I, Zanetti S, Schito AM, Fadda G, Sechi LA (2003). Incidence of virulence determinants in clinical *Enterococcus faecium* and *Enterococcus faecalis* isolates collected in Sardinia (Italy). *J. Med. Microbiol.* 52:491-498.
- Dworniczek E, Wojciech L, Sobieszczansk, B, Seniuk A (2005). Virulence of *Enterococcus* isolates collected in Lower Silesia (Poland). *Scand. J. Infect. Dis.* 37:630-636.
- Eaton TJ, Gasson MJ (2001). Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Appl. Environ. Microbiol.* 67:1628-1635.
- Extremina CI, Costa L, Aguiar AI, Peixe L, Fonseca AP (2011). Optimization of processing conditions for the quantification of enterococci biofilms using microtitre-plates. *J. Microbiol. Methods.* 84:167-173.
- Grammenou P, Spiliopoulou I, Sazakli E, Papapetropoulou M (2006). PFGE analysis of enterococci isolates from recreational and drinking water in Greece. *J. Water Health.* doi:10.2166/wh.2006.007.
- Hacker J, Hentschel, Dobrindt U (2003). Prokaryotic Chromosomes and Disease. *Science* 301:790-793.
- Hallgren A, Saeedi B, Nilsson M, Monstein HJ, Isaksson B, Hanberger, H, Nilsson L (2003). Genetic relatedness among *Enterococcus faecalis* with transposon-mediated high-level gentamicin resistance in Swedish intensive care units. *J. Antimicrob. Chemother.* 52:162-167.
- Hancock LE, Perego M (2004). The *Enterococcus faecalis* *fsr* two-component system controls biofilm development through production of gelatinase. *J. Bacteriol.* 186:5629-5639.
- Hegstad K, Mikalsen T, Coque TM, Werner G, Sundsfjord A (2010). Mobile genetic elements and their contribution to the emergence of antimicrobial resistant *Enterococcus faecalis* and *Enterococcus faecium*. *Clin. Microbiol. Infect.* 16:541-554.
- Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jorgensen A, Molin S, Tolker-Nielsen T (2003). Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol. Microbiol.* 48:1511-1524.
- Kristich CJ, Li YH, Cvitkovitch DG, Dunny GM (2004). Esp-independent biofilm formation by *Enterococcus faecalis*. *J. Bacteriol.* 186(1):154-163.
- Laverde Gomez JA, Hendrickx APA, Willems RJ, Top J, Sava I, Huebner J, Witte W, Werner G (2011). Intra- and interspecies genomic transfer of the *Enterococcus faecalis* pathogenicity island. *Plos One.* 6(4):e16720. Doi:10.1371/journal.pone.0016720.
- Loo CY, Corliss DA, Ganeshkumar N (2000). *Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes. *J. Bacteriol.* 182:1374-1382.
- Macovei L, Ghosh A, Thomas VC, Hancock LE, Mahmood S, Zurek L (2009). *Enterococcus faecalis* with the gelatinase phenotype regulated by the *fsr* operon and with biofilm-forming capacity are common in the agricultural environment. *Environ. Microbiol.* 11(6):1540-1547.
- Mannu L, Paba A, Daga E, Comunian R, Zanetti S, Dupre' I, Sechi LA (2003). Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin. *Int. J. Food Microbiol.* 88:291-304.
- Marinho AR, Martins PD, Ditmer EM, d'Azevedo PA, Frazzon J, Van Der Sand ST, Frazzon APG (2013). Biofilm formation on polystyrene under different temperatures by antibiotic resistant *Enterococcus faecalis* and *Enterococcus faecium* isolated from food. *Braz. J. Microbiol.* 44(2):423-426.
- Mohamed JA, Huang DB (2007). Biofilm formation by enterococci. *J. Med. Microbiol.* 56:1581-1588.
- Mohamed JA, Huang W, Nallapareddy SR, Teng F, Murray BE (2004). Influence of origin of isolates, especially endocarditis isolates, and various genes on biofilm formation by *Enterococcus faecalis*. *Infect. Immun.* 72:3658-3663.
- Ogier JC, Serror P (2008). Safety assessment of dairy microorganisms: The *Enterococcus* genus. *Int. J. Food Microbiol.* 126:291-301.
- Paganelli FL, Willems RJ, Leavis HL (2012). Optimizing future treatment of enterococcal infections: attacking the biofilm. *Trends Microbiol.* 20:40-49.
- Palmer KL, Kos VN, Gilmore MS (2010). Horizontal gene transfer and the genomics of enterococcal antibiotic resistance. *Curr. Opin. Microbiol.* 13:632-639.
- Patterson JE, Kelly CC (1998). Pulsed-field gel electrophoresis as an epidemiologic tool for enterococci and streptococci. *Methods Cell Sci.* 20:233-239.
- Paulsen IT, Banerjee L, Myers GSA, Nelson KE, Seshadri R, Read TD, Fouts DE, Eisen JA, Gill SR, Heidelberg JF, Tettelin H, Dodson RJ, Umayam L, Brinkac L, Beanan M, Daugherty S, DeBoy RT, Durkin S, Kolonay J, Madupu R, Nelson W, Vamathevan J, Tran B, Upton J, Hansen T, Shetty J, Khouri H, Utterback T, Radune D, Ketchum KA, Dougherty BA, Fraser CM (2003). Role of Mobile DNA in the Evolution of Vancomycin-Resistant *Enterococcus faecalis*. *Science.* 299:2071-2074.
- Pillai SK, Sakoulas G, Eliopoulos GM, Moellering RCJ, Murray BE, Inouye RT (2004). Effects of Glucose on *fsr*-Mediated Biofilm Formation in *Enterococcus faecalis*. *J. Infect. Dis.* 190:967-970.
- Pillai SK, Sakoulas G, Gold HS, Wennersten C, Eliopoulos GM (2002). Prevalence of the *fsr* locus in *Enterococcus faecalis* infections. *J. Clin. Microbiol.* 7:2651-2652.
- Qin X, Singh KV, Weinstock GM, Murray BE (2000). Effects of *Enterococcus faecalis* *fsr* genes on production of gelatinase and a serine protease and virulence. *Infect. Immun.* 68(5):2579-2586.
- Qin X, Singh KV, Weinstock GM, Murray BE (2001). Characterization of *fsr*, a regulator controlling expression of gelatinase and serine protease in *Enterococcus faecalis* OG1RF. *J. Bacteriol.* 183(11):3372-3382.
- Ramadhan AA, Hegedus E (2005). Biofilm formation and *esp* gene carriage in enterococci. *J. Clin. Pathol.* 58:685-686.
- Rice LB, Carias LL, Rudin S, Laticova V, Wood A, Hutton-Thomas R (2005). *Enterococcus faecium* Low-Affinity *pbp5* Is a Transferable Determinant. *Antimicrob. Agents Chemother.* 49:5007-5012.
- Roberts JC, Singh KV, Okhuysen PC, Murray BE (2004). Molecular epidemiology of the *fsr* locus and of gelatinase production among different subsets of *Enterococcus faecalis* isolates. *J. Clin. Microbiol.* 42:2317-2320.

- Rohlf FJ (1993). NTSYS-pc. Numerical Taxonomy and Multivariate Analysis System, Exeter Publishing, Setauket, NY.
- Sandoe JA, Witherden IR, Cove JH, Heritage J, Wilcox MH (2003). Correlation between enterococcal biofilm formation in vitro and medical-device-related infection potential in vivo. *J. Med Microbiol.* 52:547-550.
- Shankar V, Baghdayan AS, Huycke MM, Lindahl G, Gilmore MS (1999). Infection-derived *Enterococcus faecalis* strains are enriched in *esp*, a gene encoding a novel surface protein. *Infect. Immun.* 67(1):193-200.
- Singh KV, Nallapareddy SR, Murray BE (2007). Importance of the *ebp* (endocarditis and biofilm-associated pilus) locus in the pathogenesis of *Enterococcus faecalis* ascending urinary tract infection. *J. Infect. Dis.* 195:1671-1677.
- Sneath PHA, Sokal RR (1973). Numerical taxonomy. W.H. Freeman and Company, San Francisco.
- Stepanovic S, Vukovic D, Hola V, Bonavebura GD, Djukic S, Cirkovic I, Ruzicka F (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS.* 115:891-899.
- Tendolkar PM, Baghdayan AS, Gilmore MS, Shankar N (2004). Enterococcal Surface Protein, *Esp*, Enhances Biofilm Formation by *Enterococcus faecalis*. *Infect. Immun.* 72:6032-6039.
- Tendolkar PM, Baghdayan AS, Shankar N (2005). The N-Terminal domain of enterococcal surface protein, *Esp*, is sufficient for *Esp*-mediated biofilm enhancement in *Enterococcus faecalis*. *J. Bacteriol.* 187(17):6213-6222.
- Tendolkar PM, Baghdayan PM, Shankar N (2006). Putative surface proteins encoded within a novel transferable locus confer a high-biofilm phenotype to *Enterococcus faecalis*. *J. Bacteriol.* 188(6): 2063-2072.
- Toledo-Arana A, Valle J, Solano C, Arrizubieta MJ, Cucarella C, Lamata M, Amorena B, Leiva J, Penades JR, Lasa I (2001). The enterococcal surface protein, *Esp*, is involved in *Enterococcus faecalis* biofilm formation. *Appl. Environ. Microbiol.* 67:4538-4545.
- Top J, Paganelli FL, Zhang X, van Schaik W, Leavis HL, van Luit-Asbroek M, van der Poll T, Leendertse M, Bonten MJM, Willems RJL (2013). The *Enterococcus faecium* enterococcal biofilm regulator, *ebrB*, regulates the *esp* operon and is implicated in biofilm formation and intestinal colonization. *PLoS ONE* 8(5):e65224. doi:10.1371/journal.pone.0065224.
- Tsikrikonis G, Maniatis AN, Labrou M, Ntokou E, Michail G, Daponte A, Stathopoulos C, Tsakris A, Pournaras S (2012). Differences in biofilm formation and virulence factors between clinical and fecal enterococcal isolates of human and animal origin. *Microb. Pathogenesis.* 52:336-343.
- Van Schaik W, Top J, Riley DR, Boekhorst J, Vrijenhoek JEP, Schapendonk CME, Hendrickx APA, Nijman IJ, Bonten MJM, Tettelin H, Willems RJL (2010). Pyrosequencing-based comparative genome analysis of the nosocomial pathogen *Enterococcus faecium* and identification of a large transferable pathogenicity island. *BMC Genomics.* 11(239):1-18.
- Van Wamel WJB, Hendrickx APA, Bonten MJM, Top J, Posthuma G, Willems RL (2007). Growth condition-dependent *esp* expression by *Enterococcus faecium* affects initial adherence and biofilm formation. *Infect. Immun.* 75(2):924-931.
- Weng PL, Ramli R, Shamsudin MN, Cheah YK, Hamat RA (2013). High genetic diversity of *Enterococcus faecium* and *Enterococcus faecalis* clinical isolates by Pulsed-Field Gel Electrophoresis and Multilocus Sequence Typing from a Hospital in Malaysia. *BioMed Res. Int.* Article ID:938937.
- Yoshida A, Kuramitsu HK (2002). Multiple *Streptococcus mutans* genes are involved in biofilm formation. *Appl. Environ. Microbiol.* 68:6283-6291.
- Zhu X, Wang Q, Zhang C, Cheung GSP, Shen Y (2010). Prevalence, Phenotype, and Genotype of *Enterococcus faecalis* Isolated from Saliva and Root Canals in Patients with Persistent Apical Periodontitis. *Basic Res.-Biol.* 36(12):1950-1955.

Full Length Research Paper

Biosynthesis, optimization, purification and characterization of gold nanoparticles

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Many microorganisms produce intracellular metal nanoparticles. Aqueous gold, when exposed to several actinomycetal strains, become thereby, leading to the formation of gold nanoparticles. The use of microorganisms in the synthesis of nanoparticles is emerging as an eco-friendly and exciting approach gold for recovery. *Streptomyces hygroscopicus* was used for the biosynthesis of gold nanoparticles. UV and visible spectroscopic studies of biofilms revealed better synthesis of nanoparticles. It was observed that better biosynthesis of gold nanoparticles occurred when cell biomass treated with 10^{-3} and 10^{-4} mM HAuCl_4 solution as compared to other dilutions. The pH 7.0 was found to be optimum for the biosynthesis of gold nanoparticles. The TEM study reveals that there was evidence of gold nanoparticles synthesized by *S. hygroscopicus*. The results demonstrate that spherical gold nanoparticles in the range of 10 to 20 nm were observed at pH value of 7.0. The actinomycetal biomass and various concentration of aqueous HAuCl_4 solution were incubated, it was found that 10^{-4} concentration shows excellent colour of the actinomycetal biomass.

Key words: Biosynthesis, optimization, gold nanoparticles, purification, characterization.

INTRODUCTION

Chemical production processes for metal nanoparticles are not regarded as being environmentally friendly (Sastry et al., 2003; Gamez et al., 2002) and generally yield only spherical nanoparticles. On the other hand, a wide variety of geometric, metal nanoparticles were produced by both prokaryotic and eukaryotic organisms including bacteria, fungi and yeasts (Sastry et al., 2003; Ahmad et al., 2003; Mukherjee, 2001). This bioreduction of metal particles is regarded as an organism's survival mechanism against toxic metal ions and occurs via an active or passive process or a combination of the two (Duran et al., 2005; Ibrahim et al., 2001). Such a biological route involving micro-organisms provides great advantages over traditional methods, as it has the

potential to be cost-effective, simple and environmentally friendly. The primary advantage of biological route is the ability, in theory to manipulate the properties of the nanoparticles by gaining control over the mechanism that determines their size and shape (Ahmad et al., 2003; Mukherjee, 2001). Many microorganisms, both unicellular and multicellular are known to produce inorganic materials either intracellularly or extracellularly (Mann, 1996; Lloyd, 2003) often of nanoscale dimensions and of exquisite morphology and hierarchical assembly. Some well-known examples of microorganisms synthesizing inorganic materials include magnetotactic bacteria (which synthesize magnetite nanoparticles) (Ahmad et al., 2003) and actinomycetes such as the extremophilic actino-

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mycete; *Thermomonospora* sp. (Ahmad et al., 2003) and the alkalotolerant actinomycete *Rhodococcus* sp. (Fortin and Beveridge, 2000). Both live microorganisms and dead microorganisms are gaining importance by virtue of their facile assembly of nanoparticles. Gold particles of nanoscale dimensions may be readily precipitated within bacterial cells by incubation of the cells with Au³⁺ ions (Klaus et al., 2001).

The bacterium *Pseudomonas stutzeri* AG259 isolated from a silver mine, when placed in a concentrated aqueous solution of AgNO₃, led to the reduction of the Ag⁺ ions and to the formation of silver nanoparticles of well-defined size and distinct morphology within the periplasmic space of the bacteria (Mukherjee et al., 2001). Eukaryotic organisms such as fungi may be used to grow nanoparticles of different chemical compositions and sizes. A number of different genera of fungi have been investigated in this regard and it has been shown that fungi are extremely good candidates in the synthesis of gold (Mandal et al., 2006; Mukherjee et al., 2001) or silver (Ahmad et al., 2003; Duran et al., 2005; Gardea-Torresdey et al., 2002; Varshney et al., 2009) particles. Several attempts of synthesis of metal nanoparticles have been made by researchers. Very recently, it was also shown that *Fusarium oxysporum* produced optoelectronic material Bi₂O₃ nanocrystals in the size between 5 to 8 nm extracellularly with quasispherical morphology and good tunable properties. When bismuth nitrate was added as precursor, the as-synthesized nanocrystals were in monoclinic and tetragonal phases (Uddin et al., 2008). *F. oxysporum* (Mukherjee et al., 2002), *Colletotrichum* sp. (Shankar et al., 2003) and *Trichothecium* sp. (Ahmad et al., 2005) produced extracellular gold nanoparticles with spherical, triangular and hexagonal morphologies with 5 to 200 nm in size. It was also intriguing to observe that the silver nanoparticles produced by *T. asperellum* (Mukherjee et al., 2008), *T. viride* (Fayaz et al., 2010), *F. oxysporum* (Senapati et al., 2004), *P. chrysosporium* (Vigneshwaran et al., 2006), *F. solani* (Ingle et al., 2009), *F. semitectum* (Basavaraja et al., 2008), *F. acuminatum* (Shankar et al., 2003), *A. fumigatus* (Bhainsa and D'Souza, 2006), *C. versicolor* (Sanghi and Verma, 2009), *A. niger* (Gade et al., 2008), *P. glomerata* (Birla et al., 2009), *P. brevicompactum* (Shaligram et al., 2009), *C. cladosporioides* (Balaji et al., 2009), *P. fellutanum* (Kathiresan et al., 2009) and *V. volvacea* (Philip, 2009) were predominantly spherical with pyramidal, rod-like and triangular morphologies in the size of 5 to 200 nm.

The purification of water-soluble gold nanoparticles is particularly difficult because the nanoparticles and the impurities have similar solubility, often making standard purification techniques (that is, precipitation, extraction, chromatography, centrifugation or dialysis) inadequate or inefficient (Brust et al., 1995; Kanaras et al., 2002; Weare et al., 2000; Brust et al., 1994). Effective purification of nanoparticles is, therefore, a necessary step for controlling the quality and characteristics of nanoparticle

products (Dalwadi et al., 2005).

MATERIALS AND METHODS

Actinomycetal isolate

The *S. hygrosopicus* metal tolerant actinomycetal isolate was obtained from soil samples of Eastern Balaghat ranges of Maharashtra, India.

Preparation of biomass

S. hygrosopicus was grown in 500 ml Erlenmeyer flask containing 100 ml of sterile Malt Extract Glucose Yeast Extract Peptone (MGYP) both supplemented with griseofulvin at 50 µg/ml. Incubation was with shaking (200 rpm) at 35°C for 4 days. The flasks were removed from the shaker and placed at 5 to 10°C, to let the mycelium settle. The supernatant fluid was discarded and 100 ml of sterile distilled water was added for washing the cells. The flasks were kept at 5 to 10°C for 30 min to let the mycelium settle again. The supernatant fluid was poured off slowly to discard. 100 ml sterile distilled water was again added to the flask, and this procedure was repeated three more times. The mycelial mass was then separated from the sterile distilled water by centrifugation (1500 rpm) for 10 min; the mycelial pellets were weighed and used for the synthesis of gold nanoparticles.

Preparation of metal stock solutions

333.79 g of HAuCl₄ in 1000 ml of distilled water were used to obtain the 10 M.

Exposure of biomass to metal solutions

Five grams of actinomycetal wet biomass were exposed to 50 ml of a sterilized aqueous solution of HAuCl₄ at varying concentrations in 250 ml Erlenmeyer flasks and the flasks placed on a shaker at 200 rpm and incubated at 35°C for 4 days.

Characterization of metal nanoparticles

Visual observations

Samples of the reaction mixtures were verified visually for a possible colour change after 12, 24, 48 and 72 h of incubation (Table 1). The change in colour from pale yellow to a pinkish appearance was indicative of the formation of gold nanoparticles.

U. V. and visible spectroscopy

Biosynthesis of metal ions was also monitored by taking 2 ml aliquots of reaction mixture at different time intervals and centrifuging them at 5000 rpm for 10 min. The centrifuged biomass was washed twice with double distilled water and biofilms were prepared. The biofilms were dried in an oven at 45°C for 1 h and examined by spectroscopic analysis using an SL 159 U. V. and visible spectrophotometer (300 to 800 nm). The biomass samples showing the desired color change were used for further studies.

Preparation of samples for SEM

The presence of nanoparticles was confirmed by SEM. The metal

Table 1. Visual observation on *S. hygroscopicus* biomass colour after treatment with various HAuCl₄ solutions.

Molarity	Colour of biomass (control)	Colour change in biomass			
		After 12 h	After 24 h	After 48 h	After 72 h
10 ⁻¹	Yellowish white	Yellowish white	Yellowish white	Yellowish white	Yellowish white
10 ⁻²	Yellowish white	Yellowish white	Yellowish white	Yellowish white	Faint pink
10 ⁻³	Yellowish white	Faint pink	Purple	Purple	Violet
10 ⁻⁴	Yellowish white	Yellowish white	Yellowish white	Yellowish white	Faint pink
10 ⁻⁵	Yellowish white	Yellowish white	Yellowish white	Yellowish white	Yellowish white

treated biomass was centrifuged at 1500 rpm for 20 min and washed twice with double distilled water. The washed biomass was sonicated and homogenized using homogenizer. The homogenate was applied onto glass slide as an uniform thin layer and fixed in 2.5% glutaraldehyde for 12 h at room temperature. After fixation, the sample was subjected to dehydration in increasing concentrations of alcohol namely, 25, 50, 75 and 100% for 15 min each time. After platinum sputtering coating samples were examined by SEM.

Scanning electron microscopy (SEM)

The prepared biofilms were mounted onto carbon-coated copper grid. Micrographs were obtained using a JEOL (6360) JED-2300 analysis station operating at 200 kV.

Transmission electron microscopy (TEM)

The biomass samples were dispersed in water, left for 5 min in an ultra sonicator and, then, left to rest for 10 min. One drop of suspension was placed onto a grid of copper coated with 300 mesh palladium and carbon. Grids were examined using Zeiss CEM902 microscope at 80 kV.

Optimization of the biosynthesis of gold and silver nanoparticles

Effect of metal concentration

10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ mM solutions of HAuCl₄ were prepared 50 ml volumes of each solution were transferred into 250 ml Erlenmeyer flasks. The metal solutions were sterilized at 121°C for 30 min. The freshly prepared and washed *S. hygroscopicus* biomass with solution without incubation was kept in refrigerator to serve as control.

Two gram of freshly prepared and washed biomass were added to each flask and the flask incubated at 35°C (150 rpm) for 72 h. Every 12 h, a 2 ml sample was collected, centrifuged at 1500 rpm at for 20 min, the biomass (pellet) recovered, biofilms prepared, and absorbance biofilm was determined at 550 nm with the help of U. V. visible spectrophotometer.

Effect of pH

50 ml volumes of the HAuCl₄ (10⁻⁴ mM) solution were placed into seven 250 ml Erlenmeyer flasks. pH of the metal solution was adjusted with 0.1 N NaOH to 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, respectively. The metal solution was sterilized at 121°C for 30 min. The freshly prepared, washed *S. hygroscopicus* cell biomass with

metal solution without incubation was kept at refrigerator as a control.

2 g of freshly prepared and washed biomass were added to each one of the aforementioned seven flasks and the flasks were incubated at 35°C (150 rpm) for 72 h. After 12, 24, 48 and 72 h of incubation, a 2 ml sample was collected from each flask, centrifuged at 1500 rpm at 10°C for 20 min and the biomass (pellet) recovered, biofilms were prepared as described earlier and absorbance was determined at 550 nm with the help of U. V. visible spectrophotometer.

Partial purification of gold nanoparticles

The HAuCl₄ treated and washed biomass samples containing nanoparticles were collected, placed in crucibles and sequentially subjected to drying at room temperature for 24 h, at 45°C for 1 h, at 60°C for 1 h and, lastly at 200°C overnight. The ashes so obtained contained partially purified gold nanoparticles.

RESULTS AND DISCUSSIONS

It was found that the yellowish colour of *S. hygroscopicus* biomass when exposed to various HAuCl₄ solutions changed gradually (Table 1). A dark violet colour appeared over time under some conditions (Figure 1). The formation of such a violet colour is indicative of the intracellular formation of gold nanoparticles. It was also observed that after 72 h treatment, the aqueous HAuCl₄ solution was colorless, thereby indicating that the extracellular reduction of the HAuCl₄ ions has not occurred. The significant colour change after 72 h was observed only with the 10⁻⁴ mM solution.

Effect of HAuCl₄ metal at various concentrations on biosynthesis of nanoparticles

The effect of HAuCl₄ concentration on synthesis of gold nanoparticles by *S. hygroscopicus* was studied by U. V. visible spectroscopy. Absorbance of HAuCl₄ treated biofilm after 72h exposure at concentration of 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ mM was determined at different wavelength and given in Figure 2. It was observed that maximum absorption at critical wavelength (550 nm) was obtained at 10⁻³ and 10⁻⁴ mM HAuCl₄ concentration as compared to 10⁻¹, 10⁻² and 10⁻⁵ mM HAuCl₄ concentrations.

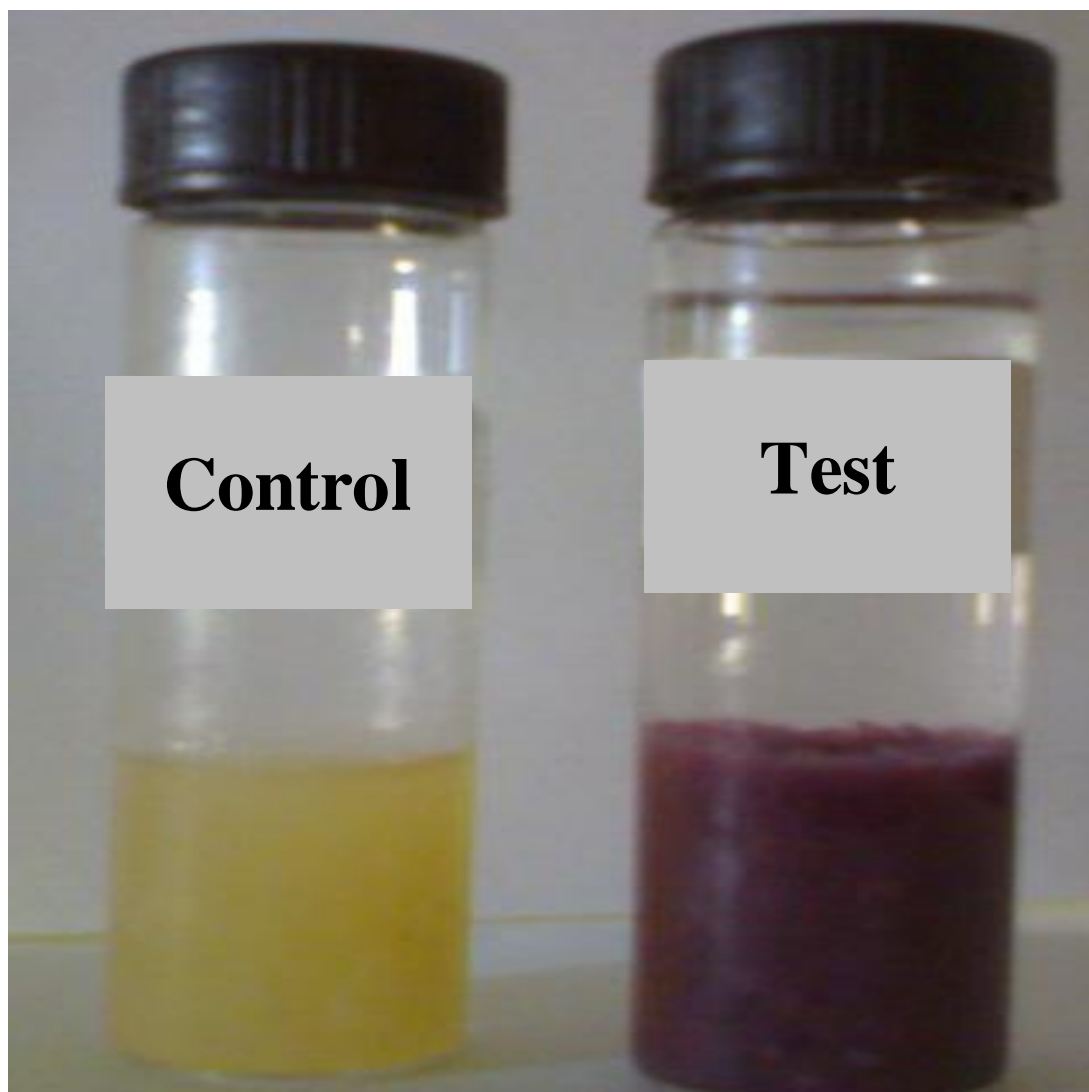


Figure 1. *S. hygrosopicus* biomass (control) before exposure to the 10^{-4} mM aqueous HAuCl₄ solution, (test) after exposure to 10^{-4} mM aqueous solution HAuCl₄ for 72 h.

Effect of pH on biosynthesis of nanoparticles

The effect of pH on synthesis of gold nanoparticles by actinomycetes was studied using U. V. visible spectroscopy. *S. hygrosopicus* was exposed for 72 h to 10^{-4} mM HAuCl₄ solution adjusted at various pH namely, 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. At the end absorbance was determined at different wavelength and given in Figure 2. It was found that optimum pH for the biosynthesis of HAuCl₄ nanoparticles was 7.0 (Figure 3). The pH was found to be an important parameter affecting gold nanoparticle synthesis. Variations in pH during exposure to Au-ions had an impact on the size, shape and number of particles produced per cell (Gericke and Anthony, 2006; Suryawanshi and Deshmukh, 2008; Kathiresan et al., 2009). The optimum gold accumulation

by microbial cells normally occurs in the pH range of 2 to 6 (Joerger and Klaus, 2000) and changes in the pH has an effect on the size of gold nanoparticles (Mukherjee et al., 2001). *Lactobacillus* sp. showed that changes in the pH could have an effect on the size distribution of gold nanoparticles (Nair and Pradeep, 2002). Typical bright field transmission electron microscopy of aqueous 10^{-4} HAuCl₄ treated *S. hygrosopicus* biomass before and after 72 h exposure are given in Figure 4. The TEM image clearly shows presence of well-separated and almost spherical-shaped metal nanoparticles in the biomass after 72 h exposure; whereas, aggregate of metals are seen in the metal treated biomass at 0 h of exposure. Biosynthesized gold nanoparticles by *S. hygrosopicus* were partial purified and characterized by SEM. It was found that gold nanoparticles were almost spherical in

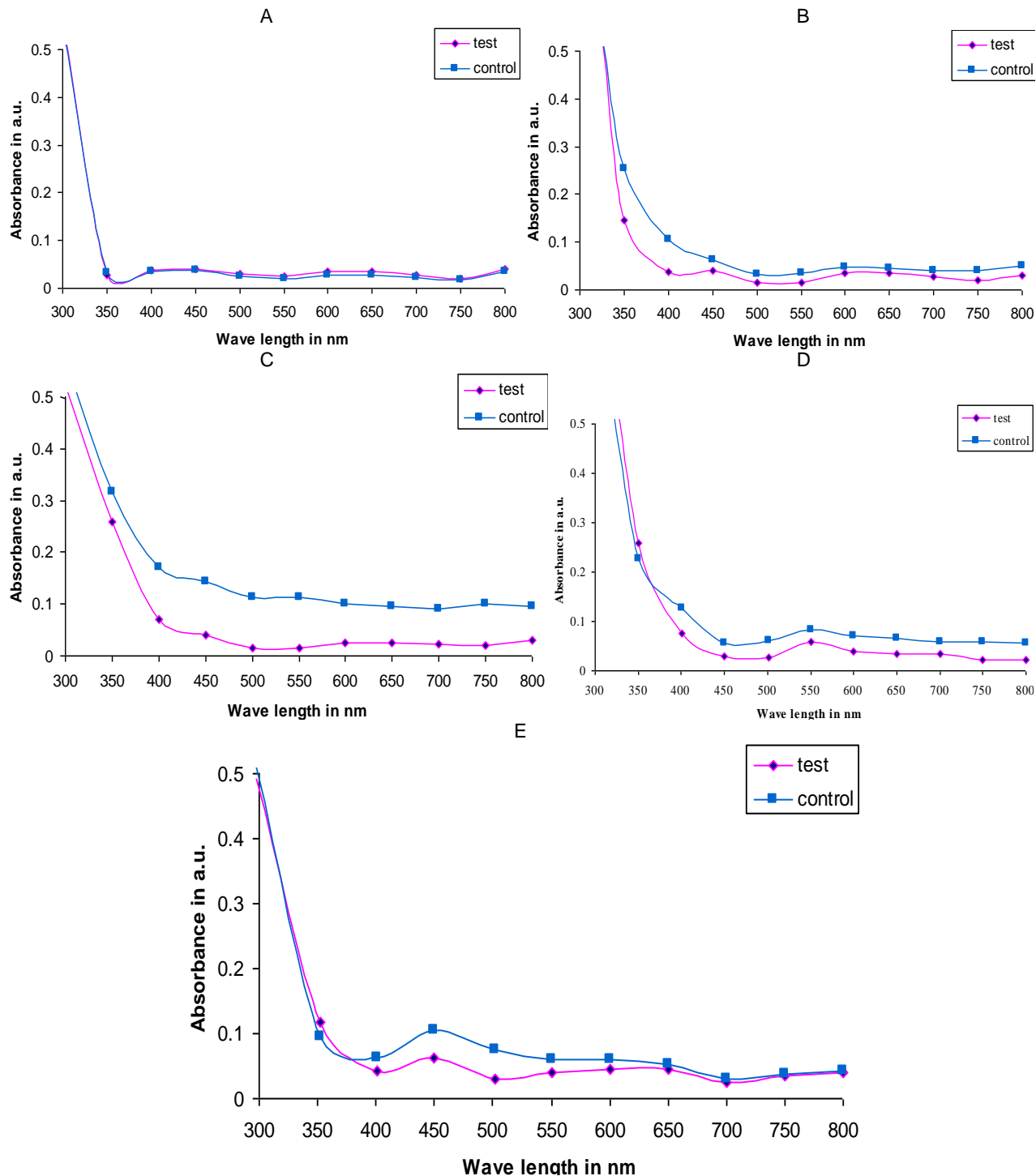


Figure 2. U. V. visible absorbance of HAuCl_4 various concentration treated *S. hygroscopicus* biofilm after 72 h. (A = with 10^{-1} mM, B = with 10^{-2} mM, C = with 10^{-3} mM, D = with 10^{-4} mM and E = with 10^{-5} mM).

shape and monodispersed (Figure 5). The characterization of HAuCl_4 treated actinomycetal biomass after 72 h exposure was carried out by XRD and metal nanoparticles size was determined. The XRD pattern of the HAuCl_4 treated sample (Figure 6) corresponds to that of

pure gold nanoparticles. The size gold nanoparticles was ranging from 15 to 30 nm in diameter.

Present investigation reports actinomycete *S. hygroscopicus* have ability to synthesize good dispersed gold nanoparticles. The results agree with those reports

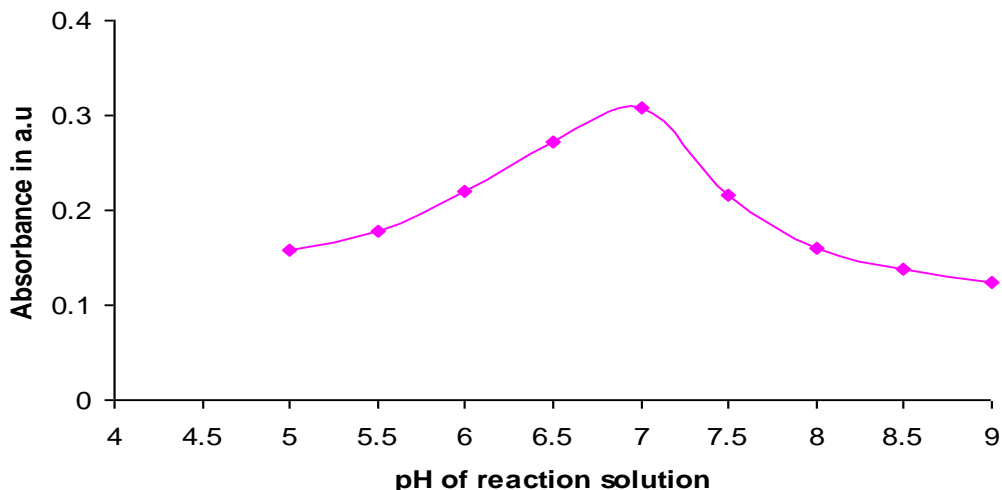


Figure 3. UV visible absorbance (550 nm) of HAuCl_4 (10^{-3} mM) treated *S. hygroscopicus* biofilm at various pH after 72 h.

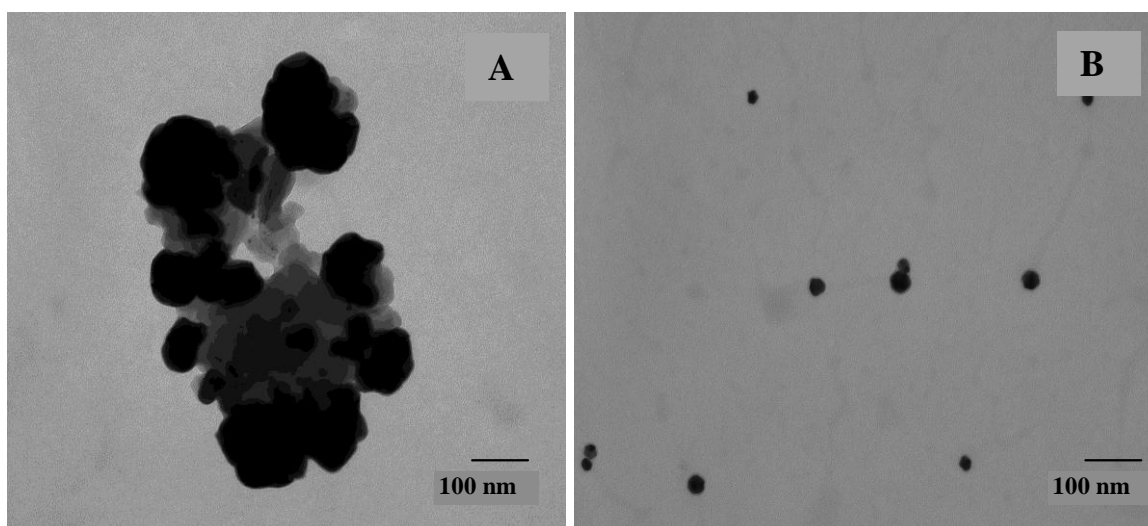


Figure 4. Characterization of nanoparticles by transmission electron microscopy: TEM image showing the gold crystals (A) before exposure to 10^{-4} aqueous solution HAuCl_4 . (B) showing gold nanoparticles (after exposure to 10^{-4} aqueous solution HAuCl_4), synthesized by using *S. hygroscopicus*.

for gold nanoparticles synthesized by different methods (Ahmad et al., 2003; Shankar et al., 2003; Mukherjee et al., 2001; Nair and Pradeep, 2002). The size of gold nanoparticles using Scherrer equation was also determined by different research workers (Mukherjee et al., 2001). Silver nanoparticles have been characterized using XRD by various investigators (Balaji et al., 2009). The size of silver nanoparticles can also be determined by laser diffraction (Sadowski, 2008). X-ray diffraction (XRD), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were applied for the characterization of the gold nanoparticles assisted by bacterium *E. coli* (Liangwei, 2007). The synthesis of

intracellular nanoparticles was studied by Mukherjee et al. (2001) by giving evidence of intracellular generation of gold nanoparticles provided by X-ray diffraction analysis of *Verticillium* biofilm deposited on Si substrate.

Conclusion

Gold nanoparticles are synthesized by the biomass of the bacterium, *S. hygroscopicus*. This is an economical, efficient, eco-friendly and simple process of biosynthesis of nanoparticles. The effect on pH and metal concentration of the synthesis of gold nanoparticles is studied.

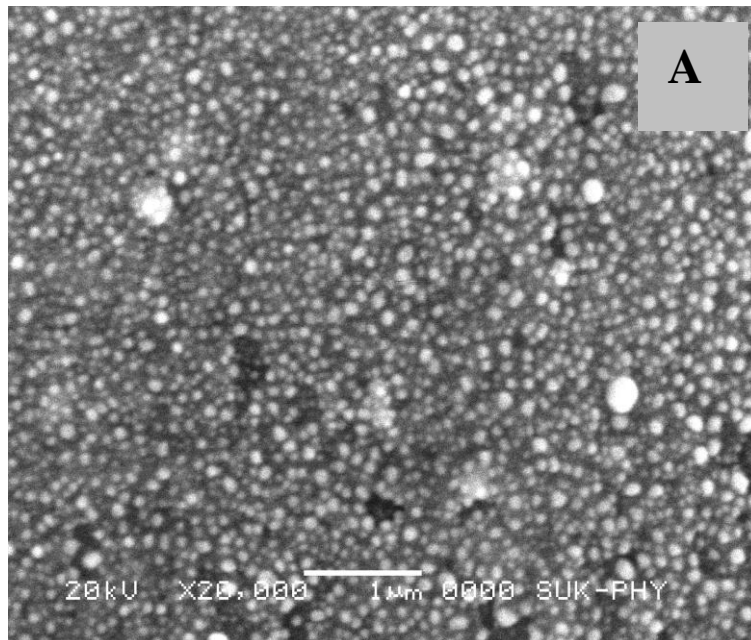


Figure 5. SEM image showing purified gold nanoparticles synthesized by *S. hygroscopicus*.

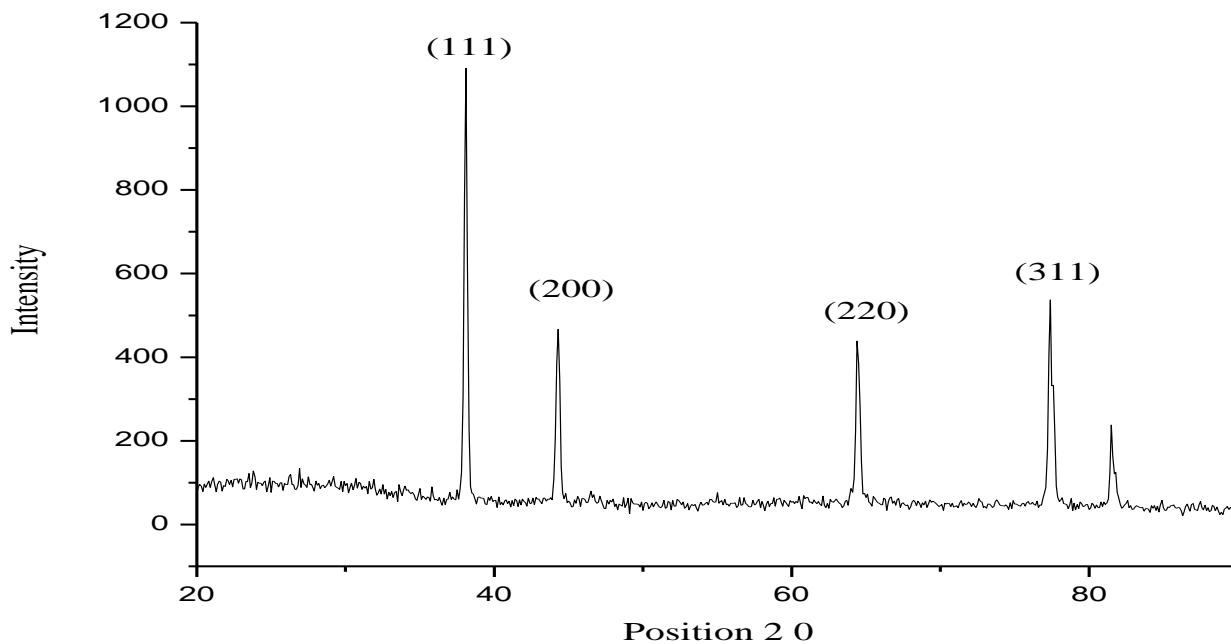


Figure 6. X-ray diffraction pattern of the gold nanoparticles.

The metal nanoparticles are partially purified by ignition method.

REFERENCES

Ahmad A, Mukherjee P, Senapati S, Mandal D, Khan MI, Kumar R

(2003). Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium oxysporum* *Colloids Surf. B: Biointerfaces*, 28:313.
 Ahmad A, Senapati S, Khan IM, Kumar R, Ramani R, Srinivas V, Sastry M (2003). Intracellular synthesis of gold nanoparticles by a novel alkalotolerant actinomycete, *Rhodococcus* species. *Nanotechnology* 14:824.
 Ahmad A, Senapati S, Khan MI, Kumar R, Sastry M (2003). Extracellular biosynthesis of monodisperse gold nanoparticles by a

- novel extremophilic actinomycete, *Thermomonospora* sp. *Langmuir* 19:3550.
- Ahmad A, Senapati S, Khan MI, Kumar R, Sastry MJ (2005). Extra-/intracellular biosynthesis of gold nanoparticles by an alkalotolerant fungus, *Trichothecium* sp. *Biomed Nanotechnol.* 1:47-53.
- Ahmad A, Senapati S, Khan MI, Ramani R, Srinivas V, Sastry M (2003). Intracellular synthesis of gold nanoparticles by a novel alkalotolerant actinomycete, *Rhodococcus* species. *Nanotech.* 14: 824.
- Balaji DS, Basavaraja S, Deshpande R, Mahesh BD, Prabhakar BK, Venkataraman A (2009). Extracellular biosynthesis of functionalized silver nanoparticles by strains of *Cladosporium cladosporioides* fungus. *Colloids Surf B Biointerfaces.* 68:88-92.
- Basavaraja S, Balaji SD, Lagashetty A, Rajasab AH, Venkataraman A (2008). Exacellular biosynthesis of silver nanoparticles using the fungus *Fusarium semitectum*. *Mat. Res. Bull.* 43:1164-1170.
- Bhainsa KC, D'Souza SF (2006). Extracellular biosynthesis of silver nanoparticles using the fungus *Aspergillus fumigatus*. *Colloids Surf B Biointerfaces.* 47:160-164.
- Birla SS, Tiwari VV, Gade AK, Ingle AP, Yadav AP, Rai MK (2009). Fabrication of silver nanoparticles by *Phoma glomerata* and its combined effect against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Lett. Appl. Microbiol.* 48:173-179.
- Brust M, Bethell D, Schiffrin DJ, Kiely CJ (1995). Novel gold-dithiol networks with non-metallic electronic properties. *Adv. Mater.* 7:795-797.
- Brust M, Walker M, Bethell D, Schiffrin DJ, Whyman RJ (1994). Synthesis of Thiol Derivatized Gold Nanoparticles in a 2-phase Liquid-Liquid system. *J. Chem. Commun.* 5:801-802.
- Dalwadi G, Heather AE, Yan C (2005). Comparison of diafiltration and tangential flow filtration for purification of nanoparticle suspensions. *Pharm. Res.* 5:7781-7782.
- Duran N, Marcato PD, Alves OL, De Souza GH, Esposito E (2005). Mechanistic aspects of biosynthesis of silver nanoparticles by several *Fusarium oxysporum* strains. *J. Nanobiotechnol.* 3:8.
- Duran N, Marcato PD, Alves OL, De Souza GI, Esposito E (2005). Mechanistic aspects of biosynthesis of silver nanoparticles by several *Fusarium oxysporum* strains. *J. Nanobiotechnol.* 3:8.
- Fayaz M, Balaji K, Girilal M, Yadav R, Kalaichelvan PT, Venkatesan R (2010). Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: a study against Gram-positive and Gram-negative bacteria. *Nanomedicine* 6:103-109.
- Fortin D, Beveridge TJ, In: Baeuerien E (2000). *Biomining*. From Biology to Biotechnology and Medical Applications, Wiley-VCH, Weinheim, 7
- Gade AK, Bonde PP, Ingle AP, Marcato P, Duran N, Rai MK (2008). Exploitation of *Aspergillus niger* for synthesis of silver nanoparticles. *J. Biobased Mater. Bioenergy* 3:123-129.
- Gamez G, Gardea-Torresdey JL, Tiemann KJ, Parsons J, Dokken K, Jose YM (2002). Recovery of gold (III) from multi-elemental solutions by alfalfa biomass. *Adv. Environ. Res.* 7: 563.
- Gardea-Torresdey JL, Parsons JG, Gomez E, Peralta-Videa J, Troiani HE, Santiago P, Jose-Yacamán M (2002). Formation and Growth of Au Nanoparticles inside Live Alfalfa Plants. *Nano Lett.* 2:397.
- Gericke M, Anthony P (2006). Microbial production of gold nanoparticles. *Gold Bulletin* 39:1.
- Ibrahim S, Ahmad A, Baba B (2001). Bioaccumulation of silver and the isolation of metal-binding protein from *P. diminuta*. *Brazil Arch. Biol. Technol.* 44: 223-225.
- Ingle A, Gade A, Pierrat S, Sonnichsen C, Rai M (2008). Mycosynthesis of silver nanoparticles using the fungus *Fusarium acuminatum* and its activity against some human pathogenic bacteria. *Curr. Nanosci.* 4:141-144.
- Ingle A, Rai M, Gade A, Bawaskar M (2009). *Fusarium solani*: a novel biological agent for the extracellular synthesis of silver nanoparticles. *J. Nanopart. Res.* 11:2079-2085.
- Joerger RT, Klaus CG (2000). Biologically produced silver-carbon composite materials for optically functional thin film coatings. *Adv. Mater.* 12:407-409.
- Kanaras AG, Kamounah FS, Schaumburg K, Kiely CJ, Brust M (2002). Thioalkylated Tetraethylene Glycol: a New Ligand for Water Soluble Monolayer Protected Gold Clusters. *J. Chem Soc. Chem. Commun.* pp. 2294-2295.
- Kathiresan K, Manivannan S, Nabeel MA, Dhivya B (2009). Studies on silver nanoparticles synthesized by a marine fungus, *Penicillium fellutanum* isolated from coastal mangrove sediment. *Colloids Surf B Biointerfaces* 71:133.
- Kathiresan K, Manivannan S, Nabeel MA, Dhivya B (2009). Studies on silver nanoparticles synthesized by a marine fungus, *Penicillium fellutanum* isolated from coastal mangrove sediment. *Colloids and Surfaces B: Biointerfaces.* 71:133-137.
- Klaus T, Joerger R, Olsson E (2001). Granqvist CG Bacteria as workers in the living factory: Metal-accumulating bacteria and their potential for materials science. *Trends Biotechnol.* 19(1):15-20.
- Lloyd JR (2003). Microbial metal reduction. *FEMS Microbiol. Rev.* 27:411.
- Mandal D, Bolander ME, Mukhopadhyay D, Sarkar G, Mukherjee P (2006). The use of microorganisms for the formation of metal nanoparticles and their application. *Appl Microbiol Biotechnol.* 69: 485.
- Mann S (1996). *Biomimetic Materials Chemistry*. VCH Publishers, Weinheim.
- Mukherjee P (2001). Bioreduction of $AuCl_4^-$ Ions by the Fungus, *Verticillium* sp. and Surface Trapping of the Gold Nanoparticles Formed. *Chem. Int. Edn.* 40:3585.
- Mukherjee P, Ahmad A, Mandal D, Senapati S, Sainkar SR, Khan MI, Parischa R, Ajaykumar PV, Alam M, Kumar R (2001). Fungus-mediated synthesis of silver nanoparticles and their immobilization in the mycelial matrix: A novel biological approach to nanoparticle synthesis. *Nano Lett.* 1:515-519.
- Mukherjee P, Ahmad A, Mandal D, Senapati S, Sainkar SR, Khan MI, Ramani R, Parischa R, Ajaykumar P, Alam M, Sastry M, Kumar R (2001). Bioreduction of $AuCl_4^-$ ions by the fungus, *Verticillium* sp. and surface trapping of the gold nanoparticles formed. *Angew Chem Int. Ed.* 40:3585-3588.
- Mukherjee P, Ahmad A, Mandal D, Senapati S, Sainkar SR, Khan MI, Ramani R, Parischa R, Ajaykumar PV, Alam M, Sastry M, Kumar R (2001). Bioreduction of $AuCl_4^-$ ions by the fungus, *Verticillium* sp. and surface trapping of the gold nanoparticles formed. *Angew. Chem. Int. Ed.* 40:3585-3588.
- Mukherjee P, Roy M, Mandal BP, Dey GK, Mukherjee PK, Ghatak J, Tyagi A K, Kale SP (2008). Green synthesis of highly stabilized nanocrystalline silver particles by a non-pathogenic and agriculturally important fungus *T. asperellum*. *Nanotechnology* 19:075-103.
- Mukherjee P, Senapati S, Mandal D, Ahmad A, Khan MI, Kumar R, Sastry M (2002). Extracellular synthesis of gold nanoparticles by the fungus *Fusarium oxysporum*. *Chem. Biochem.* 3:461-463.
- Nair B, Pradeep T (2002). Coalescence of nanoclusters and formation of submicron crystallites assisted by *Lactobacillus* strains. *Cryst. Growth Des.* 2:293-298.
- Philip D (2009). Biosynthesis of Au, Ag and Au-Ag nanoparticles using edible mushroom extract. *Spectrochim Acta. Part A.* 73:374-381.
- Sanghi R, Verma P (2009). Biomimetic synthesis and characterisation of protein capped silver nanoparticles. *Bioresour Technol.* 100:501-504.
- Sastry M, Ahmad A, Khan IM, Kumar R (2003). Biosynthesis of metal nanoparticles using fungi and actinomycete. *Curr. Sci.* 85:162-170.
- Senapati S, Mandal D, Ahmad A, Khan MI, Sastry M, Kumar R. (2004). Fungus mediated synthesis of silver nanoparticles: a novel biological approach. *Ind J Phys.* 78A:101-105.
- Shankar SS, Ahmad A, Pasricha R, Sastry M (2003). Bioreduction of chloroaurate ions by geranium leaves and its endophytic fungus yields gold nanoparticles of different shapes. *J. Mat. Chem.* 13:1822-1826.
- Shankar SS, Ahmad A, Pasricha R, Sastry M (2003). Bioreduction of chloroaurate ions by geranium leaves and its endophytic fungus yields gold nanoparticles of different shapes. *J. Mat. Chem.* 13:1822.
- Suryawanshi ML, Deshmukh AM (2008). Studies on aquatic actinomycetes from Shivaji Sagar, Ph. D. thesis, Shivaji University, Kolhapur, India.
- Uddin I, Adyanthaya S, Syed A, Selvaraj K, Ahmad A, Poddar P (2008). Structure and microbial synthesis of sub-10 nm Bi_2O_3 nanocrystals. *J. Nanosci. Nanotechnol.* 8:3909-3913.
- Varshney RA, Mishra N, Bhadauria S, Gaur MS (2009). A novel microbial route to synthesize silver nanoparticles using fungus

Hormoconis Resinae. *Digest J. Nanomaterials Biostructures*, 4(2):349-355.
Vigneshwaran N, Kathe AA, Varadarajan PV, Nachane RP, Balasubramanya RH. (2006) Biomimetics of silver nanoparticles by white rot fungus, *Phaenerochaete chrysosporium*. *Colloids Surf B Biointerfaces*. 53:55-59.

Weare WW, Reed SM, Warner MG, Hutchison JE (2000). Improved synthesis of small (dCORE-1.5 nm) phosphine-stabilized gold nanoparticles. *J. Am. Chem. Soc.* 122:12890-12891.

Full Length Research Paper

Bovine Shiga toxin producing *Escherichia coli* O157:H7 of Bangladesh: Is it capable of causing diseases similar to clinical strains?

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Escherichia coli O157:H7 is a predominant serotype of Shiga toxin producing *E. coli* (STEC) and is responsible for many outbreaks worldwide. Until recently, there is no comparative study on the bovine and clinical isolates as no STEC O157:H7 has been isolated from patients in Bangladesh. In the present study, the local bovine isolates were compared with a reference clinical strain to investigate whether the bovine isolates are capable of producing same degree of illness as the clinical strain. Two local isolates (CD-11 and CD-17) of bovine origin and a reference clinical strain (*E. coli* O157:H7 NCTC 12079) were investigated for the presence of virulence genes by polymerase chain reaction (PCR), Shiga toxin production by VTEC-RPLA, invasive property by Congo red binding and Sereny's keratoconjunctivitis, and other enterotoxic, cytotoxic and mouse lethal activities. Both the local isolates and the reference clinical strain showed the presence of *eae* and *stx*₂ genes and were found to be non-invasive. The isolates also produced enterotoxin, cytotoxin and mouse lethality similar to that found with the reference strain. All these results strongly suggest that the local bovine STEC O157:H7 isolates have potential to cause diseases similar to clinical STEC O157:H7 strain, which might lead to any outbreak in Bangladesh.

Key words: Bovine, *Escherichia coli*, STEC O157:H7, Bangladesh.

INTRODUCTION

The bacterium *Escherichia coli* O157:H7 has been reported as the predominant serotype of Shiga toxin producing *E. coli* (STEC) (Armstrong et al., 1996; Besser et al., 1999; Tarr et al., 2005). Cattles are considered to be the principal natural reservoirs of the organisms, excreting the bacteria in their feces (Gansheroff and O'Brien, 2000; Molina et al., 2003). Consumption of foods, particularly undercooked ground beef and raw milk has been associated with large food poisoning outbreaks, in which this organism was identified as the etiologic agent (WHO, 1997). The first outbreak of STEC O157:H7

was recorded in the United States in 1982 and other outbreaks occurred later in the United Kingdom, continental Europe, Africa, New Zealand and Japan over the next decade (CDC, 1982; Coombes et al., 2011; Pennington, 2010). STEC O157:H7 infections cause hemorrhagic colitis and hemolytic uremic syndrome (HUS), which includes thrombocytopenia and acute renal failure.

The pathogenicity of STEC O157:H7 is associated with various virulence factors, such as Shiga toxins 1 and 2 (Stx1 and Stx2), that are encoded by *stx*₁ and *stx*₂ genes,

respectively. Stx1 is antigenically similar to Shiga enterotoxin produced by *Shigella dysenteriae* type 1. Stx2 is heterogeneous (Stx2c, Stx2d, Stx2e and Stx2f) and immunologically different from Stx1 (Nakao and Takeda, 2000). Shiga toxins are A₁B₅ toxins that halt protein synthesis in the host cells, a process that may lead to an apoptotic cell death, and cause the vascular endothelial damage observed in patients with hemorrhagic colitis and the HUS (Paton and Paton, 1998). Another virulence factor is the protein intimin (encoded by the *eae* gene) which is responsible for the intimate attachment of the bacterium to the intestinal epithelial cells and causes the formation of attaching and effacing (A/E) lesions in the intestinal mucosa (Kaper et al., 1998). On the other hand, Stx exhibits cytotoxic effects on Vero and other cell lines. Hence, Stx is known as verotoxin (Vtx) and STEC are synonymously called verotoxin producing *E. coli* (VTEC). Stx is also a potent enterotoxin which is demonstrated by its ability to provoke fluid accumulation in rabbit ileal loops and is probably responsible for causing diarrhea (Blanco et al., 1991; Nataro and Kaper, 1998; Ferreira et al., 2002).

A number of studies were performed in different countries to compare the virulence factors of bovine and human STEC O157:H7 strains. Comparative studies in Ireland, Sweden and Japan revealed that cattle isolates possessing same phenotypic and genotypic traits as human clinical isolates have potential for causing human disease (Lenahan et al., 2009; Aspan and Eriksson, 2010; Lee et al., 2011). Kim et al. (1999) suggested that *E. coli* O157:H7 strains isolated from diseased humans were members of a different lineage than strains typically isolated from healthy cattle. Bono et al. (2012) reported that cattle harbor one lineage of STEC O157:H7 subtypes which is rarely found in clinically ill humans. Thus, it might be possible that bovine STEC O157:H7 strains sharing many qualities with human clinical strains might not be associated with human diseases.

In Bangladesh, which is one of the developing countries, there is no report of any outbreak caused by the STEC O157:H7. In fact, most of the outbreaks were more often reported from the industrialized countries than from developing countries because of the advanced surveillance and reporting systems in the industrialized countries. However, there are few reports on the prevalence of the Shiga-toxin producing *E. coli* (STEC) in Bangladesh (Islam et al., 2007, 2008, 2010). All these reports included the isolation and molecular characterizations of these organisms from the diarrheal patients, slaughtered animals, raw meat and other food samples. Apart from these reports, no detail studies were reported describing the enteropathogenicity or virulence properties of the locally isolated STEC O157:H7 from bovine origin in Bangladesh. Also there is no comparative study on the bovine and clinical isolates as no STEC O157:H7 has been isolated from patients in the recent past in Bangladesh. Therefore, the question arises on how viru-

lent the bovine STEC O157:H7 isolates are or whether the bovine isolates are capable of producing same degree of illness as the clinical STEC O157:H7. In the present study, we tried to find out the answers to the above questions, where the virulence potential of the local bovine *E. coli* O157:H7 isolates was compared with a reference clinical strain, *E. coli* O157:H7 NCTC 12079.

MATERIALS AND METHODS

Bacterial strains

A reference clinical strain *E. coli* O157:H7 NCTC 12079 and a negative control strain *E. coli* K-12 were obtained from the Department of Microbiology, University of Dhaka, Bangladesh. Two bovine STEC O157:H7 (CD-11 and CD-17) were isolated from the fresh feces of 18 healthy cattle from six different dairy farms around Dhaka city, Bangladesh and were included in the present study. The isolation and detection procedures of the bovine strains are described below:

One gram of each bovine feces sample was placed in 9 mL of trypticase soy broth (TSB) supplemented with 20 mg/mL novobiocin (Wako, Japan), and incubated at 37°C for 16-18 h. The enriched samples were streaked onto sorbitol MacConkey agar (Oxoid, England) plates supplemented with 0.5 mg/L cefixime and 1.5 mg/L potassium tellurite (Sigma, Germany) and incubated as above. After incubation, non-sorbitol fermenting colonies were streaked onto eosine methylene blue (EMB) agar (Oxoid, England) and 4-methylumbelliferyl-β-D-glucuronide (MUG) agar (Difco, USA) and incubated at 37°C for 18-22 h. Colonies showing green metallic sheen on EMB agar and no fluorescence on MUG agar, were characterized by indole production, citrate utilization, methyl red, Voges-Proskauer, triple sugar iron and oxidase tests as described by Cappuccino and Sherman (2011). Presence of the O157 and H7 antigens in biochemically positive colonies were investigated by latex agglutination test kit (Wellcolex™, Remel, USA) and the two STEC O157:H7 strains (CD-11 and CD-17) were re-confirmed by PCR targeting the *rfb*_{O157} and *fliC*_{H7} genes (Figure 1) (Paton and Paton, 1998; Gannon et al., 1997). The description of each primer pair is given in Table 1.

Molecular detection of virulence genes by PCR

PCR was performed to detect the major known virulence genes *eae*, *stx*₁, and *stx*₂ according to the previous studies (Kawasaki et al., 2005; Vidal et al., 2004). The details of the nucleotide sequence and size of the PCR amplicon for each primer pair are listed in Table 1. Template DNA was prepared by boiling DNA method as described by Radu et al. (2000). One bacterial colony was suspended in 1 ml of distilled water and boiled in a water bath for 10 min. After centrifugation at 10,000 rpm for 5 min, the supernatant was used as template DNA. PCR assay was performed using a thermal cycler (Bio-Rad, USA) in a total volume of 25 µL containing 2 µL of template DNA and 23 µL of master mix composed of 1 x PCR buffer (Takara, Japan), 0.5 µM (each) primer set, 0.2 mM each of the four deoxynucleotide triphosphates (dNTP mixture, Takara, Japan) and 0.5 U of TaKaRa Ex Taq™ polymerase (Takara, Japan). Chromosomal DNA of *E. coli* K-12 was used as negative control. The optimized PCR conditions for the genes assayed are shown in Table 2. The PCR amplicons were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide and photographed using a gel documentation machine (Bio-Rad, USA). DNA markers (Invitrogen, USA) were used as size references.

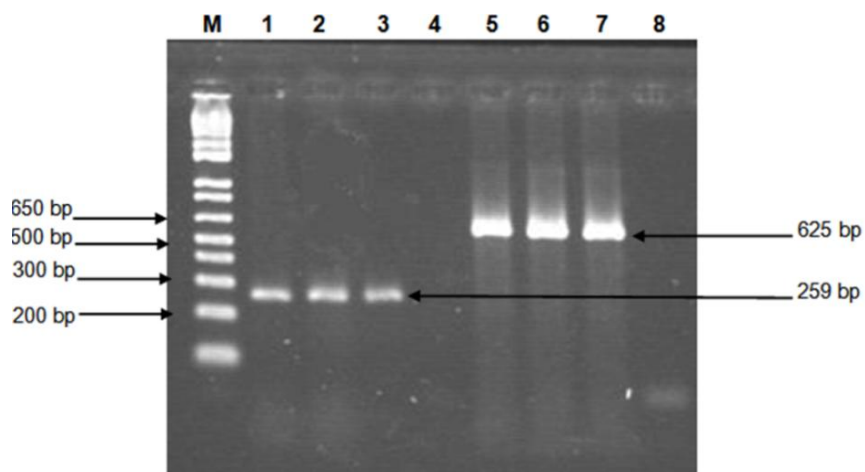


Figure 1. Agarose gel electrophoresis showing 259 and 625 bp PCR amplification products of *rfb*_{O157} and *fliC*_{H7} genes respectively. M = 1 kb plus DNA ladder; 1, 5 = CD-11; 2, 6 = CD-17; 3, 7 = *E. coli* O157:H7 NCTC 12079; 4, 8 = *E. coli* K-12.

Table 1. Primers used in the study.

Target gene	Primer name	Primer sequence (5'→3')	Amplicon size (bp)	Reference
<i>rfb</i> _{O157}	O157F	CGGACATCCATGTGATATAGG	259	Paton and Paton (1998)
	O157R	TTGCCTSTGTACAGCTAATCC		
<i>fliC</i> _{H7}	FLICH7-F	GCGCTGTCGAGTTCTATCGAG	625	Gannon et al. (1997)
	FLICH7-R	CAACGGTGACTTTATCGCCATTCC		
<i>eae</i>	VS8	GGCGGATTAGACTTCGGCTA	150	Kawasaki et al. (2005)
	VS9	CGTTTTGCCACTATTGCC		
<i>stx</i> ₁	LP30	CAGTTAATGTGGTGGCGAAGG	348	Vidal et al. (2004)
	LP31	CACCAGACAAATGTAACCGCTC		
<i>stx</i> ₂	LP41	ATCCTATTCCCGGGAGTTTACG	584	Vidal et al. (2004)
	LP42	GCGTCATCGTATACACAGGAGC		

Table 2. PCR conditions used for the detection of various genes of STEC O157:H7.

Stage	PCR assay			
	<i>rfb</i> _{O157}	<i>fliC</i> _{H7}	<i>eae</i>	<i>stx</i> ₁ and <i>stx</i> ₂
Initial denaturing	94°C: 10 min	94°C: 10 min	94°C: 10 min	94°C: 10 min
Denaturing	94°C: 1 min	94°C: 30 s	94°C: 20 s	94°C: 1 min
Annealing	56°C: 1 min	65°C: 30 s	60°C: 30 s	55°C: 1 min
Extension	72°C: 1 min	72°C: 75 s	72°C: 30 s	72°C: 1 min
Cycle no.	35 cycles	35 cycles	35 cycles	35 cycles
Final extension	72°C: 7 min	72°C: 7 min	72°C: 7 min	72°C: 7 min

Detection of Shiga toxin production

The types of Shiga toxin produced by each isolate of STEC O157:H7 were determined using VTEC-Reverse Passive Latex Agglutination (RPLA) test kit (Oxoid TD960) following the procedure described in the kit manual.

Congo red binding test

Trypticase soy agar containing 0.01% (w/v) Congo red was streaked with both bovine and reference clinical strains and incubated at 37°C for 18 h. After incubation, colonies were examined for the presence (colonies with dark red center) or absence

(colonies without dark red center) of Congo red binding (Sharma et al., 2006).

Animal maintenance

All the animal experiments were undertaken following the ethical issues set the Faculty of Biological Sciences, University of Dhaka (Reference number 5210). All efforts were made to minimize the sufferings of the animals.

Sereny's keratoconjunctivitis test

This test was performed according to the procedure as reported previously (Sereny, 1955). Briefly, 20 μ L of an overnight culture of the STEC O157:H7 strains (both bovine isolates and reference clinical) or *Shigella flexneri* 2a containing approximately 5×10^8 cells/ml in phosphate buffer saline (PBS) was inoculated into one of the eyes of a guinea pig and the other eye served as the negative control. A total of four guinea pigs were included in each group and the *S. flexneri* 2a inoculated eyes served as the positive control. All guinea pigs were observed daily for 5 days for any inflammatory responses in the eyes of the animals.

Preparation of live cells

Ten milliliters of Brain Heart Infusion (BHI) broth was inoculated with 5 colonies of the pure culture of each strain (bovine or reference clinical) and incubated at 37°C for 6 h with shaking. One milliliter BHI broth containing approximately 10^5 - 10^6 cfu was used as inoculum for the enterotoxicity assay (Sanyal et al., 1975).

Preparation of culture filtrate

Fifty milliliters conical flasks containing 10 ml of BHI broth were inoculated with 5 colonies of the pure cultures of each strain (bovine isolates or reference clinical) and incubated at 37°C for 20 h with shaking (100 rpm). The cultures were centrifuged at 10,000 rpm for 10 min at 10°C and each supernatant was filtered through a Millipore membrane (0.45 μ m pore diameter) and preserved at -20°C. These culture filtrates were used for enterotoxicity, cytotoxicity and mouse lethality assays.

Enterotoxicity assay

Detection of enterotoxic ability of Stx produced by the STEC O157:H7 strains was tested in the rabbit ileal loop (RIL) following the procedure of Sanyal et al. (1975). One milliliter each of a 6 h grown bacterial cultures or the culture filtrates prepared above, were tested in loops of an adult rabbit ileum (New Zealand White variety, weighing 1.8-2.0 kg). Each sample was tested in duplicate rabbits following laparotomy and live cells or culture filtrate of *Vibrio cholerae* 569B and the culture filtrate of *E. coli* K-12 or the BHI broth were inoculated as positive and negative controls, respectively. The inoculated rabbits were euthanized after 18 h and the volume of fluid accumulation per cm of gut in each rabbit was measured. Fluid accumulation ≥ 0.5 ml/cm was considered as positive.

Cytotoxicity assay

Cytotoxicity of the STEC O157:H7 culture filtrates were tested on HeLa and MDCK (Medin-Derby canine kidney) cells (Konowalchuk

et al., 1977). HeLa or MDCK cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Freshly trypsinized cells were counted, suspended in growth medium (approximately 1×10^5 cells/ml) and distributed in 0.1 ml volume into 96-well cell culture plates. Monolayers of cells were established by 18 to 20 h of incubation at 35°C in a 5% CO₂ atmosphere and 0.1 ml volume of each culture filtrate was added to the cell monolayers. The plates were incubated for an additional 18 to 20 h and checked for any change in cell morphology. BHI medium was used as negative control and each sample was checked in duplicate wells.

Mouse lethality assay

Five groups of Swiss Albino mice, each comprising of six animals, were injected intraperitoneally with the culture filtrates (0.1 ml each) of the STEC O157:H7 strains (Tesh et al., 1993). Negative control animals received the *E. coli* K-12 culture filtrate or BHI broth only. All mice were kept in cages with free access to food and sterile water, monitored daily for 10 days and any change in behavior or sickness was recorded.

RESULTS AND DISCUSSION

Shiga toxin producing *E. coli* (STEC) are food-borne pathogens that cause hemolytic colitis and a serious sequel, HUS. The largest outbreaks of STEC are due to a single *E. coli* serotype, O157:H7, although STEC non-O157:H7 serotypes also cause similar diseases (Khan et al., 2002). There are a number of comparative studies between human disease and bovine-associated STEC O157 in different countries. A study on cattle and human clinical isolates of STEC O157 showed that cattle isolates possessed all the virulence traits that are typical for the human pathogenic strains. On the other hand, the human clinical isolates had different combinations of Stx-variants as compared to the cattle isolates (Nielsen and Scheutz, 2002). In another study in Czech Republic, it was reported that the phenotypic and genotypic characteristics of both bovine and human STEC O157 isolates were identical or closely related which supported the pathogenic potential of the bovine isolate for humans (Bielaszewska et al., 2000). An investigation of STEC O157 strains isolated from human sporadic infections with those of cattle found that human clinical isolates constitute a small fraction of bovine isolates (Roldgaard et al., 2004). However, Baker et al. (2007) demonstrated that STEC O157:H7 isolates from healthy cattle were less virulent than those from human disease outbreaks. Bangladesh is an endemic zone for diarrheal diseases and in recent years, STEC non-O157:H7 has also been isolated from hospitalized patients. Although the STEC non-O157:H7 is an uncommon pathogen among the hospitalized patients with diarrhea, however, the low prevalence of STEC non-O157:H7 in Bangladesh might be due to acquired immunity against the pathogen in the population (Islam et al., 2007). Apart from the few reports on the isolation and virulence study of STEC O157:H7 from slaughtered animals, no STEC O157:H7 has been

Table 3. Detection of Congo red binding, Sereny’s test, enterotoxigenic activity, cytotoxic activity, mouse lethality assay and presence of virulence genes in the STEC O157:H7 strains.

STEC O157:H7	Congo red binding	Sereny’s test	Enterotoxigenic activity (ml/cm)	Cytotoxic activity	Mouse lethality assay	<i>eae</i> gene	<i>stx1</i> gene	<i>stx2</i> gene	Toxin detection by RPLA
CD-11	-	-	2.2	+	+	+	-	+	Stx 2
CD-17	-	-	2.0	+	+	+	-	+	Stx 2
NCTC 12079	-	-	2.4	+	+	+	+	+	Stx 1 and 2
<i>E. coli</i> K-12	ND	ND	-	-	-	-	-	-	-
<i>V. cholera</i> 569B	ND	ND	3.0	ND	ND	ND	ND	ND	ND

ND = not done; ‘+’ = positive result; ‘-’ = negative result.

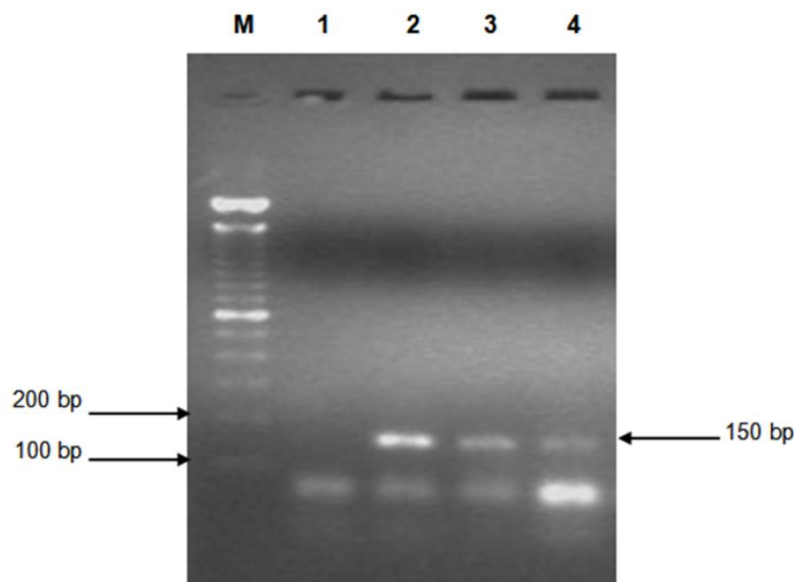


Figure 2. Agarose gel electrophoresis showing 150 bp PCR amplification products of *eae* gene. M = 100 bp DNA ladder; 1 = *E. coli* K-12; 2 = *E. coli* O157:H7 NCTC 12079; 3 = CD-11; 4= CD-17.

isolated either from diarrheal patients or other sources in Bangladesh (Islam et al., 2008). Therefore, not much is known about the virulence properties of this pathogen isolated either from the patients or the cattle. In the present study, the pathogenicity and virulence potential of two bovine STEC O157:H7 isolates were compared to that of a reference clinical *E. coli* O157:H7 NCTC 12079 strain to understand the virulence potential of the local bovine isolates.

Table 3 summarizes comparative virulence traits of local bovine isolates (CD-11 and CD-17) and the reference clinical strain *E. coli* O157:H7 NCTC 12079. PCR is generally considered to be the most sensitive means of determining whether a fecal specimen or a food sample contains STEC (Paton and Paton, 1998). In our PCR assay, the presence of the main virulence genes (*eae*, *stx1* and *stx2*), which have been widely used by

other researchers, were investigated and only the *stx2* gene was detected in both bovine isolates. On the other hand, both *stx1* and *stx2* genes were detected in the reference strain (Figures 2 to 4). It has been reported that the *stx2* gene was more common in bovine than the *stx1* in most of the studies performed in the USA, Japan and European countries (Ding et al., 2011). Epidemiologic data suggest that STEC O157 strains that express Stx2 are more important than Stx1 in the development of HUS and strains that express Stx2 alone are more likely to be associated with the progression to HUS than strains that produce both Stx1 and Stx2 (Griffin and Tauxe, 1991; Griffin, 1995). Numerous investigators have also shown strong association between the carriage of *eae* gene and the capacity of STEC causing severe human disease, especially HUS (Suardana et al., 2011). In this study, this important virulence gene was also detected by PCR in

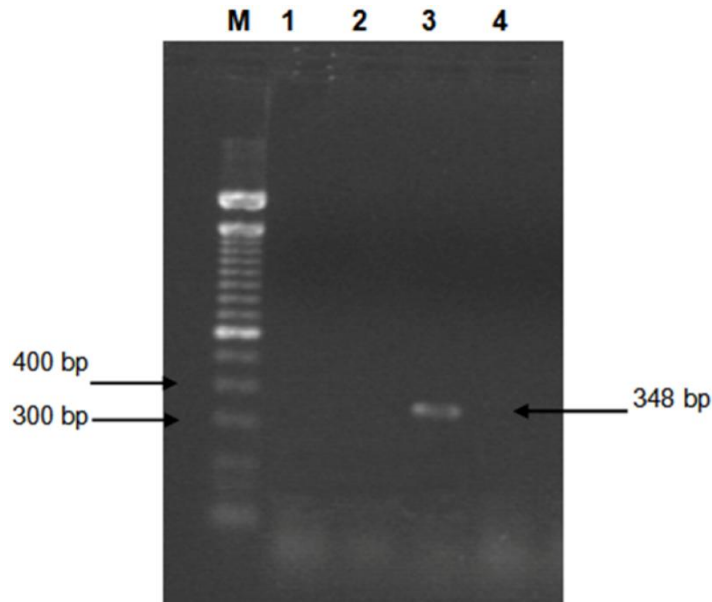


Figure 3. Agarose gel electrophoresis showing 348 bp PCR amplification products of *stx*₁ gene. M = 100 bp DNA ladder; 1 = CD-11; 2 = CD-17; 3 = *E. coli* O157:H7 NCTC 12079; 4 = *E. coli* K-12.

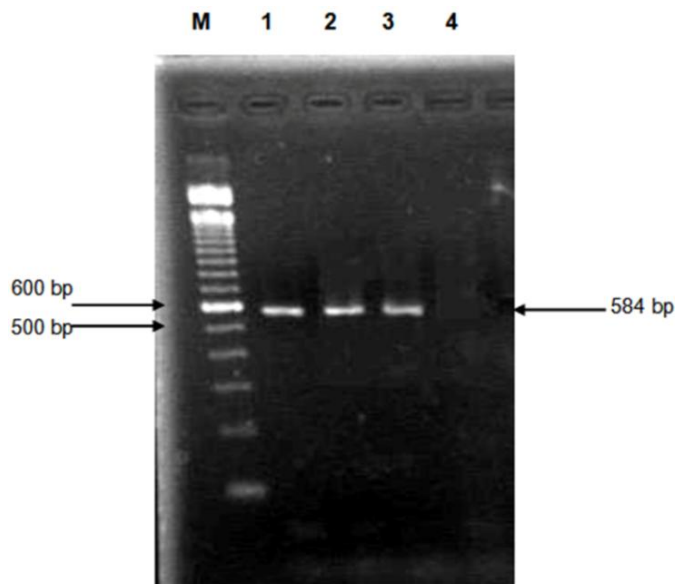


Figure 4. Agarose gel electrophoresis showing 584 bp PCR amplification products of *stx*₂ gene. M = 100 bp DNA ladder; 1 = CD-11; 2 = CD-17; 3 = *E. coli* O157:H7 NCTC 12079; 4 = *E. coli* K-12.

both the STEC O157:H7 isolates and reference strain, which gave evidence that these strains had potency to colonize the intestine and induce attaching-effacing lesions and also cause cytopathic effects in intestinal epithelial cells. Beutin et al. (2004) reported that Shiga

toxin type 1c and 2d were found to be present only in *eae* negative STEC strains, and type 2 was significantly more frequent in *eae*-positive STEC strains. Heuvelink et al. (1998) reported that bovine strains of STEC O157 share virulence factors with human strains, including *stxs* and *eae*,

and may be considered potential human pathogens. Our isolates were also evaluated for Shiga toxin production through VTEC-RPLA and the results were 100% in concordance with PCR results.

In the Congo red binding test, all of the three STEC O157:H7 strains (both bovine and reference clinical) produced colourless (non-Congo red binding) colonies, which indicated that the strains were non-invasive (Uhlich et al., 2002). The Congo red binding test was again supported by the Sereny's test, where none of these three strains could produce keratoconjunctivitis in guinea pig eyes, indicating non-invasive nature of the strains. However, the positive control *S. flexneri* 2a strain showed fully developed keratoconjunctivitis with purulence in guinea pig eyes within three days.

In the ligated rabbit ileal loop assay, both live cells and culture filtrates prepared from the bovine isolates and the reference clinical strain, induced fluid accumulation in the large gut of rabbits almost in the same range as that of the positive control *V. cholerae* 569B strain (Table 3). This is probably due to the release of sufficient amount of enterotoxin produced by the live cells *in vivo* in the large gut or *in vitro* during the 20 h period of cultivation of the STEC strains. The fluid accumulation in the large gut also indicated the potential of the bovine isolates to produce diarrhea in the host system in the same range as the reference clinical strain. Again in the cytotoxicity assay, the culture filtrates prepared from the bovine strains changed the cell morphology of both HeLa and MDCK cells, indicating the cytotoxicity of the bovine STEC O157:H7 strains.

The degree of cytotoxicity of the bovine culture filtrates was the same with the reference clinical culture filtrate. Also in the mouse lethality assay, all mice which received culture filtrates prepared from the bovine or reference clinical STEC O157:H7 strain became sick in two days. All these animals were unable to move, probably due to paralysis of the hind legs and died between 3-6 days after the culture filtrate injection, which could be due to Shiga toxin production by both bovine and the reference clinical strains (Obata et al., 2008). However, the negative control animals were found to be in good health and survived the mouse lethal activity assay.

All these findings clearly indicate that the bovine STEC O157:H7 isolates, CD-11 and CD-17, are as virulent as the reference clinical STEC O157:H7 strain. In a recent report, it was found that STEC O157:H7 increased its pathogenicity in the animal model after the passage through the gastrointestinal tract of the same host (Fernandez-Brando et al., 2012). This is a matter of concern, as the bovine STEC strains, if passaged through a suitable host, might increase its pathogenicity. Although no one single animal model fully represents the spectrum of STEC illness, however, the results of the study strongly suggest that the bovine STEC O157:H7 isolates have potential to cause disease similar to the clinical STEC O157:H7 strains, which might also lead to

any future outbreak in Bangladesh. Further studies are required with large number of isolates from various sources for better understanding of the virulence potential of local STEC O157:H7. In addition, more virulence characteristics and clonal relatedness of isolates can be included in these studies.

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REFERENCES

- Armstrong GL, Hollingsworth J, Jr Morris JG (1996). Emerging foodborne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. *Epidemiol. Rev.* 18:29-51.
- Aspan A, Eriksson E (2010). Verotoxigenic *Escherichia coli* O157:H7 from Swedish cattle; isolates from prevalence studies versus strains linked to human infections - A retrospective study. *BMC Vet. Res.* 6:7.
- Baker DR, Moxley RA, Steele MB, Lejeune JT, Christopher-Hennings J, Chen DG, Hardwidge PR, Francis DH (2007). Differences in virulence among *Escherichia coli* O157:H7 strains isolated from humans during disease outbreaks and from healthy cattle. *Appl. Environ. Microbiol.* 73:7338-7346.
- Besser RE, Griffin PM, Slutsker L (1999). *Escherichia coli* O157:H7 gastroenteritis and hemolytic uremic syndrome: an emerging infectious disease. *Annu. Rev. Med.* 50:355-367.
- Beutin L, Krause G, Zimmermann S, Kaulfuss S, Gleier K (2004). Characterization of shiga toxin-producing *Escherichia coli* strains isolated from human patients in Germany over a 3-year period. *J. Clin. Microbiol.* 42:1099-1108.
- Bielaszewska M, Schmidt H, Liesegang A, Prager R, Rabsch W, Tschape H, Cizek A, Janda J, Blahova K, Karch H (2000). Cattle can be a reservoir of sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157:H- strains and a source of human diseases. *J. Clin. Microbiol.* 38:3470-3473.
- Blanco J, Blanco M, Garabal JI, González EA (1991). Enterotoxins, colonization factors and serotypes of enterotoxigenic *Escherichia coli* from humans and animals. *Microbiologia* 7:57-73.
- Bono JL, Smith TP, Keen JE, Harhay GP, McDanel TG, Mandrell RE, Jung WK, Besser TE, Gerner-Smith P, Bielaszewska M, Karch H, Clawson, ML (2012). Phylogeny of Shiga Toxin-Producing *Escherichia coli* O157 isolated from cattle and clinically ill humans. *Mol. Biol. Evol.* 29:2047-2062.
- Cappuccino JG, Sherman N (2011). *Microbiology: A Laboratory Manual*. 9th edn. Pearson/Benjamin Cummings Publishing Company Inc, San Francisco, CA. pp. 147-209.
- CDC (1982). Isolation of *E. coli* O157:H7 from sporadic cases of hemorrhagic colitis -United States. *Morb. Mortal. Wkly. Rep.* 31:580-585.
- Coombes BK, Gilmour MW, Goodman CD (2011). The evolution of virulence in non-O157 shiga toxin-producing *Escherichia coli*. *Front. Microbiol.* 90:1-3.
- Ding H, Huang L, Mao X, Zou Q (2011). Characterization of *stx2* and its variants in *Escherichia coli* O157:H7 isolated from patients and animals. *Afr. J. Biotechnol.* 10:12991-2998.
- Fernandez-Brando RJ, Miliwebsky E, Mejias MP, Baschkier A, Panek CA, Abrey-Recalde MJ, Cabrera, G, Ramos MV, Rivas M, Palermo MS (2012). Shiga toxin-producing *Escherichia coli* O157:H7 show an increased pathogenicity in mice after the passage through the gastrointestinal tract of the same host. *J. Med. Microbiol.* 61:852-859.
- Ferreira AJ, Elias WP Jr, Pelayo JS, Giraldo R, Pedrosa MZ, Scaletsky

- ICA (2002). Culture supernatant of Shiga toxin-producing *Escherichia coli* strains provoke fluid accumulation in rabbit ileal loops. *FEMS Immunol. Med. Microbiol.* 19:285-288.
- Gannon VP, D'Souza S, Graham T, King RK, Rahn K, Read S (1997). Use of the flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic *Escherichia coli* strains. *J. Clin. Microbiol.* 35:656-662.
- Gansheroff LJ, O'Brien AD (2000). *Escherichia coli* O157:H7 in beef cattle presented for slaughter in the U.S.: Higher prevalence rates than previously estimated. *Proc. Natl. Acad. Sci. U.S.A.* 97:2959-2961.
- Griffin PM (1995). *Escherichia coli* O157:H7 and other enterohemorrhagic *Escherichia coli*. In Blaser et al. (eds) *Infections of the gastrointestinal tract*, Raven Press, New York. pp. 739-761.
- Griffin PM, Tauxe RV (1991). The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiol. Rev.* 13:60-98.
- Heuvelink AE, van den Biggelaar FL, de Boer E, Herbes RG, Melchers WJ, Huis in't Veld JH, Monnens LA (1998). Isolation and characterization of verocytotoxin-producing *Escherichia coli* O157 strains from Dutch cattle and sheep. *J. Clin. Microbiol.* 36:878-882.
- Islam MA, Heuvelink AE, de Boer E, Sturm PD, Beumer R R, Zwietering MH, Faruque AS, Haque R, Sack DA, Talukder KA (2007). Shiga toxin-producing *Escherichia coli* isolated from patients with diarrhoea in Bangladesh. *J. Med. Microbiol.* 56:380-385.
- Islam MA, Mondol AS, Azmi IJ, de Boer E, Beumer RR, Zwietering MH, Heuvelink AE, Talukder KA (2010). Occurrence and characterization of Shiga toxin-producing *Escherichia coli* in raw meat, raw milk, and street vended juices in Bangladesh. *Foodborne Pathog. Dis.* 7:1381-1385.
- Islam MA, Mondol AS, de Boer E, Beumer RR, Zwietering MH, Talukder KA, Heuvelink AE. (2008). Prevalence and genetic characterization of Shiga toxin-producing *Escherichia coli* isolated from slaughtered animals in Bangladesh. *Appl. Environ. Microbiol.* 74:5414-5421.
- Kaper JB, Elliott S, Sperandio V, Perna TP, Maythaw GF, Blatner FR (1998) Attaching and effacing intestinal histopathology and the locus of enterocyte effacement. In Kaper JB, O'Brien AD (eds) *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains, ASM press, Washington DC. pp. 163-82.
- Kawasaki S, Horikoshi N, Okada Y, Takeshita K, Sameshima T, Kawamoto, S (2005). Multiplex PCR for simultaneous detection of *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in meat samples. *J. Food Prot.* 68:551-556.
- Khan A, Yamasaki S, Sato T, Ramamurthy T, Pal A, Datta S, Chowdhury NR, Das SC, Sikdar A, Tsukamoto T, Bhattacharya SK, Takeda Y, Nair GB (2002). Prevalence and genetic profiling of virulence determinants of non-O157 Shiga toxin-producing *Escherichia coli* isolated from cattle, beef, and humans, Calcutta, India. *Emerg. Infect. Dis.* 8:54-62.
- Kim J, Niefeldt J, Benson AK (1999). Octamer-based genome scanning distinguishes a unique subpopulation of *Escherichia coli* O157:H7 strains in cattle. *Proc. Natl. Acad. Sci. U.S.A.* 96:13288-13293.
- Konowalchuk J, Speirs JI, Stavric S (1977). Vero response to a cytotoxin of *Escherichia coli*. *Infect. Immun.* 18:775-779.
- Lee K, French NP, Hara-Kuda Y, Iyoda S, Kobayashi H, Konishi YS, Tsubone H, Kumagai S (2011). Multivariate analyses revealed distinctive features differentiating human and cattle isolates of Shiga toxin-producing *Escherichia coli* O157 in Japan. *J. Clin. Microbiol.* 49:1495-1500.
- Lenahan M, O'Brien SB, Byrne C, Ryan M, Kennedy CA, McNamara EB, Fanning S, Sheridan JJ, Sweeney T (2009). Molecular characterization of Irish *E. coli* O157:H7 isolates of human, bovine, ovine and porcine origin. *J. Appl. Microbiol.* 107:1340-1349.
- Molina PM, Parma AE, Sanz ME (2003). Survival in acidic and alcoholic medium of shiga toxin-producing *Escherichia coli* O157: H7 and non-O157: H7 isolates in Argentina. *BMC Microbiol.* 3:17.
- Nakao H, Takeda T (2000). *Escherichia coli* Shiga toxin. *J. Nat. Toxins* 9:299-313.
- Nataro JP, Kaper JB (1998). Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* 11:142-201.
- Nielsen EM, Scheutz F (2002). Characterisation of *Escherichia coli* O157 isolates from Danish cattle and human patients by genotyping and presence and variants of virulence genes. *Vet. Microbiol.* 88:259-273.
- Obata F, Tohyama K, Bonev AD, Kolling GL, Keepers TR, Gross LK, Nelson MT, Sato S, Obrig TG (2008). Shiga toxin 2 affects the central nervous system through receptor globotriaosylceramide localized to neurons. *J. Infect. Dis.* 198:1398-1406.
- Paton AW, Paton JC (1998). Detection and characterization of Shiga toxinigenic *Escherichia coli* by using multiplex PCR assays for *stx*₁, *stx*₂, *eaeA*, enterohemorrhagic *E. coli hlyA*, *rfb*_{O111}, and *rfb*_{O157}. *J. Clin. Microbiol.* 36:598-602.
- Pennington H (2010). *Escherichia coli* O157. *Lancet* 376:1428-1435.
- Radu S, Rusul G, Ling OW, Purwait E, Mustakim M, Lihan S (2000). Rapid isolation and detection of *Escherichia coli* O157:H7 by use of rainbow agar O157TM and PCR assay. *Southeast Asian J. Trop. Med. Public Health* 31:77-79.
- Roldgaard BB, Scheutz F, Boel J, Aabo S, Schultz AC, Cheasty T, Nielsen EM, Olsen KE, and Christensen BB (2004). VTEC O157 subtypes associated with the most severe clinical symptoms in humans constitute a minor part of VTEC O157 isolates from Danish cattle. *Int. J. Med. Microbiol.* 294:255-259.
- Sanyal SC, Singh SJ, Sen PC (1975). Enteropathogenicity of *Aeromonas hydrophila* and *Plesiomonas shigelloides*. *J. Med. Microbiol.* 8:195-198.
- Sereny B (1955). Experimental shigella keratoconjunctivitis shigellosa. *Acta Microbiol. Acad. Sci. Hungar.* 2:293-296.
- Sharma KK, Soni SS, Meharchandani S (2006). Congo red dye agar test as an indicator test for detection of invasive bovine *Escherichia coli*. *Vet. Archiv* 76:363-366.
- Suardana W, Artama WT, Asmara W, Daryono BS (2011). Adherence pheno-genotypic of *Escherichia coli* O157:H7 isolated from beef, feces of cattle, chicken and human. *Indones. J. Biotechnol.* 16:46-52.
- Tarr PI, Gordon CA, Chandler WL (2005). Shiga toxin producing *Escherichia coli* and haemolytic uremic syndrome. *Lancet* 365:1073-1086.
- Tesh VL, Burriss JA, Owens JW, Gordon VM, Wadolkowski EA, O' Brien AD (1993). Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. *Infect. Immun.* 61:3392-3402.
- Uhlich GA, Keen JE, Elder RO (2002). Variations in the *csxD* promoter of *Escherichia coli* O157:H7 associated with increased virulence in mice and increased invasion of HEp-2 cells. *Infect. Immun.* 70:395-399.
- Vidal R, Vidal M, Logos R, Levine M, Pradom V (2004). Multiplex PCR for diagnosis of enteric infections associated with diarrheagenic *Escherichia coli*. *J. Clin. Microbiol.* 42:1787-1789.
- WHO (1997). Consultation on the prevention and control of *Enterohaemorrhagic Escherichia coli*. Geneva, Switzerland. p. 39.

Full Length Research Paper

Response of forage quality in Persian clover upon co-inoculation with native *Rhizobium leguminosarum* symbiovar (sv.) *trifoli* RTB₃ and plant-growth promoting *Pseudomonas florescence* 11168 under different levels of chemical fertilizers

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The excessive use of chemical fertilizers has generated several environmental problems. In order to evaluate the effects of co-inoculation of *Pseudomonas*, native *Rhizobium* with nitrogen and phosphorus levels on forage quality in Persian clover (*Trifolium rosapinatum* L. cv.Br₇₃), this study was carried out in experimental fields of Lorestan Agricultural Research Center (Borujerd station) Lorestan province, Iran in 2011. A factorial experiment in the form of complete randomized block design with three replications was studied. Experimental treatments include: four levels of chemical fertilizers: F₀ = N₀ + P₀, F₁ = N₂₅ + P₅₀ (25 Kg/ha Urea, 46% N₂) + 50 Kg/ha Super phosphate triple, 46% P₂O₅), F₂ = N₅₀ + P₁₀₀ and F₃ = N₇₅ + P₁₅₀ for sites 1 and 2. The biological fertilizers include: control (no bacterium), *Pseudomonas florescence*, native *Rhizobium* and co-inoculation (*Pseudomonas* + native *Rhizobium*). In these studies, some characteristics such as: crude protein (CP); dry matter digestible (DMD), water soluble carbohydrates (WSC), crude fiber (CF), acid detergent fiber (ADF), ash and neutral detergent fiber (NDF) were assessed. Results show that biological fertilizers were significantly (p<0.05) affected with regards to CP, WSC and ADF but chemical fertilizers were significantly (p<0.01) affected with regards to CP, DMD, ASH, CF, ADF and NDF. Interaction between chemical and biological fertilizers showed that CP (α=0.05) and WSC (α=0.01) were significant. Crude protein percentage tended (r=-0.35; α=0.1) and (r=-0.73; α=0.01) negatively correlated with ADF and CF percentages respectively. In this study, F₂S₃ (native rhizobium with reduced application of chemical fertilizers) treatment as compared to F₂S₁ (no application of biological fertilizers with recommended chemical fertilizers) increased positive indexes such as: CP (4.58%); DMD (4.38%) and ASH (3.25%) and decreased negative indexes such as CF (-0.77%) and NDF (-6.4%). Therefore, it is recommended for low input sustainable agriculture (LISA) and high quality of forage.

Key words: Low input sustainable agriculture (LISA), Persian clover, forage quality, *Pseudomonas*, native *Rhizobium*, chemical fertilizers.

INTRODUCTION

The diazotroph bacteria such as symbiotic bacteria (*Rhizobium sp.*) are beneficial microorganisms in the root zone of the legume being reported as very essential for plant establishment and growth, especially under unbalanced conditions (Braea, 1997).

The effect of fertilizers, bacteria *Azospirillum lipoferum* and *pseudomonas flouresence* on rice yield showed that application of looks/of nitrogen with *pseudomonas* and without *azospirillum* had the highest yield of 5733 kg/ha. In this study, nitrogen fertilizer with bacteria *Pseudomonas flouresence* and *A. lipoferum* had significant effect on harvest index at $P < 0.01$ level (Khorshidi et al., 2011). Solubility of phosphorus is an important features as growth stimulant for this bacteria (Alipour and Malakouti, 2003; Kolb and Martin, 1998). Another useful role of stimulating growth bacteria for plants is to reduce or eliminate the harmful effects of pathogen agents, through the phenomenon of induced systemic resistance (ISR) in plants. Salicylic acid produced by plant growth stimulants bacteria could induce systemic resistance phenomenon in rhizobpher (Maurhofer et al., 1998).

The role of micro-organism such as plant- growth-promoting rhizobacteria (PGPR) as modifiers of soil fertility and facilitators of plant establishment is being considered (Requena et al., 1997). Application of PGPR to different crop-production systems has been proposed. Some PGPR have been described by several authors to promote emergence of host plants and have named emergence-promoting rhizobacteria (EPR) (de Freitas and Germida, 1990). These effects may be of great use in encouraging plant emergence in soils with a poor structure, such as those in arid or semi-arid zones.

Many rhizobacteria have been shown to produce antibiotics that inhibit the growth of an antagonistic fungi (Shahverdi et al., 2012; Mirsheari et al., 2012) and bacterium *P. fluorescens* (Trevisan) Migula F113, for example, has been shown to control the soft rot potato pathogen *Erwinia carotovora* subspecies *atroseptica* by producing the antibiotic 2,4-diacetylphloroglucinol (DAPG) (Whipps 2001). Three glucanase-producing actinomycetes, when used separately or more effectively in combination, could significantly promote plant growth and therefore inhibit the growth of *Pythium aphanidermatum* (El Tarabily et al., 2009). Other major antibiotics produced by *Bacillus cereus* are phenazine- e-carboxylic acid and phenazine-1-carboxamide; 2, 4-diacetyl phloroglucinol (phl) (Dunne et al., 1998), pyoluteorin (Nowak-Thompson et al., 1999), zwittermicin A (Emmert et al., 2004), gluconic acid, 2-hexyl-5-propyl

resorcinol (Cazorla et al., 2006) and kanosamine (Milner et al., 1996).

In forage crop such as Persian clover, total biomass used with animal, forage quality is important. In many reports, crude protein (CP), dry matter digestible (DMD) and metabolism energy (ME) have been mentioned as forage quality indexes that were assessment with nitrogen percentage and acid detergent fiber (ADF). Amount of protein in forage and protein yield have direct relationships with nitrogen in soil (Hasanvand et al., 2009). Pholsen (2004) reported that organic and chemical fertilizers had no affect on ADF, NDF and DMD percentages of sorghum forage. In this report, protein percentage had significant effect.

The effective strain of Rhizobia in alfalfa (Gholipour et al., 2008) and bean (Khodshenas, 2006) was reported. These can affect economic production and decrease nitrogen fertilizer application.

Persian clover (*Trifolium rosapinatum* L.) is a native forage (Fabaceae) of Turkey, Iraq, Afghanistan and Iran (Taylor, 1985) that foragers consume with hay, silage and green yield. Total Persian clover-cropped area in Iran is about 42.000 hectares and about 45% belong to Lorestein province (West central in Iran). Clover plants require large amounts of mineral nutrients such as N, P and K for their growth and development (Yazdi et al., 1996; Zamanian et al., 2002; Fraser et al., 2009). The annual biological nitrogen fixation (BNF) rate, which is the result of symbiosis between clover and rhizobia ranges broadly from 85 to 360 kg.ha⁻¹ (Russell et al., 1973; Ardakani et al., 2009a).

This study was carried out to integrate chemical and biological fertilizers (special native strain) for increasing forage quality, Low Input Sustainable Agriculture (LISA), safe agro ecosystem, environment and food in Persian clover as main forage in the region.

MATERIALS AND METHODS

The trials were conducted in Lorestan Agricultural research central at Borujerd Agricultural research station, Lorestan province, Iran (longitude: 48°55' E, latitude: 33°40' N, height 1476 m) in 2011. Climate of these area is characterized by moderate summers and cold and humid winter. The mean annual temperature is about 14°C, the average precipitation is about 400 mm and average evapotranspiration is about 1500 mm. Mean annual maximum air temperature was 39°C (in July) and minimum was -10°C (in January) Mirzae, 1995). Before planting, combined soil (samples to 0-30 and 30-60 cm depth were collected and their physical

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Abbreviation: CP, Percentages of crude protein; DMD, dry matter digestible; WSC, water soluble carbohydrates; CF, crude fiber; ADF, acid detergent fiber; NDF, ash and neutral detergent fiber.

Table 1. Some soil properties of the experimental fields.

Field	Silt (%)	Sand (%)	Clay (%)	Depth	K(av.) P.P.M.	P(av.) P.P.M.	O.C.	pH of paste	EC $\times 10^3$
Place 1	50	33	17	0-30	340	13.8	1.15	7.39	1.42
				60.30	140	2.8	0.66	7.60	0.69
Place 2	47	29	24	0-30	290	9.0	1.10	7.51	1.39
				30-60	160	5.4	0.49	7.65	0.86

Table 2. Analysis of variance for forage quality indexes.

S.O.V	d.f	CP	DMD	DMD	WSC	ADF	ASH	CF	NDF
Place	1	11.416	37.058	37.058	0.009	0.500	6.255*	93.183**	83.373
Place*Block	4	11.330	14.151	14.151	3.459	14.983*	0.954	3.919	13.937
Chemical Fertilizer	3	366.297**	141.924**	141.924**	1.066	22.101**	5.514**	117.250**	220.443**
Chemical Fertilizer* Place	3	2.578	3.234	3.234	0.405	4.567	1.290	11.842	19.848
Chemical Fertilizer* Place*Block	12	12.384	15.621	15.621	3.657	12.238**	0.717	9.026	17.538
Biological Fertilizer	3	59.168*	5.719	5.719	10.438*	14.800*	2.061	4.170	47.85
Chemical Fertilizer* Biological Fertilizer	9	10.697*	10.203	10.203	1.801**	10.635	1.671	20.036	38.162
Biological Fertilizer* Place	3	3.644	2.253	2.253	2.198	0.450	0.784	7.220	1.574
Biological Fertilizer* Place* Chemical Fertilizer	9	4.042	1.308	1.308	1.406	0.654	0.451	5.766	11.898
(CV%)		20.07	5.931	5.931	0.167	6.06	11.66	15.35	18.82

ns, *, **no significant at 5 and 1% probability levels based on Duncan, respectively. CP: Crude protein percentage; DMD: Dry Matter Digestible Percentage, ADF; Acid Detergent Fiber Percentage, CF: Crude Fiber Percentage, NDF: Natural Detergent Fiber Percentage, WSC: Water Soluble Carbohydrates Percentage, ASH: ASH Percentage.

and chemical properties tested. Specifically, our tests included determination of soil texture using the hydrometry method (Gee and Bauder, 1986), pH and salinity of a saturated paste (Rhoades, 1982), organic C (wet oxidation method) (Nelson and Sommers, 1973), the concentration of available P (sodium bicarbonate extraction method) (Olsen, 1954) and available K (flam photometer method, emission spectrophotometry) (Knudsen et al., 1982) were determined (Table 1).

The study was conducted in a factorial arrangement in the form of randomized complete block design (RCBD) with three times. Experimental treatments including: Four levels of chemical fertilizers: $F_0 = N_0 + P_0$, $F_1 = N_{25} + P_{50}$ (25 kg/h Urea, 46% N_2) + 50 Kg/h super phosphate triple, 46% P_2O_5 , $F_2 = N_{50} + P_{100}$ and $F_3 = N_{75} + P_{150}$. The biological fertilizers include: control (no bacterium), *Pseudomonas fluorescence* 11168 strain (plant growth promoting rhizobacteria-PGPR), native *Rhizobium* (*Leguminosarum symbiovar* (sv.) *trifoli* RTB3 strain) and co-inoculation (*Pseudomonas* + native *Rhizobium*).

The *P. fluorescence* was prepared from gene bank of Soil and Water Research Institute of Tehran, Iran and native *Rhizobium* was isolated from Lorestan province fields under Persian clover by the Biology Department of Tehran Water and Soil Institution with a population of 10^8 CFU.ml⁻¹. Before inoculation, the seeds surface was mixed with 10% sugar completely for more adhesion of inoculums. Finally, the seeds were inoculated and mixed thoroughly with inoculants. Plot consisted of 4 rows in 5 m long with 50 cm spaced between rows and 15 kg.ha⁻¹ seed rates. In each place, the crops were harvested three times during the experiment (3 cut of forage were carried out). In the second cutting (about 10% flowering), the two middle rows were used for sampling and mea-

sured parameters such as forage quality was assessed. Forage quality indexes included: percentage of crude protein (CP); dry matter digestible (DMD), water soluble carbohydrates (WSC), crude fiber (CF), acid detergent fiber (ADF), ash and neutral detergent fiber (NDF). These indexes were assessed by Near Infrared Reflectance spectroscopy method (NIR) (Jafari et al., 2003; Hofman et al., 1999).

Chemical nitrogen fertilizers were included (urea 46%N) and triple super phosphate (46% P_2O_5). Field was irrigated due to environment condition and soil moisture. Weeding was done by hand. Data was analyzed with SAS statistical software and using Duncan's multiple range test (DMRT) for mean comparison.

RESULTS AND DISCUSSION

Result of this study showed that there was affect only on CF ($\alpha=0.01$) and ASH ($\alpha=0.05$) (Table 2). The amount of CF was 25.99 and 24.02% in site 1 and 2, respectively. Also, ASH in site 1 was more than the other place.

In some reports, the effect of climate on forage quality was not significant while soil and fertility effects were significant. It was related to water contents and maintenance in these soils (Arzani et al., 2010; Zaboli et al., 2010).

The biological fertilizers could affect significantly ($\alpha=5\%$) CF, WSC and ADF. The data indicated that the highest CP (22.84%) and DMD (65.94%) belong to native

Table 3. Comparison of mean values of forage quality indexes.

Field	CP	DMD	WSC	ADF	ASH	CF	NDF
Place 1	21.2634 ^a	64.3608 ^a	19.0418 ^a	34.5867 ^a	8.8740 ^a	24.0227 ^b	41.815 ^a
Place 2	20.9538 ^a	60.9538 ^a	19.0227 ^a	34.4424 ^a	8.3635 ^b	25.9931 ^a	39.957 ^a
B1	20.436 ^{ab}	65.034 ^a	19.5196 ^{ab}	34.3094 ^b	8.6240 ^a	25.262 ^a	41.00 ^a
B2	19.538 ^b	64.698 ^a	19.6288 ^a	33.8408 ^b	8.4060 ^a	25.282 ^a	38.857 ^a
B3	22.847 ^a	65.948 ^a	18.7394 ^b c	34.2583 ^b	8.4137 ^a	25.092 ^a	41.983 ^a
B4	22.373 ^a	64.549 ^a	18.2412c	35.6496 ^a	9.0314 ^a	24.396 ^a	41.692 ^a
F1	19.666 ^b	63.539 ^b	19.454 ^a	34.4850 ^{ab}	8.2081 ^b	26.217 ^a	36.980 ^b
F2	20.09 ^b	64.847 ^b	19.1931 ^a	34.3314 ^b	8.4969 ^b	25.780 ^a	42.832 ^a
F3	18.382 ^b	63.088 ^b	18.7310 ^a	35.7839 ^a	8.4585 ^b	26.323 ^a	40.41 ^{ab}
F4	27.056 ^a	68.454 ^a	19.1594 ^a	33.4577 ^b	9.3117 ^a	21.711 ^b	43.678 ^a

CP, Crude protein percentage; DMD, dry matter digestible percentage; ADF, acid detergent fiber percentage; CF, crude fiber percentage; NDF, natural detergent fiber percentage; WSC, water soluble carbohydrates percentage; ASH, ASH percentage; F, chemical fertilizers; B, biological fertilizers .

Table 4. Ortoogonal comparisons in forage quality Indexes.

NDF	CF	ASH	ADF	WSC	DMD	CP	%
38.22	34.61	8.06	33.98	19.08	65.18	22.23	B1F3
39.8	32.89	7.84	33.98	18.66	64.69	22.77	B2F2
38.91	34.16	8.94	34.73	18.74	65.03	24.13	B3F2
44.93	36.62	8.36	34.13	19.16	64.93	23.13	B4F2
*	ns	ns	ns	ns	ns	ns	Prob.

CP, Crude protein percentage; DMD, dry matter digestible percentage; ADF, acid detergent fiber percentage; CF, crude fiber percentage; NDF, natural detergent fiber.

Rhizobium and lowest negative indexes such as ADF(34.25%) and NDF(38.85%) belong to *Pseudomonas* (data not shown). Also the ortogonal comparisons in forage quality Indexes showed that no significant differences between B1F3 and B2F2, B3F2 and B4F2 treatments except in NDF index (Table 4).

The above mentioned amounts comparison to control treatment increased (9.18% in CP and 1.38% in DMD respectively). While ADF decreased (-5.63%) in comparison with the control treatment. In many reports, CP and DMD percentages are positive indexes of forage quality. Most of researchers suggested that CP and DMD percentages were quality indexes in forage crop that were affected by organic and chemical fertilizers (Ahmed et al., 2012).

The effects of biological fertilizers on these indexes indicated that the highest positive indexes such as CP, DMD, WSC and ASH as compared to control (S1) were 10.55 and 1.38% (in native *rhizobium*); 0.56 (in

Pseudomonas) and 4.55% (in co-inoculation) while negative indexes such as ADF, CF and NDF belonged to S2, S4 and S2 which were -1.35%, -3.56% and -5.53%, respectively (Table 3). Interaction between biological x chemical fertilizers showed that CP ($\alpha = 0.05$) and WSC ($\alpha = 0.01$) were significant (Table 2). The highest CP (28.91%) was observed in F₄S₃. Mentioned treatment had 21.9% more than F₁S₁ (control). The other characteristics such as DMD; ASH; NDF; CF and ADF were 9.9; 3.5, 7.83, 20.65, 16.58 and -10.5%, respectively. The antigenic and synergic effects of co-inoculation were observed in this study. The antigenic effect was observed on WSC. The amounts of this effect were 18.24% while it was 18.73% in native *rhizobium* and 19.62% in *Pseudomonas*. The synergic effect was observed on ADF too (Table 3).

Rodeles (1999) reported that co-inoculation with *rhizobium* and *azotobacter* in *Vicia faba* L. as compared to *Rhizobium* had 100% increase on action of nitrogenas

Table 5. Correlation between forage quality Indexes.

	CP	DMD	WSC	ADF	ASH	CF	NDF
DMD	0.69706**	1.0000					
WSC	-0.27941	0.14412	1.0000				
ADF	-0.35588	-0.73824**	-0.23529	1.0000			
ASH	0.56176**	0.38529	-0.15882	-0.06176	1.0000		
CF	-0.73824**	-0.46765	0.14706	0.22059	-0.82941**	1.0000	
NDF	0.52059	0.73824**	-0.10588	-0.39706	0.63529**	-0.48235	1.0000

CP, Crude protein percentage; DMD, dry matter digestible percentage; ADF, acid detergent fiber percentage; CF, crude fiber percentage; NDF, natural detergent fiber percentage; WSC, water soluble carbohydrates percentage; ASH, ASH percentage.

enzyme. He added that co-inoculation with *Rhizobium* + *Azospirillum* and *Rhizobium* + *Azotobacter* changed concentration and distribution of micro and macro elements such as: Mg, Ca, K, P, Fe, Cu, Zn, Mn and B as compared to *Rhizobium* application alone. Also, Tilak (2004) reported the synergic effect of *Rhizobium* + *Pseudomonas* on numbers of nodule in peagon pea and added that co-inoculate with these bacteria increased to 85% in nodulation while it was 50% in *Rhizobium* application alone. In many researches, CP; Phosphorus content in forage (P), cell wall content in forage; DMD; CF; In vitro digestible of forage; crude energy and energy of metabolism (Em) were mentioned for assessment of forage quality. In this study, chemical fertilizers were more effective on indexes. Moreover, in these indexes, CP is the most important that was affected by chemical and biological fertilizers; because it is related to nitrogen content in soil (Ali et al., 2009; Poormoradi et al., 2010).

The influence of PGPR on dry matter accumulation in chick pea (*C. arietinum* L.) yield under field conditions has been thoroughly studied (Rokhzadi et al., 2008). Studies have shown that a combined inoculation of *Azospirillum* spp., *Azospirillum chroococcum* 5, *Mesorhizobium ciceri* SWR17 and *Pseudomonas fluorescens* P₂₁ improved nodulation, increased dry matter accumulation in roots and shoots, grain yields, biomass and protein yield of chick-pea by a significant margin. Parmar and Dadarwal (1999) studied co-inoculation of the rhizobacteria with effective *Rhizobium* strains of chickpea and observed a significant increase in nodule weight, root and shoot biomass and total plant nitrogen when grown either in sterilized chillum jars or under pot culture conditions. The *Rhizobium* stimulatory *Pseudomonas* sp. "CRP55b" showed maximum increase in all the symbiotic parameters.

Ahmed et al. (2004) and Cox et al. (1998) reported contradictory effects of chemical and organic fertilizers on qualities of forage crop with multi-cutting. In this crop, chemical fertilizers effect was seen on forage quality only in first cutting but organic fertilizers had effect on throughout plant growth period (throughout cutting). This is related to leaching and fixation of chemical fertilizers,

they added that by application of organic and chemical fertilizers in grass and legume, amount of the CF in grass did not any change while it decreased in legume by organic fertilizer.

Most researchers believed that increase of yield is due to increase of photosynthesis rate and photosynthesis matter translocation time. In other reports, increase in P absorption was related to biological fertilizers application too (Ali et al., 2009; Mirzakhani et al., 2009; Ardakani et al., 2000b; Hazarika et al., 2000).

Correlation coefficients among variables are presented in Table 5. Crude protein percentage tended ($r=-0.35$; $\alpha=0.1$) and ($r=-0.73$; $\alpha=0.01$) negatively correlated with ADF and CF percentages respectively. In addition, CP was positively correlated with DMD ($r=0.69$; $\alpha=0.002$) and ASH ($r=0.56$; $\alpha=0.02$). Gadberry et al. (2005) reported that ADF tended to be negatively correlated with CP ($\alpha=0.08$). Also, negative correlation of CP with WSC indicated that with increase of fertility nitrogen and phosphorus caused improvement in growth of plant and protein content. Negative correlation between DMD and CP with ADF and also CF with CP, ASH and DMD indicated the efficiency of integrated chemical and biological fertilizers application. Others reported these results in application of organic fertilizers integrated with chemical fertilizers, for example Gadberry et al. (2005) in Bermuda grass and James (1997) in alfalfa hay; vegetative hay of corn and tall fescue hays.

Conclusions

In this study, F₂S₃ (native *Rhizobium* with reduced application of chemical fertilizers) treatment as compared to F₂S₁ (no application of biological fertilizers with recommended chemical fertilizers) increased positive indexes such as: CP (4.58%); DMD (4.38%) and ASH (3.25%) and decreased negative indexes such as CF (-0.77) and NDF (-6.4%). Also, application of F₂S₃ was decreased, 1050 ton/year Urea, and 2100 ton/year. Superphosphate triple in Persian clover in Iran, so F₂S₃ is recommended for low input sustainable agriculture (LISA) and high qua-

lity of forage.

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REFERENCES

- Ahmed SA, Halim RA, Ramlan MF (2012). Evaluation of the use of farmyard manure on a Guinea Grass (*panicum maximum*)-stilo (*Stylosanthes guianensis*) mixed pasture. *Pertanila J. Trop. Agric. Sci.* 35(1):55-65.
- Ali FS, Zayed T, Abdol- Mohsen E (2009). Optimisation of nitrogen fertilizer levels for maximum colonization of mycorrhiza on roots of coriander plants. *Afr. Crop Sci. Conf. Proceed.* 9:117-122.
- Alipour Z, Malakouti M (2003). Role of Plant Growth Promoting Rhizobacteria (PGPR) on Growth and Plant Health. Technical Publication No. 309, Soil and Water Research Institute (WSI) of Iran. (in Farsi). (These are added)
- Ardakani MR, Pietsch G, Moghaddam A, Raza A, Fridel JK (2009b). Response of root properties to symbiosis between lucerne (*Medicago sativa*), Rhizobia and Mycorrhiza under dry organic farming conditions. *Am. J. Agric. Biol. Sci.* 4(4):266-272.
- Ardakani MR, Pietsch G, Raza A, Moghaddam A, Fridel JK (2009a). Nitrogen fixation and yield of lucerne (*Medicago sativa* L.), as affected by co-inoculation with *Sinorhizobium melioli* and *Abusular mycorrhiza* under dry organic farming conditions. *American-Eurasian J. Agric. Environ. Sci.* 6(2):173-183.
- Arzani J, Sahragard P, Saedi K (2010). Comparison of phenological stages on forage quality of rangelands species in rangeland of Saral, kordestan Iranian J. Rang. 4(2):160-167.
- Braea JM, Azcon - Aguilar C, Azcon R (1997). VAM improve both symbiotic N₂-fixation and N-uptake from soil as assessed with a N-technique under field conditions. *New Phytol.* 106:717-725.
- Cazorla FM, Duckett FT, Bergstrom S, Noreen R, Odik R (2006). Biocontrol of avocado Dematophora root rot by the antagonistic *Pseudomonas fluorescens* PCL 1606 correlates with the production 2-hexyl-5-propyl resorcinol. *Mol. Plant Microb. Interact.* 19:418-428.
- Cox WJ, Rcherney D, Hancher JJ (1998). Row spacing hybrid and plant density effects on corn silage yields and quality. *Jour. Of production Agriculture* 11:129-134.
- De Freitas JR, Germida J (1990). Plant growth promoting rhizobacteria for winter wheat. *Can. J. Microbiol.* 36:265-272.
- Dunne C, Moenne-Loccoz J, McCarthy H, Higgins J, Powell DN, Dowling F (1998). Combining proteolytic and phloroglucino-producing bacteria for improved control of *Pythium mediated* damping off of sugar beet. *Plant Pathol.* 47:299-307.
- EI Tarabily KA, Nassar GE, Sivasithampam K (2009). Plant growth promotion and biological control of *Pythium aphanidermatum*, a pathogen of cucumber, by endophytic actinomycetes. *J. Appl. Microbiol.* 106:13-26.
- Emmert EA, Klimowicz AK, Thomas MG, Handelsman J (2004). Genetic of zwittericin a production by *Bacillus cereus*. *Appl. Environ. Microbiol.* 70:104-115.
- Fraser J, McCarney D, Najda H, Mir Z (2009). Yield potential and forage quality of annual forage legumes in Southern Alberta and Northeast Saskatchewan. *Can. J. Plant Sci.* 84:143-185.
- Gadberry TR, Troxel TR, Jenning JA (2005). Influence of protein content of bermudagrass hay on Invitro matter digestibility and the Arkansas TDN equation. *Arkansas Animal Sci Dep Report.*, <http://arkansasagnews.uark.edu/535-23.pdf>.
- Gholipour H, Nabizdeh A, Nasrolahzadeh A (2008). Nitrogen fixation in greenhouse condition with native Rhizobia strains. *Res. Agron. Sci.* 2(2):78-83.
- Hasanvand M, Jafari AA, Sepahvand A, Nakhjavan S (2009). Study for yield and quality traits in 6 climatic populations of common vetch (*Vicia sativa*) grown under optimum and dry land farming system in Lorestan, Iran. *Iranian J. Rang. Desert Res.* 16(4):518-535.
- Hazarika DK, Taluk Dar NC, Phodkan AK, Saikia VN, Das BC, Deka PC (2000). Influence of *Vesicular arbuscular mycorrhiza* fungi and phosphate solubilizing bacteria on nursery establishment and growth of tea seedling in Assam. *Symp. (12). Assam Agricultural Uni., Jorhat.*
- Hofman PC, Brehmand NM, Bauman M (1999). Prediction of laboratory and In Situ protein fractions in legume and grass silages using Near-Infrared Reflectance spectrometry. *J. Dairy Sci.* 82(4):764-770.
- Jafari A, Connolly A, Frolich A, Walsh EK (2003). A note on estimation of quality in perennial ray grass by NIR. *Irish J. Agri. Food Res.* 212:193-299.
- James B, Reeves C (1997). Relationship between crude protein and determination of non dispensable lignin. *Dairy Sci.* 80:692-699.
- Khodshenas M, Dadpoo M, Kharasi K (2006). Efficiency of *rhizobium* strains of N₂-fixation in fields of Bean. 8th Iranian Congress of Soil Sci., Iran, Rasht.
- Khorshidi YR, Ardakani MR, Ramezanpour MR, Khavazi K, Zargari K (2011). Response of Yield and Yield Components of Rice (*Oryza sativa* L.) to *Pseudomonas fluorescens* and *Azospirillum lipoferum* under different nitrogen levels. *American-Eurasian J. Agric. Environ. Sci.* 10(3):387-395.
- Kolb W, Martin P (1998). Influence of Nitrogen on the Number of N₂-Fixation and Total Bacteria in the Rhizosphere. *Soil Biol. Biochem.* 20:221-225.
- Maurhofer M, Reimann C, Schmidli-sacherer S, Heeb D, Haas G (1998). Salicylic Acid Biosynthetic Genes Expressed in *Pseudomonas fluorescens* Strain P3 Improve the Induction of System Resistance in Tobacco Against Tobacco Necrosis Virus. *Phytopathol.* 88:678-684.
- Milner J, Silo-Suh L, Lee JC, Caldry J (1996). Production of kanosamine by *Bacillus cereus* UW 85. *Appl. Environ. Microbiol.* 62:3061-3065.
- Mirshakari B, Shahverdi M, Ardakani MR, Rashidi V, Asadi Rahmani H, Nasrollahi M (2012). Investigation effect of biological fertilizers at different levels of chemical fertilizer to decrease of Persian clover sooty blotch (*Cymadothea trifolii* pers & wolp (1935). First Symposium of Forecasting and Monitoring in plant protection (12-15Feb.). (Abstract). Borujerd, Iran.
- Mirzae A (1995). The climate of Silakhor pain. The first symposium of agriculture in Silakhor pain (3-5 Sep). (Abstract). Azad Uni Of Borujerd. Borujerd, Iran.
- Mirzakhani MR, Ardakani MR (2009). Response of safflower to co-inoculation with *Azotobacter chroocoum* and *Glomus intraradites* under different level of nitrogen and phosphorus. *Am. J. Biol. Sci.* 4(3):255-261.
- Nowak-Thompson B, Chaney J, Wings S, Gould JE (1999). Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. *J. Bacteriol.* 181:2166-2174.
- Parmar N, Dadarwal KR (1999). Stimulation of nitrogen fixation and induction of flavonoid like compounds by rhizobacteria. *J. Appl. Microbiol.* 86:3-44.
- Pholsen S, Sukseri A (2004). Effect of organic amendment and chemical fertilizer on growth, yield and food quality of forage sorghum (*S. bicolor* L. Moench). *Pak. J. Biol. Sci.* 7(4):651-657.
- Poormoradi S, Jafari AA (2010). Assessment of yield and forage quality of *Trifolium repens* L. varieties in northern Alborz. *Iranian. J. Desert Rang.* 17(4):615-623.
- Requena N, Jimenez I, Toro M, Barea J (1997). Interaction between plant promoting, Arbuscular mycorrhizal fungi and *Rhizobium* spp. in

- the rhizosphere of Anthyllis, a model legume for regeneration in Mediterranean semi-arid ecosystems. *New Phytol.* 136:667-677.
- Rodeles B, Gonzalez-Lopez Martinez-Toledo MV, Pozo C (1999). Influence of *Rhizobium/ Azotobacter* and Rh/ *Azospirillum* combined inoculation on mineral composition of fabaceae (*Vicia faba* L.). *Biology and Fertility of Soil.* 29:169. linkinghub. elsevier. com/ retrieve/pii/ S0929139398001577.
- Rokhzadi A, Asgazadeh F, Darvish G, Nour-Mohammed E (2008). Influence of plant growth promoting rhizobacteria on dry matter accumulation and yield of chick pea (*Cicer arietinum* L.) under field conditions. *American-Eurasian J. Agric. Environ. Sci.* 3:253-257.
- Russell EW (1973). *Soil conditions and plant growth.* 10th edition, Longman, London.
- Shahverdi M, Mirshekari B, Ardakani MR, Rashidi V, Asadi Rahmani H, Nasrollahi M, Rahjo V (2012). Relative efficiency of biological fertilizers to chemical fertilizers application on rust clover (*Uromyces trifolii-repentis*-Cast. Liro) damage. First Symposium of Forecasting & Monitoring in plant protection (12-15 Feb.). (Abstract). Borujerd, Iran.
- Taylor NL (1985). *Clover science and Technology.* American Society of Agronomy. Inc. Madison, Wisconsin, U.S.A.
- Tilak KV, Ranganak N, Manoharachar C (2004). Synergistic effect of plant growth promoting Rhizobacteria and *Rhizobium* on nodulation and N₂-fixation by Pigeon Pea (*Cajanus cajan* L.). *Europ. J. Soil Sci.* 57:67-71.
- Whipps JM (2001). Microbial Interactions and biocontrol in the rhizosphere. *J. Exp. Bot.* 52:487-511.
- Yazdi SB, poostini K (1996). *Principle in plant production.* Uni. Cent. Press., Tehran, Iran. 300.
- Zaboli MA, Chanbari J, Nori S (2010). Effect of phenological period on forage quality in *Aeluropus littoralis* L. and *A. lagopoides* in pasture of Hamon Leak. *Iran. J. Rang.* 4(3):126-135.
- Zamanian M, Moghadam A, zamani M (2002). *Persian clover. Seed and plant Improv. Inst.(SPII) press., Karadj. Iran.*

Full Length Research Paper

Quantification of reserpine content and antibacterial activity of *Rauvolfia serpentina* (L.) Benth. ex Kurz

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Reserpine is well known bioactive compound isolated from *Rauvolfia serpentina*. The aim of this study was to quantify reserpine content and evaluate the antibacterial activity of methanol extracts of *R. serpentina* against *Salmonella typhimurium*, *Escherichia coli*, *Citrobacter freundii*, *Proteus vulgaris*, *Enterococcus faecalis* and *Staphylococcus aureus*. Roots of *R. serpentina* were collected from Gadarpur and Uttarakhshi of Uttarakhand State, India. The antibacterial activity of the methanol extracts was evaluated by determination of minimum inhibitory concentration (MIC) and the diameter of zone of inhibition (ZOI) against both Gram positive and Gram negative bacteria using agar well diffusion method. The study reveals that reserpine content was higher (0.37%) in the sample collected from Gadarpur, whereas it was found to be 0.31% in sample collected from Uttarakhshi. The highest zone of inhibition (13 mm) with lowest MIC (625 µg) was observed against *Staphylococcus aureus* and highest MIC (10 mg) was observed against *Escherichia coli*, whereas *Proteus vulgaris* was observed resistant to tested extracts upto 10 mg. *R. serpentina* contain good amount of reserpine and exhibited strong antibacterial activity against most of the tested human pathogenic bacteria. Therefore, the results of the study support the folklore claim of the plant species.

Key word: High performance thin layer chromatography (HPTLC), reserpine, ciprofloxacin, zone of inhibition, minimum inhibitory concentration.

INTRODUCTION

Many bacteria and fungi produce human diseases which are currently controlled through the massive use of synthetic bactericides and fungicides. Some of them are resistance to synthetic drugs and caused therapeutic problem (Guillemot, 1999). Plants extracts are one of the options that have recently received attention and expected that it will be active against synthetic drug resistant pathogens. Therefore, the search for plant based new antibacterial and antifungal agents are imperative. *Rauvolfia serpentina* extract have been used to treat infections for thousands of years in Indian system

of medicines. It is used for the treatment of fever, anxiety, epilepsy, snake bite, rheumatism, insanity, eczema, intestinal disorders, psychiatric disorders, nervous disorders, cardiovascular disorder, bacterial infections and in the management of hypertension schizophrenia (Kirtikar and Basu, 1993; Gaur, 1999; Joshi and Kumar, 2000; Manuchair, 2002).

Reserpine has highly complex pattern of activity and is the main biological active phytochemical of the commercial drug Sarpagandha prepared from *R. serpentina*. Indole alkaloids such as reserpine, ajmaline and ajma-

licine were determined from *R. serpentina* and *R. vomitoria* by high performance layer chromatography (HPLC) and high performance thin layer chromatography (HPTLC) (Klushnichenko et al., 1994; Srivastava et al., 2006).

In pharmaceutical industries, reserpine is in great demand and mainly extracted from *Rauvolfia* species. Pharmacological studies demonstrate that *Rauvolfia* possesses cardiovascular (Anitha and Kumari 2006), antihypertensive (Von Poser et al., 1990), antiarrhythmic (Kirillova et al., 2001), antiinflammatory (Rao et al., 2012), antipyretic (Amole and Onabanjo, 1999), antidiabetic (Campbell et al., 2006), anticancer (Bemis et al., 2006), hypoglycaemic and hypolipidemic (Qureshi et al., 2009), hepatoprotective (Gupta et al., 2006a), sedative (Weerakoon et al., 1998), antihistaminase (Sachdev et al., 1961), mosquito larvicidal (Das and Chandra, 2012), antibacterial (Ahmed et al., 2002) and antidiarrhoeal (Ezeigbo et al., 2012) activities.

It is also reported that *R. tetraphylla* leaves have potent antibacterial activity against Gram positive and Gram negative bacteria which might be due to the presence of alkaloids (Abubacker and Vasantha, 2011). But no scientific investigation has so far been reported in literature regarding antibacterial activity of *R. serpentina* cultivated in Uttarakhand. Due to high market demand, *R. serpentina* has been introduced for cultivation in the state of Uttarakhand, India, in recent years and successfully grown at farms field with excellent biomass and seeds production capacity.

It is important to analyze the main contents of *R. serpentina* before recommending them for large scale cultivation and medicinal uses. Standardization of herbal drug is also a scientific interest in the herbal drug industry. Considering that, present study was designed to quantify the reserpine content in *R. serpentina* roots and also evaluate antibacterial activity.

MATERIALS AND METHODS

Roots of *R. serpentina* were collected from Gadarpur farm of Herbal Research and Development Institute, Uttarakhand, India (designated as RS I) and farmer's nursery located at Uttarakhshi (RS II). The plant materials were washed with tap water, cut in small pieces and spread over glass plate to dryness. The dried samples were grinded through pulverizer and particles passed through sieve were taken for extraction and analysis. Standard, reserpine was procured from Sigma Aldrich (Germany), precoated silica gel 60 F₂₅₄ TLC plate from Merck and all other chemicals used were HPLC grade.

Extraction and preparation of samples solution

Hundred milligrams of powdered roots of *R. serpentina* were treated with 1 ml of ammonia for 10 min and then extracted in 10 ml methanol (MeOH). Solvent was removed to dryness under vacuum. The dried extracts were dissolved with 2 ml methanol to make 50 mg/ml solution. Both the extracts were filter through 0.45 µm syringe filter and used for analysis. Accurately 15 µl of each sample was applied to TLC plate.

Preparation of standard solutions

A stock solution of reserpine (0.1 mg/ml) was prepared in methanol. Different volumes (2, 4, 6 and 8 µl) of the stock solution equivalent to 200, 400, 600 and 800ng were applied to the TLC plates. The calibration curve, correlation coefficient and regression equation were obtained using WinCATS software.

Instrumentation and chromatographic conditions

The standard and sample solutions were applied on precoated 20 ×10 cm silica gel 60 F₂₅₄ plate in the form of bands with 100 µl syringe using automatic sample applicator (Linomat 5). Samples were applied to the plate as 6 mm band, 10 mm apart from Y and 15 mm from X axis using N₂ gas. The slit dimension was 5 × 0.30 mm and scanning speed was 20 mm/s. The plate was developed in a twin trough chamber saturated with mobile phase (chloroform: toluene: ethylacetate: diethylamine). After development, the plate was dried with the help of dryer and observed under UV chamber. The well developed bands of reserpine in standard and *R. serpentina* extracts were scanned at 254 nm in absorption mode with CAMAG TLC scanner controlled by WinCATS software. The source of radiation was deuterium lamp emitting a continuous UV spectrum in the range of 190 to 400 nm.

Antibacterial activity

Antibacterial activity of *R. serpentina* extracts was determined by well diffusion method according to Deshmukh et al. (2012) with slight modifications. Bacterial cultures of *Salmonella typhimurium*, *Escherichia coli*, *Citrobacter freundii*, *Proteus vulgaris*, *Enterococcus faecalis* and *Staphylococcus aureus* were obtained from Department of Microbiology, HNB Garhwal University, Srinagar, India and used as test organism. All the bacteria were maintained on nutrient agar No. 2 (Himedia, India) at 37°C. The Gram positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*) and Gram negative bacteria (*Salmonella typhimurium*, *Escherichia coli*, *Citrobacter freundii* and *Proteus vulgaris*) were pre cultured in nutrient broth. The stock culture suspensions were diluted with sterile saline water (0.85% NaCl). The Petri dishes were flooded with Mueller Hinton Agar and after solidification of agar 0.1 ml of diluted inoculums were spread over Mueller Hinton Agar (Himedia, India) in the dishes using sterile L spreader to achieve confluent growth of test organism. Wells were then bored into the agar using a sterile 6 mm diameter cork borer. Accurately 100 µl of 6.25, 12.5, 25, 50 and 100 mg/ml crude extracts were introduced into the wells, plates were then incubated in refrigerator for about 2 h to allow the diffusion of solution in the medium. After that these plates were incubated at 37°C in incubator for 24 h. Controls were set up in parallel using the solvents that were used to dissolve the extracts. The plates were observed for minimum inhibitory concentration (MIC-lowest concentration of antibacterial that will inhibit the visible growth of microorganism) and zones of inhibition (ZOI). The effects were compared with those of 100 µl Ciprofloxacin at a concentration of 100 µg/ml (10 µg) and the zone of inhibition was measured using antibiotic zone scale.

RESULTS AND DISCUSSION

High performance thin layer chromatography (HPTLC) was used for the estimation of reserpine (structure in Figure 1) in *R. serpentina*. The standard and sample solutions were spotted in the form of band on the TLC

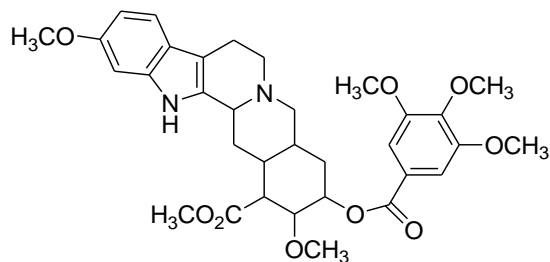


Figure 1. Structure of reserpine.

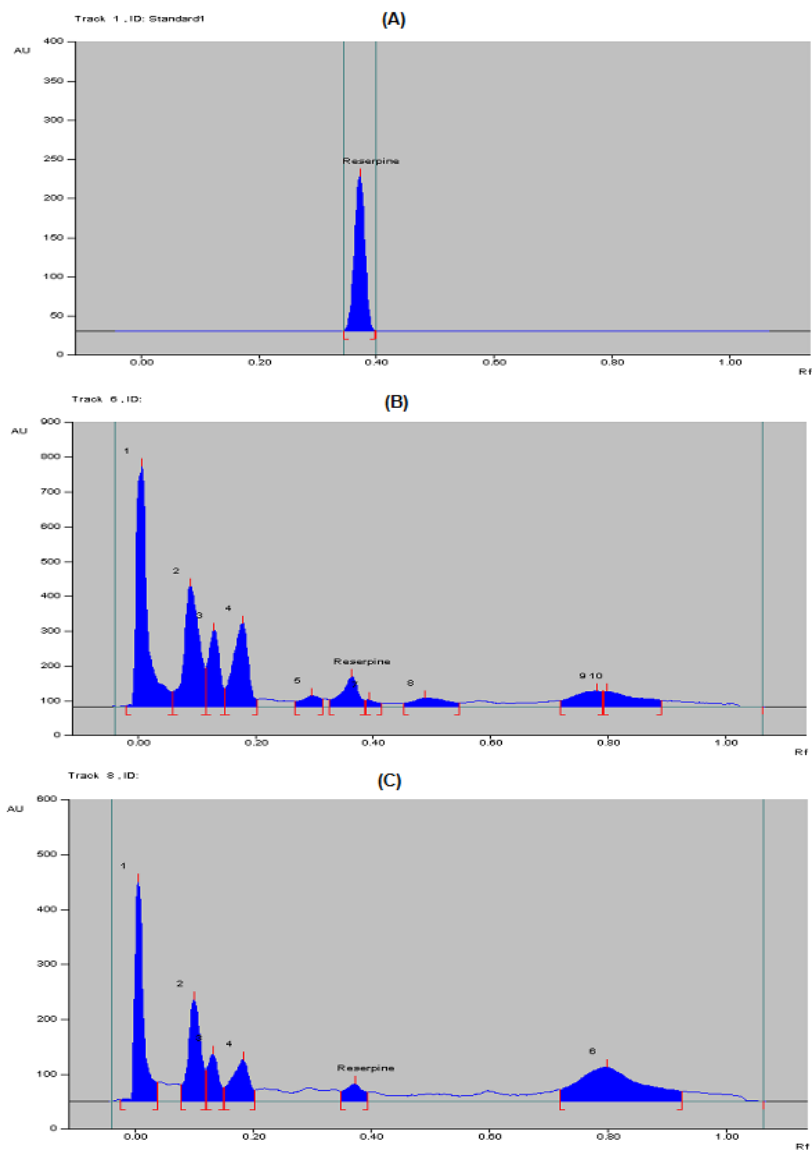


Figure 2. HPTLC Chromatograms of (A) Reserpine, (B, C) *Rauvolfia Serpentina* collected from Gadarpur (RS I) and Uttarakashi (RS II).

plates and run in different solvent systems. The mobile phase consisting of chloroform: toluene: ethylacetate: diethylamine (7:7:4:1) gave well defined bands and sharp peaks. The *r_f* value and correlation coefficient for reser-

pine was found 0.36 and 0.99, respectively. The chromatogram of standard and samples are shown in Figure 2. The bands of reserpine in samples were confirmed by comparing *r_f* values with standard. The qualitative results

Table 1. Antibacterial activity of *Rauvolfia serpentina* against different test organisms.

Bacterial test organism	Strain no.	RS I		RS II		Standard (Ciprofloxacin)	
		MIC ($\mu\text{g}/\mu\text{l}$)	ZOI (mm)	MIC ($\mu\text{g}/\mu\text{l}$)	ZOI (mm)	MIC ($\mu\text{g}/\mu\text{l}$)	ZOI (mm)
<i>Salmonella typhimurium</i>	MTCC 3224	50	8	50	6	50	-
<i>Escherichia coli</i>	MTCC 443	100	12	100	10	25	18
<i>Citrobacter freundii</i>	MTCC 4221	25	9	25	7	12.5	16
<i>Proteus vulgaris</i>	MTCC 1771	-	-	-	-	25	8
<i>Enterococcus faecalis</i>	MTCC 439	25	9	25	7	6.25	11
<i>Staphylococcus aureus</i>	MTCC 3103	6.25	13	6.25	11	50	10

MIC= minimum inhibitory concentration, ZOI= zone of inhibition.

confirmed the presence of reserpine in both the samples studied. *R. serpentina* collected from Gadarpur was found to contain 0.37% reserpine (dry weight basis) while *R. serpentina* collected from Uttarakashi contain 0.31%. Reserpine has been estimated in *Rauvolfia* species by HPLC and HPTLC. The total reserpine content in *Rauvolfia* species were found 0.06 to 3.0% (Kokate et al., 1998; Gupta et al., 2006b). Kumar et al. (2010) had also quantified the reserpine content of *R. serpentina* collected from different geographical locations of South India. They observed that the reserpine content was ranged from 0.0382 to 0.1442%. Baratto et al. (2012) quantified reserpine content in the dried stem bark of *R. sellowii* by HPLC and found 0.01% dry weight basis. Comparison with previous studies clearly shows that the *R. serpentina* cultivated in Uttarakhand has good reserpine content. This may be attributed to the ambient climatic and topographic conditions of Uttarakhand state.

The methanol extracts of *R. serpentina* exhibited excellent antibacterial activity against tested bacterial organisms as compared to the standard ciprofloxacin. The results were summarized in Table 1. Zone of inhibition are average of triplicate experiments. Sample 1 (RS I) of *R. serpentina* exhibited higher zone of inhibition than Sample 2 (RS II). The highest zone of inhibition (13 mm for RS I and 11 mm for RS II) with lowest MIC (625 μg) was observed against *Staphylococcus aureus* and highest MIC (10 mg) was observed against *Escherichia coli*, whereas *Proteus vulgaris* was observed resistant upto 10 mg of methanol extract of *R. serpentina*. It was also observed that *R. serpentina* has similar effect towards *Citrobacter freundii* and *Enterococcus faecalis*. Deshmukh et al. (2012) reported antibacterial activity of *R. serpentina* against *S. typhii*, *S. aureus*, *E. coli* and *B. subtilis*.

The methanol extract of *R. serpentina* roots was reported most effective (MIC 40 $\mu\text{g}/\mu\text{l}$) against *S. typhii*, moderate against *B. subtilis* (MIC 80 $\mu\text{g}/\mu\text{l}$) and least effective against *S. aureus* and *E. coli* (MIC 90 $\mu\text{g}/\mu\text{l}$). The petroleum ether extract of *R. serpentina* has been tested for antibacterial activity against Gram positive and negative bacteria and observed 3.0 to 7.8 mm zones of inhibition for Gram positive bacteria and 5.0 to 8.2 mm for

the Gram negative bacteria (Harisaranraj et al., 2009). Comparison of our results with these findings clearly shows that methanol extract of *R. serpentina* is more effective than petroleum ether extract. Antimicrobial activity of *R. tetraphylla* has also been reported, its methanol extract showed 0.25 to 100 mg/ml minimum inhibitory concentration against bacterial pathogens and 0.5 to 100 mg/ml against fungal pathogens (Shariff et al., 2006). Our results demonstrated that methanol extract of *R. serpentina* has concentration dependent antibacterial activity against most of the tested organism.

Conclusion

R. serpentina cultivated in Uttarakhand has good reserpine content and also exhibited moderate to strong antibacterial activity against tested human pathogenic bacteria. Therefore, the species is recommended for large scale cultivation. The results of the study support the folklore claim along with the development of new antimicrobial drugs from the plant. The antibacterial activity of *R. serpentina* may be attributed to the various phytochemical constituents present in the crude extract. Therefore, further work is needed to isolate the active principle from the plant extract which may have even more potency.

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REFERENCES

- Abubacker MN, Vasantha S (2011). Antibacterial activity of ethanolic leaf extract of *Rauvolfia tetraphylla* (Apocyanaceae) and its bioactive compound reserpine. Drug Invention Today 3(3):16-17.

- Ahmed S, Amin MN, Anjum A, Haque ME (2002). *In vitro* antibacterial activity of *Rauwolfia serpentina* and its tissue culture. Niger. J. Nat. Prod. Med. 6:45-49.
- Amole OO, Onabanjo AO (1999). Reserpine: The effect and uses of *Rauwolfia vomitoria*. J. Chemother. 3:45-47.
- Anitha S, Kumari BDR (2006). Stimulation of reserpine biosynthesis in the callus of *Rauwolfia tetraphylla* L. by precursor feeding. Afr. J. Biotechnol. 5:659-661.
- Baratto LC, Campos FR, Santos RPCM (2012). A validated method using RP-HPLC for quantification of reserpine in the Brazilian tree *Rauwolfia sellowii* Müll. Arg. (Apocynaceae). Quim. Nova 35(2):408-410.
- Bemis DL, Capodice JL, Gorroocurn P, Katz AE, Buttyan R (2006). Antiproliferative activity of β -carboline alkaloid enriched extract from *Rauwolfia vomitoria*. Int. J. Oncol. 29:1065-1073.
- Campbell JIA, Mortensen A, Molgaard P (2006). Tissue lipid lowering effect of a traditional Nigeian antidiabetic infusion of *Rauwolfia vomitoria* foliage and *Citrus aurantium* fruit. J. Ethnopharmacol. 104: 379-386.
- Das D, Chandra G (2012). Mosquito larvicidal activity of *Rauwolfia serpentina* L. seeds against *Culex quinquefasciatus* Say. Asian Pac. J. Trop. Med. 5(1):42-45.
- Deshmukh SR, Ashrit DA, Patil BA (2012). Extraction and evaluation of indole alkaloids from *Rauwolfia serpentina* for their antimicrobial and antiproliferative activities. Int. J. Pharm. Pharm. Sci. 4(5):329-334.
- Ezeigbo II, Ezeja MI, Madubuike KG, Ifenkwe DC, Ukwani IA, Udeh NE, Akomas SC (2012). Antidiarrhoeal activity of leaf methanolic extract of *Rauwolfia serpentina*. Asian Pac. J. Trop. Biomed. 2(6):430-432.
- Gaur RD (1999). The Flora of the District Garhwal, North West Himalaya. Trans Media, Srinagar, Garhwal, India.
- Guillemot D (1999). Antibiotic use in humans and bacterial resistance. Curr. Opin. Microbiol. 2:494-498.
- Gupta AK, Chitme H, Dass SK, Misra N (2006a). Hepatoprotective activity of *Rauwolfia serpentina* rhizome in paracetamol intoxicated rats. J. Pharmacol. Toxicol. 1:82-88.
- Gupta MM, Srivastav A, Tripathi AK, Misra H, Verma RK (2006b). Use of HPTLC, HPLC and densitometry for qualitative separation of indole alkaloids from *Rauwolfia serpentina* roots. J. Planar. Chromatogr. 19: 282-287.
- Harisaranraj R, Suresh K, Babu SS, Achudhan VV (2009). Phytochemical based strategies for pathogen control and antioxidant capacities of *Rauwolfia serpentina* extracts. Rec. Res. Sci. Technol. 1(2):67-73.
- Joshi N, Kumar N (2000). *Rauwolfia*. In: Aromatic and medicinal Plants in Central Himalayas. Kumar, N. (Ed.). Defence Agricultural Research Laboratory, Pithoragarh, Uttarakhand, India.
- Kirillova NV, Smirnova MG, Komov VP (2001). Sequential isolation of superoxide dismutase and ajmaline from tissue culture of *Rauwolfia serpentina* Benth. Prikl. Biokhim. Mikrobiol. 37:181-185.
- Kirtikar KR, Basu BD (1993). Indian Medicinal Plants. 2nd Edn., Dehra Dun Publishers, India.
- Klushnichenko VE, Yakimov SY, Bychkova TP, Kuzovkina IN, Vulf AN, Miroshnikov AI (1994). Separation of indole alkaloids from *R. serpentina* and *R. vomitoria* by HPLC and TLC methods. Pharm. Chem. J. 28:855-859.
- Kokate CK, Purohi AP, Gokhale SB (1998). Pharmacognosy (Pub.) DK Furia. Nirali Prakashan, Pune, India.
- Kumar HV, Nirmala SS, Rajendra CE (2010). Reserpine content of *Rauwolfia serpentina* in response to geographical variation. Int. J. Pharma. Bio. Sci. 1(4):429-434.
- Manuchair E (2002). Reserpine. In: Pharmacodynamics basis of Herbal Medicine. Ebadi, MS (Ed.) CRC Press, Boca Raton.
- Qureshi SA, Nawaz A, Udani SK, Azmi B (2009). Hypoglycaemic and hypolipidemic activities of *Rauwolfia serpentina* in Alloxan induced diabetic rats. Intl. J. Pharmacol. 5(5):323-326.
- Rao GB, Rao PU, Rao ES, Rao TM, Rao M, Praneeth VSD (2012). Evaluation of *in-vitro* antibacterial activity and antiinflammatory activity for different extracts of *Rauwolfia tetraphylla* L. root bark. Asian Pac. J. Trop. Biomed. 2(10):818-821.
- Sachdev KS, Aiman R, Rajapurkar MV (1961). Antihistaminase activity of serpentine. Br. J. Pharmacol. Chemother. 16(2):146-152.
- Shariff N, Sudarshana MS, Umesha S, Hariprasad P (2006). Antimicrobial activity of *Rauwolfia tetraphylla* and *Physalis minima* leaf and callus extracts. Afr. J. Biotechnol. 5(10):946-950.
- Srivastava A, Tripathi AK, Pandey R, Verma RK, Gupta MM (2006). Quantitative determination of reserpine, ajmaline and ajmalicine in *Rauwolfia serpentina* by reverse phase high performance liquid chromatography. J. Chromatogr. Sci. 44:557-560.
- Von Poser G, Andrade HH, Da Silva KV, Henriques AT, Henriques JA (1990). Genotoxic, mutagenic and recombinogenic effects of *Rauwolfia* alkaloids. Mutat. Res. 232:37-43.
- Weerakoon SW, Arambewela LSR, Premakumara GAS, Ratnasooriya WD (1998). Sedative activity of the crude extract of *Rauwolfia densiflora*. Phamiaceut. Biol. 36(5):360-361.

Full Length Research Paper

Serotypes and antimicrobial resistance of invasive *Streptococcus pneumoniae* isolates from East Algeria (2005-2011)

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Streptococcus pneumoniae is one of the most common bacterial causes of morbidity and mortality worldwide causing life threatening infections such as meningitis, pneumonia and bacteremia. Antibiotic resistance in *S. pneumoniae* has increased worldwide but there are few data in Algeria and more information is needed about serotype distribution of invasive *S.pneumoniae* isolates. From 2005 to 2011, a total of 100 non-duplicate invasive *S. pneumoniae* isolates were identified at the University Hospital from East Algeria. Antibiotic resistance was determined by the Clinical and Laboratory Standards Institute (CLSI) disk diffusion test and the minimum inhibitory concentration of beta-lactams and erythromycin were determined using the E test method (AB BIODISK). Eighty three (83) serotypes were determined by agglutination by latex particles and/or by the Neufeld test using monovalent antisera (Statens Serum Institute). Among the 100 isolates, 57% were non-susceptible to penicillin (PNSP), 46% were intermediate and 11% were resistant (MIC range 2-4 µg/ml). Resistance rates to other antibiotics were as follow: erythromycin (22%), tetracycline (20%), cotrimoxazol (51%). All the strains were susceptible to chloramphenicol, vancomycin and levofloxacin. The predominant serotypes were 14, 19F, 23F, and 6B accounting for 50.6% of tested strains. Non-penicillin susceptibility was associated with serotype 14 (88.23%), 6B (80.00%), 19F (61.53%), and 23F (57.14%). In children ≤ 5 years of age, the rate of this serotypes were 14 (23.33%), 19F (13.33%), 23F (13.33%) and 6B (10%). Pneumococcal vaccination is not compulsory in Algeria. The theoretical coverage of PCV13 added up to 74.19%. Continual surveillance of antibiotic susceptibility and serotype distribution is recommended in order to plan future treatment and preventive strategies.

Keywords: *Streptococcus pneumoniae*, serotype distribution, antibiotic resistance, invasive infection, pneumococcal conjugate vaccine.

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) is one of the most frequent causes of serious invasive infections, such as, meningitis, bacteremia and pneumonia and is the

major cause of morbidity and mortality worldwide. In 2005, WHO estimated that 1.6 million people die of pneumococcal diseases every year, including the deaths

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of nearly one million children aged < 5 years, most of whom live in developing countries (vaccine for childhood immunization-WHO position paper, 2007). The capsule is the main virulence factor and there are 93 known antigenically distinct capsular polysaccharide serotypes of *S. pneumoniae* (Henrichsen, 1995; Bentley et al., 2006; Calix and Nahm, 2010). The prevalence of penicillin resistance has been increasing worldwide (Jenkins et al., 2005; Yang et al., 2008; Hoban et al., 2005; Varon, 2012). Penicillin resistance is usually associated with resistance to other antibiotics, particularly, macrolide and the emergence of multidrug resistance *S. pneumoniae* has been observed in various countries making therapeutic options more difficult (Song et al., 2004b; Jenkins et al., 2005; Johnson et al., 2006; Zhou et al., 2011; Charfi et al., 2012). Many studies have shown that levels of antibiotic resistance are directly proportional to antibiotic consumption in the community (Bronzwaer, et al., 2002; van de Sande-Bruinsma et al., 2008).

The resistance of *S. pneumoniae* to antibiotics is gradually becoming a serious problem, which underlines the urgent need for vaccines to control pneumococcal diseases. At present, three pneumococcal conjugate vaccines are available for children. Introduction of heptavalent pneumococcal conjugate vaccine (PCV7) for infants led to substantial reductions in the incidence of invasive pneumococcal disease (IPD) in the United States and other industrialized countries (Varon, 2012; Myint et al., 2013). However, the increase in the rate of invasive pneumococcal disease (IPD) cases caused by non-vaccine strains has been a concern (Ingels et al., 2012; Van der Linden et al., 2012). Although PCV7 continues to effectively decrease the pneumococcal disease burden, the incidence of IPD caused by non-PCV serotypes has increased among vaccinated children, and these strains are often highly resistant to commonly used antimicrobials (Tyrrell et al., 2009; Azzari et al., 2012; Gant et al., 2012; Pichon et al., 2013, Tóthpál et al., 2012).

The aim of this study was to characterize the epidemiology of children and adult IPD in University Hospital from Constantine. 100 strains of *S. pneumoniae* were isolated from patients with invasive infections across the period of 2005-2011. In Algeria, the pneumococcal conjugate vaccine was not introduced yet in the national program of immunization. In order to evaluate the potential contribution of a pneumococcal conjugate vaccine, antibiotic susceptibility and multi-drug resistance were investigated and serotype distribution was analyzed. Furthermore, the theoretical coverage of the 7-, 10- and 13-valent conjugate vaccines was evaluated.

MATERIALS AND METHODS

Bacterial strains and species identification

A total of 100 *S. pneumoniae* clinical isolates were collected from

January 2005 to December 2011 in the University Hospital Ibnbadis from Constantine, Algeria. All the non-duplicate invasive *S. pneumoniae* isolates recovered from adults and children were included. Isolates were obtained from cerebrospinal fluid (CSF), blood and pleural fluid and when an isolate was recovered from CSF and blood, it was categorized as meningitis. Bacterial strains were grown on Columbia sheep blood agar and incubated at 37°C under a 5% CO₂ atmosphere for 20-24 h. All isolates were originally identified as *S. pneumoniae* based on colony morphology, Gram staining, α-hemolysis and optochin susceptibility.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was determined on Mueller-Hinton agar by standard disk diffusion procedure according to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI). A total of 15 antibiotics were tested including oxacillin (screening), penicillin, amoxicillin, cefotaxime, imipenem, erythromycin, clindamycin/lincosamide, tetracycline, chloramphenicol, cotrimoxazole, vancomycin, rifampicin, levofloxacin, ciprofloxacin and linezolid. Minimum inhibitory concentration (MIC) is determined using E test method ((AB BIODISK) for penicillin, amoxicillin, cefotaxime, imipenem and erythromycin. The CLSI criteria for MIC were applied to classify the isolates as susceptible (S), intermediate (I), or resistant (R) (both the CLSI 2007 and the CLSI 2011 criteria for penicillin) (CLSI, 2007; CLSI, 2011).

S. pneumoniae ATCC 49619 was used as the quality control strain and was included in each set of tests to ensure the accuracy of the results. Multi-drug resistant (MDR) was defined as resistance to three or more classes of antibiotics used in this study.

Serotyping

Eighty three (83) isolates were serotyped using rapid latex agglutination (Pneumotest kits) and the capsule reaction test used antisera from the Statens Serum Institut (Copenhagen, Denmark). The isolates that reacted negatively with the antisera were classified as non-typeable.

The coverage of the PCV7, PCV10 and PCV13 vaccines was estimated by calculating the percentage of isolates that expressed the serotypes included in the vaccine.

Statistical methods

All data was analyzed with the software WHONET 5. The χ^2 test was used for comparing proportion of PNSP in the two age groups; P value of < 0.05 was considered to be statistically significant.

RESULTS

Antimicrobial-susceptibility

100 clinical isolates responsible from invasive pneumococcal diseases (IPD) (cerebrospinal fluid n= 75, blood n=22, pleural fluid n= 3) were analyzed and the more clinical presentation was meningitis (75%). Of 100 isolates, sex ratio was 2.8 (74 males and 26 females). 54 strains were isolated from adults (\geq 18 years) and 46 were from children (\leq 17 years), among them, 31 were under 5 years of age (31/46, 67.39%) (Table 1).

The global non-susceptible rate of *S. pneumoniae* to

Table 1. Distribution of 100 pneumococcal strains according to type of sampling and age.

Sample	≤5years	6 to17 years	18 to 40 years	>41 years	Total
CSF	24	10	19	8	61
CSF + Blood	0	3	8	3	14
Blood	7	2	6	7	22
Pleural fluid	0	0	0	3	3
Total	31	15	33	21	100

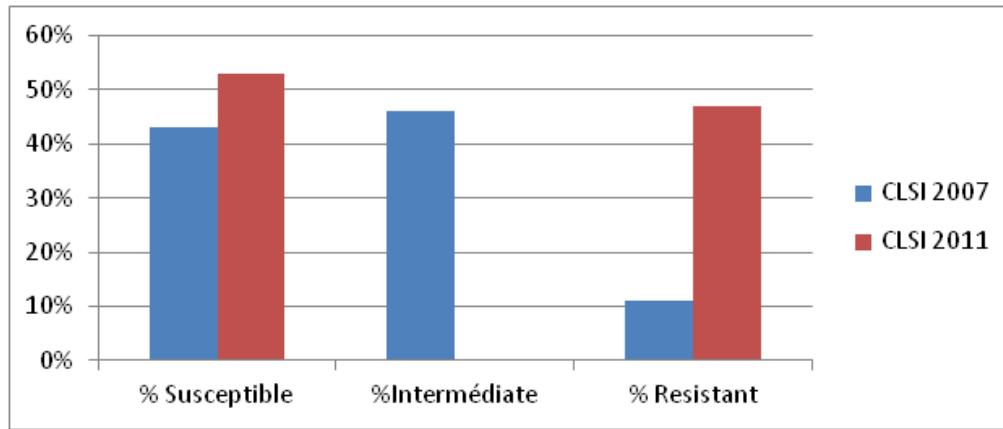


Figure 1. Frequency of resistance to penicillin according to CLSI standards.

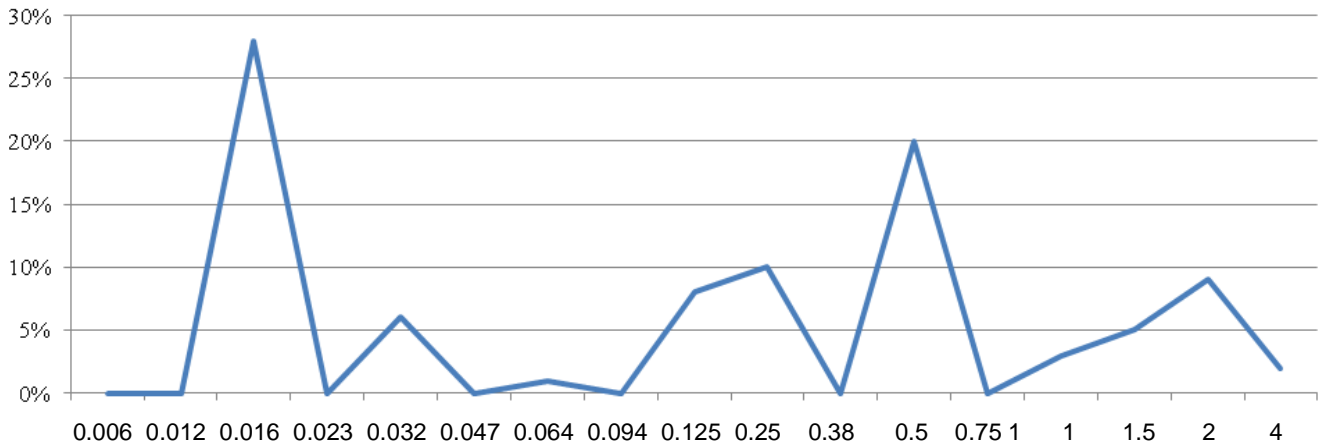


Figure 2. MICs (µg/ml) of 100 pneumococcal isolates for penicillin.

penicillin (R+I) was 57%, using the CLSI 2007 criteria, the penicillin intermediate rate was 46% and resistant rate was 11% (Figure 1).

The MICs of penicillin to most *S. pneumoniae* strains ranged from 0.012 µg/ml to 0.023 µg/ml and from 0.38µg/ml to 0.75 µg/ml (Figure 2). The penicillin non-susceptible rate of pediatric isolates was 80. 42% (37/46) with 15.21% (7/46) penicillin-resistant strains (MIC

ranged between 2 - 4 µg/ml). There were 29.62% penicillin-intermediate strains and 7.4% penicillin-resistant strains among adult isolates (MIC = 2 µg/ml).

The non-susceptible rates to amoxicillin and cefotaxime were 9 (2% of resistant strains), and 8% respectively without any identified resistant strain for cefotaxime. All the isolates were susceptible to imipenem (Table 2).

The non-susceptible rates to erythromycin, tetracycline

Table 2. Susceptibility and MICs of 100 pneumococcal isolates to 5 antibiotics.

Antimicrobial agent	Sample source age	Range	MIC ($\mu\text{g/ml}$)			MIC ($\mu\text{g/ml}$)		
			CLSI 2007			CLSI 2011		
			%S	%I	%R	%S	%I	%R
Penicillin	Adults	0.012- 2	62.96	29.62	7.4	72.22	/	27.77
	Children	0.064- 4	19.56	65.21	15.21	30.43	/	69.56
Amoxicillin	Adults	0.016- 1.5	92.59	7.4	0			
	Children	0.064- 4	89.13	6.52	4.34			
Cefotaxime	Adults	0.016- 1.5	94.44	5.55	0			
	Children	0.064- 1.5	89.13	10.86	0			
Imipenem	Adults	0.002- 0.38	100	0	0			
	Children	0.002- 0.12	100	0	0			
Erythromycin	Adults	0.016- > 256	79.62	0	20.37			
	Children	0.064- > 256	76.08	0	23.91			

MIC, Minimum inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute.

and trimethoprim-sulfamethoxazole were respectively 22, 20 and 51% (Figure 3).

The MICs of 100% resistant *S. pneumoniae* for erythromycin (22 strains) were above 256 $\mu\text{g/ml}$ and 100% were resistant to clindamycin (MLS_B phenotype). All the isolates were susceptible to chloramphenicol, vancomycin, rifampicin, levofloxacin and linezolid.

Of 100 isolates, the rate of MDR was 20% and among PNSP isolates (57 strains), 29.8% (17/57) were resistant to erythromycin and 33.33% (19/57) were MDR. Among erythromycin resistant strains, 77.27% (17/22) were PNSP and 77.27% (17/22) were MDR.

Serotype distribution

The serotype distribution of 83 clinical isolates is shown in Figure 4. 82 serotypes were identified and one strain was non-typeable. The most prevalent serotypes were 14, 19F, 23F, and 6B accounting for 50.60% (42/83) of the tested strains.

In children \leq 5 years of age, the rate of these serotypes were 14 (22.58%), 23F (12.90%), 19F (12.90%), and 6B (9.60%), and a total of 19 pneumococcal isolates expressed the serotypes included in PCV7, so the coverage of PCV7 was 61.29% (19/31).

In this group of age, there were 2 strains expressing serotypes 1 and 7F (3.2% for each). There were no strains expressing serotype 5, furthermore, the coverage of PCV10 was 67.74% (21/31). On the other hand, the coverage of PCV13 added up to 74.19% (23/31).

Non-vaccine serotypes, such as serotypes 9A, 10A, 11, 12A, 24F, 33F and 35B are expressed in small proportions (3.22% each) (Figure 5).

Non-penicillin susceptibility was associated with serotypes 14 (88.23%), 23F (80%), 6B (80.00%) and 19F (61.53%) whilst serotype 18C was identified in three strains which were PNSP.

Serotype 19A was identified in three strains and two of

them were PNSP isolated from meningitis, among them, one strain was isolated in a 2 year old children. Serotype 1 was identified for three strains; one strain was isolated in children under 5 years of age and was PNSP. Serotype 35B was found mostly in meningitis and none was PNSP. Non-susceptibility to penicillin was observed in other serotypes such as serotypes 9N, 16, 29, 12A, 47F and 24F, rarely isolated (Table 3).

The most resistant isolates to erythromycin were serotype 19F (31.81%, 7/22) and 14 (22.72%, 5/22). MDR were most frequent among serotype 14 and 19F (35%, 7/20 and 30%, 6/20 respectively). There were other MDR serotypes such as serotype 19A (2/20), and serotypes 7F, 6B, 3, 10A and 9N (1/20 for each).

DISCUSSION

The resistance of *S. pneumoniae* to antibiotics varied over time, among different regions, age, serotypes, sources of the strains, and treatment of IPD presents a difficult challenge because of the fast distribution of the penicillin non-susceptible strains worldwide (Felmingham et al., 2002; Reinert, 2009). Despite its importance, a few studies on the serotype distribution and antimicrobial resistance of invasive *S. pneumoniae* diseases (IPD) were investigated in Algeria.

In the present study, the penicillin non-susceptible rate was very high and increased to 57%, when the break-point of CLSI 2007 was adopted, the penicillin intermediate rate was 46% and resistant rate was 11% whereas, no isolate was found to have intermediate susceptibility to penicillin and resistance rate was 47% based on the 2011 CLSI criteria. These rates of PNSP placed Algeria among countries with the highest levels of penicillin resistance, due at least in part to the misuse of this antibiotic over an extended period of time.

Application of the 2011 breakpoints showed higher resistance rates for meningitis than non-meningitis in our

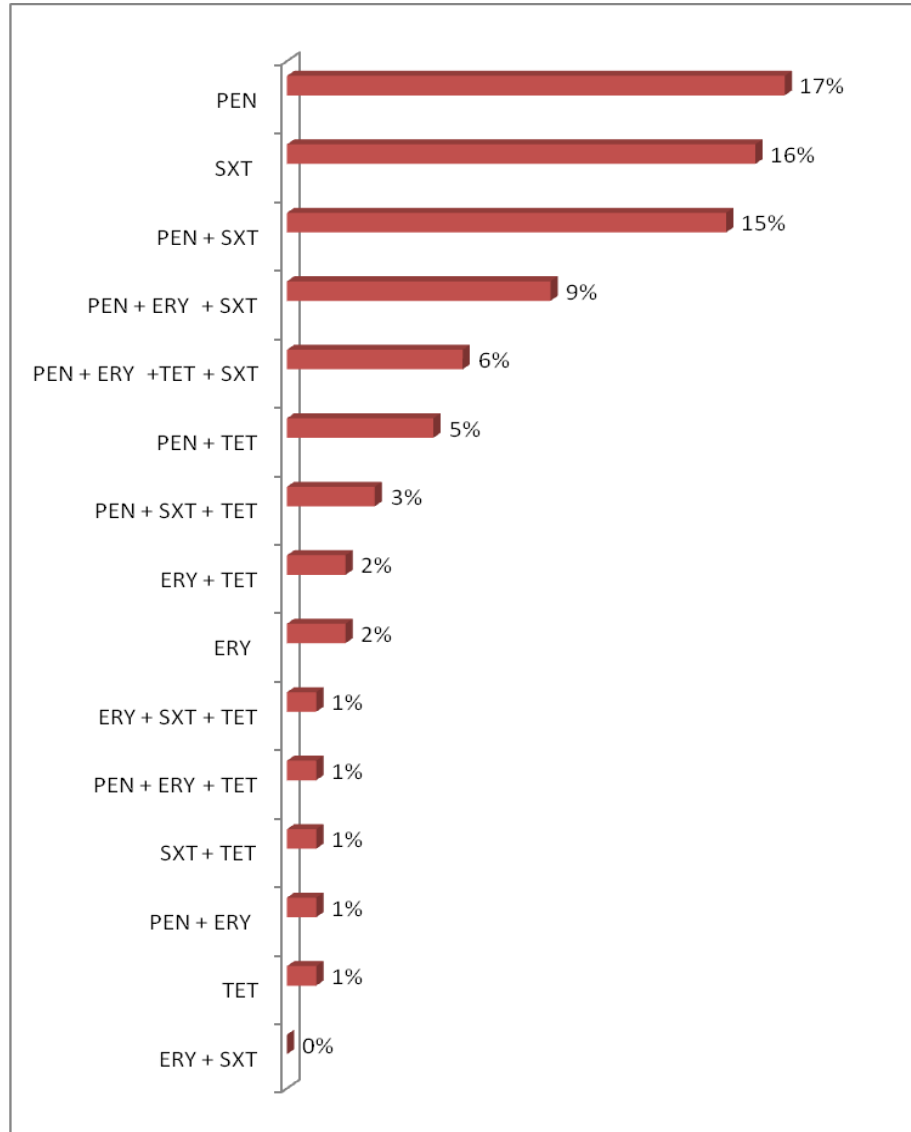


Figure 3. Antibiotic resistant pattern of 100 pneumococcal isolates. PEN, penicillin; ERY, erythromycin; TET, tetracycline; SXT, cotrimoxazole.

study. In the USA, the proportion of resistant meningial isolates increased from 10.7% under the pre-2008 breakpoints to 27.5% under the 2008 breakpoints. However, according to the new non-meningial breakpoints, all isolates were susceptible to penicillin while the majority expressed intermediate resistance (CDC, 2008). There was a significant difference of resistance to penicillin in the two age groups ($P < 0.05$) in present study. Such antimicrobial susceptibility differences between isolates from children and adults have previously been reported in other studies (Hoban et al., 2005; Varon, 2012).

Previously reported rates of PNSP in Algeria cannot be compared with our results, because the criteria were different. Generally, prevalence of PNSP increased over time and the rate observed in our study was higher than

those reported by Smati et al. (1994) and Tali-Maamar et al. (2012) (12.5% in all isolates and 23.5% in meningitis respectively).

Prevalence rates of penicillin non-susceptible varied widely among countries that did not include PCV7. In Asia, Song et al. (2004a) demonstrated that Asians had the world's highest level of antimicrobial resistance in *S.pneumoniae*. The rates of penicillin resistance amongst clinical strains were 71.4% in Vietnam, 68.8% in Thailand and 54.8% in Korea. In France in 2002, the rate of penicillin non-susceptible pneumococci (PNSP) reached up to 50% of all strains isolated (Varon, 2012). Moderately high rates of PNSP were showed in southern and eastern Mediterranean region (25% in 2003-2005) (Borg et al., 2009) and reached 40.5% in Spain (García-

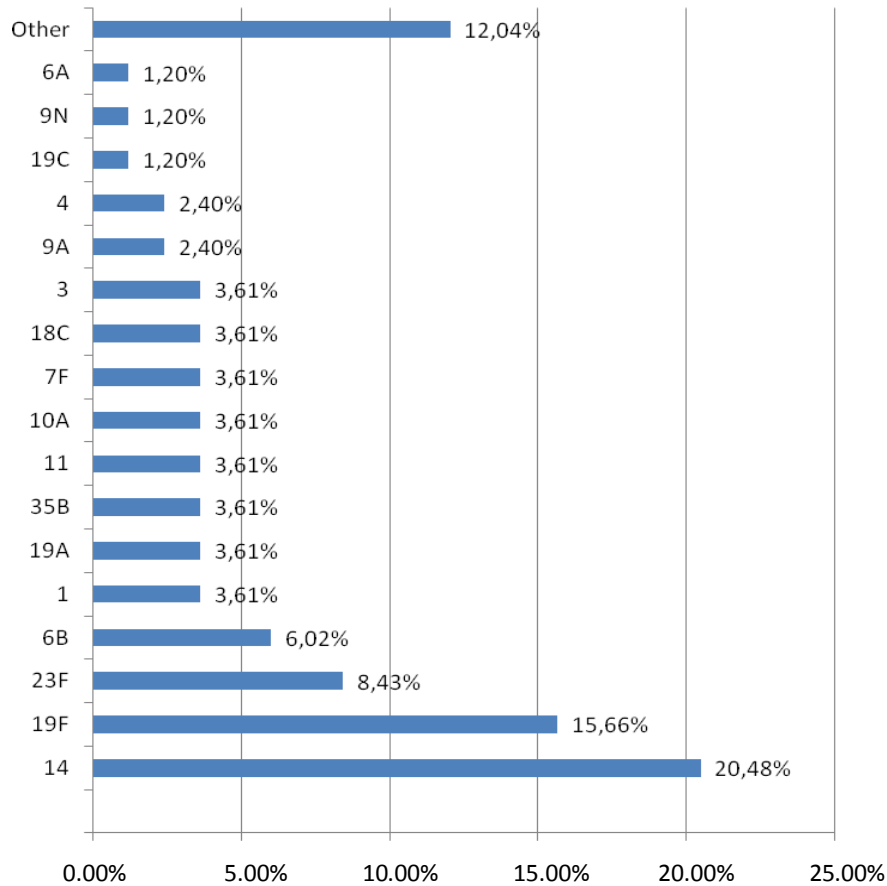


Figure 4. Serotype distribution of 83 pneumococcal isolates. Other includes serotypes 12A, 16, 24F, 47F, 33F, 39, 29, 21, 48 and one non-typeable strain.

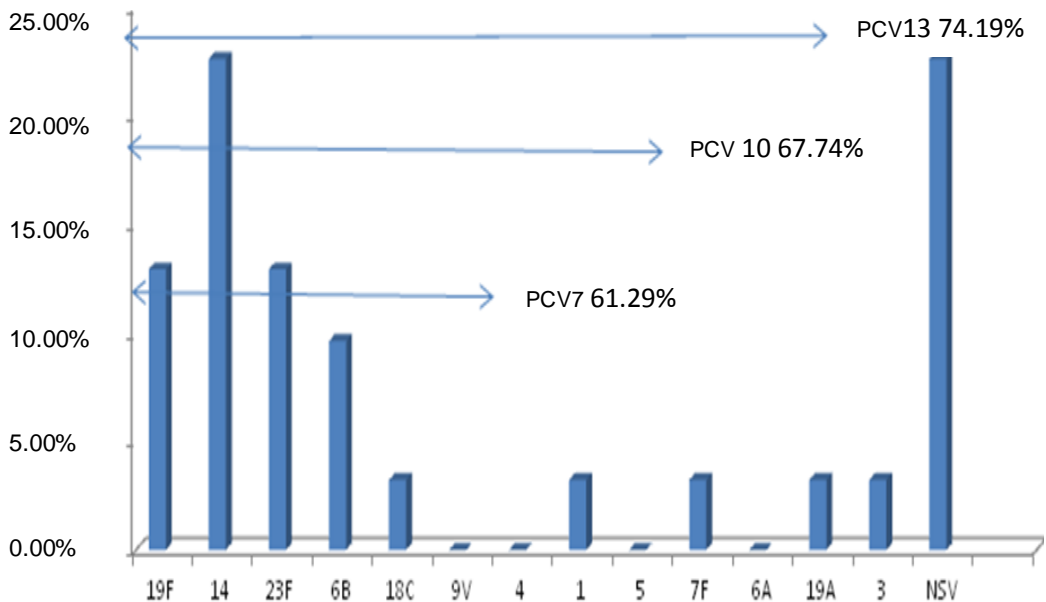


Figure 5. Distribution of serotypes in children ≤5 years of age and vaccine coverage (n=30/31). NSV, Non-serotype-vaccine; PCV, Pneumococcal vaccine; PCV7, 4, 6B, 9V, 14, 18C, 19F, 23F; PCV10, 4, 6B, 9V, 14, 18C, 19F, 23F + 1, 5, 7F; PCV13, 4, 6B, 9V, 14, 18C, 19F, 23F + 1, 3, 5, 6A, 7F, 19A.

Table 3. Distribution of the predominant serotypes of *S.pneumoniae* PNSP, ENSP, and MDR from adults and children.

Serotype	Number of isolates %	PNSP%		ENSP		MDR%	
		Adults	Children	Adults	Children	Adults	Children
14	17	23.52	64.70	11.76	17.64	11.763	23.52
19F	13	23.07	38.46	23.07	30.76	23.07	23.07
23F	7	0	54.14	0	0	0	0
6B	5	20	60	40	0	0	25
1	3	0	33.33	0	0	0	0
3	3	0	33.33	0	0	0	33.33
19A	3	0	66.66	0	66.66	0	66.66
35B	3	0	0	0	0	0	0
11	3	66.66	33.33	0	0	0	0
10A	3	0	66.66	0	33.33	0	33.33
7F	3	33.33	33.33	0	33.33	0	33.33
18C	3	33.33	66.66	0	0	0	0

PNSP: penicillin non-susceptible pneumococcus, ENSP: erythromycin non-susceptible pneumococcus, MDR: multidrug resistance.

Suárez et al., 2006). In African countries, PNSP rates also varied. Rates of 48.5 and 50.4% have been reported respectively in Morocco, and Tunisia as in Algeria (Elmdaghri et al., 2012; Smaoui et al., 2009). PNSP rates reported for Kenya, Uganda, Tanzania and Ethiopia were as low as 0% in 2003-2007 (Mudhune et al., 2009), rates of 0.5, 12 and 27.3% have been reported respectively in South Africa (Silberbauer et al., 2011), Ghana (Holliman et al., 2007) and Senegal (Manga et al., 2008). Differences in rates of pneumococcal penicillin resistance between countries have been shown to be associated with levels of antimicrobial consumption (van de Sande-Bruinsma et al., 2008).

Introduction of PCV7 was associated with substantial declines in PNSP prevalence (Farrell et al., 2007; van de Sande-Bruinsma et al., 2008; Varon, 2012). In France, the rate of the strains with decreased susceptibility to penicillin decreased from 50 to 30% over a 6-years study (2002-2007). The decrease was even more marked in children less than 2 years of age: 64% of PNSP in 2002 to 41% in 2007 (Varon, 2012). In agreement, our study shows higher rates of resistance strains relative to countries that implement the vaccine

The rates of strains with decreased susceptibility to other beta-lactams in our study were higher than those showed in two previous Algerian's studies. An Algerian's study in 2003 did not identify any strains resistant to amoxicillin or cefotaxime (Ramdani-Bougoussa and Rahal, 2003). A later study, published in 2012, identified 4.2% as cefotaxime resistant in meningitis (Tali-Maamar et al., 2012). In Tunisia (Smaoui et al., 2009), these rates were a bit higher, in IPD of children under 5 years of age, the rates of resistance to amoxicillin and cefotaxime were 11.4 and 5.7% respectively.

In post vaccine period, a study on the antimicrobial susceptibility of *S. pneumoniae* in eight European countries indicated that the resistance rate to cefotaxime was

5.1% (Reinert et al., 2005) while, the rate of strains with decreased susceptibility to other beta lactams remained high in France (20% to amoxicillin and 10% to cefotaxime) (Varon, 2012)

The rates of resistance to erythromycin, was 22% in this study, While resistance rate was higher for erythromycin (31%) in a previous Algerian's study because criteria were different (Tali-Maamar et al., 2012) whereas the rate of resistance reported in 1994 was low (Smati et al., 1994).

The prevalence of macrolide resistance in *S. pneumoniae* increased worldwide but was highly variable between countries, and was mainly due to widespread use of macrolides, mostly azithromycin (Hyde et al., 2001; Dias and Canica, 2004).

Many Asian countries showed extremely high prevalence rates of macrolide resistance (> 88,3% during 2000-2002 (Song et al., 2004b). In the United States, the rate of resistance to erythromycin was 25% in 2000 (Whitney et al., 2000). The highest rates of 43.6, 46.1, and 53.7% respectively has also been reported from Spain, France and Greece (Reinert et al., 2005; Daikosa et al., 2008). In Australia, an increase in resistance was remarkable for erythromycin (3.5% in 2000, 11% in 2008) (Hoenigl et al., 2010). In Africa, an increase of resistance rates was also noticed for erythromycin in Morocco (9.4% in 1998-2001, 12.2% in 2002-2005 and 14.4% in 2006-2008) (Benbachir et al., 2012).

Introduction of PCV7 was followed by declines in erythromycin non-susceptible pneumococci (Kyaw et al. 2006; Farrell et al., 2008; Tyrrell et al., 2009; Varon, 2012). In France in 2007, around 30% of isolated *S. pneumoniae* strains were resistant to macrolides compared to 50% in 2001 (Varon, 2012) whereas, erythromycin resistance continued to rise in the post PCV7 years (Horacio et al., 2012).

In this study, rates of resistance to tetracycline, cotrimoxazole and chloramphenicol were respectively 20, 51 and 0% and the rates of 30, 43 and 5.8% respectively, were observed in recent Algerian study (Tali-Maamar et al., 2012). Highest rates of resistance were seen in African's and Asian's countries (Holliman et al., 2007; Liu et al., 2008; Charfi et al., 2012; Thomas et al., 2013). In Ghana, although most isolates of IPD were resistant to tetracycline and cotrimoxazole (85% and 63%), resistance to tetracycline and cotrimoxazole remained high even though these agents were no longer used for empirical treatment of chest infection in the region (Holliman et al., 2007).

The resistance to cotrimoxazole was noted to be high in Asia (> 85%), probably because of its widespread use for presumptive treatment of pneumonia (Thomas et al., 2013). In our study, cotrimoxazole resistance was high; nevertheless this antibiotic was not delivered without prescription in Algeria and is used as the second line of defense in treating bacterial acute lower respiratory tract infections after amoxicillin failure. While, all strains were susceptible to chloramphenicol, this antibiotic is rarely used nowadays. The resistant rates to chloramphenicol, in Morocco and Tunisia were relatively low (8.1 and 17.2% respectively) (Elmdaghri et al., 2012; Smaoui et al., 2009). The resistant rates to chloramphenicol increased to 68.2% in Senegal; chloramphenicol was the most frequently used antibiotics (Manga et al., 2008).

Vancomycin and levofloxacin showed 100% of efficacy in the present study; vancomycin is not recommended for monotherapy in meningitis and fluoroquinolones are rarely used for empiric therapy of community-acquired pneumonia in Algeria. These drugs may be important alternatives for use in the treatment of infections caused by multidrug-resistant *S. pneumoniae*, but the spread of fluoroquinolone-resistant clones may cause rapid increase in resistance with widespread use of these agents as has been reported from Honk Kong (Ho et al., 2004).

The rate of multidrug resistance strains (MDR) is relatively low in our study compared to those found in Asian countries (up to 71.4%) (Lee et al., 2010), and the rate of MDR in PNSP was so low, whereas penicillin resistance is an important marker for the presence of MDR. Introduction of PCV7 in several countries was followed by decline in prevalence in PNSP and in MDR; the overall rate of invasive MDR isolates declined by 59% in USA (Kyaw et al., 2006).

Natural fluctuations in serotypes responsible for IPD occurred over time. The pattern of predominant IPD associated serotypes varied with age and country (Mehr and Wood, 2012). Globally, seven serotypes account for the bulk of IPD disease (1, 5, 6A, 6B, 14, 19F and 23F).

S. pneumoniae serotypes identified in our study were similar to those reported in some countries before introduction of PCV7 (Reinert et al., 2010). An Algerian's authors have reported the variation in time of circulating

SP serotypes. A study from 1996 to 2000 showed that serotypes 1 and 5 were the most frequent in both adults and children, while serotype 19 and 23 were rare (Ramdani-Bouguessa and Rahal, 2003). In contrast, a study from 2001-2010 in children under 5 years of age, showed that serotypes 14, 23F, 19F, 6B and 1 were common (Tali-Maamar et al., 2012).

In our study, a correlation between serotypes and antimicrobial resistance patterns was observed. The four most common serotypes (14, 23F, 6B and 19F) were associated with high rates of resistance to penicillin. The highest rates of resistance tended to occur in the most prevalent serotypes.

Similar results were also reported in many countries before introduction of PCV7. In Tunisia, the most prevalent serotypes for invasive pneumococcal isolates in children were 14, 23F, 4 and 19F; serotype 14 was the most prevalent serotypes in IPD and was highly penicillin non-susceptible (Charfi et al., 2012). A study from South Africa reported that the most common serotypes in IPD in children < 5 years of age were 14, 1, 6A/6B, 19F and 23F and penicillin non-susceptibility was observed in serotypes 14, 19F, 6A and 23F (Silberbauer et al., 2011).

In Australia, the most common serotypes causing IPD were 14, 19F, 6B, and 18C, and the most common PNSP IPD serotypes were serotypes 19F and 9V (Watson et al., 2007).

In China, the most prevalent serotypes were 19F, 14, 23F, 6B and 19A and the most prevalent serotypes of PNSP were 19F, 14, and 23F (Yang et al., 2008).

In Brazil, serotypes 14, 3, 23F, 19F, and 6B were the most prevalent serotypes and 86% of serotypes 14, 23F, 6B and 19F were PNSP (de O Menezes et al., 2011).

Following introduction of PCV7, there has been a steady increase in the incidence of non-PCV7 serotypes. The replacement of vaccine serotypes by non-vaccine serotypes observed in invasive infections partly reflects the modified distribution of serotypes colonizing the nasopharynx of young children (Cohen et al., 2010). Most of the rise in non-PCV7 IPD is attributable to serotype 19A (Munoz-Almagro et al., 2009; Tyrrell et al., 2009; Azzari et al., 2012; Rosen et al., 2011; Horacio et al., 2012; Ingels et al., 2012; Bautista-Marquez et al., 2013).

In the USA, the incidence of 19A IPD in children < 5 years of age rose from 2.6 cases per 100,000 population (pre-PCV7 period; 1998-1999) to 9.3 cases per 100,000 population (post-PCV7 period; 2005) (MMWR, 2008). Serotype 19A is now one of the most common causes of IPD in young children from developed countries (Fenoll et al., 2009; Bettinger et al., 2010; Kaplan et al., 2010). Changes in *S. pneumoniae* serotype distribution after the introduction of PCV7 cannot be automatically assumed to be due to PCV7, because temporal changes in serotype distribution were observed in some countries pre-PCV7 (Jefferies et al., 2010). However, the emergence of serotype 19A was reported before the introduction or widespread use of PCV7 in some countries (Choi et al., 2008;

Shin et al., 2011).

Serotype 19A is particularly important in the epidemiology of IPD because of its potential for invasiveness and its propensity to acquire resistance and MDR (Kyaw et al., 2006; Farrell et al., 2007). Serotype 19A was greatly exposed to selection pressure of antibiotics: 85% of serotype 19A pneumococci were PNSP in French's study (Varon, 2012).

In USA, the proportion of IPD caused by PNSP 19A increased from 20.4 in 2004 to 43.7% in 2008 (Beall et al., 2011). Furthermore, most PNSP serotype 19A isolates were also resistant to other antibiotics or were MDR. One of the most significant findings from this study was the presence of serotype 19A (3.61%) and 66.6% of 19A showed high resistance rates to several antibiotics including penicillin.

We demonstrate in our study that PCV13 provided good coverage for invasive pneumococcal isolates for the children ≤ 5 years of age (74.19%). The theoretical vaccinal coverage for PNSP in children was evaluated at 62.1, 66.7 and 72.4% for PCV7, PCV10 and PCV13 respectively in previous study in Algeria.

These results represented an additional contribution to our current understanding of burden invasive pneumococcal disease in one of developing countries. Continual surveillance of antibiotic susceptibility and serotype distribution is recommended. These results suggest that the expanded coverage offered by PCV13 will provide additional protection against pneumococcal disease in Algeria.

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REFERENCES

- Azzari C, Moriondo M, Cortimiglia M, Valleriani C, Canessa C, Indolfi G, Ricci S, Nieddua B, Maurizio de Martino F, Resti M (2012). Potential serotype coverage of three pneumococcal conjugate vaccines against invasive pneumococcal infection in Italian children. *Vaccine* 30:2701-2705.
- Bautista-Marquez A, Richardson V, Ortiz-Orozco O et al (2013). Prevalence of pneumococcal disease, serotype distribution, and antimicrobial susceptibility in Mexican children younger than 5 years of age. *Arch. Med. Res.* 44:142-150.
- Beall BW, Gertz RE, Hulkower RL, Whitney CG, Moore MR, Brueggemann AB (2011). Shifting genetic structure of invasive serotype 19A pneumococci in the United States. *J. Infect. Dis.* 203:1360-1368.
- Benbachir M, Elmadaghri N, Belabbes H, Haddioui G, Benzaid H, Zaki B (2012). Eleven-year surveillance of antibiotic resistance in *Streptococcus pneumoniae* in Casablanca (Morocco). *Microb. Drug Resist.* 18(2):157-160.
- Bentley SD, Aanensen DM, Mavroidi A, Saunders D, Rabinowitz E, Collins M et al (2006). Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. *PLoS Genet.* 2(3):31.
- Bettinger JA, Scerfele DW, Kliner JD, Halperin SA, Vaudry W, Law B et al. (2010). The effect of routine vaccination on invasive pneumococcal infections in Canadian children immunization monitoring program, Active 2000-2007. *Vaccine* 28:2130-2136.
- Borg MA, Tiemersma E, Scicluna E, Van de Sande-Bruinsina N, de Kraker M, Monen J et al (2009). Prevalence of penicillin and erythromycin resistance among *Streptococcus pneumoniae* isolates reported by laboratories in the southern and eastern Mediterranean region. *Clin. Microb. Infect.* 15:232-237.
- Bronzwaer SL, Cars O, Buchholz U, Mølsted S, Goetsch W, Veldhuijzen IK et al (2002). A European Study on the relationship between antimicrobial use and antimicrobial resistance. *Emerg. Infect. Dis.* 8:278-82.
- Calix J, Nahm MH (2010). A new pneumococcal serotype 11E has a variability inactivated WCJE gene. *J. Infect. Dis.* 202(1):29-38.
- Centers for Diseases Control and Prevention (CDC) (2008). Effects of new penicillin susceptibility breakpoints for *Streptococcus pneumoniae*- United States 2006-2007. *MMWR- Morb Mortal Wkly Rep.* 57:1353-1355.
- Charfi F, Smaoui H, Kechrid A (2012). Non-susceptibility trends and serotype coverage by conjugate pneumococcal vaccines in a Tunisian pediatric population: A 10-year study. *Vaccine* 30:18-24.
- Choi EH, Kim SH, Eun BW, Kim SJ, Kim NH, Lee J, Lee HJ (2008). *Streptococcus pneumoniae* serotype 19A in children, South Korea. *Emerg. Infect. Dis.* 14:275-81.
- Clinical and Laboratory Standards Institute (2007). Performance standards for antimicrobial susceptibility testing In: Sixteen informational supplement CLSI. M100-S16.
- Clinical and Laboratory Standards Institute (2011). Performance standards for antimicrobial susceptibility testing In: Twenty-first informational supplement CLSI. M100-S21;31(1).
- Cohen R, Levy C, Bonnet E, Grondin S, Desvignes V, Lecuyer A, Fritzell B, Varon E (2010). Dynamic of pneumococcal nasopharyngeal carriage in children with acute otitis media following PCV7 introduction in France. *Vaccine* 28:6114-21.
- Daikosa GL, Koutsolioutsoub A, Tsiodrasc S (2008). Evolution of macrolide resistance in *Streptococcus pneumoniae* clinical isolates in the prevaccine era. *Diagn. Microbiol. Infect. Dis.* 60:393-398
- de O Menezes AP, Campos LC, dos Santos MS, Azevedo J, Dos Santos RC, Carvalho Mda G et al (2011). Serotype distribution and antimicrobial resistance of *Streptococcus pneumoniae* prior to introduction of the 10-valent pneumococcal conjugate vaccine in Brazil, 2000-2007. *Vaccine* 29(6):1139-44.
- Dias R, Canica M (2004). Emergence of invasive erythromycin-resistant *Streptococcus pneumoniae* strains in Portugal: contribution and phylogenetic relatedness of serotype 14. *J. Antimicrob. Chemother.* 54:1035-1039.
- Elmadaghri N, Benbachir M, Belabbes H, Zaki B, Benzaid H (2012). Changing epidemiology of pediatric *Streptococcus pneumoniae* isolates before vaccine introduction in Casablanca (Morocco). *Vaccine* 30:46-50.
- Farrell DJ, Felmingham D, Shackcloth J, Williams L, Maher K, Hope R et al (2008). Non-susceptibility trends and serotype distributions among *Streptococcus pneumoniae* from community-acquired respiratory tract infections and from bacteraemia in the UK and Ireland, 1999 to 2007. *J. Antimicrob. Chemother.* 62(2):87-95.
- Farrell DJ, Klugman KP, Pichichero M (2007). Increased antimicrobial resistance among nonvaccine serotypes of *Streptococcus pneumoniae* in the pediatric population after the introduction of 7-valent pneumococcal vaccine in the United States. *Pediatr. Infect. Dis. J.* 26:123-8.
- Felmingham D, Reinert RR, Hirakata Y, Rodloff A (2002). Increasing prevalence of antimicrobial resistance among isolates of *Streptococcus pneumoniae* from the PROTEKT surveillance study and comparative in vitro activity of the ketolide, telithromycin. *J. Antimicrob. Chemother.* 50(1):25-37
- Fenoll A, Granizo JJ, Aguilar L, Giménez MJ, Aragonese-Fenoll L, Hanquet G et al (2009). Temporal trends of invasive *Streptococcus pneumoniae* serotypes and antimicrobial resistance patterns in Spain from 1979 to 2007. *J. Clin. Microbiol.* 47(4):1012-20.
- Gant CM, Rosingh AW, López-Hontangas JL, van der Heijden M, González-Morán F, Bijlsma JJ et al. (2012). Serotype distribution and antimicrobial resistance of invasive pneumococcal disease strains in

- the Comunidad Valenciana, Spain, during the winter of 2009-2010: low PCV7 coverage and high levofloxacin resistance. *Antimicrob. Agents Chemother.* 56(9):4988-9.
- García-Suárez Mdel M, Villaverde R, Caldeilla AF, Méndez FJ, Vázquez F (2006). Serotype distribution and antimicrobial resistance of invasive and non-invasive pneumococcal isolates in Asturias, Spain. *Jpn J. Infect. Dis.* 59(5):299-05.
- Henrichsen J (1995). Six newly recognized types of *Streptococcus pneumoniae*. *J. Clin. Microbiol.* 33(10):2759-62.
- Ho PL, Que TL, Chiu SS, Yung RW, Ng TK, Tsang DN, Seto WH, Lau YL (2004). Fluoroquinolone and other antimicrobial resistance in invasive pneumococci, Hong Kong, 1995-2001. *Emerg. Infect. Dis.* 10(7):1250-7.
- Hoban D, Baquero F, Reed V, Felmingham D (2005). Demographic analysis of antimicrobial resistance among *Streptococcus pneumoniae*: worldwide results from PROTEKT 1999-2000. *Int. J. Infect. Dis.* 9(5):262-273.
- Hoenigl M, Fussi P, Feierl G et al (2010). Antimicrobial resistance of *Streptococcus pneumoniae* in Southeast Austria, 1997-2008. *Int. J. Antimicrob. Agents* 36:24-27.
- Holliman RE, Liddy H, Johnson JD, Adjei O (2007). Epidemiology of invasive pneumococcal disease in Kumasi, Ghana. *Trans. R. Soc. Trop. Med. Hyg.* 101(4):405-13.
- Horacio AN, Diamantino-Miranda J, Aguiar SI et al (2012). Serotype changes in adult invasive pneumococcal infections in Portugal did not reduce the high fraction of potentially vaccine preventable infections. *Vaccine* 30:218-24.
- Hyde TB, Gay K, Stephens DS, et al (2001). Macrolide resistance among invasive *Streptococcus pneumoniae* isolates. *JAMA* 286:1857-1862.
- Ingels H, Rasmussen J, Andersen PH, Harboe ZB, Glismann S, Konradsen H, et al (2012). Impact of pneumococcal vaccination in Denmark during the first 3 years after PCV introduction in the childhood immunization programme *Vaccine* 30:3944-3950.
- Jefferies JM, Smith AJ, Edwards GF, McMenamin J, Mitchell TJ, Clarke SC (2010). Temporal analysis of invasive pneumococcal clones from Scotland illustrates fluctuations in diversity of serotype and genotype in the absence of pneumococcal conjugate vaccine. *J. Clin. Microbiol.* 48:87-96.
- Jenkins SG, Farrell DJ, Patel M et al (2005). Trends in anti-bacterial resistance among *Streptococcus pneumoniae* isolated in the USA, 2000-2003: PROTEKT US years 1-3. *J. Infect.* 51:355-63.
- Johnson DM, Stilwell MG, Fritsche TR, et al (2006). Emergence of multidrug-resistant *Streptococcus pneumoniae*: report from the SENTRY Antimicrobial Surveillance Program (1999-2003). *Diagn Microbiol Infect Dis.* 56:69-74.
- Kaplan SL, Barson WJ, Lin PL, Stovall SH, Bradley JS, Tan TQ et al (2010). Serotype 19A is the most common serotype causing invasive pneumococcal infections in children. *Pediatrics* 125(3):429-36.
- Kyaw MH, Lynfield R, Schaffner W, Craig AS, Hadler J, Reingold A et al (2006). Effect of introduction of the pneumococcal conjugate vaccine on drug-resistant *Streptococcus pneumoniae*. *Active Bacterial Core Surveillance of the Emerging Infections Program Network.* *N. Engl. J. Med.* 354(14):1455-63.
- Lee S, Lee K, Kang Y, Bae S (2010). Prevalence of serotype and multidrug-resistance of *Streptococcus pneumoniae* respiratory tract isolates in 265 adults and 36 children in Korea, 2002-2005. *Microb. Drug Resist.* 16(2):135-42.
- Liu Y, Wang H, Chen M, Sun Z, Zhao R, Zhang L et al (2008). Serotype distribution and antimicrobial resistance patterns of *Streptococcus pneumoniae* isolated from children in China younger than 5 years. *Diagn. Microbiol. Infect. Dis.* 61(3):256-63.
- Manga NM, Ndour CT, Diop SA, Ka-Sall R, Dia NM, Seydi M, et al (2008). Adult purulent meningitis caused by *Streptococcus pneumoniae* in Dakar, Senegal. *Med. Trop.* 68(6):625-628.
- Mehr S, Wood N (2012). *Streptococcus pneumoniae* - a review of carriage, infection, serotype replacement and vaccination. *Paediatr. Respir. Rev.* 13:258-264.
- MMWR (2008). Invasive pneumococcal disease in children 5 years after conjugate vaccine introduction-eight states 1998-2005. *Morb Mortal Weekly Report.* 57:144-148.
- Mudhune S, Wamae M, (2009). Network surveillance for pneumococcal Disease in the East African Region. Report on invasive disease and meningitis due to *Haemophilus influenzae* and *Streptococcus pneumoniae* from the Network for Surveillance of Pneumococcal Disease in the East African Region. *Clin. Infect. Dis.* 48(2):147-52.
- Munoz-Almagro C, Esteva C, de Sevilla MF et al (2009). Emergence of invasive pneumococcal disease caused by multidrug-resistant serotype 19A among children in Barcelona. *J. Infect.* 59:75-82.
- Myint TT, Madhava H, Balmer P, Christopoulou D, Attal S, Menegas D et al (2013). The Impact of 7-valent pneumococcal conjugate vaccine on invasive pneumococcal disease: A Literature Review. *Adv. Ther.* 30:127-51.
- Pichon B, Ladhani SN, Slack MP, Segonds-Pichon A, Andrews NJ, Waight PA et al (2013). Changes in the molecular epidemiology of *Streptococcus pneumoniae* causing meningitis following the introduction of pneumococcal conjugate vaccination in England and Wales. *J. Clin. Microbiol.* 51:820-827.
- Pneumococcal conjugate vaccine for childhood immunization-WHO position paper (2007). *Wkly Epidemiol Rec.* 82(12):93-104.
- Ramdani-Bouguessa N, Rahal K (2003). Serotype distribution and antimicrobial resistance of *Streptococcus pneumoniae* isolated in Algiers, Algeria. *Antimicrob. Agents Chemother.* 47:824-6.
- Reinert RR (2009). The antimicrobial resistance profile of *Streptococcus pneumoniae*. *Clin. Microbiol. Infect.* 15(3):7-11.
- Reinert RR, Paradiso P, Fritzell B (2010). Advances in pneumococcal vaccines: the 13-valent pneumococcal conjugate vaccine received market authorization in Europe. *Expert Rev Vaccines* 9:229-36.
- Reinert RR, Reinert S, van der Linden M, Cil MY, Al-Lahham A, Appelbaum P (2005). Antimicrobial susceptibility of *Streptococcus pneumoniae* in eight European countries from 2001 to 2003. *Antimicrob. Agents Chemother.* 49(7):2903-2913.
- Rosen JB, Thomas AR, Lexau CA et al (2011). Geographic variation in invasive pneumococcal disease following pneumococcal conjugate vaccine introduction in the United States. *Clin Infect. Dis.* 53:137-143.
- Shin J, Baek JY, Kim SH, Song JH, Ko KS (2011). Predominance of ST320 among *Streptococcus pneumoniae* serotype 19A isolates from 10 Asian countries. *J. Antimicrob. Chemother.* 66(5):1001-1004.
- Silberbauer EJ, Ismail N, Gottberg AV, Hoosen AA (2011). Serotype and antimicrobial profile distribution of invasive pneumococcal isolates in the pre-vaccine introduction era in Pretoria, South Africa, 2005 through 2009. *Diagn. Microbiol. Infect. Dis.* 71:309-311.
- Smaoui H, Amri J, Hajji N, Kechrid A (2009). Antimicrobial susceptibility and serotype distribution of *streptococcus pneumoniae* isolates in children in Tunis. *Archives de Pédiatrie* 16:220-226.
- Smati F, Laouar H, Khalifa F, Bentchouala C, Hacini A, Lezzar A (1994). Resistance of *Streptococcus pneumoniae* to penicillin. *Med. Mal. Infect.* 24:1190-2.
- Song JH, Jung SI, Ko KS, Kim NY, Son JS, Chang HH, Ki HK, Oh WS, Suh JY, Peck KR, Lee NY, Yang Y, Lu Q, Chongthaleong A, Chiu CH, Lalitha MK, Perera J, Yee TT, Kumarasinghe G, Jamal F, Kamarulzaman A, Parasakthi N, Van PH, Carlos C, So T, Ng TK, Shibl A (2004a). High prevalence of antimicrobial resistance among clinical *Streptococcus pneumoniae* isolates in Asia (an ASNORP study). *Antimicrob. Agents Chemother.* 48:2101-2107.
- Song JH, Chang HH, Suh JY, Ko KS, Jung SI, Oh WS et al (2004b). Macrolide resistance and genotypic characterization of *Streptococcus pneumoniae* in Asian countries: a study of the Asian network for surveillance of resistant pathogens (ANSORP). *J. Antimicrob. Chemother.* 53:457-63.
- Tali-Maamar H, Laliem R, Bentchouala C, Touati D, Sababou K, Azrou S et al (2012). Reprint of: Serotyping and antibiotic susceptibility of *Streptococcus pneumoniae* strains isolated in Algeria from 2001 to 2010. *Vaccine* 30(6):25-31.
- Thomas K, Mukkai KL, Veeraraghavan B et al (2013). Invasive pneumococcal disease associated with high case fatality in India. *J. Clin. Epidemiol.* 66:36-43.
- Tóthpál A, Laub K, Kardos S, Nagy K, Dobay O (2012). Changes in the serotypes of Hungarian pneumococci isolated mainly from invasive infections: a review of all available data between 1988 and 2011. *Acta Microbiol. Immunol. Hung.* 59(3):423-33.
- Tyrrell GJ, Lovgren M, Chui N, Minion J, Garg S, Kellner JD, Marrie TJ (2009). Serotypes and antimicrobial susceptibilities of invasive

- Streptococcus pneumoniae* pre- and post-seven valent pneumococcal conjugate vaccine introduction in Alberta, Canada, 2000-2006. *Vaccine* 27(27):3553-3560.
- van de Sande-Bruinsma N, Grundmann H, Verloo D, Tiemersma E, Monen J, Goossens H et al (2008). Antimicrobial drug use and resistance in Europe. *Emerg. Infect. Dis.* 14:1722-30.
- Van der Linden M, Weib S, Falkenhorst G, Siedler A, Imöhl M, von Kries R (2012). Four years of universal pneumococcal conjugate infant vaccination in Germany: impact on incidence of invasive pneumococcal disease and serotype distribution in children. *Vaccine* 30(40):5880-5885.
- Varon E (2012). Epidemiology of *Streptococcus pneumoniae*. *Med. Mal. Infect.* 42:361-365.
- Watson M, Brett M, Brown M, Stewart MG, Warren S (2007). Pneumococci responsible for invasive disease and discharging ears in children in Sydney, Australia. *J. Med. Microbiol.* 56:819-823.
- Whitney CG, Farley MM, Hadler J, Harrison LH, Lexau C, Reingold A et al (2000). Increasing prevalence of multidrug-resistant *Streptococcus pneumoniae* in the United States. *N. Engl. J. Med.* 343:1917-1924.
- Yang F, Xu XG, Yang MJ, Zhang YY, Klugman KP, McGee L (2008). Antimicrobial susceptibility and molecular epidemiology of *Streptococcus pneumoniae* isolated from Shanghai, China. *Int. J. Antimicrob. Agents* 32:386-91.
- Zhou L, Yu SJ, Gao W, Yao KH, Shen A, Yang Y (2011). Serotype distribution and antibiotic resistance of 140 pneumococcal isolates from pediatric patients with upper respiratory infections in Beijing, 2010. *Vaccine* 29:7704-7710.

Full Length Research Paper

Evaluation of faecal coliform levels in the discharges from the city of El Jadida, Morocco

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In order to assess the fecal coliform contents in the raw sewage from the city of El Jadida, the principal component analysis of these contents coupled with the physicochemical parameters of water (temperature, pH, electrical conductivity and total suspended solids) was carried out. The bacteriological analysis of samples taken at the level of collector SIDI DAWI of the city of El Jadida between May 2008 and June 2010 shows that the effluent were characterized by fecal coliforms contents ranging from 2.22×10^7 and 9.64×10^7 CFU/100 mL with an average of 6.38×10^7 CFU/100 mL. These values were far above the recommended norm by WHO for irrigation water which is 10^3 CFU/100 mL. Fecal coliforms have a positive and highly significant correlation with temperature, pH and total suspended solids. Also, poorly significant and negative correlations were observed with electrical conductivity. Among the physicochemical parameters studied, only the electrical conductivity appeared as the parameter to be monitored during the treatment of wastewater to reduce the load of fecal coliforms. A physical treatment is necessary especially for decantation followed by biodenitrification.

Key words: Wastewater, pollution, physicochemical parameters, fecal coliform, El Jadida, PCA.

INTRODUCTION

Raw sewage consists of all waters likely to contaminate the environments in which they are discharged. It contains pollutants and by-products of human use, either of domestic or industrial origin (Asano and Cotruvo, 2004). Sometimes, dirty wastewater drains into surface watersheds and into the sea (Hammer, 1986; Tchobanoglous and Burton, 2003). When this happens, the environment is as much at risk as people. The pathogens in raw sewage can contaminate ecosystems and thus may cause a health risk for humans and animals.

In Morocco, the state of the environment continues to

deteriorate due to the overexploitation of natural resources, air pollution, desertification, climate change and development of the industry (Salama et al., 2013).

In the context of limited water resources, wastewater in Morocco has not only a new limitation of the available resource but also an affront to human health and environmental quality in general.

Pollution by non-purified wastewater also affects the quality of sea water. The composition of wastewater from household can be extremely variable and depends on three factors, which are the original composition of drinking water, the various uses by individuals who can

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Figure 1. Geographical situation of the site of study.

provide a nearly infinite number of pollutants, and finally the users themselves who will reject the organic matter in wastewater (urine, feces) (Salama et al., 2013).

Other studies conducted by Lamghari (LamghariMoubarrad, 2005; LamghariMoubarrad and Assobhei, 2007) on the parasitological characterization of the wastewater from the city El Jadida in Morocco, the impact of partially treated wastewater on coastwater and sediments and on the infantile population of the discharge area showed that pollution detected in the effluent of wastewater as well as at the coast of El Jadida, is in fact a problem for the environment of the city. It is therefore necessary to take preventative measures to minimize this real danger: simple measures such as increasing public awareness about the contamination threats to more serious measures like the comprehensive treatment of wastewater before it is dumped into the ocean.

Our study aimed to evaluate faecal coliform levels in wastewater rejected on the coast from the city of El

Jadida between 2008 and 2010 by coupling with physicochemical parameters in Principal component analysis (PCA).

The physicochemical parameters studied are the main growth factors of fecal coliforms in an ecosystem and it is temperature, pH, electrical conductivity and total suspended solids.

MATERIALS AND METHODS

Studied zone

The city of El Jadida is the second industrial pole of Morocco and is located on the Atlantic coast of Morocco between Casablanca (90 km southwest of Casablanca) and Jorf Lasfar (one of the largest ports in Africa) (Figure 1). It covers the area of 2480 hectares, with latitude of 27 m and it has 4 urban districts (Chofqi, 2004).

In August, there was the famous Moussem of Moulay Abdellah Amghar, which attracts over one hundred thousand visitors. It is bounded in the north by the Atlantic Ocean, to the east by the rural commune Haouzia, to the south by the rural commune OueldHcine and to the west by the rural commune MlyAbdellah and the Atlantic



Figure 2. Sampling zone of wastewaters to SIDI DAOUI (El Jadida).

Ocean.

Wastewater sampling

The wastewater that is dumped into the sea comes from domestic origin or a mixture with industrial wastewaters (95 and 5%). Samples of wastewater were collected (one per month) and stored at 4°C (Figures 2 and 3).

Analytical Methods

Physico-chemical parameters

The pH and temperature of the wastewater samples were measured at the collection site. Electrical conductivity and total suspended solids, were analyzed in the laboratory according to the methods prescribed in AFNOR (French national organization for standardization) handbook (AFNOR, 1999).

Bacteriological parameters

The bacteriological analysis of the various samples of wastewater consisted of an enumeration of the indicator germs for fecal contamination (fecal coliforms, FC) (Salama et al., 2013).

Enumeration of fecal coliforms

Fecal coliforms or thermotolerant coliforms are a sub-group of total coliforms able to ferment lactose at a temperature of 44.5°C.

The most important species of this bacterial group is *Escherichia coli* (*E. coli*) and to a lesser extent some species of the genera *Citrobacter*, *Enterobacter* and *Klebsiella* (Elmund et al., 1999; Emmanuel et al., 2004).

The spatio-temporal evolution of the abundances of FC was assessed by counting the colonies on yellow-orange Tergitol agar and triphenyltetrazolium chloride (TTC - Tergitol 7). The inoculated Petri dishes were incubated at 44.5°C for 24 h (AFNOR, 2001).

Statistical study

Statistical analysis was based on Principal Component Analysis (PCA). PCA is a data analysis tool that helps to explain the structure of correlations or covariance using linear combinations of the original data. Its use helps to reduce and interpret data in a small scale (Lagarde, 1995; Mayo, 1995). Matrices intermediate correlations, correlations between variables and axes and projection of variables in space axes F1 and F2 were obtained with XLSTAT 2010 software.

For the treatment of data by PCA, we used the variables such as temperature, pH, electrical conductivity, total suspended solids and fecal coliform as individuals and 15 samples taken at the level of collector SIDI DAWI.

Table 2 shows the correlation coefficients between the variables and the first two axes F1 and F2. Figure 4 reveals to us the projection of variables in space axes F1 and F2. The correlation matrices between the variables studied are shown in Table 3.

RESULTS

Qualitative aspect of the effluent of collector SIDI DAOUI

Table 1 shows changing physicochemical parameters (temperature pH, electrical conductivity and total suspended solids) and bacteriological parameters (Fecal Coliforms) studied between May 2008 and June 2010.

The effluent temperature was between 25.1 and 29.2°C. The minimum and maximum pH values ranged from 6.1 to 6.9 respectively. The maximum value of the recorded electrical conductivity was 3.57 mS/cm and the minimum value was 1.52 mS/cm.

The total suspended solids oscillate between a maximum value of 860.4 mg/L and a minimum value of 403.5 mg/L. The bacteriological analysis showed that effluents were characterized by levels of Fecal Coliforms

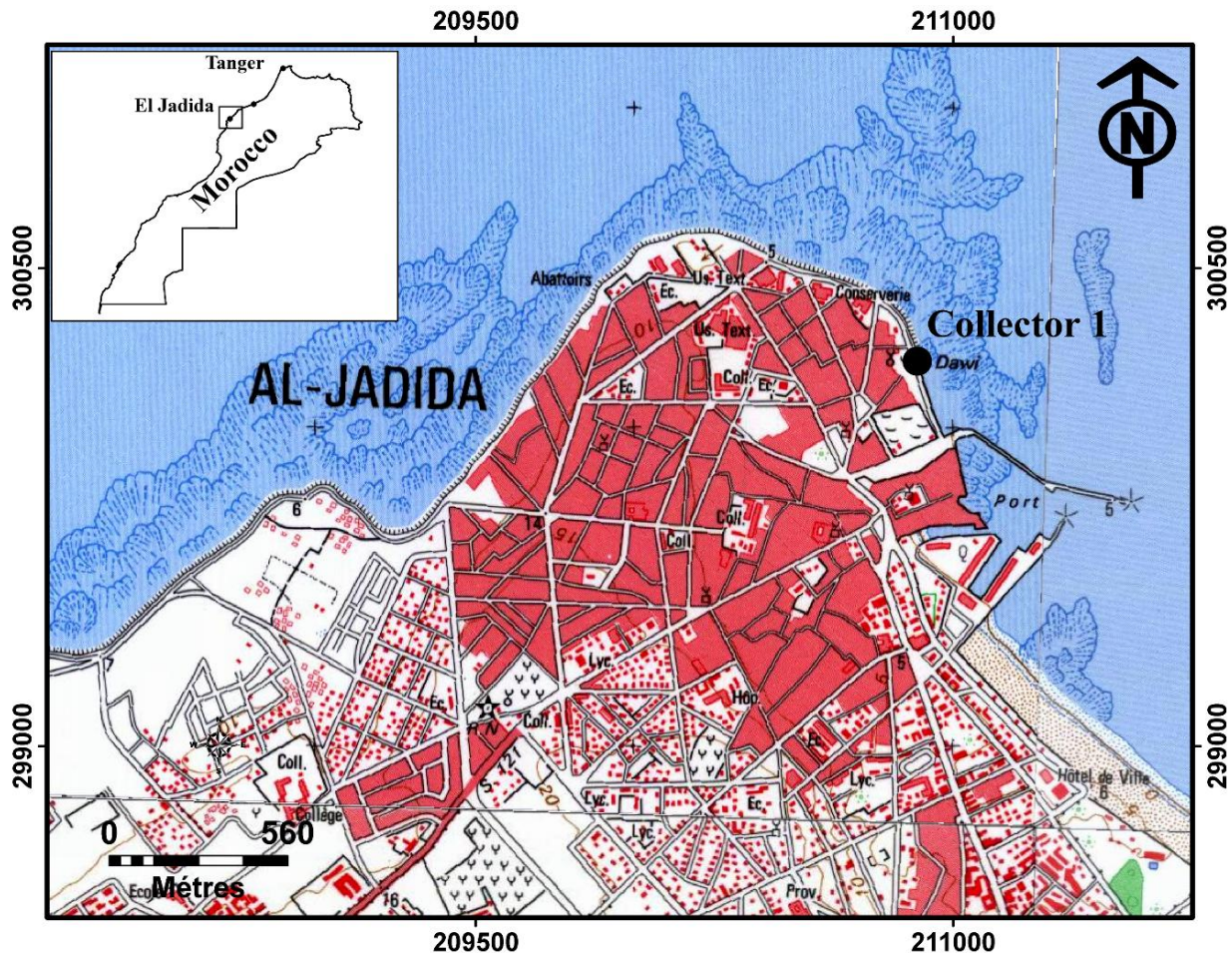


Figure 3. Location of sampling sites in study zone: Collector 1 (SIDI DAWI collector) (Salama et al., 2012).

Table 1. The average values of physicochemical and bacteriological parameters of raw wastewater in the collector SIDI DAWI.

Parameter	Maximum	Minimum	Average	Standard deviation
T (°C)	29.20	25.10	27.20	1.12
pH	6.90	6.10	6.30	0.25
EC (mS/cm)	3.57	1.52	2.13	0.83
TSS (mg/L)	860.40	403.50	677.61	143.82
FC (CFU/100 mL)	9.64×10^7	2.22×10^7	6.38×10^7	2.12×10^7

Table 2. Correlations between variables and the principal axes.

Parameter	F1	F2
T (°C)	0.860	-0.165
pH	0.735	0.432
EC	-0.386	0.895
TSS	0.922	0.103
FC	0.950	0.078

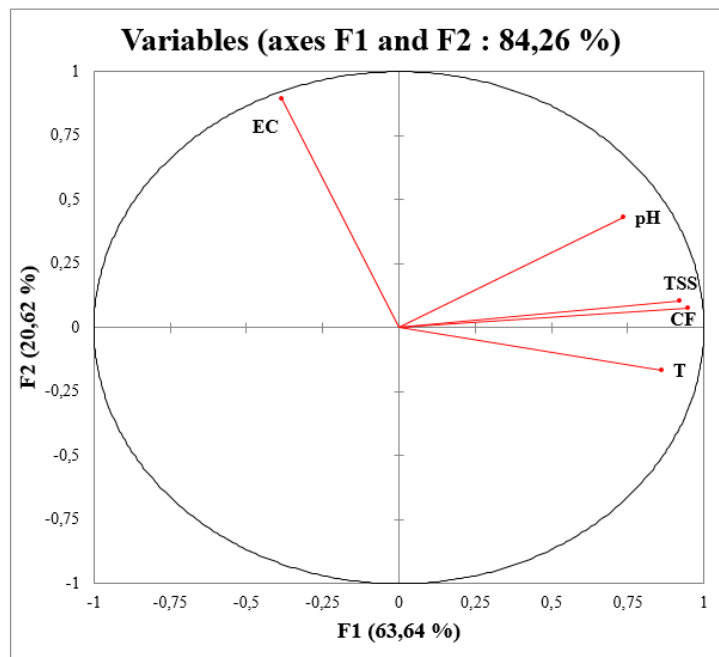


Figure 4. Projection of the variables in the space of the axes F1 and F2.

Table 3. The inter-elementary correlations matrix.

Variable	T(°C)	pH	EC	TSS	FC
T°C	1				
pH	0.592	1			
EC	-0.429	0.006	1		
TSS	0.660	0.564	-0.233	1	
FC	0.704	0.608	-0.276	0.990	1

ranging from 2.22×10^7 and 9.64×10^7 CFU/100 mL with an average of 6.38×10^7 CFU/100 mL.

DISCUSSION

The projection of the variables in the space of the axes F1 and F2 showed that parameters fitted at a probability of more than 84.26%. The probability of factorial design is acceptable (Athamena, 2006).

The axis F1 is formed by the temperature, pH, total suspended solids and fecal coliform. The axis F2 is formed by electrical conductivity (Table 2 and Figure 4).

The axis F1 corresponds to a disorder effluent (concentrations of TSS are very high) due to the degradation of organic matter. The latter are considered as vectors of pollution because many pollutants, including heavy metals are absorbed by these particles.

A highly significant positive correlation was observed

between fecal coliform, temperature and pH (Table 3). The temperature and pH had a very important role in the solubility of the salts and especially gas.

The recorded temperatures at the effluent were below 30 (JORA, 1993) and 35°C considered as the limit value of direct discharge into the receiving environment (MEMEE, 2002).

These results confirm the work of Mayo (1995) who showed that the increase in pH affected the abundance of fecal coliforms that is to say, the basic pH led to a net decrease in the survival of fecal coliforms.

Another highly significant positive correlation was observed between fecal coliform and TSS (Table 3). The TSS takes into account all the colloidal materials, insoluble minerals or organic solids. Suspended particles included silt, clay, organic matter, inorganic fine particles and microorganisms (Santé Canada, 2003; Schnitzer and Kahn, 1972).

The most important effect of TSS, related to health is

probably its ability to protect bacteria and virus against disinfection solutions or heat, if exposed a too short time (Hudson, 1962). The microbial load of fecal coliforms is very important at the level of the effluents of the collector SIDI DAWI. This bacterial load exceeded that recommended as maximum by WHO for irrigation water, which is of the order 10^3 CFU/100 mL (WHO, 1986).

A negative and weakly significant correlation was observed between fecal coliforms and electrical conductivity (Table 3). The electrical conductivity is probably one of the simplest and most important for the quality control of wastewater. It reflects the overall degree of mineralization and it provides information on the salinity. These results confirm the work of Chedad and Assobhi (2007) which showed that salinity is a factor causing stress which undergoes bacteria of fecal pollution in the salty middle, where the bacteria must establish the osmotic balance between the external middle and its cytoplasm.

Conclusion

The bacteriological analyses show that the effluent collector of the city of El Jadida is characterized by levels of fecal coliforms ranging from 2.2×10^7 to 9.64×10^7 CFU/100 mL. The statistical results show that fecal coliform have a positive and highly significant correlation with temperature, pH and TSS, and conversely negative and weakly significant correlations with the electrical conductivity.

Among the physical parameters studied, only conductivity proves a convincing parameter in wastewater treatment to reduce the load of fecal coliform. In the field of quality control of water, the conductivity measurement proves a compelling setting for the treatment of wastewater. Use wastewater for agricultural purposes also reduces the pressure on the environment, but there are factors that must be considered, including the presence of pathogens. The treatment of raw sewage is essential to limit the potential impacts of such activities on the environment and the health of producers and consumers.

REFERENCES

- AFNOR (French Association of Standardisation) (1999). Techniques, the quality of the water, French Association for Standardization. Collection of French standards. Standard Test Methods for Water. Paris, France.
- AFNOR (French Association of Standardisation) (2001). Standard Test Methods for Water. In :Collection of French standards (6th edition). Defense, Paris.
- Asano T, Cotruvo JA (2004). Groundwater recharge with reclaimed municipal wastewater: health and regulatory considerations. *Water Res.* 38:1941-1951.
- Athamena M (2006). Study of thermal resources of all immigrant south Setif, Magister Thesis, Faculty of Engineering Sciences, University of Batna. p. 131.
- Chedad K, Assobhi O (2007). Study of survival of bacteria of fecal contamination (fecal coliforms) in the waters of the oyster lagoon area of Oualidia (Morocco), *Bulletin of the Scientific Institute of Rabat, Section Life Sciences.* 29-79.
- Chofqi A (2004). Highlighting the mechanisms of contamination of groundwater by leachate from uncontrolled discharge (El Jadida Morocco):Geology, hydrogeology, Geoelectric, Geochemistry and Epidemiology. Thesis Univ. El Jadida, Morocco. p. 250.
- Elmund GK, Allen MJ, Rice EW (1999). Comparison of Escherichia coli, total coliform and fecal coliform populations as indicators of wastewater treatment efficiency. *Water Environ. Res.* 71:332-339.
- Emmanuel E, Théléys K, Mompoin M, Blanchard JM, Perrodin Y (2004). Evaluation of environmental hazards associated with the discharge of urban wastewater in the Bay of Port-au-Prince, Haiti Submitted: Book "Water and Environment" of the "Environmental Law" Network of the University Agency of the Francophony(AUF). Port-au-Prince. p. 15.
- Hammer MJ (1986). Water and wastewater technology, United States.
- Hudson HE (1962). High-quality water production and viral disease. *J. Am. Water Works Assoc.* 54:1265.
- JORA (Official Journal of the Republic of Algeria) (1993). Discharge standards in the receiving environment. 46:7-12.
- Lagarde J (1995). Initiation into the data analysis. Ed. Dunod. Paris, 157.
- LamghariMoubarrad FZ (2005). Parasitological characterization of wastewater of El Jadida, their impact on the coast (water and sediment) and infant population of the discharge area. State thesis, Faculty of Sciences, El Jadida, Morocco. p. 200.
- LamghariMoubarrad FZ, Assobhei O (2007). Health risks of raw sewage with particular reference to Ascaris in the discharge zone of El Jadida (Morocco). *Desalination* 215(1): 120-126.
- Mayo AW (1995). Modelling coliform mortality in waste stabilization ponds. *J. Environ. Eng.* 121(2):140-152.
- MEMEE (Minister of the Energy, Mines, Water and Environment) (2002). « Moroccan standards, Official Bulletin of Morocco », N° 5062 du 30 Ramadan 1423. Rabat.
- Salama Y, Chennaoui M, Mountadar M, Rihani M, and Assobhei O (2013). The physicochemical and bacteriological quality and environmental risks of raw sewage rejected in the coast of the city of el Jadida (Morocco). *Carpath. J. Earth Environ. Sci.* 8(2):39-48.
- Salama Y, Mountadar M, Rihani M, Assobhei O (2012). Evaluation physicochemical and bacteriological of raw sewage from the city of El Jadida (Morocco). *Science Lib.* 4(120906).
- Santé Canada (2003). Recommendations for the quality of drinking water in Canada turbidity. Supporting documentation. Prepared by the Federal-Provincial-Territorial Committee on Drinking Water, Ottawa, Ontario. p.36.
- Schnitzer M, Kahn SH (1972). Humic substances in the environment, Marcel Dekker, New York, N.Y. p. 204.
- Tchobanoglous G, Burton FL (2003). Wastewater engineering treatment and reuse. Boston: McGraw-Hill. graywater summer. 15:7-68.
- WHO (1986). Quality guidelines for drinking water: health and supporting documentation Geneva criteria. 2 Edition.

Full Length Research Paper

Diversity and biopotential of endophytic actinomycetes from three medicinal plants in India

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Three medicinal plants, *Aloe vera*, *Mentha arvensis* and *Ocimum sanctum* were explored for endophytic actinomycetes diversity, plant growth promoting and antimicrobial activity. Endophytic actinomycetes were most commonly recovered from roots (70% of all isolates) followed by stems (17.5%) and leaves (12.5%), respectively. Single genus *Streptomyces* ranked first (60% of all isolates) followed by *Micromonospora* (25%), *Actinopolyspora* (7.5%), and *Saccharopolyspora* (7.5%). The highest numbers of endophytic actinomycetes were isolated from *Ocimum sanctum* (45%). Out of 22 isolates tested, 12 showed the ability to solubilize phosphate in the range of 5.4-16.5 mg/100 ml, while 16 isolates produced indole-3 acetic acid (IAA) ranging between 8.3-38.8 µg/ml. Nine isolates produced the amount of hydroxamate-type of siderophore ranging between 5.9-64.9 µg/ml and only four isolates were able to produce catechol-type of siderophore in the range of 11.2-23.1 µg/ml. Of the nine, interestingly, eight endophytic actinomycetes (88.9%) showed a significant antagonistic activity against one or more phytopathogenic fungi indicating their possible role as plant biocontrol agents. An extended infection of root tissues of *Ocimum sanctum* by *Saccharopolyspora* O-9 was observed using transmission electron microscope (TEM).

Key words: Endophytic actinomycetes, antifungal activity, indole-3-acetic acid, medicinal plants, phytopathogenic fungi, siderophores.

INTRODUCTION

Actinomycetes represent a high proportion of the soil microbial biomass and have the potential to produce a diverse range of secondary metabolites including various antibiotics, anticancer and immunosuppressive agents and plant growth hormones (Strobel and Daisy, 2003; Fiedler et al., 2008; Schulz et al., 2009) that play an important role in agriculture and pharmaceutical industry. Development of pathogen resistance against existing medicines and emergence of new diseases compelled the search for novel secondary metabolites (Strobel and Daisy, 2003). Endophytic actinomycetes are the microbes

that reside in healthy tissues of living plants without causing clinically detectable symptoms of disease and can be isolated from the surface-sterilized plant tissues (Nimnoi and Pongslip, 2009).

Endophytic actinomycetes have attracted attention in the search for novel bioactive natural compounds that can be used as new drugs replacing those against which pathogenic strains have rapidly acquired resistance. The association of actinomycetes with plants is found to confer many advantages such as the production of antimicrobials, extracellular enzymes, phytohormones and

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siderophores. They also help in phosphate solubilization and plant protection against abiotic and biotic stresses (Bailey et al., 2006; Clegg and Murray, 2000). Endophytic actinomycetes have been isolated from stem and root interior of many plants, such as snakevine, tomato, banana, neem, wheat, etc (Castillo et al., 2002; Cao et al., 2004, 2005; Verma et al., 2009; Coombs and Franco, 2003). The endophytic *Streptomyces* NRRL 30562 obtained from the snakevine produced novel peptide antibiotic with wide-spectrum activity against many pathogenic fungi and bacteria (Castillo et al., 2002). Endophytic actinomycetes are considered to be potential biocontrol agents as they can colonize the interior of the host plant avoiding competition by other microbes in the soil. *Streptomyces* S30 isolate was found to be an effective biocontrol agent to the root pathogen *Rhizoctonia solani* (Cao et al., 2004; Kunoh, 2002). Many actinomycetes isolates of *Azadirachta indica* were strong inhibitors of potential filamentous fungal pathogens *Pythium* spp., and *Phytophthora* spp. (Verma et al., 2009). Many endophytic *Streptomyces* sp. can enhance crop yield through protection of their host against pathogens as in the case of 'take all disease' of wheat (Coombs, 2002; Coombs and Franco, 2003).

Actinomycetes are known for their ability to promote plant growth by producing indole-3-acetic acid (IAA) to help root development or by producing siderophore to bind Fe^{3+} from the environment and help to improve nutrient uptake (Leong, 1986). Medically important herbs and ethnopharmacologically used plants are wide spread in the Indian subcontinent. However, to the best of our knowledge, there are very few recent reports regarding the microbiological studies on the endophytic actinomycetes residing in the medicinal plants. Thus, this habitat deserves close examination for potential and novel microbes that could produce compounds with desired bioactivities. The present study was undertaken to isolate endophytic actinomycetes from selected medicinal plants and evaluate their potential for plant growth promoting and antimicrobial activity.

MATERIALS AND METHODS

Sampling

Thirty six healthy plants of *Aloe vera*, *Mentha arvensis* and *Ocimum sanctum* were randomly selected from herbal garden, Punjab Agricultural University, Ludhiana district (30.9° North and 75.85° East; Elevation, 247 m) of northern India. These plants species have medicinal value due to their rich contents of functional flavonoids, anthraquinones, saponins, sterols, phenols and essential oils. The samples were dug out carefully to ensure that maximal amount of root, shoot and leaf materials were collected. Samples were placed in plastic bags and brought to the laboratory in an ice-box and used to screen actinomycetes within 48 h of collection.

Surface sterilization and isolation

The root, stem and leaf segments were washed in running tap water

to remove adhered epiphytes and soil debris. After drying in sterile conditions, tissue surfaces were sterilized by using 70% (v/v) ethanol for 5 min and sodium hypochlorite solution (0.9% w/v available chlorine) for 20 min. Surface-sterilized tissues were washed thrice in sterile distilled water. In order to reduce the opportunity for emergence of endophytic fungi from the tissue, the samples were soaked in 10% $NaHCO_3$ solution for 10 min to disrupt the growth of the fungi. Each root, shoot and leaf were cut into small pieces (0.5-1.0 cm) and placed on tryptic soy agar (TSA) medium and incubated at 28°C for 20-25 days to record the microbial growth. Effectiveness of surface sterilization was tested by the method of Schulz et al. (1993).

Identification of actinomycetes

Cultural and morphological characteristics, including presence of aerial mycelia, spore mass color, distinctive reverse colony color, color of diffusible pigments and spore chain morphology were used as identification characters (Cao et al., 2005). Visual observation of both morphological and microscopic characteristics using light microscopy and Gram-stain properties were also performed. All morphological characters were observed on TSA and the criteria used for classification and differentiation was as follows: (i) Aerial mass color: The mass color of mature sporulating aerial mycelium was observed following growth on TSA plates. The aerial mass was classified according to Bergey's manual of systematic bacteriology. (ii) Substrate mycelium: Distinctive colors of the substrate mycelium were recorded. (iii) Diffusible pigments: The production of diffusible pigment was also considered. (iv) Spores chain morphology: The shape of the spore chains observed under light microscope was also used as an important step in the identification. (v) Biochemical screening: Physiological criteria such as ability to degrade casein, starch, esculin, Tween 80, tyrosine, xanthine and hypoxanthine as substrates by the various actinomycetes strains were also used for genus confirmation.

Plant growth promoting traits of endophytic actinomycetes isolates

Phosphate solubilization

Endophytic actinomycetes isolates were inoculated on Pikovskaya medium supplemented with tricalcium phosphate and incubated at 28°C for seven days. The halo zone around the colony was presumptive confirmation of phosphate solubilization. Quantitative estimation of phosphate solubilization in broth was carried out using Erlenmeyer flasks (250-ml) containing 50 ml of Pikovskaya medium. The selected isolates were grown in liquid Pikovskaya medium for 7 days for the analysis of released phosphate content in the culture filtrate. The total amount of phosphate solubilized by actinomycetes isolates was estimated by Jackson (1973) method.

Indole acetic acid (IAA) production

The production of IAA by 40 actinomycetes isolates was determined according to the method of Gordon and Weber (1951). The isolates were grown on yeast malt extract agar at 28°C for 5 days. Eight millimeter diameter agar discs were inoculated into 100 ml of yeast malt extract broth containing 0.2% L-tryptophan and incubated at 28°C with shaking at 125 rpm for seven days. Cultures were centrifuged at 11,000 rpm for 15 min. One milliliter of the supernatant was mixed with 2 ml of the Salkowski reagent. Development of pink color indicated IAA production. Optical density (OD) was taken at 530 nm using a spectrophotometer (ELICO, UV-VIS Spectrophotometer- SL 159). The level of IAA produced was

estimated by comparison with an IAA standard.

Siderophores production

The endophytic actinomycetes isolates were inoculated on chrome azurol S (CAS) agar medium and incubated at 28°C for five days (Schwyn and Neilands, 1987). The colonies with orange zones were considered as siderophore producing isolates. The active isolates (width of orange zone on CAS plate > 20 mm) were cultured on glycerol yeast broth and incubated at 28°C with shaking at 125 rpm for 10 days. Catechol-type siderophores were estimated by Arnow (1937) method and hydroxamate siderophores were estimated by the Csaky (1948) test.

In vitro antagonistic assay

The endophytic actinomycetes isolates were evaluated for their antagonistic activity against nine pathogenic fungi *Aspergillus niger*, *Aspergillus flavus*, *Alternaria brassicicola*, *Botrytis cinerea*, *Fusarium oxysporum*, *Penicillium digitatum*, *Penicillium pinophilum*, *Phytophthora drechsleri* and *Colletotrichum falcatum* by dual-culture *in vitro* assay. Fungal discs (8 mm in diameter), 5 days old on PDA at 28°C were placed at the center of PDA plates. Two actinomycete discs (8 mm) 5 days old, grown on yeast malt extract agar were incubated at 28°C placed on opposite sides of the plates, 3 cm away from fungal disc. Plates without the actinomycete disc served as controls. All the plates were incubated at 28°C for 14 days and the colony growth inhibition (%) was calculated by using the formula, $C - T/C \times 100$, where C is the colony growth of pathogen in control and T is the colony growth of pathogen in dual culture. The zone of inhibition was measured between the pathogen and actinomycete isolates (Khamna et al., 2009).

Transmission electron microscopy (TEM)

Electron microscopy (TEM) of *Saccharopolyspora* O-9 inoculated root sample of *Ocimum sanctum* was carried out according to the method by Wescott et al. (1987). *Saccharopolyspora* O-9 was grown on plates containing TSA medium and incubated at 28°C for 7 to 10 days until luxuriant sporulation had occurred. Spores were harvested by scraping them off from the plate with a sterile loop and suspending them in 2 ml sterile H₂O. Approximately 30 *Ocimum sanctum* seeds were placed in a sterile Petri dish and treated separately with 2 ml of the actinomycete spore suspensions and water as a control. After mixing the seeds well in the spore suspensions, the Petri dish was left on an angle in the laminar flow cabinet overnight to evaporate the water and coat the spores onto the seeds. The seeds were grown in pots with approximately 110-120 g of sterilized field soil per pot and seeds were sown at a depth of 1 cm. Plants were grown for six weeks in a glasshouse with watering as required. Roots were then harvested and immersed in water so that adhering soil could be removed with a fine sable-hair brush. Pieces of roots (about 1 cm long) were selected and fixed for 24 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C. The tissue was rinsed in same buffer three times and then fixed in 2% (w/v) osmium tetroxide for 1 h. After three rinses in buffer, the tissues were dehydrated via a graded alcohol series and treated with propylene oxide. Infiltration with spur's resin mix was performed for two days using graded resin prepared in propylene oxide and cured for 16 h at 60°C. Thin sections (70-90nm) were cut on Leica ultra microtome and examined after staining with 2% uranyl acetate with transmission electron microscope (Hitachi H-7650, Tokyo, Japan).

RESULTS AND DISCUSSION

Isolation and identification of endophytic actinomycetes

A total of 40 isolates of endophytic actinomycetes were isolated from the root, stem and leaf tissues of three medicinal plants of India, *A. vera*, *M. arvensis* and *O. sanctum*. Most of the actinomycetes were identified as *Streptomyces* sp., by morphological characteristics, which was consistent with the other reports from different hosts (Coombs and Franco, 2003; Cao et al., 2004; Verma et al., 2009; Shenpagam et al., 2012). Out of the 40 isolates, the majority (70%) was recorded from roots followed by stems (17.5%) and leaves (12.5%) respectively.

Kafur and Khan (2011) isolated only one actino-mycete from leaf samples of *M. arvensis*. *Streptomyces* was the dominant genus (n=24, 60% of isolates) followed by *Micromonospora* sp. (n=10, 25%), *Actinopolyspora* sp. (n=3, 7.5%) and *Saccharopolyspora* sp. (n=3, 7.5%). Shenpagam et al. (2012) also isolated *Streptomyces* spp. from leaf and root samples of *O. sanctum*. The isolates were obtained most frequently from roots and less frequently from terrestrial parts, and this may be because of prevalence of actinomycetes in rhizospheric soil (Sardi et al., 1992). Based on colony and cultural characteristics, various subgroups were identified and among *Streptomyces* sp., the subgroup *Streptomyces cinereus* (n=4) was most frequently isolated followed by *Streptomyces albosporus*, *Streptomyces aureus*, *Streptomyces griseofuscus*, *Streptomyces roseosporus*, *S. viridis* (n=3 for each), respectively. *S. albosporus* and *S. griseofuscus* were obtained from the roots of all the three medicinal plants (Table 1).

The results also revealed that the surface treatment was adequate for the isolation of endophytic actinomycetes, as surface sterilized imprinted Petri plate (control) did not produce any growth. Thus, all the actinomycetes recorded in this experiment must have been endophytic and not the epiphytic.

Phosphate solubilization

Out of 40 isolates, twelve (30%) were observed to solubilize phosphate as they formed a clear zone around the colony on Pikovskaya medium. The amount of phosphate solubilized by actinomycete isolates obtained from medicinal plants fall in the range from 4.2 to 16.5 mg/100 ml (Table 2). The maximum amount of phosphate solubilization was shown by *S. albosporus* A4 (16.5 mg/100 ml). These results are in accordance with some earlier report (Hamdali et al., 2008) where high amount of phosphate solubilizing activity by *Streptomyces cavourensis* (83.3 mg/100 ml) followed by *Streptomyces griseus* (58.9 mg/100 ml) and *Micromonospora aurantiaca* (39 mg/100

Table 1. Genera and number of endophytic isolates of actinomycetes recovered from root, stem and leaf tissues of *A. vera*, *M. arvensis* and *O. sanctum*.

Actinomycetes group	No. of endophytic actinomycetes isolated from								
	<i>Aloe vera</i>			<i>Ocimum sanctum</i>			<i>Mentha arvensis</i>		
	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf
<i>Streptomyces albosporus</i>	1	0	0	1	0	0	1	0	0
<i>S. aureus</i>	0	0	0	1	0	0	1	0	1
<i>S. cinereus</i>	1	0	0	1	1	0	0	0	1
<i>S. globisporus</i>	0	0	0	1	0	0	0	1	0
<i>S. griseofuscus</i>	1	0	0	1	0	0	1	0	0
<i>S. roseosporus</i>	0	0	0	1	0	0	0	1	1
<i>S. viridis</i>	1	0	0	1	0	0	0	1	0
<i>S. griseorubroviolaceus</i>	0	0	0	1	1	0	1	0	0
<i>Actinopolyspora</i> spp.	0	0	0	1	0	0	2	0	0
<i>Micromonospora</i> spp.	1	0	0	2	1	1	3	1	1
<i>Saccharopolyspora</i> spp.	1	0	0	1	0	0	1	0	0
Total	6	0	0	12	3	1	10	4	4

Table 2. Plant growth promoting traits of endophytic actinomycetes isolated from medicinal plants.

Isolate	IAA production ($\mu\text{g/ml}$)	Phosphate solubilization ($\text{mg}/100 \text{ ml}$)
<i>Saccharopolyspora</i> A1	-	7.5
<i>Streptomyces viridis</i> A3	-	15.1
<i>S. albosporus</i> A4	22.4	16.5
<i>S. cinereus</i> A6	15.6	5.4
<i>S. griseofuscus</i> A8	-	12.5
<i>Micromonospora</i> A9	9.1	-
<i>Streptomyces roseosporus</i> M1	30.2	5.8
<i>S. griseofuscus</i> M2	34.5	-
<i>S. cinereus</i> M5	8.3	-
<i>S. albosporus</i> M7	18.8	8.4
<i>Saccharopolyspora</i> M13	11.1	-
<i>Streptomyces cinereus</i> O1	20.1	13.8
<i>S. aureus</i> O2	35.4	-
<i>Micromonospora</i> O6	9.0	4.2
<i>Saccharopolyspora</i> O9	17.2	14.9
<i>Streptomyces albosporus</i> O11	18.8	11.0
<i>S. globisporus</i> O13	10.7	-
<i>Micromonospora</i> O14	-	11.6
<i>Streptomyces viridis</i> O15	15.7	-
<i>S. griseorubroviolaceus</i> O17	38.8	-
<i>S. griseofuscus</i> O18	14.2	-
<i>S. aureus</i> O20	19.6	-
CD@5%	0.97	0.61

ml) was reported. Microbial solubilization of mineral phosphate might be either due to the acidification of external medium or the production of chelating substances that increase phosphate solubilization (Welch et al., 2002; Whitelaw, 1999). Hence, P-solubilizing actinomycetes play an important role in the improvement of plant

growth.

Indole acetic acid production

Eighteen (45%) out of 40 isolates produced the phyto-

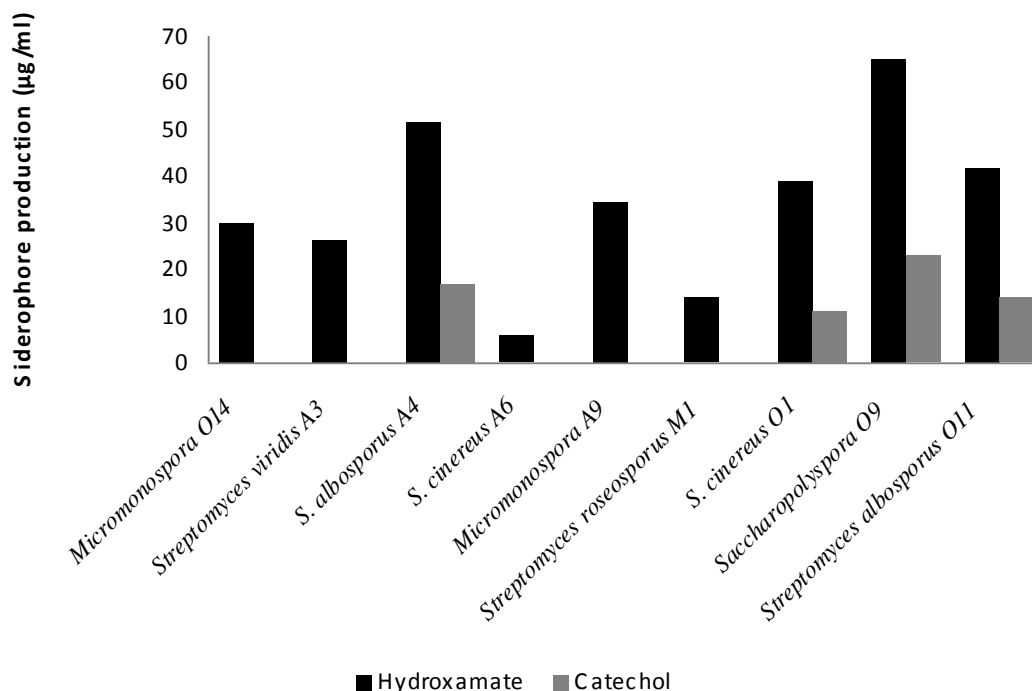


Figure 1. Siderophores production by different actinomycete isolates.

hormone indole acetic acid (IAA) and 14 of these belonged to *Streptomyces* sp. The range of IAA production was 9.0 - 38.8 µg/ml. The maximum IAA was produced by *S. griseorubroviolaceus* O-17. While *S. cinereus* M5 isolate produced the minimum yield of IAA (Table 2). Nimnoi et al. (2010) isolated endophytic actinomycetes from *Aquilaria crassna* and found that *Nocardiajiangxiensis* produced maximum IAA (15.14 µg/ml) whereas *Actinomadura glauciflava* produced minimum yield of IAA (9.85 µg/ml). The maximum IAA production was found to be greater in our study as compared to that reported by Nimnoi and Pongslip (2009). Soil and rhizosphere actinomycetes have shown potential to produce IAA and promoted the plant growth. Khamna et al. (2009) reported most active strains from the rhizospheres of *Cymbopogon citratus* and *Cymbopogon mangga*. *Streptomyces* CMU-HOO9 from *C. citratus* rhizosphere soil showed high ability to produce IAA. It is possible that high levels of tryptophan will be present in root exudates of medicinal plants and enhance IAA biosynthesis. Nimnoi and Pongslip (2009) demonstrated that the isolates of IAA synthetic bacteria enhanced root and shoot development of *Raphanus sativus* and *Brassica oleracea* more than fivefold when compared with the control. The IAA producing actinomycetes such as *Micromonospora*, *Nocardia*, *Actinomadura* and *Streptosporangium* have been reported to increase dry weight of corn, cucumbers, tomato, sorghum and carrot (El-Tarabily et al., 1997; Mishra et al., 1987). The presence of endophytic actinomycetes that produce IAA may have an important role in plant

growth.

Siderophore production

Siderophore production was detected in nine actinomycetes isolates. The active isolates grew on CAS agar and an orange halo formed around the colonies. Most of them were *Streptomyces* sp. and *Saccharopolyspora* O-9 isolated from *Ocimum sanctum* showing high siderophore production ability. The 8 isolate *Saccharopolyspora* O-9 produced catechol (23.1 µg/ml) and hydroxamate (64.9 µg/ml) on glycerol yeast broth (Figure 1). This finding is in agreement with that obtained by Nimnoi et al. (2010) that eight endophytic actinomycetes isolates produced hydroxamate type of siderophore in the range of 3.21-39.40 µg/ml and one isolate produced catechol type siderophore (4.12 µg/ml). Khamna et al. (2009) showed that *Streptomyces* CMU-SK 126 isolated from *Curcuma mangga* rhizospheric soil exhibited high ability for siderophore production and produced catechol type (16.19 µg/ml) as well as hydroxamate type (54.9 µg/ml) siderophores. Siderophores are produced by various soil microbes to bind Fe³⁺ from the environment, transport it back to microbial cell and make it available for growth (Neilands and Leong, 1986; Mishra et al., 1987). Microbial siderophores may also be utilized by plants as an iron source (Bar-Ness et al., 1991; Wang et al., 1993; Robinson et al., 2002; Schulz et al., 2009). The role of siderophores produced by rhizospheric or endo-

Table 3. Antagonistic activity of endophytic actinomycetes isolated from medicinal plants against fungal plant pathogens.

Actinomycete isolates	Percentage inhibition (%) ^a								
	1	2	3	4	5	6	7	8	9
<i>Micromonospora</i> O14	0	0	0	62.2 ± 0.1	19.5 ± 0.1	12.6 ± 0.1	0	16 ± 0.1	16.1 ± 0.1
<i>Streptomyces viridis</i> A3	0	0	0	39.6 ± 0.2	22.8 ± 0.1	45.8 ± 0.1	0	0	17.4 ± 0.1
<i>S. albosporus</i> A4	11.4 ± 0.2	14.5 ± 0.1	0	63.5 ± 0.1	60.6 ± 0.1	19.8 ± 0.2	0	69.3 ± 0.3	57.9 ± 0.1
<i>S. cinereus</i> A6	0	0	0	42.4 ± 0.08	0	17.6 ± 0.1	34.5 ± 0.2	0	0
<i>Micromonospora</i> A9	0	0	0	40.3 ± 0.2	0	48.5 ± 0.2	0	13.9 ± 0.1	0
<i>Streptomyces cinereus</i> O1	0	0	0	25.3 ± 0.1	15.2 ± 0.1	0	0	0	42.6 ± 0.1
<i>Saccharopolyspora</i> O9	13.2 ± 0.2	28.6 ± 0.1	26.5 ± 0.2	71.4 ± 0.2	30.5 ± 0.1	51.4 ± 0.1	17.8 ± 0.1	56.4 ± 0.1	49.9 ± 0.1
<i>Streptomyces albosporus</i> O11	16.7 ± 0.1	17.5 ± 0.2	0	44.6 ± 0.2	18.6 ± 0.1	59.5 ± 0.2	0	53.5 ± 0.2	53.4 ± 0.2

^aMean ± standard error from triplicate samples. 1, *Aspergillus niger*; 2, *C. falcatum*; 3, *Aspergillus flavus*; 4, *Alternaria brassicicola*; 5, *Penicillium digitatum*; 6, *Fusarium oxysporum*; 7, *Penicillium Pinophilum*; 8, *Phytophthora drechsleri*; 9, *Botrytis ciner*.

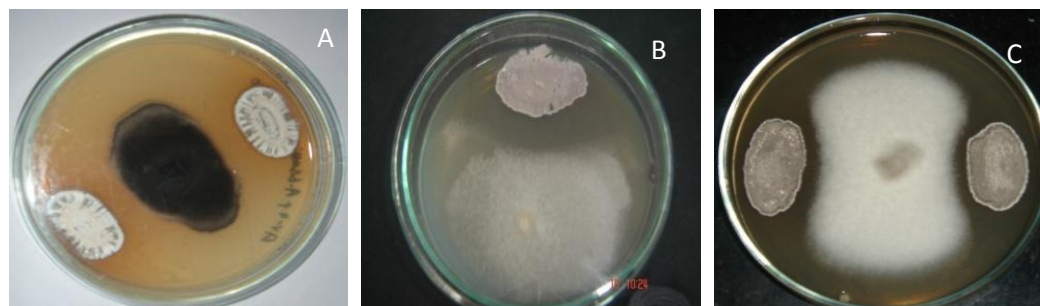


Figure 2. Antagonism of *Saccharopolyspora* O9 against (A) *Alternaria brassicicola* (B) *Botrytis cinerea* (C) *Fusarium oxysporum*.

phytic microbes has drawn more attention, for the metabolites that may be involved in promoting the growth substances and antagonise the phytopathogen (Bailey et al., 2006).

Antifungal activities

A total of eight endophytic actinomycetes isolates

have strong inhibitory activity against *Alternaria brassicicola* and *Fusarium oxysporum* (Table 3). *Saccharopolyspora* O-9 from *O. sanctum* strongly inhibited all the pathogenic fungi, and maximum percent inhibition was observed against *A. brassicicola* (71.4%). Many isolates of endophytic *Streptomyces* exhibited antagonism against either one or more than one tested phytopathogens. *Streptomyce albosporus* O-11

and *S. albosporus* A-4 antagonized all the tested fungi except *A. flavus* and *P. pinophilum* (Figure 2). Taechowisan and Lumyong (2003) isolated 59 endophytic actinomycetes from roots of *Zinger officinale* and *Alpinia galanga* and tested against *Candida albicans* and phytopathogenic fungi. *Streptomyces aureofaciens* CMUAc130 was the most effective in antifungal activity. Biocontrol effects of endophytic actinomycetes both *in vitro*

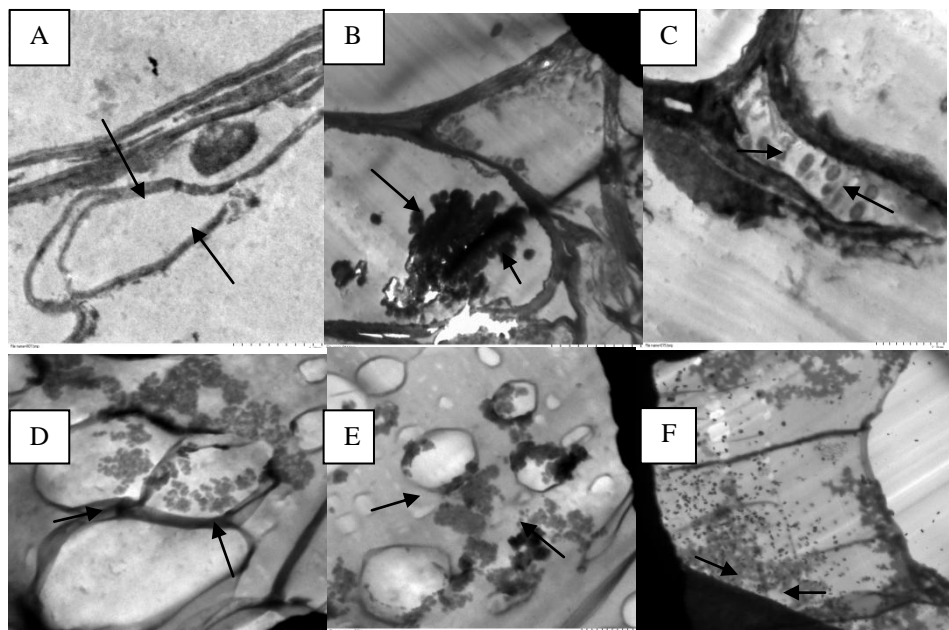


Figure 3. Electron micrographs of *Ocimum sanctum* roots infected with *Saccharopolyspora* O-9. A-C: Infection into middle lamella; D-E: hypervacuolation and vesicle formation; F: presence of spores inside the cell.

and *planta* have been reported. In another study, thirty eight strains of endophytic actinomycetes isolated from surface sterilized wheat and barley roots were tested for their antagonistic activity to wheat root pathogens *Gaeumannomyces graminis*, *Rhizoctonia solani* and *Pythium* sp. It was observed that 17 of 38 isolates displayed statistically significant activity *in planta* against *G. garminis* (Coombs et al., 2004). Zhao et al. (2011) interestingly reported that 59 out of 60 strains showed antagonistic activity against at least one of the 11 indicator organisms. Thirty eight *Streptomyces* isolates inhibited the growth of at least five indicator organisms. SAUK6015, a strain most similar to *Nonomuraea roseola*, was a very potent inhibitor of pathogens *Exerohilum turcicum* and *Curvularia lunata*, whereas SAUK6030, a strain most similar to *Micromonospora chokoriensis* was a potent inhibitor of *Curvularia lunata* (Zhao et al., 2011). The medicinal plants host numerous *Streptomyces* strains expected to produce a wide variety of bioactive metabolites. The antagonistic activity displayed in this study further indicates that endophytic *Streptomyces* hosted by medicinal plants are a key source of bioactive compounds. The ability of isolates to inhibit the growth of fungal pathogens is implication of the diffusible secondary metabolites secreted by actinomycetes.

Transmission electron microscopy of *Saccharopolyspora* O-9 inoculated *O. sanctum*

Transmission electron micrographs depicted an extended

infection of the root tissues of *O. sanctum* by *Saccharopolyspora* O-9. The infection had been observed beneath the cell membrane as well as deep inside the cell. An elaborate vacuole and vesicle formation has also been observed in the infected tissue. The hyphae of *Saccharopolyspora* O-9 were also found within the middle lamella of infected root tissues (Figure 3). This finding corroborates the result obtained by Clark and Matthews (1987) and de Almeida et al. (2009) who had observed migration of the microorganism from the cytoplasm to inside of the vacuole and the vesicles formation.

The present study reveals that medicinal plants provide a rich source of diversity of endophytic actinomycetes. The presence of actinomycetes inside these plants confer many advantages to host plants such as production of phytohormone (IAA), siderophores, ability to solubilize the phosphate as well as protection against plant pathogens.

The metabolites produced by endophytic actinomycetes may enhance the fitness and growth of the host and indirectly affect the harmful microbial population. Further investigations are required to understand the other forms of relationship between endophytic actinomycetes and host plant that may be useful in pharmacological and agricultural fields in the future.

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REFERENCES

- Arnou LE (1937). Colorimetric estimation of the components of 3, 4-dihydroxy phenylalanine tyrosine mixtures. *J. Biol. Chem.* 118:531-535.
- Bailey BA, Bae H, Strem MD, Roberts DP, Thomas SE, Crozier J, Samuels GJ, Choi IY, Holmes KA (2006). Fungal and plant gene expression during the colonization of cacao seedlings by endophytic isolates of four *Trichoderma* species. *Planta* 224:1449-1464.
- Bar-Ness E, Chen Y, Hadar Y, Marschner H, Romheld V (1991). Siderophores of *Pseudomonas putida* as an iron source for dicot and monocot plants. *Plant Soil* 130:231-241.
- Cao L, Qiu Z, You J, Tan H, Zhou S (2004). Isolation and characterization of endophytic *Streptomyces* strain from surface sterilized tomato (*Lycopersicon esculentum*) roots. *Lett. Appl. Microbiol.* 39:425-430.
- Cao L, Qiu Z, You J, Tan H, Zhou S (2005). Isolation and characterization of endophytic *Streptomyces* antagonists of fusarium wilt pathogen from surface sterilized banana roots. *World J. Microbiol. Biotechnol.* 24:147-52.
- Castillo UF, Strobel GA, Ford EJ, Hess WM, Porter H, Jensen JB, Albert H, Robinson R, Condon MAM, Teplow DB, Stevens D and Yaver D (2002) Munumbicins, wide spectrum antibiotics produced by *Streptomyces* NRRL 30562, endophytic on *Kennedia nigricans*. *Microbiology* 148:2675-2685.
- Clark CA, Matthews SW (1987) Histopathology of sweet potato root infection by *Streptomyces ipomoea*. *Phytopathology* 77:1418-1423.
- Clegg C, Murray P (2002). Soil microbial ecology and plant root interaction. In: Gordon AJ (ed) 6th edn, IGER Innovations. pp. 36-39.
- Coombs JT (2002). Antibiotics for wheat. *Science Now! The national science forum*, 20-22 August, 2001. <http://www.abc.net.au/science/news/stories/s351442.htm> (viewed 24-3-06).
- Coombs JT, Franco CM (2003). Isolation and identification of actinobacteria from surface sterilized wheat roots. *Appl. Environ. Microbiol.* 69:5603-5608.
- Coombs JT, Michelson TPP, Franco CMM (2004). Evaluation of endophytic actinobacteria antagonists of *Gaeumannomyces graminis var. tritici* in wheat. *Biol. Control* 29:359-366.
- Csaky TZ (1948). On the estimation of bound hydroxylamine in biological materials. *Acta Chem. Scand.* 2:450-454.
- de Almeida CV, Andreote FD, Yara R, Andre F, Tanaka FAO Azevedo JL, de Almeida M (2009). Bacteriosomes in axenic plants: endophytes as stable endosymbionts. *World J. Microbiol. Biotechnol.* 25:1757-1764.
- EI-Tarabily KA, Hardy GE St, Sivasithamparam K, Hussein AM, KurtbOke ID (1997). The potential for the biological control of cavity spot disease of carrots caused by *Pythium coloratum* by streptomycete and non streptomycete actinomycetes in western Australia. *New Phytol.* 137:495-507.
- Fiedler HP, Bruntner C, Riedlinger J, Bull AT, Knutsen G, Goodfellow M, Jones A, Maldonado L, Pathom-aree W, Beil W, Schneider K, Keller S, Sussmuth RD (2008). Proximicin A, B and C, novel aminofuran antibiotic and anticancer compounds isolated from marine strains of the actinomycetes *Verrucosispora*. *J. Antibiot. (Tokyo)*. 61:158-163.
- Gordon SA, Weber RP (1951). Colorimetric estimation of Indoleacetic acid. *Plant Physiol.* 26:192-195.
- Hamdali H, Hafidi M, Virolle M J, Ouhdouch Y (2008). Rock phosphate-solubilizing actinomycetes screening for plant growth promoting activities. *World J. Microbiol. Biotechnol.* 24:2565-2675.
- Jackson ML (1973) Estimation of phosphorous content. In: *Soil Chemical Analysis*, Prentice Hall, New Delhi (India). pp. 134-82.
- Kafur A. Khan AB (2011). Isolation and screening of endophytic actinomycetes against human bacterial pathogens. *Ecoscan*.1:173-177.
- Khamna S, Yokota A, Lumyong S (2009). Actinomycetes isolated from medicinal plant rhizosphere soil: diversity and screening of antifungal compound, indole-3-acetic acid and siderophore production. *World J. Microbiol. Biotechnol.* 25:649-655.
- Kunoh H (2002). Endophytic actinomycetes: Attractive biocontrol agents. *J. Gen. Plant Pathol.* 68:249-252.
- Leong J (1986). Siderophores: their biochemistry and possible role in the biocontrol of plant pathogens. *Ann. Rev. Phytopathol.* 24:187-209.
- Mishra SK, Taft WH, Putnam AR, Ries SK (1987). Plant growth regulatory metabolites from novel actinomycetes. *J. Plant Growth Reg.* 6:75-84.
- Neilands JB, Leong J (1986) Siderophores in relation to plant growth and disease. *Ann Rev Phytopathol.* 37:187-208.
- Nimnoi P, Pongsilp N (2009). Genetic diversity and plant growth promoting ability of indole-3-acetic acid (IAA) synthetic bacteria isolated from agricultural soil as well as rhizosphere, rhizoplane and root tissue of *Ficus Religiosa L.*, *Leucaena Leucocephala* and *Piper sarmentosum* Roxb. *Res. J. Agric. Biol. Sci.* 5:29-41.
- Nimnoi P, Pongsilp N, Lumyong S (2010). Endophytic actinomycetes isolated from *Aquilaria crassna* Pierreex Lec and screening of plant growth promoters production. *World J. Microbiol. Biotechnol.* 26:193-203.
- Sardi P, Saracchi M, Quaroni S, Petrolini B, Borgonovi GE, Merli S (1992). The isolation of endophytic streptomyces strains from surface-sterilized roots. *Appl. Environ. Microbiol.* 58:2691-2693.
- Schulz B, Wanke U, Draeger S (1993). Endophytes from herbaceous and shrubs: effectiveness of surface sterilization methods. *Mycol. Res.* 97:1447-1450.
- Schulz D, Nachtigall J, Riedlinger J, Schneider K, Poralla K, Imhoff JF, Beil W, Nicolson G, Fiedler HP, Sussmuth RD (2009). Piceamycin and its N-acetylcysteine adduct is produced by *Streptomyces* sp. GB 4-2. *J. Antibiot. (Tokyo)*. 62:513-518.
- Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophore. *Annu Biochem* 160:47-56 p. GB 4-2. *J. Antibiot (Tokyo)*. 62:513-518.
- Shenpagam NH, Devi DK, Isduja G, Sandhya R (2012). Isolation of endophytic actinomycetes from medicinal plants and its mutational effect on biocontrol activity. *Int. J. Pharm. Sci. Res.* 3:4338-4344.
- Strobel GA, Daisy B (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiol. Mol. Biol. Rev.* 67:491-302.
- Taechowisan T, Lumyong S (2003). Activity of endophytic actinomycetes from roots of *Zingiber officinale* and *Alpinia galanga* against phytopathogenic fungi. *Ann. Microbiol.* 53:291-298.
- Verma VC, Gond SK, Kumar A, Mishra A, Kharwar RN, Gnage A (2009). Endophytic Actinomycetes from *Azadirachta indica* A. Juss.: Isolation, diversity, and anti-microbial activity. *Microbial Ecol.* 57:749-756.
- Wang Y, Brown HN, Crowley DE, Szanislo PJ (1993) Evidence for direct utilization of a siderophore, ferrioxamine B, in axenically grown cucumber. *Plant Cell Environ.* 16:579-585.
- Welch SA, Taunton AE, Banfield JF (2002). Effect of microorganism and microbial metabolites on apatite dissolution. *Geomicrobiol. J.* 19:343-367.
- Wescott SW III, Beer SV, Israel HW (1987) Interactions between actinomycete-like organisms and young apple roots grown in soil conducive to apple replant disease. *Phytopathology* 77:1071-1077.
- Whitelaw MA (1999) Growth promotion of plants inoculated with phosphate-solubilizing fungi. *Adv. Agron.* 69:99-144.
- Zhao K, Penttinen P, Guan T, Xiao J, Chen Q, Xu J, Lindstrom K, Zang L, Zhang X, Strobel GA (2011). The diversity and anti-microbial activity of endophytic isolated from medicinal plants in Panxi Plateau, China. *Curr. Microbiol.* 62:182-190.

Full Length Research Paper

High-performance liquid chromatography (HPLC) Identification of five new phenolic compounds involved in the olive tree (*Olea europea* var. Sigoise) resistance to *Verticillium dahliae*

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Verticillium wilt is a vascular disease caused by *Verticillium dahliae* which represents a serious threat for olive growing in Algeria. Many studies have shown the potential involvement of phenolic compounds in the reaction of plants to pathogens. Our study shows that the presence of *Verticillium* wilt induces a high production of polyphenols in infected olive trees compared to uninfected ones. The presence of high concentrations of flavonoids (3.45%) and alkaloids (0.44%) in the infected trees suggests that flavonoids and alkaloids may play a role in the olive tree resistance to *verticillium* wilt. The high performance liquid chromatography (HPLC) analysis showed the presence of five phenolic compounds: oleuropeine, luteonine, catechin, and for the first time verbascoside, apigenine-7-glycoside and some derivatives hydroxycinnamic compounds. These substances are good resistance markers and should help to make efficient strategies for the biocontrol of *verticillium* wilt.

Key words: *Olea europea* var. Sigoise, *verticillium* wilt, *Verticillium dahliae*, phenolic compounds, resistance, HPLC.

INTRODUCTION

The olive tree (*Olea europea*) is in full expansion in many countries. This tree, grown for its fruit, is sensitive to a great number of diseases such as *Verticillium* wilt which causes considerable losses in productivity. In recent years, deteriorations of the olive tree due to *Verticillium* wilt have worsened. This disease can propagate very quickly due to the large dissemination of the disease-causing agent and its long survival in the ground (Serrhini and Zeroual, 1995). Plants have complex mechanisms to protect themselves against pathogens. Phenolic secondary metabolites, which are considered as involved in the special organoleptic properties of oil, have been shown to

play a role in the resistance of some olive (*Olea europea* L.) varieties to oil autoxidation (Botia et al., 2001). In addition, some reports (Ruiz-Barba et al., 1991; Marsilio and Lanza, 1998) have shown that some phenolic substances of olive trees may inhibit the growth of bacteria, such as *Lactobacillus plantarum*, *Leuconostoc mesenteroides* and fungi like *Phytophthora* (Del Rio et al., 2003). Similarly, the phenolic metabolism of the olive tree is considered as a plant-response to the infection by *Verticillium dahliae* (Daayf, 1993). Thus, increasing the endogenous levels of these secondary metabolites can improve the resistance properties of the plant and be

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used as a natural alternative for preventing plant diseases.

Methods for detecting and recognizing phenolic compounds rely mainly on chromatographic separation, using the HPLC analysis (El Modafar et al., 1993; El Modafar and El Boustani, 2001) which allows their successful identification.

In Algeria, little is known about the resistance of *Olea europea* var. Sigoise. The aim of this work was to detect the presence of phenolic compounds potentially involved in the resistance of *Olea europea* var. Sigoise to verticillium wilt and determine the chemical nature of these compounds using HPLC method.

MATERIALS AND METHODS

Isolation of the pathogen

To isolate the pathogen, sections from the stem of infected olive tree were superficially disinfected with 95% ethyl alcohol for 30 s, rinsed three times with sterile distilled water, dried on sterile filter paper and plated on Potato Dextrose Agar (PDA) medium amended with streptomycin (100 p.p.m.) and incubated at 25°C in the dark, for two weeks (Tsrar et al., 1998). The isolates were identified as *V. dahliae*, based on the description of Hawksworth and Talboys (1970).

Plant material

The study was carried out with 4 years old infected and uninfected olive trees (*Olea europea* var. Sigoise) grown in a commercial plantation, located in Tlemcen, Algeria. The stems were washed, dried with paper towel, cut into approximately 1 cm squares, dried in an oven at 60°C for at least 24 h, crushed and degreased in a Soxhlet, before use. All analyses were conducted in triplicate, and the results were based on dry weight per 100 grams of sample.

Yields extraction

Tannins extraction

Powdered material (100 g) was extracted at 4°C using 500 ml of a mixture of acetone-water (25/45, v/v) for 4 days (Bruneton, 1999). The extracts were filtered under vacuum through filter paper and the acetone was evaporated under reduced pressure. Subsequently, dichloromethane (2 × 25 ml) was used for the extraction of lipids and pigments from the aqueous extracts using a separating funnel. Afterward, the aqueous phase was extracted with 25 ml of ethyl acetate. This process was repeated four times. After filtration, the organic phases (ethyl acetate) containing tannins were recovered and concentrated to dryness under vacuum, using a rotary evaporator. The residue obtained after evaporation was kept at 4°C and used for further investigation.

Flavonoids extraction

A quantity of 10 g of dried material was extracted with 100 ml of methanol and 5 g of calcium carbonate by boiling for 1 h (Danguet and Foucher, 1982). After filtration, through Whatman filter paper,

the methanol was evaporated under reduced pressure to eventually give an aqueous extract. Subsequently, the dry extract was recovered with 50 ml of boiling water. The aqueous extract was filtered and subjected to solvent fractionation, firstly with diethyl ether, then ethyl acetate and finally n-butanol, using separating funnel (pyrex). All fractions were concentrated, dried to constant weight in an oven at 45°C and kept at 4°C.

Extraction of alkaloids

An amount of 10 g of dried sample was mixed with 250 ml of HCl 2% and 110 ml of ethyl acetate. After cold soaking (4 °C) for 10 h, the mixture was filtered and basified with NH₄OH. The basic aqueous phase was extracted twice with ethyl acetate until no alkaloid was detected in the aqueous phase. The alkaloid residue was obtained by decantation and evaporation of the organic phase (Bruneton, 1999).

Crude extraction

The dried powder of olive stem (10.0 g) was extracted in triplicate, using EtOH (96% v/v) at room temperature, under stirring. The aqueous suspension of the concentrated EtOH extract was evaporated to dryness and used for all investigations (Kukic et al., 2008).

Total phenol content analysis

The total phenolic content (TPC) was determined using Folin Ciocalteu reagent (Singleton and Rossi, 1965). Briefly, 5 µl of the crude extract was added to 1.70 ml of distilled water and 300 µl of Folin Ciocalteu reagent (previously diluted 3-fold in distilled water). The mixture was allowed to stand for 3 min, then 0.5 ml of Na₂CO₃ (20%, w/v) was added to the mixture. After 1 h in the dark at room temperature, the absorbance was measured at 760 nm. The results were expressed as gallic acid equivalents (mg of gallic acid/mg dry weight extract).

HPLC analysis

HPLC analyses of polyphenols were performed by RP-HPLC coupled with diode array detection DAD using a Symmetry C18 column (5 µm, 100 × 4.6 mm) (Waters ref. WAT186002616) equipped with a membrane degasser, a Rheodyne injector 7725i (La Jolla, the USA) and a binary pump 1525 (flow rate, 0.75 mL min⁻¹; T = 30 °C; volume injected: 10 µl). The mobile phase was made up of two solvents: methanol (A) and acidified distilled water with acetic acid 1% (v/v) (B). The gradient was performed in three steps: step 1, from 5 to 45 % of A in B for 25 min; step 2, from 45 to 100 % of A in B for 3 min; step 3, isocratic to 100 % of A for 2 min. The total time of elution was 35 min. Each polyphenol was identified by its retention time and UV-vis spectrum after comparison with standards. Empower software was used to control the device.

RESULTS

Identification of *Verticillium dahliae*

The symptoms from infected plants were very similar to those described for verticillium wilt of olive tree, including foliar chlorosis and necrosis, stunting and vascular

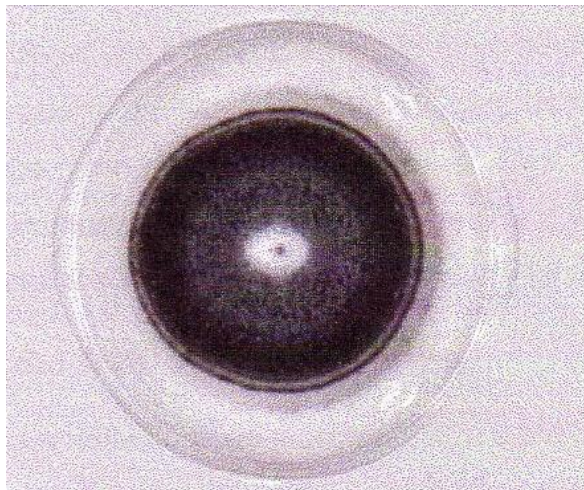


Figure 1. Colony type of *Verticillium dahliae*.

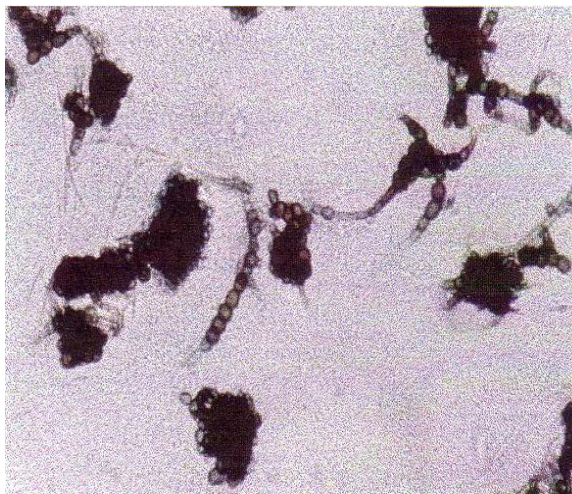


Figure 2. Microsclerotia of *V. dahliae* on PDA medium (40 x).

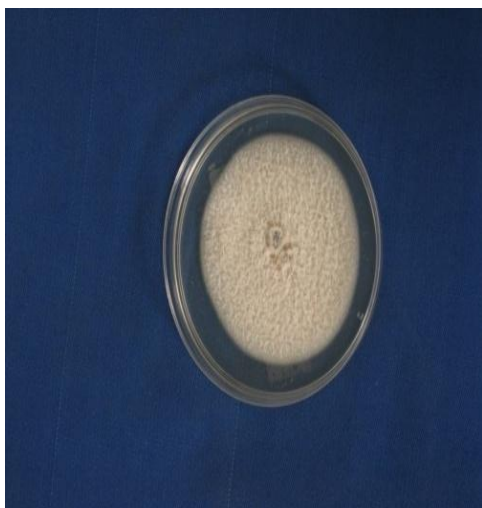


Figure 3. Hyaline colony of *V. dahliae*.

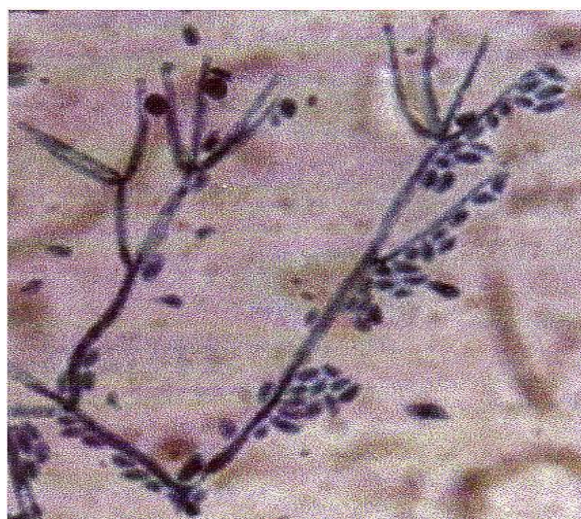


Figure 4. Mycelium and conidiophores of *V. dahliae* (40 x)

discoloration.

Fungal colonies were black (Figure 1) and produced microsclerotia characteristic of *V. dahliae* (Figure 2). The color of the colony change from black to white after many transfers (Figure 3). The presence of single-celled oval conidia and distinct verticillate conidiophores confirmed the isolate of *V. dahliae* (Figure 4).

Total phenol content

Figure 5 shows the total phenol content in a whole stem from uninfected and infected olive plants. The total phenol contents in the whole stems of the infected plants (780 mg/g) was practically double that measured in the uninfected plants (440 mg/g).

Yields extraction

The yields of tannins, flavonoids and alkaloids are presented in Figure 6. The yield of tannins in whole stem from infected and uninfected olive plants was 0.1 and 0.3 %, respectively.

The yield of flavonoids and alkaloids was higher in infected plants: (3.45 % and 2 %) for flavonoids and (0.44 % and 0.04 %) for alkaloids content in uninfected and infected plants respectively.

Identification of phenolic compounds by HPLC

The data (retention time, λ_{max} in the visible region, and

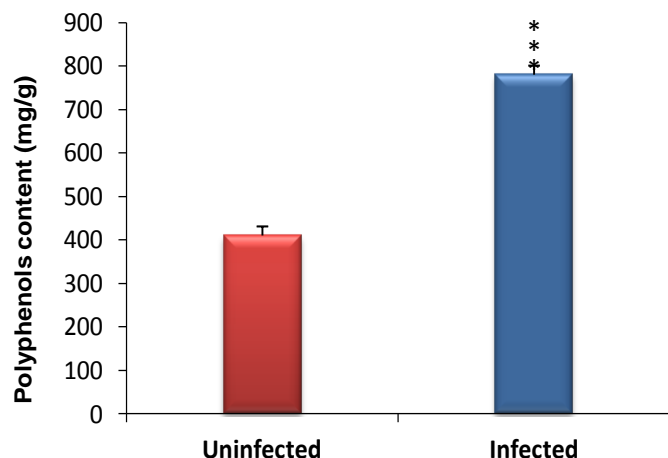


Figure 5. Polyphenols content of uninfected and infected olive-tree.

Table 1. Retention time (R_t), wavelengths of maximum absorption in the visible region (λ_{max}) and tentative identification of phenolic compounds in whole stem and leaf of olive-tree.

Peak	R_t (min)	λ_{max} (nm)	Tentative identification
1	10,364	288	Flavonoid (N.D.)
2	14,541	288	N.D.
3	15,084	327	N.D.
4	15,680	322	Hydroxycinnamic derivative
5	15,710	331	Verbascoside
6	16,748	349	Luteoline-7-glucoside
7	17,117	288	Flavonoid (N.D.)
8	17,196	322	N.D.
9	18,598	280	Oleuropein
10	18,710	337	Apigenine-7-glucoside
11	20,000	332	N.D.
12	20,495	281	Catechin
13	21,000	330	N.D.
14	21,696	287	Flavonoid (N.D.)
15	22,000	333	N.D.

tentative identification) obtained for the phenolic compound peaks in the HPLC-DAD analyses are presented in Table 1 and Figure 7a. HPLC studies point to five phenolic compounds determined in olive stem extracts: oleuropein ($t_R = 18.59$ min, maximum absorbance at 280 nm), luteolin-7-glucoside ($t_R = 16.74$ min, maximum absorbance at 349 nm), verbascoside ($t_R = 15.71$ min, maximum absorbance at 290 nm), apigenine-7-glucoside ($t_R = 18.71$ min, maximum absorbance at 263 and 337 nm) and catechin ($t_R = 20.49$ min, maximum absorbance at 281 nm) (Figure 7b). The retention time and absorption spectrum (obtained by means of a UV/vis DAD) were identical to those obtained for the corresponding standards.

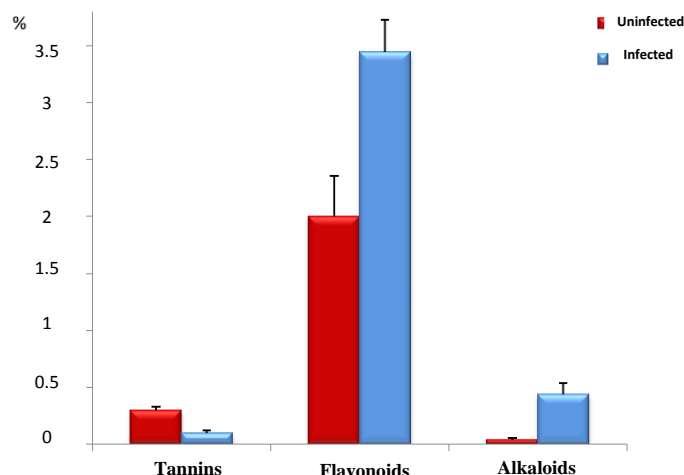


Figure 6. Tannins, flavonoids and alkaloids contents of uninfected and infected olive-tree.

DISCUSSION

V. dahliae was isolated from the whole stem and leaf, presenting the typical symptoms of Verticillium wilt, according to Vigouroux (1975). These include xylem browning and leaf rolling to their inner face, with color changing from yellow to brown. Then, the dry leaves became brittle and fell.

V. dahliae isolate produced black microsclerotia as resting structures and was identified according to the published descriptions of Hawksworth and Talbot (1970) and Goud et al. (2003). The color of colonies mainly changed from black to white on PDA medium. The hyaline isolates lost their ability to produce microsclerotia, most probably due to many transfers and long maintenance in several laboratories under different conditions (Smith, 1965).

The obtained results show that total polyphenols were present in infected olive trees at higher levels than in uninfected olive trees; and the difference was statistically significant ($p < 0.001$). However, the difference in tannin and flavonoid yields was significant ($p < 0.05$).

This is certainly due to the type of analysis which shows that the total polyphenols synthesis was better after *V. dahliae* infection. The TPP content obtained confirms this idea because the TPP analysis gives a quantitative result whereas the yield gives a qualitative one.

In a previous work, El Boustani et al. (1998, 2nd International Electric Conference of Synthetic Organic Chemistry, personal communication), showed that inoculation of the olive twigs by a conidial suspension of *V. dahliae* resulted in important modifications in flavone and phenol levels. These findings suggest that the first step of the response mechanism to infection in olive plants is a rapid accumulation of phenols at the infection site, thus reducing or slowing the pathogen growth, as

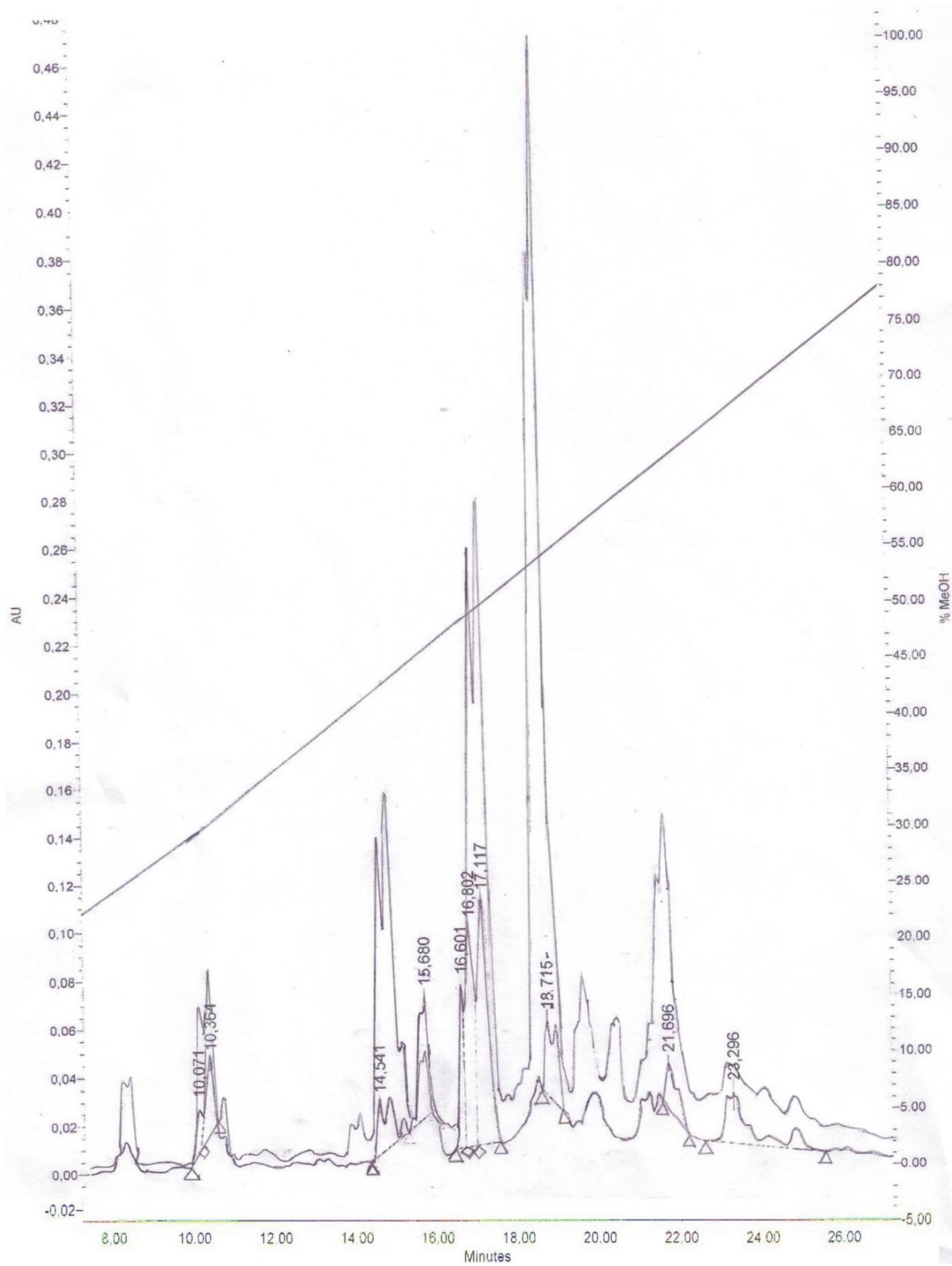


Figure 7a. Chromatogram (zoom) recorded at 350 nm showing the phenolic compounds profiles identified and not identified of olive-tree stem (*Olea europea* var. Sigoise).

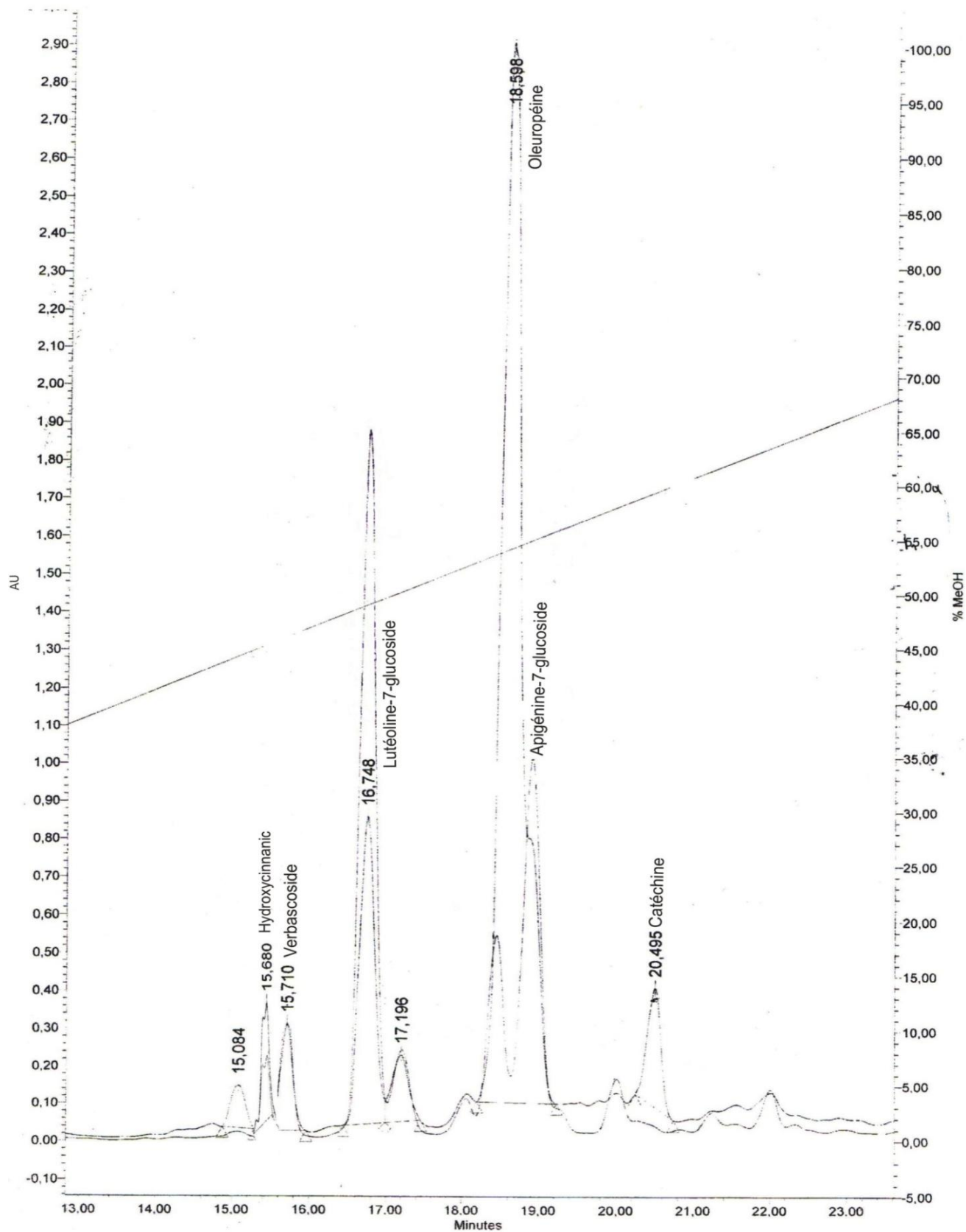


Figure 7b. Chromatogram (zoom) recorded at 350 nm showing the phenolic compounds profiles identified of olive-tree stem (*Olea europea* var. Sigoise).

reported for other vegetal materials (Matern and Kneusel, 1988; Del Rio et al., 2004). Therefore, in contrast to flavonoids and alkaloids, the tannin content of the uninfected sample was higher than that of the infected one. This suggests that tannins, which are constitutive substances, mainly present in the bark, were synthesized and used initially by the olive plant in its defense against pathogens before transforming into flavonoids. Tannins were found in tropical plants at high concentrations, by Makkar and Becker (1998), because their synthesis is promoted by light, whereas flavonoids and alkaloids are inducible compounds, since they are not produced directly during the photosynthesis, but result from further chemical reactions. Our results are in agreement with those of Corbaz (1990), whose study results show that the young leaves at the cotton plant are often resistant to *V. dahliae* and become sensitive as they grow older. This phenomenon might be ascribed to the inhibition of mycelium growth in young tissues, which contain higher concentrations of substances such as tannins than those in the old leaves.

In selective extractions, that concentrations of alkaloids in infected olive plants were higher than in uninfected ones also suggest that alkaloids may have a role in the response mechanism of olive plants to *Verticillium* wilt. Williams and Charles (2006) showed that alkaloids have antibiotic properties; these nitrogenous substances, synthesized from amino acids, salt out the cyanhydric acid when the plants are damaged (Hopkins, 2003). Our results show that the concentration of flavonoids was higher in infected olive trees compared to the uninfected ones. Similar results were found in potato plants inoculated with *V. dahliae*, which induced a production of flavonol glycosides two to three times higher than in the uninoculated plant (El Hadrami et al., 2011). Likewise, Daayf (1993) showed that infection of the cotton plant by *V. dahliae* stimulated the accumulation of phenolic compounds (flavones and flavonols). All these results highlighted that tannins, as constitutive substances, should be first used by the olive tree in their reaction to pathogen attacks; whereas flavonoids are inducible molecules, since they are obtained from tannin degradation.

Our main findings were that the HPLC analysis revealed the presence of three new phenolic compounds in infected olive stems, namely verbascoside, apigenine-7-glycoside and hydroxycinnamic derivatives which have, to our knowledge, never been isolated before, from this variety of olive tree.

Conclusion

This study strongly suggests that some of the secondary metabolites, especially flavonoids and alkaloids, present in olive plants act as phytoanticipins and/or phytoalexins in the plant's natural defense mechanism, as it has been

established for other phenolic secondary metabolites in different plant materials infected by pathogenic fungi. The HPLC analysis revealed the presence of three new phenolic compounds, namely verbascoside, apigenine-7-glycoside and hydroxycinnamic derivatives.

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REFERENCES

- Botia JM, Ortuno A, Benavente-Garcia O, Baidez AG, Frias J, Marcos D, Del Rio JA (2001). Modulation of the biosynthesis of some phenolic compounds in *Olea europea* L. fruits: their influence on olive oil quality. *J. Agric. Food Chem.* 49(1):355-358.
- Bruneton J (1999). Pharmacognosie. Phytochimie. Plantes médicinales. Technique & Documentation. 3^{ème} Ed. Lavoisier, Paris. pp. 370-401.
- Corbaz R (1990). Principes de phytopathologie et de lutte contre les maladies des plantes. Ed. Presses polytechniques et universitaires romandes. pp. 103-226.
- Daayf F (1993). La verticilliose du cotonnier. Pouvoir pathogène et diversité génétique de *Verticillium dahliae*, réactions de la plante à l'infection. Doctorat dissertation, University of Montpellier, France. pp. 14-20.
- Danguet JC, Foucher JP (1982). Les flavonoïdes d'*Arbutus unedo* L. (Ericacées). *Plants Médicinales et Phytothérapie.* 16 (3):185-191.
- Del Rio JA, Baidez AG, Botia JM, Ortuno A (2003). Enhancement of phenolic compounds in olive plants (*Olea europea* L.) and their influence on resistance against *Phytophthora* sp. *Food Chem.* 83:75-78.
- Del Rio JA, Gomez P, Baidez AG, Arcas MC, Botia JM, Ortuno A (2004). Changes in the levels of polymetoxylflavones and flavonones as a part of the defence mechanism of *Citrus sinensis* (cv. Valencia late) fruits against *Phytophthora citrophthora*. *J. Agric. Food Chem.* 52:1913-1917.
- El Hadrami A, Adam R, Daayf F (2011). Biocontrol treatments confer protection against *Verticillium dahliae* infection of potato by inducing antimicrobial metabolites. *Mol. Plant Microbe Interact.* 24(3):328-335.
- El Modafar C, Clériveret A, Fleuriet A, Macheix JJ (1993). Inoculation of *Platanus acerifolia* with *Ceratocystis fimbriata* f. sp. platani induces scopoletin and umbelliferone accumulation. *Phytochemistry* 34:1271-1276.
- El Boustani E (2001). Cell wall-bound phenolic acid and lignin contents in date palm as related to its resistance to *Fusarium oxysporum*. *Biol. Plant* 44:125-130.
- Goud JK, Termoshuizen AJ, Gams W (2003). Morphology of *Verticillium dahliae* and *V. tricorpus* on semi-selective media used for the detection of *V. dahliae* in soil. *Mycol. Res.* 107:822-830.
- Hawksworth DL, Talboys PW (1970). *Verticillium dahliae*. C.M.I. Descriptions of Pathogenic Fungi and Bacteria, Section 26, Sheat No 256, Commonwealth Mycological Institute, Kew, UK.
- Hopkins WG (2003). Physiologie végétale. Ed. De Boeck, University of Bruxelles, Belgium. p. 203.
- Kucic J, Popovic V, Petrovic S, Mucaji P, Ciric A, Stojkovic D, Sokovic M (2008). Antioxidant and antimicrobial activity of *Cynara cardunculus* extracts. *Food Chem.* 107:861-868.
- Makkar HPS, Becker K (1998). Do tannins in leaves of trees and shrubs from Africa and Himalayan regions differ in level and activity? *Agro. For. Syst.* 40:59-68.
- Marsilio V, Lanza B (1998). Characterisation of an oleuropein degrading strain of *Lactobacillus plantarum*. Combined effects of compounds

- present in olive fermenting brines (phenols, glucose and NaCl) on bacterial activity. *J. Sci. Food Agric.* 76:520-524.
- Matern U, Kneusel RE (1988). Phenolic compounds in plant disease resistance. *Phytoparasitica* 16:153.
- Ruiz-Barba JL, Garrido-Fernandez A, Jimenez-Diaz R (1991). Bactericidal action of oleuropein extracted from green olives against *Lactobacillus plantarum*. *Lett. Appl. Microbiol.* 12: 65-68.
- Serrhini MN, Zeroual A (1995). La verticilliose de l'olivier au Maroc. *Sciences & Techniques. Olivae* 58:58-61.
- Singleton VL, Rossi JA (1965). Colorimetry of total phenolics with Phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Viticulture* 16:144-148.
- Smith HC (1965). The morphology of *Verticillium albo-atrum*, *V. dahliae*, and *V. tricorpus*. *New Zealand J. Agric. Res.* 8:450- 478.
- Tsrer L, Erlich O, Amitai S, Hasanovsky M (1998). *Verticillium* wilt of paprika caused by a highly virulent isolate of *Verticillium dahliae*. *American Phytopathological Society. Plant Dis.* 82:437-439.
- Vigouroux A (1975). *Verticillium dahliae* agent d'un dépérissement de l'olivier en France. *Ann. Phytopathol.* 7(1):37-44.

Full Length Research Paper

Stress sensitivity assays of bacteriophages associated with *Staphylococcus aureus*, causal organism of bovine mastitis

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Bacteriophages can provide an alternative measure for the control of *Staphylococcus aureus*, the major causal agent for bovine mastitis. This study looked at the sensitivity of six phages towards simulated environmental and formulation stresses. Phages Sabp-P1, Sabp-P2 and Sabp-P3 showed the most stable replication rates at increasing temperatures (45 to 70°C), in comparison to phages Sabp-P4, Sabp-P5 and Sabp-P6. The effect of temperature on storage of phages showed that 4°C was the minimum temperature at which phages could be stored without a significant reduction in their lytic and replication abilities. Furthermore, all phages showed varying levels of sensitivity to chloroform exposure, with Sabp-P5 exhibiting the highest level of reduction in activity (74.23%) in comparison to the other phages. All six phages showed optimal lytic ability at pH 6 to 7 and reduced activity at any pH above or below pH 6 to 7. Exposure of phages to varying glycerol concentrations (5 to 100%) produced variable results. All six phages were most stable at a glycerol concentration between 10 to 15%. Three of the six isolated phages, Sabp-P1, Sabp-P2 and Sabp-P3, performed optimally during the *in vitro* assays and have considerable potential for *in vivo* applications to treat mastitis-infected dairy cattle.

Key words: Bacteriophage, biological control, bovine mastitis, sensitivity assays, *Staphylococcus aureus*.

INTRODUCTION

Staphylococcus aureus-induced bovine mastitis in dairy herds is one of the most widespread and destructive diseases of dairy cows. It has far reaching consequences that affect milk quality and yield, health of the dairy cow, and the economics associated with the processing of milk and milk products. Under optimal conditions, the natural defence mechanisms of the cow itself, in conjunction with cultural control measures, may prove adequate in curbing disease onset and severity. However, the real problem arises when these intrinsic defences and cultural measures are compromised by infection. Antibiotic therapy has traditionally served as the next option to achieve effective control of the disease. Overall benefits of antibiotic therapy include a more rapid elimination of

bacterial pathogens than self-cure, a reduced probability of chronic recurrent infections, a reduced depression in milk yield and a more rapid return to an acceptable somatic cell count and hence to saleable milk (Barkema et al., 2006; Nickerson, 2009).

Despite the documented success associated with antibiotic usage, it remains debatable as to whether this therapy is indeed positive in the long term. There are several conflicting views on this (Murchan et al., 2004; Borm et al., 2006; Sandgren et al., 2008; Nickerson, 2009; Blowey and Edmondson, 2010; Vanderhaeghen et al., 2010), many of which revolve around *S. aureus* and its ability to develop antibiotic resistance. In the long term, the cure rate of antibiotic treatments against this

pathogen is low and, therefore, the disease cannot be effectively eliminated and/or controlled in infected herds by using antibiotics alone (Sutra et al., 1993; Carter and Kerr, 2003; Murchan et al., 2004; Shi et al., 2010).

Antibiotic resistance can be attributed to several factors, ranging from structural features that protect the bacterium, to production of chemicals that can neutralise antibiotics (Almeida et al., 1996; Herbert et al., 2000; Blowey and Edmondson, 2010; Cabrera et al., 2011; Villar et al., 2011). The spread of virulent methicillin-resistant *S. aureus* (MRSA) (Goñi et al., 2004; Murchan et al., 2004; Nickerson, 2009; Shi et al., 2010; Vanderhaeghen et al., 2010), coupled with the development of resistance to two new antibiotics (daptomycin and linezolid) recently approved for clinical use against Gram-positive bacteria (Mangili et al., 2005), has shown that *S. aureus* is indeed a formidable pathogen.

Due to these treatment limitations, research focus into the control of bovine mastitis has shifted to alternative therapies, such as the development of vaccines (Sutra, 1993; Herbert et al., 2000; Pereira et al., 2011) and biological control options, such as the use of botanical extracts (Akinyemi et al., 2005; Fawole, 2009) or phage therapy (O'Flaherty et al., 2005b; Sulakvelidze and Barrow, 2005; Gill et al., 2006a; García et al., 2007; Jones et al., 2007; García et al., 2008; Synott et al., 2009). Considering the wealth of information at our disposal, phage therapy appears to be one of the most sustainable measures for control of bovine mastitis. Several studies have identified various phages with lytic capabilities towards *S. aureus* (O'Flaherty et al., 2005a; Gill et al., 2006a; García et al., 2007; García et al., 2008; Synott et al., 2009). While each study did present noteworthy results, it must be noted that there are limitations which must be overcome. Phage inactivation can be triggered by milk proteins and fats (O'Flaherty et al., 2005b; Gill et al., 2006b), aggregation of *S. aureus* cells within milk (O'Flaherty et al., 2005b), and intrinsic immune factors within the cow itself (O'Flaherty et al., 2005a). While these limitations do exist, the solutions to these problems are merely a product of time. Further studies into the detailed effects of whey proteins on bacterial activity and aggregation, proper delivery of phage cocktails into animal tissues, and the development of phage formulations to facilitate optimal delivery and activity within intramammary tissues, are ongoing.

The primary focus of the current study was to investigate phage sensitivity, *in vitro*, towards simulated environmental and chemical stresses. This study aimed to contribute to phage formulations for *in vivo* application.

MATERIALS AND METHODS

Bacterial host strains and their isolation

Strain SaB1 of *S. aureus* was used for phage isolation and propagation. This strain was isolated from raw bovine milk collected

from dairy farms in the province of KwaZulu-Natal, RSA (Republic of South Africa). SaB1 was initially isolated on blood agar. Strain identification to distinguish *S. aureus* from other staphylococcal strains was confirmed by hemolysis on blood agar, catalase reaction, Gram reaction and arrangement of bacterial cells upon Gram staining. This was followed by culturing on Baird-Parker agar and results were confirmed using a coagulase test (Bactident® Coagulase, Merck). SaB1 was maintained on tryptone soy agar (TSA) or tryptone soy broth (TSB).

Phage isolation, propagation and purification

Raw bovine milk samples collected from dairy farms in the province of KwaZulu-Natal, RSA (Republic of South Africa) served as the initial material from which to isolate phages. These milk samples were chosen at random and included milk with both high (>400,000 cells.ml⁻¹) and low somatic cell counts (SCC). Phages were isolated using the spot-plate method on double-layer agar (Sambrook et al., 1989; Harley and Prescott, 1993). Overnight SaB1 cultures (~1 x 10⁸ cfu.ml⁻¹) were incorporated into 7% (w/v) top agar that was supplemented with 1M CaCl₂. Raw milk samples were filtered through a 0.45 µm syringe filter. Filtered samples were then spotted onto the surface of the solidified top agar at 10 µl per spot. Plates were allowed to dry for 2 h, followed by incubation for 12 h at 37°C. Zones of clearing (plaques) were indicative of phage activity. Plaques were removed from top agar and soaked in phage buffer (Sambrook et al., 1989) for 12 h with gentle agitation (150 rpm) at 4°C; all phages were isolated through five rounds of plating from plaques. The resulting suspension was centrifuged using an Avanti J-26 XPI (www.beckmancoulter.com) at 10,000 g x 10 min at 4°C. The supernatant was filtered using a 0.45 µm syringe filter and stored as phage stock at 4°C. Subsequent phage was grown from this stock in liquid broth culture or using the double-layer agar method (Sambrook et al., 1989).

Isolated phages were purified through a modification of standard methods (Sambrook et al., 1989; Harley and Prescott, 1993). Filter-sterilised phage stock isolated from either liquid broth culture or double-layer agar method, were subjected to centrifugation using the Avanti J-26 XPI (Beckman-Coulter) at 75,600 g x 3 h x 10°C. The resulting phage pellets were re-suspended in fresh phage buffer and a second centrifugation was conducted (Avanti J-26 XPI (Beckman-Coulter) at 75,600 g x 3 h x 10°C). The phage pellets were then re-suspended in fresh phage buffer at 1/10 of the original volume that was processed. All stocks were stored at 4°C. Six phages were isolated and systematically named Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6. Multiplicity of infection (MOI) was calculated for each phage. Phage MOI was determined for each phage: Sabp-P1=3, Sabp-P2=3, Sabp-P3=3, Sabp-P4=0.2, Sabp-P5=0.1 and Sabp-P6=0.2.

Temperature sensitivity

Heat sensitivity

This method was a modification of that described by Harley and Prescott (1993). Optimal phage dilutions of 10⁻⁵ pfu.ml⁻¹ were prepared for each phage for this assay. Phage dilutions were carried out in phage buffer. Diluted phages, Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6, were placed into a water-bath at 45°C. 100 µl aliquots were removed at each of the following time intervals: 0min (control - room temperature, 22°C), 5, 10, 20, 30, 40, 50 and 60 min. Each aliquot was plated using the double-layer plating technique (Sambrook et al., 1989), with an overnight culture of SaB1 as the host bacterium. The experiment was repeated for temperatures 50, 55, 60, 65 and 70°C. Phage diluted in phage buffer was used as a control. All plates were incu-

bated at 37°C for 12 h. After 12 h, the number of plaques was counted and phage titre was calculated for each time and its associated temperature. All assays were carried out in triplicate.

Cold sensitivity (storage feasibility of phages)

This assay was conducted in order to see the opposite effects of heat treatment on phages and to test their storage capability over time. Optimal phage dilutions of 10^{-5} pfu.ml⁻¹ were prepared for each phage. Phage dilutions were carried out in phage buffer. This experiment was ongoing over a period of 6mo. Overnight SaB1 was used as the host bacterium. Phages Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6 were screened for stability at various storage temperatures (4, -20 and -80°C) with different durations at each temperature (1 h, 8 h, 12 h, 1 wk, 2 wk, 3 mo, and 6 mo). All phages were stored in sterile phage buffer (supplemented with 5% glycerol) for the duration of the experiment. At the end of each storage duration, at each temperature, a 100 µl aliquot of phage was removed and immediately plated using the double-layer plating technique.

An overnight culture of SaB1 was used as the host bacterium, hence final reaction contained 100 µl phage + 100 µl SaB1 (+ 1M CaCl₂). This was followed by double-layer plating as previously described (Sambrook et al., 1989). Fresh phage diluted in phage buffer was used as a control. Plates were incubated for 12 h at 37°C. The total number of plaques was counted and phage titre was calculated for each phage, at each time and at each associated temperature. The same phage sample was stored and screened for the entire duration of the experiment (6 mo). All assays were carried out in triplicate.

pH Sensitivity

This method is a modification of that described by Harley and Prescott (1993). TSB was calibrated (using 1 M HCl) according to the following pH range: 2, 4, 6, 8, 10, 12 and 14, and 900 µl of each was aliquoted into 2 ml Eppendorf vials. Phage stock was added to the calibrated TSB to bring the final phage dilution to 10^{-5} pfu.ml⁻¹, that is, approximately 2.91×10^8 , 3.04×10^8 , 3.12×10^8 , 1.9×10^7 , 1.76×10^7 and 2.6×10^7 pfu.ml⁻¹, for Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6 respectively. The vials were gently inverted in order to mix the phage within the TSB medium. All vials were left to stand at room temperature (22°C) for 60 min. At 60 min, 100 µl of phage suspension was removed and mixed with 100 µl of an overnight SaB1 culture (+ 1 M CaCl₂). This was followed by double-layer plating as previously described (Sambrook et al., 1989). Phage diluted in phage buffer was used as a control. All plates were incubated at 37°C for 12 h. After 12 h, the number of plaques was counted and phage titre was calculated for each pH. All assays were carried out in triplicate.

Chloroform sensitivity

This method is a modification of that described by Harley and Prescott (1993). Optimal phage dilutions of 10^{-5} pfu.ml⁻¹ were prepared for each phage. Phage dilutions were carried out in phage buffer (Sambrook et al., 1989). Overnight SaB1 was used as the host bacterium. 1 ml each, of phages Sabp-P1 to Sabp-P6, were treated with 5% (v/v) chloroform. This suspension was gently shaken (100 rpm) at room temperature (22°C) for 15 min, followed by centrifugation at 10,000 g × 10 min at 4°C. The aqueous phase was withdrawn and phages were titred using standard double-layer plating. Phage diluted in phage buffer was used as the control. All plates were incubated at 37°C for 12 h. After 12 h, the number of plaques was counted and phage titre was calculated. All assays

were carried out in triplicate.

Glycerol sensitivity

This assay was adapted from Santos et al. (2009). Optimal phage dilutions of 10^{-5} pfu.ml⁻¹ were prepared for each phage. Phage dilutions were carried out in phage buffer. Overnight SaB1 was used as the host bacterium. Glycerol was made up in sterile distilled water at the following concentrations (v/v): 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%. Phage stock was added to the 900 µl of each glycerol solution to bring the final phage dilution to 10^{-5} pfu.ml⁻¹. This was left at room temperature (22°C) for 1 h. Incubation was followed by standard double-plating and incubation of all plates at 37°C for 12 h. After incubation, the number of plaques was counted and phage titre was calculated. Phage diluted in phage buffer was used as a control. All assays were carried out in triplicate.

Statistical analyses

Differences between treatments were determined by analysis of variance (ANOVA) using Genstat 14th Edition.

RESULTS

Heat assay

ANOVA of the phage titres showed highly significant ($p < 0.001$) differences for phage titers at different temperatures, and a highly significant phage × temperature × exposure time interaction. It was found that an increase in temperature, coupled with increased exposure time to that temperature, reduced phage reproductive activity (Figure 1).

Phage counts dropped significantly from 0 min exposure to high temperature, to 10min exposure to the same temperature (across the complete temperature range of 45 to 70°C). After 10 min, phage reproductive activity stabilised across all temperature ranges, but remained low (Figure 1). Significant differences were noted between phage activity at each temperature and exposure time (< 0.001), as well as between each phage itself (< 0.001). In general, Sabp-P1, Sabp-P2 and Sabp-P3 were more stable, and titers remained higher than those for Sabp-P4, Sabp-P5 and Sabp-P6. Exposure to 70°C was the most damaging with a 92 to 96% reduction in phage titers. Overall results show that propagation of these phages is negatively affected by increased exposure to high temperatures.

Cold assay (storage feasibility of phages)

Storage temperature played an important role in influencing phage titre. ANOVA showed highly significant differences ($p < 0.001$) in phage counts when the phages were stored at different temperatures for different durations (Figure 2). Storage of phages at temperatures below 4°C reduced phage activity significantly. Sabp-P1,

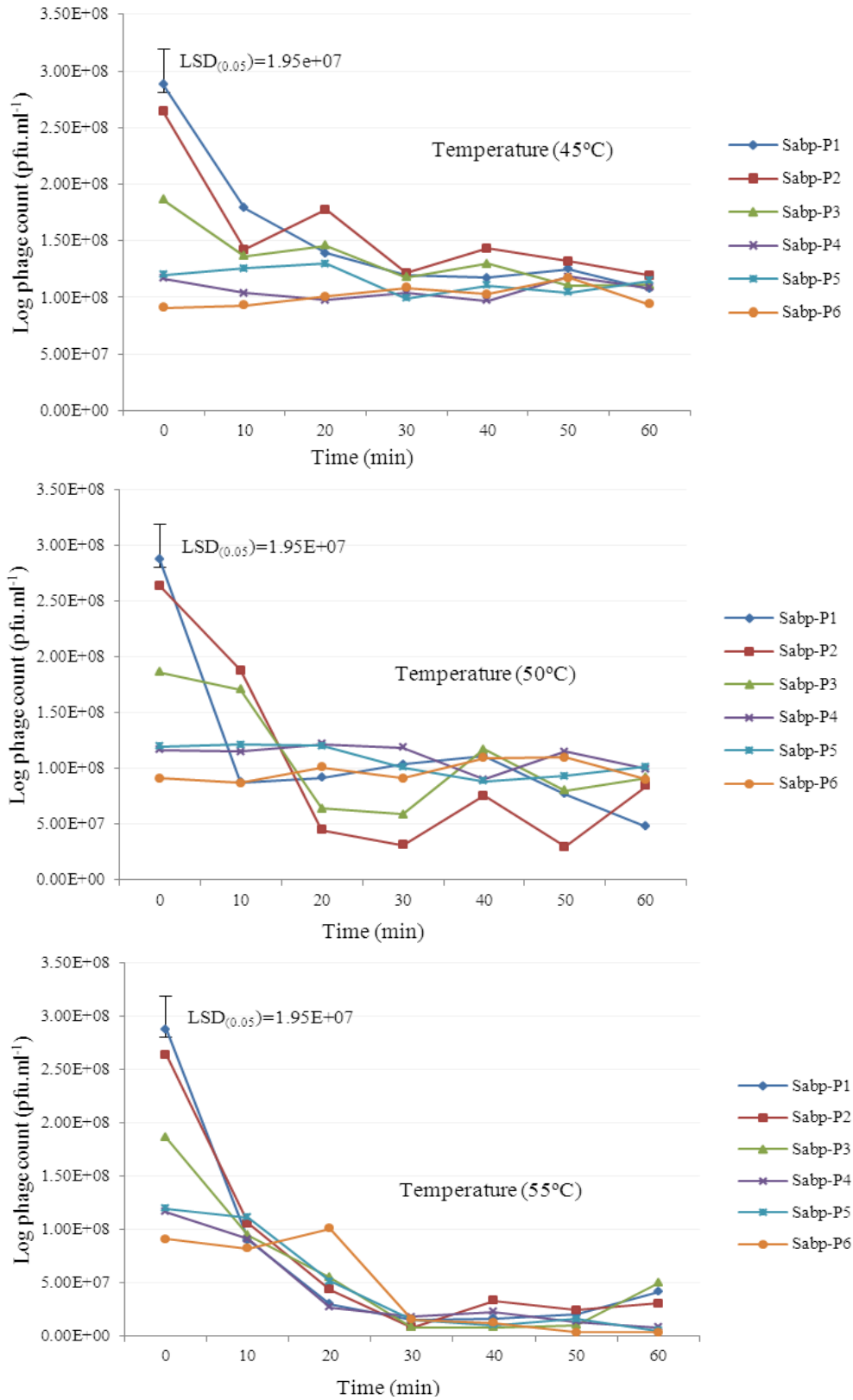


Figure 1. Heat assay screening the titres of six phages over a range of different temperatures. Sab1 was used as the host bacterium for phage propagation. Data points are the means of three independent experiments. The vertical bar represents the LSD_(0.05) when comparing any phage x temperature x time combination.

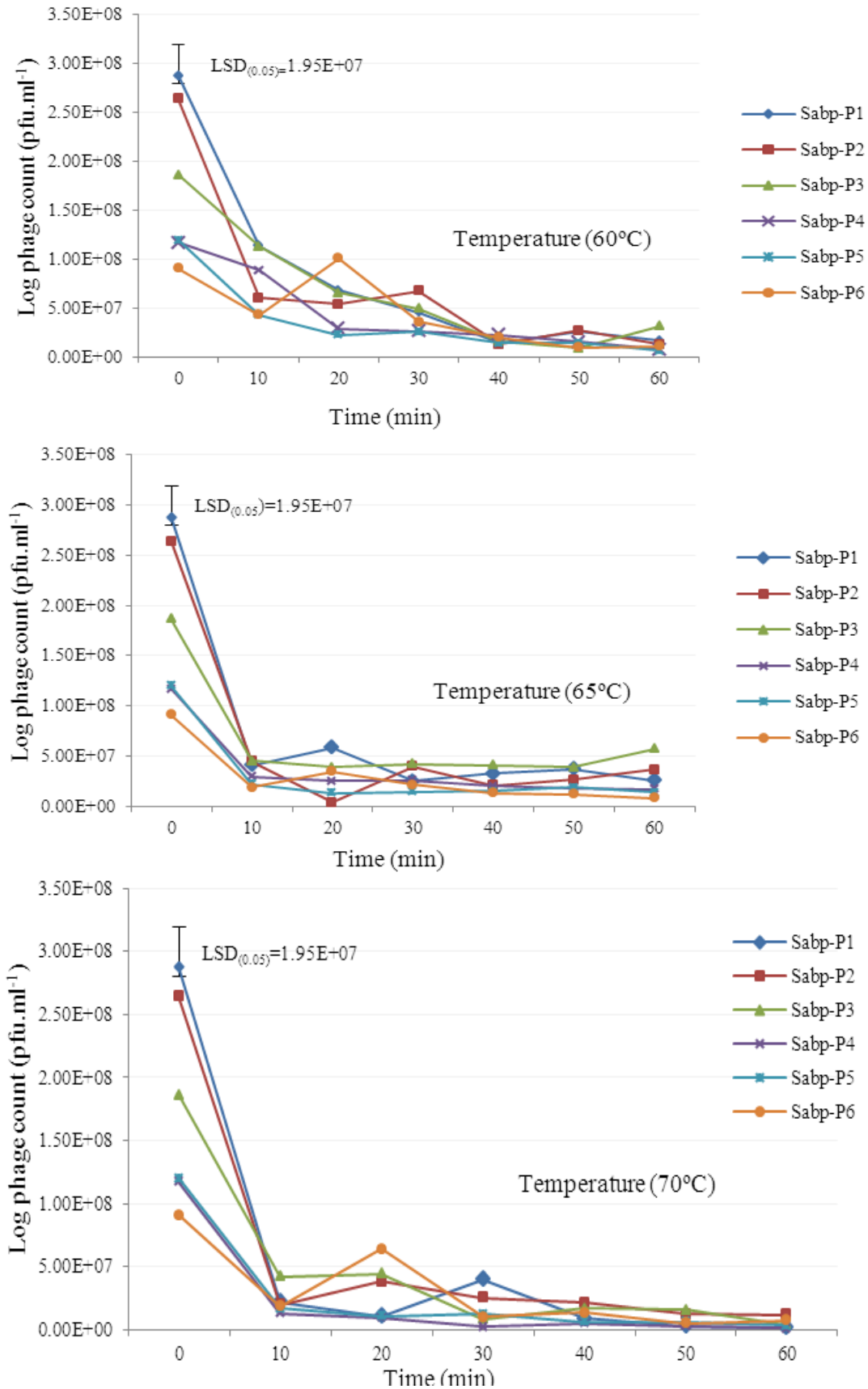


Figure 1. Contd.

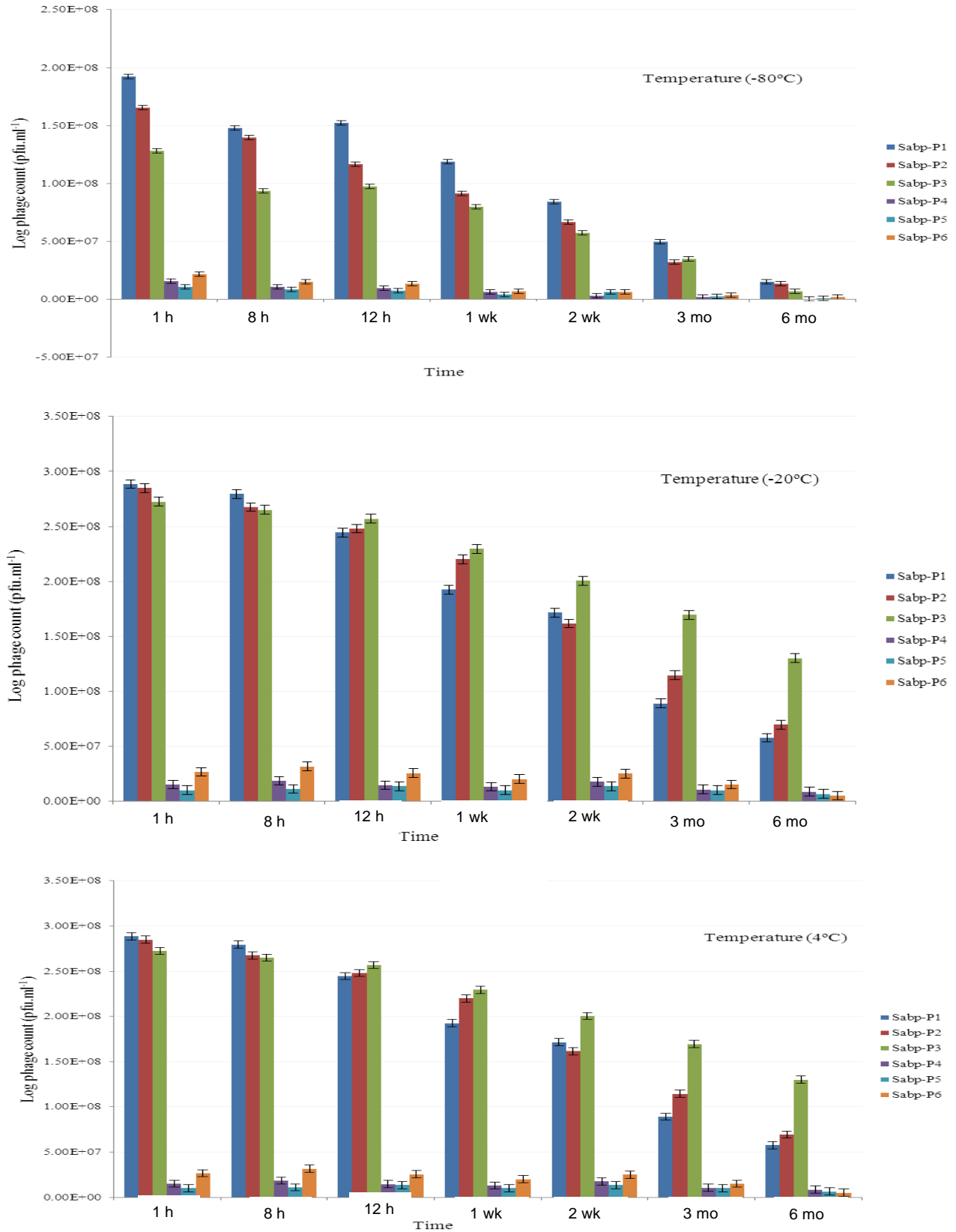


Figure 2. Screening the storage ability of six phages over a range of low temperatures. SaB1 was used as the host bacterium for phage propagation. Each bar represents the means of three independent experiments. The vertical bar represents the LSD (0.05)=3.93E+06 when comparing any phage x temperature combination.

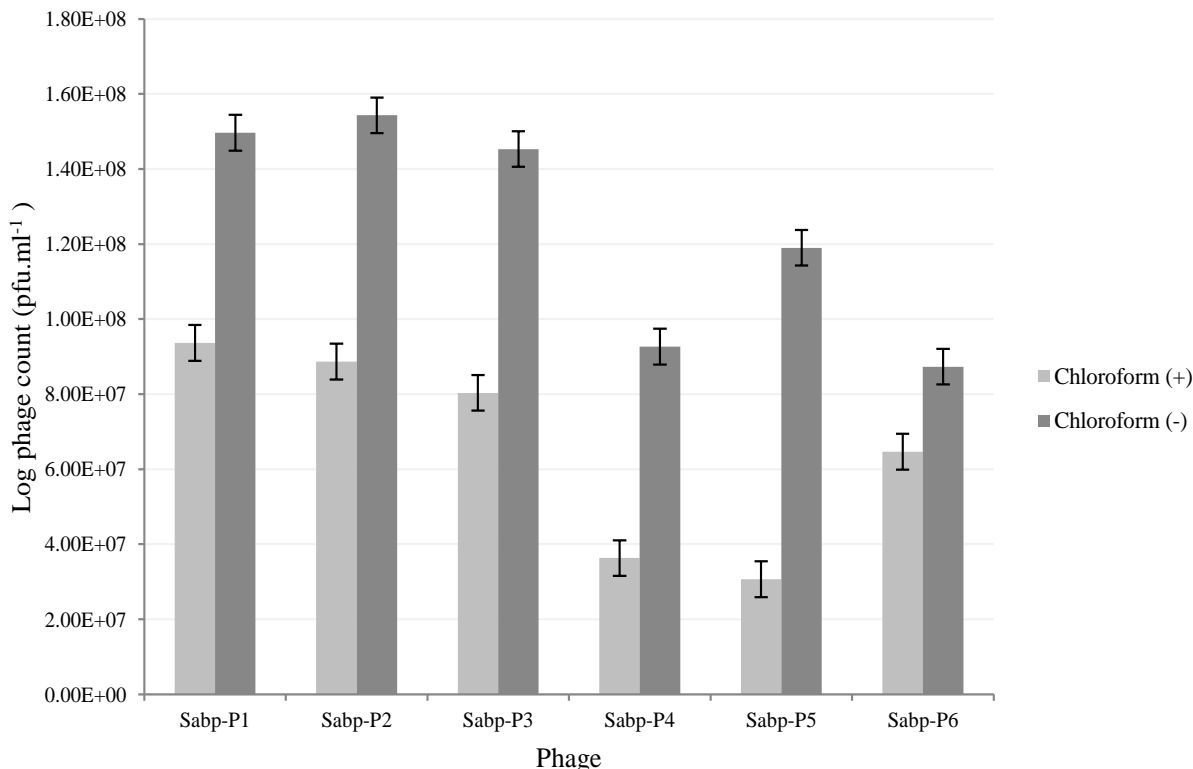


Figure 3. Effect of chloroform on phage titers. *SaB1* was used as the host bacterium for phage propagation. Each bar represents the means of three independent experiments. The vertical bar represents the LSD_(0.05)=9.5E+07 when comparing any phage x bacterium combination.

Table 1. Reduction in phage titers upon exposure to chloroform.

Phage	Reduction in phage activity (%)
Sabp-P1	37.42
Sabp-P2	42.55
Sabp-P3	44.73
Sabp-P4	60.80
Sabp-P5	74.23
Sabp-P6	25.95
Mean	47.61
LSD	9.50
CV%	0.4

Sabp-P2 and Sabp-P3 displayed more stability at all three temperature ranges (4, -20 and -80°C), although phage titre did decrease significantly at -80°C. The titres for the other three phages Sabp-P4, Sabp-P5 and Sabp-P6, were generally low throughout these studies. However, these three phages showed stability during storage at all durations and temperatures. While the study did not proceed beyond a screening period of 6mo, it appears that the optimal storage temperature for the isolated phages (in phage buffer) is in the region of 4°C.

Chloroform assay

Chloroform exposure imposed negative effects on phage titers (Figure 3). ANOVA showed that significant ($p < 0.001$) differences occurred between the titers of Sabp-P1, Sabp-P2 and Sabp-P3 in comparison to phages Sabp-P4, Sabp-P5 and Sabp-P6. Sabp-P1, Sabp-P2 and Sabp-P3 appeared more stable and titres did not reduce as significantly upon exposure to chloroform, as they did for Sabp-P4 and Sabp-P5. Sabp-P6 showed the most stability over time upon exposure to chloroform. However, a general reduction in phage titers was noted for each phage (Table 1).

pH Sensitivity

All of the screened phages (Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5, Sabp-P6) showed sensitivity to changes in the pH of the surrounding growth media (Figure 4). ANOVA showed significant differences ($p < 0.001$) in activity between phages as pH increased, as well as significant difference in interactions between the phages themselves. Sabp-P1, Sabp-P2 and Sabp-P3 showed the highest increase in phage titer between pH 6 to 7. Thereafter, a sharp decline in phage titre was noted

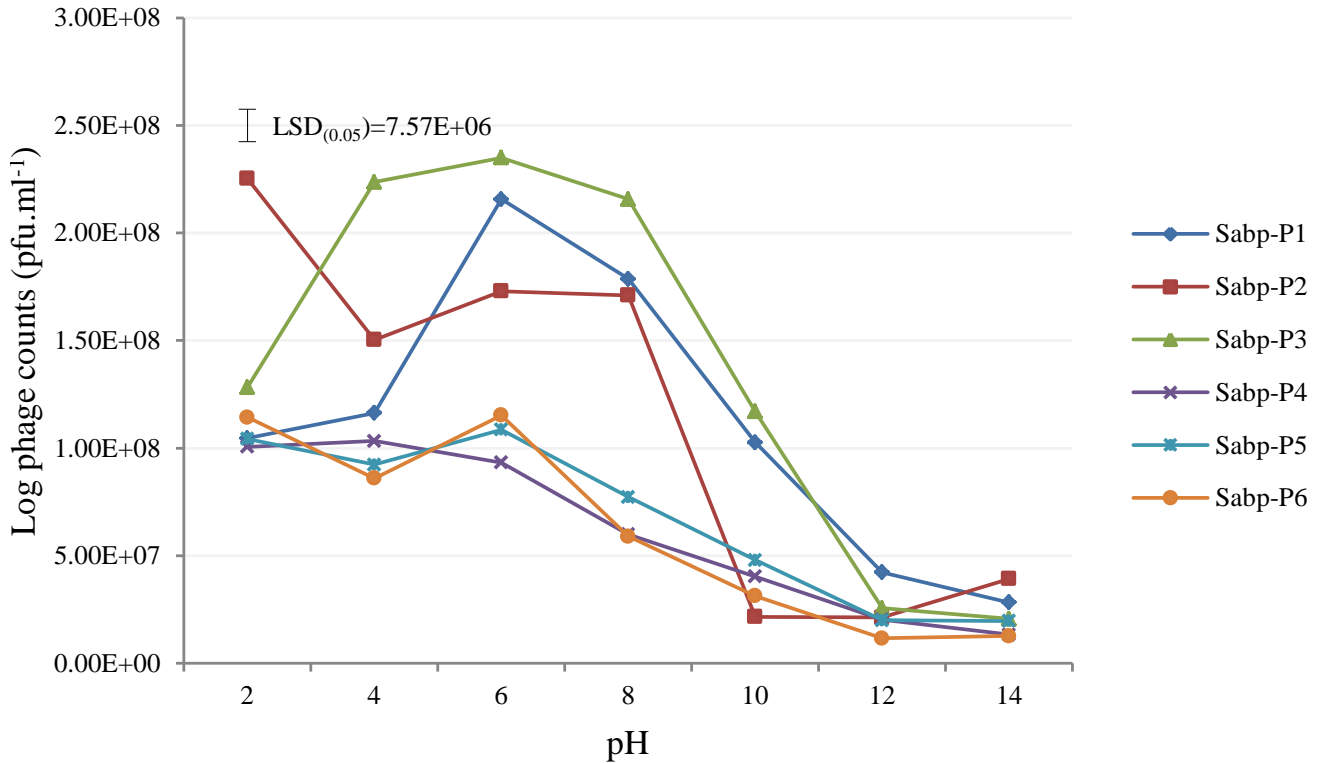


Figure 4. Effect of increasing pH on the titers of six phages. *SaB1* was used as the host bacterium for phage propagation. Data points are the means of three independent experiments. The vertical bar represents the LSD_(0.05) when comparing any phage x pH combination.

from pH 7.2 to 12. Phages, Sabp-P4, Sabp-P5 and Sabp-P6, showed a similar pattern. However, titres were significantly lower from those of Sabp-P1, Sabp-P2 and Sabp-P3. There was no significant difference in activity between Sabp-P1, Sabp-P2 and Sabp-P3, or, between activity of Sabp-P4, Sabp-P5 and Sabp-P6. However, each group differed significantly from each other.

Glycerol assay

A general trend of reduced phage activity was observed with an increasing concentration of glycerol (Figure 5). ANOVA showed significant differences ($p < 0.001$) in activity between phages as glycerol concentrations increased from 5 to 100%. Phages Sabp-P2 and Sabp-P3 are stable and able to replicate up to a glycerol concentration of 25%. Thereafter, titres dropped significantly and no further increase was noted. Phages Sabp-P1, Sabp-P4, Sabp-P5 and Sabp-P6 showed a dramatic decline in titre as glycerol concentration increased from 10% upwards. This differed significantly from the activity of Sabp-P2 and Sabp-P3 ($p < 0.001$). Exposure of the selected phages to high concentrations of glycerol resulted in a dramatic decrease in titre that ranged from 52 to 94% (Table 2).

DISCUSSION

The onset of antibiotic resistance in *S. aureus* and its implications for the treatment of bovine mastitis have raised awareness of the need for alternative control therapies. Phages provide such an alternative. However, associated with this therapy are several challenges, particularly in terms of developing stable formulations and optimizing storage and *in vivo* application of phage products. The primary objective of the current study was to evaluate phage titers upon exposure to simulated stresses that might be encountered during the formulation of the phage or during *in vivo* application of the formulated phages.

Phage activity was assessed *in vitro*, in a heat assay to determine the temperature at which the selected phages are most stable, for the longest duration. *In vivo* application of phages requires resistance (or tolerance) to high temperatures. Temperature fluctuations in an *in vivo* system could possibly arise from changes in weather patterns, physiological changes in the body of the cow itself, or even changes in the storage conditions of the phage. All six phages that were screened showed reduced lytic ability from as early as 10min exposure to 45°C. This is significant, especially in terms of formulation of phages into commercial products. Certain formulation

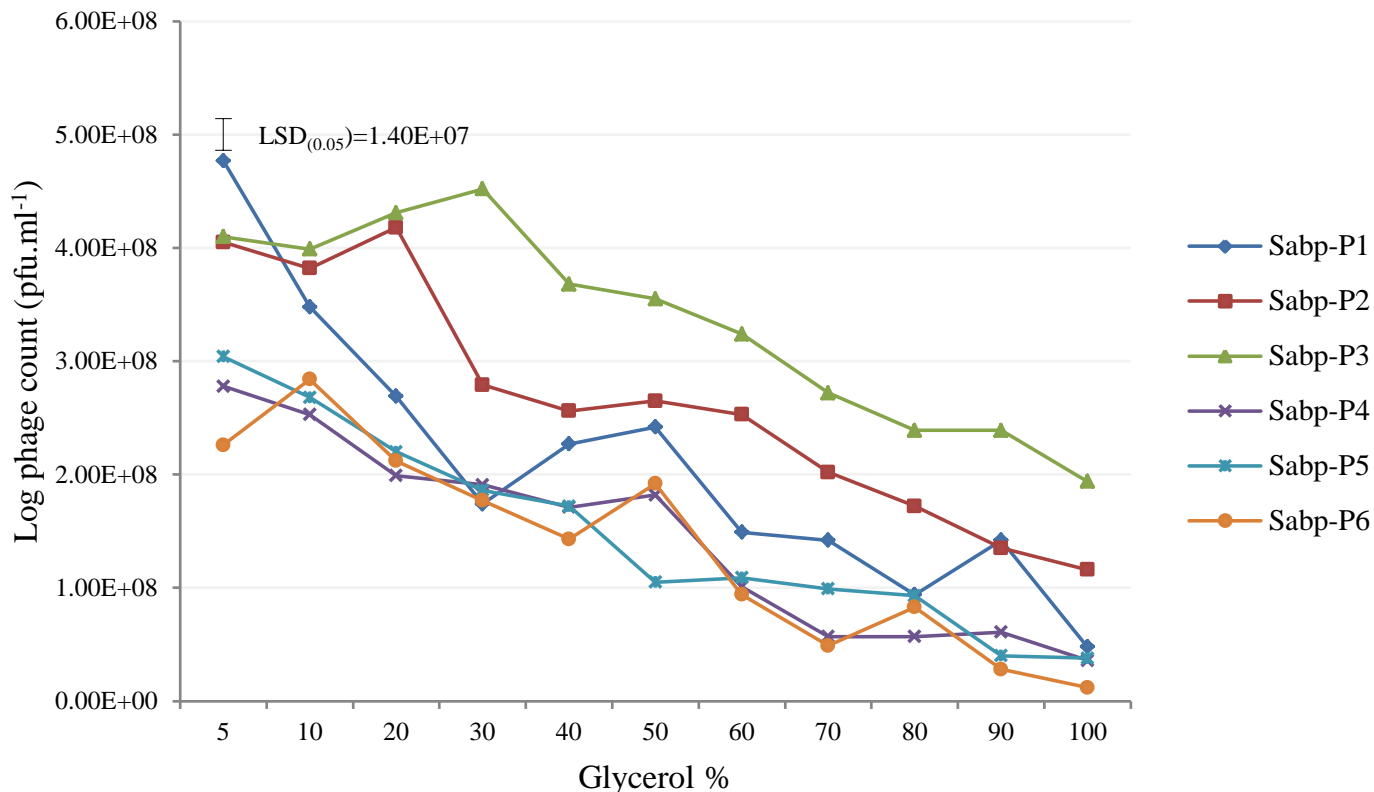


Figure 5. Effects of increasing glycerol concentration on phage titers. *SaB1* was used as the host bacterium for phage propagation. Data points are the means of three independent experiments. The vertical bar represents the LSD_(0.05) when comparing any phage x glycerol % combination.

Table 2. Reduction in phage titers after exposure to increasing concentration ranges of glycerol.

Phage	Reduction in phage activity (%)	Range of increase in glycerol concentration that limits phage growth (%)
Sabp-P1	89.94	10 - 100
Sabp-P2	71.36	25 - 100
Sabp-P3	52.68	25 - 100
Sabp-P4	87.05	10 - 100
Sabp-P5	87.5	10 - 100
Sabp-P6	94.69	10 - 100
Mean	80.54	
LSD	7.01	
CV%	1.5	

procedures may require high temperatures during the manufacturing process. This has to be undertaken with caution in order to minimize any reduction in the replication ability of the phages. Phages are primarily composed of protein (Kutter and Sulakvelidze, 2005). Any prolonged exposure to high temperature may cause denaturation of phage proteins responsible for infection of

a bacterial host. Compromised physical structure of an otherwise infective phage then reduces the control potential that that phage could have imposed on a target host. These results correlate with previous studies where phage lytic ability was shown to be reduced by prolonged exposure to high temperatures (Da Silva and Janes, 2005; Bryant et al., 2007; Chandra et al., 2011).

The selected phages also showed reduced lytic ability after exposure to temperatures below 4°C. This reduced pathogenicity could be attributed to the effect that freezing/thawing may have on phage ultrastructure. This is particularly relevant for tailed phages from the *Myoviridae* family. Delicate tail and tail fibres could become dissociated from the virus head due to changes in osmotic pressure (Jończyk et al., 2011). This sort of dissociation renders the phage ineffective as a control agent. The results of this study contradict those of Hsieh et al., (2011), where phages were found to have maintained stability even after one year of storage at -85°C. Hsieh et al., (2011) stored all phages in 7% dimethyl sulfoxide, in comparison to phage buffer (+ glycerol) in the current study. The choice of storage medium might therefore influence phage stability over time. For the purposes of an *in vivo* study, long- and short-term storage of phage products at 4°C, in a more protective medium (such as glycerol) seems the most likely way forward.

When phages are grown in large-scale liquid broth cultures, either for laboratory or *in vivo* assays, chloroform is usually added to the growing medium to kill any live bacterial cells that remained un-lysed (Sambrook et al., 1989). The isolated phages from this study showed extreme sensitivity to chloroform treatments. While phages Sabp-P1, Sabp-P2 and Sabp-P3 demonstrated significant tolerance to chloroform treatment, their lytic ability was still compromised. In light of this, an alternative method has to be employed in order to remove host cells from a culture suspension. Micro-filtration serves as one of the least-damaging methods to apply, as phage simply pass directly through the filter while un-lysed bacterial cells as well as large bacterial debris, are retained. The use of micro-filters was implemented throughout the current study.

The pH of the medium in which a phage is propagated is also an area of importance. Depending on where a phage has been isolated from, the pH of its growth medium should ideally mimic that of its natural environment in order to promote optimal phage replication. The pH of milk is between pH 6.6 to 6.7 (Blowey and Edmondson, 2010). Optimal phage replication occurred at pH 6 to 7, followed by a sharp decline at higher pH. This was also noted in a study by Da Silva and Janes (2005), where phages specific to *Vibrio* spp. (infective on oysters) were screened at various pH ranges. The *Vibrio* phages were most stable at that pH range which best mimicked the pH of the oyster system (pH 7 to 8).

The formulation of phage/s into a product that can be applied in an *in vivo* system requires storage of the phages in a medium that maintains phage stability, is itself stable over time, and does not damage the teat upon application. Dairy farmers implement both pre- and post-dipping disinfectants during the milking process (Blowey and Edmondson, 2010). Teat skin has relatively

few sebaceous glands, and continual washing and drying of the teats can remove the limited amount of fatty acids that maintain the skin barrier (Blowey and Edmondson, 2010). This can result in severe cracking/breaking of the teat skin, leaving the teat exposed to entry of pathogens. In an attempt to maintain healthy teat skin, additives are used in post-dipping formulations. Emollients (such as lanolin) and humectants (such as glycerin) are the most commonly applied additives (Blowey and Edmondson, 2010). The current study investigates the effect of glycerol concentrations on phage activity. Phage lytic ability was optimal in a suspension of up to 15% glycerol, but dropped thereafter. Phage suspension in a 10% glycerol solution could be applied as an *in vivo* treatment for the treatment of bovine mastitis. Such an application could have a two-fold benefit: (1) the protective effect of phages against *S. aureus* on teat surfaces; (2) the moisturizing effect of glycerol on sensitive teat skin. It is important to note that phages have not been shown to have any negative effects on either the actual health of the cow (O'Flaherty et al., 2005b; Gill et al., 2006a) or the quality of milk produced (Hudson et al., 2005; Garcia et al., 2008).

Conclusion

This study shows the optimum storage and growth conditions necessary in order to maintain high titre phage cultures. Slight deviations from the standard growth requirements (37°C for optimal growth; storage at 4°C, culture medium pH of 6 to 7) for the selected phages resulted in dramatic reductions in phage activity. Phages Sabp-P1, Sabp-P2 and Sabp-P3 showed more vigour and were more robust than Sabp-P4, Sabp-P5 or Sabp-P6. Sabp-P1, Sabp-P2 and Sabp-P3 would be preferred candidates when investigating *in vivo* applications of the phages as a control treatment against staphylococcal bovine mastitis.

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REFERENCES

- Akinyemi KO, Oladapo O, Okwara CE, Ibe CC, Fasura KA (2005). Screening of crude extracts of six medicinal plants used in South-West Nigerian unorthodox medicine for anti-methicillin resistant *Staphylococcus aureus* activity. BMC Complement. Alt. Med. 5:6-13.
- Almeida RA, Mathews KR, Cifrian E, Guidry AJ, Oliver SP (1996). *Staphylococcus aureus* invasion of bovine mammary epithelial cells. J. Dairy Sci. 6:1021-1026.
- Barkema HW, Schukken YH, Zadoks RN (2006). The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. J. Dairy Sci. 89:1877-1895.
- Blowey R, Edmondson P (2010). Mastitis control in dairy herds, 2nd

- edition. CAB International: UK.
- Borm AA, Fox LK, Leslie KE, Hogan JS, Andrew SM, Moyes KM et al. (2006). Effects of prepartum intramammary antibiotic therapy on udder health, milk production, and reproductive performance in dairy heifers. *J. Dairy Sci.* 89:2090-2098.
- Bryant S, Rahmanian R, Tam H, Zabetian S (2007). Effects of microwave irradiation and heat on T4 bacteriophage inactivation. *J. Experiment. Microbiol. Immunol.* 11:66-72.
- Cabrera CE, Gómez RF, Zuñiga AE, Corral RH, López B, Chávez M (2011). Epidemiology of nosocomial bacteria resistant to antimicrobials. *Colombia Med.* 42(1):117-125.
- Carter EW, Kerr DE (2003). Optimization of DNA-based vaccination in cows using green fluorescent protein and protein A as a prelude to immunization against staphylococcal mastitis. *J. Dairy Sci.* 4:1177-1193.
- Chandra M, Thankur S, Narang D, Saxena HM (2011). Isolation of a bacteriophage against *Salmonella* Dublin and determination of its physical resistance under varied *in vitro* conditions. *Afr. J. Microbiol. Res.* 5(15):2044-2047.
- Da Silva LVA, Janes ME (2005). Effect of temperature, pH, and salt content on the bacteriophage activities against virulent *Vibrio vulnificus*, attenuated *Vibrio vulnificus*, or *Vibrio parahaemolyticus*. IFT Annual Meeting, July 15-20.
- Fawole OA (2009). Pharmacology and Phytochemistry of South African Traditional Medicinal Plants used as Antimicrobials. Thesis: Master of Science, Research Centre for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Pietermaritzburg.
- García P, Madera C, Martínez B, Rodríguez A (2007). Biocontrol of *Staphylococcus aureus* in curd manufacturing using bacteriophages. *Int. Dairy J.* 17:1232-1239.
- García P, Madera C, Martínez B, Rodríguez A, Evaristo Suárez J (2008). Prevalence of bacteriophages infecting *Staphylococcus aureus* in dairy samples and their potential as biocontrol agents. *J. Dairy Sci.* 92:3019-3026.
- Gill JJ, Pacan JC, Carson ME, Leslie KE, Griffiths MW, Sabour PM (2006a). Efficacy and pharmacokinetics of bacteriophage therapy in treatment of subclinical *Staphylococcus aureus* mastitis in lactating dairy cattle. *Antimicrob. Agents Chemother.* 50(9):2912-2918.
- Gill JJ, Sabour PM, Leslie KE, Griffiths MW (2006b). Bovine whey proteins inhibit the interaction of *Staphylococcus aureus* and bacteriophage K. *J. Appl. Microbiol.* 101:377-386.
- Goñi P, Vergara Y, Ruiz J, Albizu I, Vila J, Gómez-Lus R (2004). Antibiotic resistance and epidemiological typing of *Staphylococcus aureus* from ovine and rabbit mastitis. *Int. J. Antimicrob. Agents* 23:268-272.
- Harley JP, Prescott LM (1993). Basic Laboratory and Culture Techniques: Laboratory Exercises in Microbiology, 2nd Edition. USA: W.M.C. Brown Publisher.
- Herbert A, Sayasith K, Senechal S, Dubreuil P, Lagace J (2000). Demonstration of intracellular *Staphylococcus aureus* in bovine mastitis alveolar cells and macrophages isolated from naturally infected cow milk. *FEMS Microbiol. Lett.* 1:57-62.
- Hsieh S-E, Lo H-H, Chen S-T, Lee M-C, Tseng Y-H (2011). Wide host range and strong lytic activity of *Staphylococcus aureus* lytic phage Stau2[∇]. *Appl. Environ. Microbiol.* 77(3):756-761.
- Hudson JA, Billington C, Carey-Smith G, Greening G (2005). Bacteriophages as biocontrol agents in food. *J. Food Protect.* 68(2):426-437.
- Jończyk E, Kłak M, Międzybrodzki R, Górski A (2011). The influence of external factors in bacteriophages - a review. *Folia Microbiol. (Praha)* 56(3):191-200.
- Jones JB, Jackson LE, Balogh B, Obradovic A, Iriarte FB, Momol MT (2007). Bacteriophages for plant disease control. *Ann. Rev. Phytopathol.* 45:245-62.
- Kutter E, Sulakvelidze A (2005). Bacteriophages: Biology and Applications. USA: CRC Press.
- Mangili A, Bica I, Snyderman DR, Hamer DH (2005). Daptomycin-resistant, methicillin-resistant *Staphylococcus aureus* bacteremia. *Clin. Infect. Dis.* 40:1058-1060.
- Murchan S, Aucken HM, O'Neill GL, Ganner M, Cookson BD (2004). Emergence, spread, and characterization of phage variants of epidemic methicillin-resistant *Staphylococcus aureus* 16 in England and Wales. *J. Clin. Microbiol.* 42 (11):5154-5160.
- Nickerson SC (2009). Control of heifer mastitis: Antimicrobial treatment - An overview. *Vet. Microbiol.* 134:128-135.
- O'Flaherty S, Coffey A, Meaney WJ, Fitzgerald GF, Ross RP (2005a). Inhibition of bacteriophage K proliferation on *Staphylococcus aureus* in raw bovine milk. *Lett. Appl. Microbiol.* 41:247-279.
- O'Flaherty S, Ross RP, Flynn J, Meaney WJ, Fitzgerald GF, Coffey A (2005b). Isolation and characterisation of two anti-staphylococcal bacteriophages specific for pathogenic *Staphylococcus aureus* associated with bovine infections. *Lett. Appl. Microbiol.* 41:482-486.
- Pereira UP, Oliveira DGS, Mesquita LR, Costa GM, Pereira LJ (2011). Efficacy of *Staphylococcus aureus* vaccines for bovine mastitis: A systematic review. *Vet. Microbiol.* 148:117-124.
- Sambrook J, Maniatis T, Fritsch EF (1989). Molecular Cloning: A Laboratory Manual, 2nd Edition. Cold Spring Harbour Laboratory Press, New York: Cold Spring Harbour.
- Sandgren CH, Waller KP, Emanuelson U (2008). Therapeutic effects of systemic or intramammary antimicrobial treatment of bovine subclinical mastitis during lactation. *Vet. J.* 175:108-117.
- Santos SB, Carvalho CM, Sillankorva S, Nicolau A, Ferreira EC, Azeredo J (2009). The use of antibiotics to improve phage detection and enumeration by the double-layer agar technique. *BMC Microbiol.* 9:148-157.
- Shi D, Hao Y, Zhang A, Wulan B, Fan X (2010). Antimicrobial resistance of *Staphylococcus aureus* isolated from bovine mastitis in China. *Transbound. Emerg. Dis.* 57:221-224.
- Sulakvelidze A, Barrow P (2005). Phage Therapy in Animals and Agribusiness. In Kutter E, Sulakvelidze A (eds.) Bacteriophages: Biology and Applications, USA: CRC Press, pp. 335-380.
- Sutra LAP (1993). Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Staphylococcus aureus*. *J. Med. Microbiol.* 2:79-89.
- Synott AJ, Kuang Y, Kurimoto M, Yamamichi KH, Iwano H, Tanji Y (2009). Isolation from sewage influent and isolation of novel *Staphylococcus aureus* bacteriophages with wide host ranges and potential lytic capabilities. *Appl. Environ. Microbiol.* 75 (13):4483-4490.
- Vanderhaeghen W, Cerpentier T, Adriaenssens C, Vicca J, Hermans K, Butaye P (2010). Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. *Vet. Microbiol.* 144 (1/2):166-171.
- Villar M, Marimon JM, García-Arenzana JM, de la Campa AG, Ferrándiz MJ, Pérez-Trallero E (2011). Epidemiological and molecular aspects of rifampicin-resistant *Staphylococcus aureus* isolated from wounds, blood and respiratory samples. *J. Antimicrob. Chemother.* 66(5):997-1000.

Full Length Research Paper

Antibacterial activity of the stem bark extracts of *Acacia mearnsii* De Wild

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Antibacterial activity of four different extracts from the stem bark of *Acacia mearnsii* was measured against five Gram-positive and five Gram-negative bacterial strains: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Micrococcus kristinae*, *Streptococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Klebsiella pneumonia* and *Serratia marcescens*. The extracts assessed include hexane, ethyl acetate, dichloromethane and methanol. The hexane, ethyl acetate and methanol extracts showed some inhibitory effects on the selected bacteria. The hexane extract showed some activity against four Gram-positive and two Gram-negative bacterial strains, but was not active against one Gram-positive and three Gram-negative bacterial strains. The ethyl acetate extract was effective against all the bacterial strains used in this study. The methanolic extract was effective against all the Gram-positive bacterial strains but it was not active against the Gram-negative bacterial strains except *Escherichia coli*. The dichloromethane extract was not active at all against all the bacterial strains tested.

Key words: *Acacia mearnsii*, antibacterial activity, bacteria species.

INTRODUCTION

Globally, the attractions that have led to the study of medicinal plants as a basis of pharmacologically active compounds have improved and have become greater than ever before. In some developing countries, it is acknowledged that plants are the main medicinal sources to treat infectious diseases. The human environment in these countries is crowded while sanitation is poor; therefore diseases like diarrhoea and dysentery which are caused by bacterial enteropathogens are among the core causes of morbidity and death (Alanis et al., 2005). Plants still make an input that is very important to health care, even though there is great progress in modern

medicine. The reason for this is the growing appreciation of the value of traditional medical systems, mainly of Asian origin, and the recognition of medicinal plants from the native pharmacopoeias, which have important healing power (Adebolu and Oladimeji, 2005).

A lot of work has been done on antimicrobial and phytochemical constituents of medicinal plants and utilizing them for the management of microbial infections for both topical and systemic applications as likely alternatives to formally approved chemically artificial drugs to which many infectious microorganisms have become resistant (Akinpelu and Onakoya 2006). National

and international policymakers are calling for associations linking modern and traditional remedies to bridge the gap in global public health.

Scientists on the other hand predicted that phytochemicals with sufficient antibacterial efficacy will be helpful for bacterial infections. As a result of this, there is an increase in the hunt for natural products from plants as the latest antimicrobial representatives in current times (Olajuyigbe and Afolayan, 2011).

While there is rapid growth in the arsenal of agents available to treat bacterial infections, the rise in the number of antibiotic resistant bacteria is growing faster and therefore remains beyond reach. Information from literature and accounts from ethnobotany suggest that plants are the sleeping giants of the pharmaceutical industry (Farnsworth and Morris, 1976). This is suggested because mainly through secondary metabolites, higher plants are thought to provide the natural basis of antimicrobial drugs that will produce lead compounds that may be engaged in controlling some infections worldwide (Akinpelu and Onakoya, 2006).

Fabaceae is regarded as the second largest family of medicinal plants, with over four hundred and ninety medicinal plant species of which the majority have been used as traditional medicine. Thirty one species of medicinal plants that belong to twenty genera are found in this family and these are explained in the Chinese Pharmacopeia, and a number of species are included in the Japanese Pharmacopoeia. Afterwards, it was found that these plant species have important medicinal properties, they have been used widely as pharmaceutical products (Gao et al., 2010). *Acacia*, a member of the family *Fabaceae* is a large genus with nine hundred species; about seven hundred of the species are indigenous to Australia. Other species of the same genus occur mainly in tropical and subtropical regions of Africa, Asia and America (Ahmad et al., 2011). *Acacia mearnsii* de Wild is a fast-rising leguminous tree, which is native to South Eastern Australia and was introduced to South Africa hundred and fifty years ago, primarily for the tanning industry (Fatunbi et al., 2009).

Given the effects of bacterial infestation (Faulde et al., 2001), colonization (Goldmann et al., 1978) and resistance to existing antibacterial agents (Lenski, 1998), and the risks they pose in clinics, hospitals and hospices (Frieden, 2013), we measured the antibacterial activity of the extracts of the stem bark of *Acacia mearnsii* De Wild, against bacterial species of nosocomial origin, all of which are common isolates from local hospitals.

Study area

OR Tambo District Municipality (ORTDM) is found in the Eastern Cape Province, where it occupies the eastern coastal portion of the province. This District Municipality is bordered by KwaZulu-Natal and by the Eastern Cape districts of Amatole, Chris Hani, UKhahlamba and Alfred

Nzo. The district covers 15,946.84 km² and includes seven local municipalities. It has a diversity of vegetation, from grasslands and thicket to forests and bushveld. ORTDM is believed to have the richest natural resources and the most fertile areas in the country, with good soils and climatic conditions (McCann, 2005). The inhabitants of this district municipality are about 1.7 million. About 93% of the inhabitants dwell in rural areas, whereas approximately 77% of the population is without jobs. The local language of the majority of the dwellers is isiXhosa, a Nguni language, whereas the rest of the people speak Afrikaans and English. The larger part of the region is rural with a large area of arable land. On the other hand, agriculture in the ORTDM is poorly developed and mainly subsistence (Bisi-Johnson et al., 2010).

MATERIALS AND METHODS

Hexane, ethyl acetate, methanol and dichloromethane were obtained from Sigma-Aldrich as analytical reagents. The orbital shaker used was an MRC with twelve positions for 250 ml erlenmeyer flasks. The Buchner funnel was a Corning and the filter paper was a Whatman No. 1. The rotary evaporator was a Buchi R-215 fitted with a vertical water cooled condenser. Nutrient agar was purchased from Arcos. The autoclave was an Optima B class from Prestige Medical. The water was triple distilled and passed through a deionising column. The ten bacterial strains were obtained from the National Health Laboratory Services. All nutrient media were from Arcos.

Plant collection

In a parallel study, the five plants that were most frequently mentioned and highly recommended by herbalists, traditional healers and rural dwellers were found to be *Acacia mearnsii*, *Psidium guajava*, *Teucrium kraussii*, *Strychnos henningsii* and *Xysmalobium undulatum*. This parallel study consisted of a questionnaire survey through which the data were collected on the plants that are traditionally used for the treatment of ailments of the gastro intestinal tract. The data collected included the names of the plants, the location of collection (study area), the plant parts used, the method of preparation, the method of administration, and the amounts used. In this parallel study, *A. mearnsii*, the plant species selected for the present study was reported to be the most commonly used by most traditional healers and other community members in the study area. The study also showed that the dried stem bark was the most frequently used part of the plant. The material is ground and boiled in water and the mixture may be stored for a week in a closed glass container at room temperature before use.

Acacia mearnsii was collected from Lusikisiki, in the month of July, 2011. A voucher specimen was prepared after identification and deposited at the Kei Herbarium at the Nelson Mandela Drive delivery site, Mthatha campus, Walter Sisulu University. The bark was stripped off the stem and dried in open air in the dark for at least a week, before grinding using a mortar and pestle. The ground material (2 kg) was placed in a closed glass jar and stored in the cold room at -5°C.

Extraction of plant material

Ground plant material (400 g) was sequentially extracted with 1 L of

Table 1. Test organisms used in the study; five Gram positive and five Gram negative bacteria.

Test organism	Gram strain	Diseases caused	Reference
<i>Staphylococcus aureus</i>	Positive	Atopic dermatitis, Ritter's disease, endocarditis	Khalid et al., 2011
<i>Staphylococcus epidermidis</i>	Positive	Nosocomial sepsis Intravascular catheter-associated infection	Vuong et al., 2003 Rupp et al., 2001
<i>Bacillus cereus</i>	Positive	Diarrhoeal syndrome and emetic syndrome	Granum and Lund, 1997
<i>Micrococcus kristinae</i>	Positive	Catheter-related recurrent bacteremia	Basaglia et al., 2002
<i>Streptococcus faecalis</i>	Positive	Urinary tract infections	Kau et al., 2005
<i>Escherichia coli</i>	Negative	Involved in infections of the intestinal and urinary tracts of Humans	Darfeuille-Michaud and Colombel, 2008
<i>Pseudomonas aeruginosa</i>	Negative	Wound infection in burnt patients	Khalid et al., 2011
<i>Shigella flexneri</i>	Negative	Bacillary dysentery	Sansonetti et al., 2000
<i>Klebsiella pneumonia</i>	Negative	Liver abscess	Fung et al., 2011
<i>Serratia marcescens</i>	Negative	Nosocomial infections	Sartor et al., 2000

Table 2. Test organisms used in the present study and in Olajuyigbe and Afolayan (2011).

Used only in the Present Study	Common to both present study and to Olajuyigbe and Afolayan (2011)	Used only in Olajuyigbe and Afolayan (2011)
<i>Staphylococcus epidermidis</i> (+)	<i>Staphylococcus aureus</i> (+)	<i>Bacillus subtilis</i> (+)
<i>Micrococcus kristinae</i> (+)	<i>Streptococcus faecalis</i> (+)	<i>Micrococcus luteus</i> (+)
<i>Serratia marcescens</i> (-)	<i>Bacillus cereus</i> (+)	<i>Shigella sonnei</i> (-)
	<i>Pseudomonas aeruginosa</i> (-)	<i>Salmonella typhi</i> (-)
	<i>Escherichia coli</i> (-)	<i>Enterobacter cloacae</i> (-)
	<i>Klebsiella pneumonia</i> (-)	<i>Proteus vulgaris</i> (-)
	<i>Shigella flexneri</i> (-)	

hexane, ethyl acetate, dichloromethane and methanol. All the extracts were filtered under vacuum through Whatman No. 1 filter paper. The solvent extracts were concentrated using a rotary evaporator under reduced pressure and temperature (Fawole et al., 2009). Each dry extract was later re-dissolved in its respective solvent, 0.93 g dichloromethane, 1.55 g ethyl acetate, 1.68 g methanol and 1.98 g hexane.

Test organisms

Five Gram-positive and five Gram-negative bacterial strains were used in this study (Table 1). The Gram-positive bacterial strains included *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Micrococcus kristinae* and *Streptococcus faecalis*. The Gram-negative bacterial strains included *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Klebsiella pneumonia* and *Serratia marcescens*. All the bacterial strains were obtained from the National Health Laboratory Services (NHLS).

The antibacterial activities of crude aqueous and ethanolic extracts of the stem bark of *A. mearsii* De Wild against Gram-positive and Gram-negative bacteria have been reported by Olajuyigbe and Afolayan (2011). The Gram-positive bacterial strains

included *S. aureus*, *S. faecalis*, *B. cereus*, *Bacillus subtilis* and *Micrococcus luteus*. The Gram-negative strains were *Pseudomonas aeruginosa*, *Shigella sonnei*, *Salmonella typhi*, *E. coli*, *Enterobacter cloacae*, *K. pneumonia*, *Proteus vulgaris*, and *Shigella flexneri*. Commonality with the present study includes seven test organisms: *S. aureus*, *S. faecalis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *E. coli*, *K. pneumonia* and *S. flexneri*. However, unique to the present study are three test organisms: *S. epidermidis*, *Micrococcus kristinae* and *Serratia marcescens*, while unique to the study by Olajuyigbe and Afolayan (2011) are six test organisms: *B. subtilis*, *M. luteus*, *S. sonnei*, *S. typhi*, *Enterobacter cloacae* and *Proteus vulgaris*. The comparison of the present study with that reported by Olajuyigbe and Afolayan (2011) in terms of the test organisms used is summarised in Table 2.

Antibacterial testing

The antibacterial activity was evaluated using the dilution-in-agar technique (Alanis et al., 2005). The bacterial species that were used were sub-cultured and preserved on nutrient agar that was contained in Petri dishes (Nkomo and Kambizi, 2009). Nutrient agar (60 ml) was prepared and sterilized using an autoclave at 121°C for

Table 3. Antibacterial activity of *A. mearnsii*.

Bacteria species	Gram +/-	Minimum inhibitory concentration(MIC) (mg/ml)			
		Hexane	Ethyl acetate	Dichloromethane	Methanol
<i>Staphylococcus aureus</i>	+	10	1	na	1
<i>Staphylococcus epidermidis</i>	+	10	5	na	5
<i>Bacillus cereus</i>	+	10	5	na	5
<i>Micrococcus kristinae</i>	+	10	10	na	1
<i>Streptococcus faecalis</i>	+	Na	10	na	5
<i>Escherichia coli</i>	-	10	10	na	5
<i>Pseudomonas aeruginosa</i>	-	10	10	na	na
<i>Shigella flexneri</i>	-	na	10	na	na
<i>Klebsiella pneumonia</i>	-	na	10	na	na
<i>Serratia marcescens</i>	-	na	10	na	na

Minimum inhibitory concentration (mg/ml); na: not active.

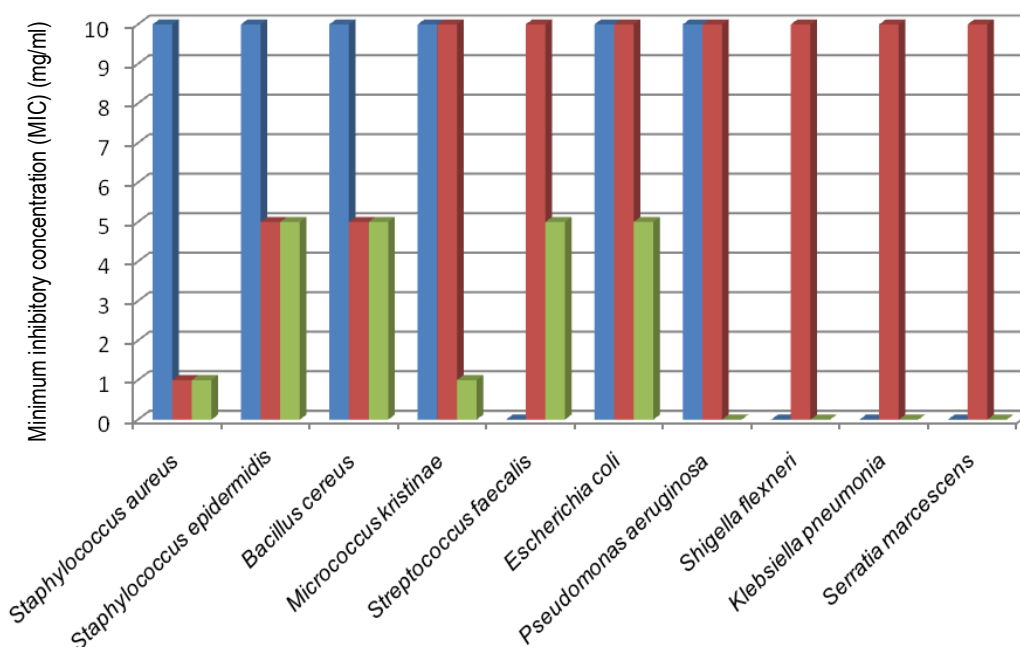


Figure 1. Antibacterial activity of the hexane (■), ethyl acetate (■) and methanol (■) extracts of the stem bark of *A. mearnsii* against ten test organisms.

15 min. The agar was distributed into 12 test tubes, after which, nine test tubes were mixed with the extract, ending up with final concentrations of 1.0, 5.0, and 10.0 mg of extract per ml of nutrient agar (Kambizi and Afolayan, 2008).

The relevant solvent was added to the remaining three test tubes containing nutrient agar and were used as controls (Nkomo and Kambizi, 2009). The control experiments that were set up consisted of inoculums without the plant extract (Banso, 2009). After this, the nutrient agar-extract and nutrient agar-solvent mixture were then poured into Petri dishes, allowed to cool and set. The test organism contained in sterilized nutrient broth was streaked in radial array on the agar plates and the plates were incubated at 37°C for 24 - 48 h; thus each treatment was replicated three times.

The concentrations of the extracts that were tested against the organisms, for suppression of growth were observed with the naked eye (Nkomo and Kambizi, 2009). The lowest concentration that

inhibited the growth of bacteria that can be visible with a naked eye was used as the minimum inhibitory concentration (MIC) (Alanis et al., 2005).

RESULTS

The results of the antibacterial activity are shown in Table 3 and are also illustrated in Figure 1. The MIC values of the dichloromethane extract are not shown in Figure 1, since the extract was not active against any of the bacterial strains. The hexane extract of the bark of *A. mearnsii* showed potent antibacterial activity against *E. coli* with MIC value of 10.0 mg/ml and the results were

almost similar to those of hexane extracts of the bark of *Ficus congensis* reported by Alaribe et al. (2011) with MIC values of 8.0 mg/ml.

The extract was not active against *K. pneumonia*. According to Alaribe et al. (2011) the same bacterial species was not inhibited by the extract. In this study, the hexane extract showed activity against four Gram-positive bacterial strains with MIC value of 10.0 mg/ml but was not active against *S. faecalis*. This extract also showed some activity against two Gram-negative bacterial strains, *E. coli* and *P. aeruginosa*, but was not active against the remaining three Gram-negative bacterial strains, *S. flexneri*, *K. pneumonia* and *S. marcescens*.

The ethyl acetate extract was effective against all bacterial strains used in this study with MIC values ranging from 1.0 and to 10.0 mg/ml. The methanolic extract was effective against all Gram-positive bacterial strains with MIC values of 1.0 and 5.0 mg/ml but it was not active against the Gram-negative bacterial strains except for *E. coli* which was susceptible at MIC value of 5.0 mg/ml. The extract from dichloromethane was not active at all against any of the bacterial strains tested. It can be concluded that dichloromethane was not a suitable solvent for extracting the active compounds in *A. mearnsii* as no active ingredients were extracted.

DISCUSSION

The inhibition of the growth of Gram-negative and Gram-positive bacteria may be dependent on several factors which include, but are not limited to: resistance to physical disruption, susceptibility to anionic detergents, the thickness of their peptidoglycan cell wall components; Gram-positive bacteria are characterised by a thick layer whereas Gram-negative are characterised by a thin layer with an impenetrable cell wall hence they are more resistant against antibodies. Gram-negative and Gram-positive bacteria normally found in the gastrointestinal tract (GIT) can cause GIT diseases, and this is of particular interest to the present study. The organisms responsible for cholera and bubonic plague are Gram-negative. The MIC for the test microorganisms varied widely against the degree of their vulnerability. The antimicrobial with a low activity against an organism has a high MIC while a highly active antimicrobial gives a low MIC (Banso, 2009).

The methanol extract was the most active, although mainly against Gram-positive bacterial strains. The ethyl acetate extract on the other hand showed the widest spectrum of antibacterial activity, although it was relatively weaker in comparison to the methanolic extract activity. The hexane extract showed no anti-bacterial activity stronger than MIC = 10 mg/ml. The inactivity of the methanol extract on the remaining gram negative bacteria might be due to the composition of these

bacterial cell walls, their resistance to disruptions and the nature of the anionic compounds present in the extract. However, more detailed work is ongoing to ascertain this. The fact that the average activity is positively correlated with the hydrophilicity of the solvent, suggests that the active compounds are hydrophilic or ionic (Darout et al., 2000). The Gram-positive bacteria were clearly more susceptible to the extracts than the Gram-negative bacteria. This may be seen as a justification for the traditional use of the bark extracts of *A. mearnsii* De Wild for the treatment of GIT diseases, such as diarrhoea and stomach ache.

Furthermore, according to Ajali and Okoye (2009), wound ulcers can be infected by *S. aureus* and *Pseudomonas auriginosa* and in their study, these organisms were found to be very sensitive to the root bark extracts of *Olax viridis*. Even in the present study, *S. aureus* and *P. auriginosa* were found to be sensitive to the bark extracts of *A. mearnsii*.

The important activity of the extracts against some enteric organisms like *E. coli* invokes curiosity. This may provide an explanation about the ethnomedicinal use of *A. mearnsii* in the management of diarrhoea (Ajali and Okoye, 2009). The observations of antibacterial activity that were shown by the stem bark extracts of *A. mearnsii* could be due to the amount of one or more compounds present in the plant material. It is also possible that different compounds may be responsible for the activity observed (Fawole et al., 2009). The traditional healers rarely use a single plant in their prescriptions. Using plant mixtures is beneficial in most cases, where different plant parts are used in combination or in series. There is no uniformity in traditional medicine with respect to the harvesting of unrefined materials, technique of production and in quality control of the finished product. Hence there is a need for pharmacological screening of medicinal plants to provide a scientific foundation for the sustained traditional use of plants to provide society with potential sources of new, effective and safe drugs (Eldeen et al., 2005).

Conclusion

The higher potency of the bark extracts of methanol and ethyl acetate are indicative of the extracts that should be used to isolate active compounds. Although, many workers have reported that water is a poor extractor of antibacterial compounds from plant materials (Ibekwe et al., 2001; Karaman et al., 2003), the results of Olajuyigbe and Afolayan (2011) suggested that water may be a good extraction medium and its extract may be as potent as alcoholic solvent extracts. This potency may, however, be due to the presence of anionic components such as thiocyanate, nitrate, chloride and sulphates along with other water soluble antibacterial compounds present in the plant material (Darout et al., 2000). However, the

inclusion of anionic components is likely to be minimised in the ethyl acetate extract. It is therefore unlikely that the potency of the ethyl acetate extract is due to the presence of anionic components, and more likely, it is due to the presence of active organic compounds. These organic compounds can be identified using the bio-autography method, followed by isolation, structure determination and structure-activity studies.

REFERENCES

- Adebolu TT, Oladimeji SA (2005). Antimicrobial activity of leaf extracts of *Ocimum gratissimum* on selected diarrhoea causing bacteria in southwestern Nigeria. *Afr. J. Biotechnol.* 4(7):682-684.
- Ahmad B, Khan I, Azam S, Bashir S, Ahmad J, Hussain F (2011). Screening of *Acacia modesta* for haemagglutination, antibacterial, phytotoxic and insecticidal activities. *J. Med. Plant. Res.* 5(14):3090-3096.
- Ajali U, Okoye FBC (2009). Antimicrobial and anti-inflammatory activities of *Olax viridis* root bark extracts and fractions. *Int. J. Appl. Res. Nat. Prod.* 2(1):27-32.
- Akinpelu DA, Onakoya TM (2006). Antimicrobial activities of medicinal plants used in folklore remedies in south-western. *J. Biotechnol.* 5:1078-1081.
- Alanis AD, Calzada F, Cervantes, JA, Torres J, Ceballos GM (2005). Antibacterial properties of some plants used in Mexican traditional medicine for the treatment of gastrointestinal disorders. *J. Ethnopharmacol.* 100:153-157.
- Alaribe CS, Shode F, Coker HAB, Ayoola G, Sunday A, Singh N, Iwuanyanwu S (2011). Antimicrobial Activities of Hexane Extract and Ducussatin from Stembark Extract of *Ficus congensis*. *Int. J. Mol. Sci.* 12(4):2750-2756.
- Banso A (2009). Phytochemical and antibacterial investigation of bark extracts of *Acacia nilotica*. *J. Med. Plant Res.* 3(2):082-085.
- Basaglia G, Carretto E, Barbarini D, Moras L, Scalone S, Marone P, De Paoli P (2002). Catheter-Related Bacteremia Due to *Kocuria Kristinae* in a Patient with Ovarian Cancer. *J. Clin. Microbiol.* 40(1):311-313.
- Bisi-Johnson MA, Obi CL, Kambizi L, Nkomo M (2010). A survey of indigenous herbal diarrhoeal remedies of O.R. Tambo district, Eastern Cape Province, South Africa. *J. Biotechnol.* 9(8):1245-1254.
- Darfeuille-Michaud A, Colomel J (2008). Pathogenic *Escherichia coli* in inflammatory bowel diseases: Proceedings of the 1st International Meeting on *E. coli* and IBD, June 2007, Lille France. *J. Croh. Col.* 2(3):255-262.
- Eideen IMS, Elgorashi EE, Van Staden J (2005). Antibacterial, anti-inflammatory, anti-cholinesterase and mutagenic effects of extracts obtained from some trees used in South African traditional medicine. *J. Ethnopharmacol.* 102(2005) 457-464.
- Farnsworth NR, Morris RW (1976). Higher plants - the sleeping giant of drug development. *Am. J. Pharm. Educ.* 148:46-52.
- Fatunbi AO, Dube S, Yakubu MT, Tshabalala T (2009). Allelopathic Potential of *Acacia mearnsii* De Wild. *World Appl. Sci. J.* 7(12):1488-1493.
- Faulde M, Sobe D, Burghardt H, Wermter R, (2001). Hospital infestation by the cluster fly, *Pollenia rudis sensu stricto* Fabricius 1794 (Diptera: Calliphoridae), and its possible role in transmission of bacterial pathogens in Germany. *Int. J. Hyg. Environ. Health* 203(3):201-204
- Fawole OA, Finnie JF, Van Staden J (2009). Antimicrobial activity and mutagenic effects of twelve traditional medicinal plants used to treat ailments related to the gastro-intestinal tract in South Africa. *South Afr. J. Bot.* 75:356-362.
- Frieden T (2013). Meeting the Challenges of Drug-Resistant Diseases in Developing Countries, Committee on Foreign Affairs Subcommittee on Africa, Global Health, Human Rights, and International Organizations, United States House of Representatives, US Department of Health and Human Services, Centers for Disease control and Prevention.
- Fung CP, Chang FY, Lee SC, Hu BS, Kuo BIT, Liu CY, Ho M, Siu LK (2011). A global emerging disease of *Klebsiella pneumoniae* liver abscess: is serotype K1 an important factor for complicated endophthalmitis? *Gut* 50(3):420-424.
- Gao T, Yaoa H, Song J, Liu C, Zhu Y, Ma X, Pang X, Xu H, Chen S (2010). Identification of medicinal plants in the family Fabaceae using a potential DNA barcode ITS2. *J. Ethnopharmacol.* 130(1):116-121.
- Goldmann DA, Leclair J, Macone A, (1978). Bacterial colonization of neonates admitted to an intensive care environment. *J. Pediatr.* 93(2):288-293
- Granum PE, Lund T (1997). *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol. Lett.* 157(1997):223-228.
- Kambizi L, Afolayan AJ (2008). Extracts from *Aloe ferox* and *Withania somnifera* inhibit *Candida albicans* and *Neisseria gonorrhoea*. *J. Biotechnol.* 7(1):012-015.
- Kau AL, Martin SM, Hayes E, Caparon MG, Hultgren SJ (2005). Enterococcus faecalis Tropism for the Kidneys in the Urinary Tract of C57BL/6J Mice. *Infect. Immun.* 73(4):2461-2468.
- Khalid A, Uzair-ur-Rehman, Sethi A, Khilji S, Fatima U, Khan MI, Waqas M, Najam-us-Saqib Q, Asad MHHBA, Farzana K, Mahmood S, Waseem A, Ismail T, Murtaza G (2011). Antimicrobial activity analysis of extracts of *Acacia modesta*, *Artemisia absinthium*, *Nigella sativa* and *Saussurea lappa* against Gram positive and Gram negative microorganisms. *Afr. J. Biotechnol.* 10(22):4574-4580.
- Lenski E (1998). Bacterial evolution and the cost of antibiotic resistance. *Int. Microbiol.* 1(4):265-270
- McCann M (2005). Programme of Support to Local Economic Development in the Eastern Cape. Eastern Cape Competitive Advantage Assessment And Training Support Project. European Consultants Organisation. EC FRAMEWORK CONTRACT AMS/451 - LOT N°9, MISSION N°2005/109496
- Nkomo M, Kambizi L (2009). Antimicrobial activity of *Gunnera perpensa* and *Heteromorpha arborescens* var. *abyssinica*. *J. Med. Plant Res.* 3(12):1051-1055.
- Rupp ME, Fey PD, Heilmann C, Gotz F (2001). characterization of the importance of staphylococcus epidermidis autolysin and polysaccharide intercellular adhesin in the pathogenesis of intravascular catheter-associated infection in a rat model. *J. Infect. Dis.* 183(7): 1038-1042.
- Sansonetti PJ, Phalipon A, Arondel J, Thirumalai K, Banerjee S, Akira S, Takeda K, Zychlisky A (2000). Caspase-1 Activation of IL-1 β and IL-18 Are Essential for *Shigella flexneri*-Induced Inflamm. *Immun.* 12(5):581-590.
- Sartor C, Giacomo V, Duvivier C, Tissot-Dupont H, Sambuc R, Drancourt M (2000). Nosocomial *Serratia marcescens* infections associated with extrinsic contamination of a liquid nonmedicated soap. *Infect. Control Hosp. Epidemiol.* 21(3):196-199.
- Vuong C, Gerke C, Somerville GA, Fischer ER, Otto M (2003). Quorum-Sensing Control of Biofilm Factors in *Staphylococcus epidermidis*. *J. Infect. Dis.* 188(5):706-718.

Full Length Research Paper

Influenza seasonality affected by the 2009 pandemic episode in Senegal

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In Senegal, the seasonality of influenza epidemics is well defined with a clear peak around August and September (in rainy season which occurs from July to October). Surprisingly, the first detection of the A(H1N1)pdm09 virus was in January 2010 and rapidly reached a high detection peak between January and February, indicating a real shift in the influenza seasonality in Senegal. Therefore, climatic factors, the host susceptibility seem insufficient to explain the epidemiology of this virus which is quite different as compared to that of seasonal viruses. Intrinsic properties of this virus may play a role in its seasonal behavior.

Key words: influenza, A(H1N1)pdm09, pandemic, seasonality, epidemiology.

INTRODUCTION

In late March of 2009, a new influenza virus, a quadruple reassortant H1N1 virus, emerged in Mexico (Neumann et al., 2009; New South Wales Public Health Network, 2009). The virus, A(H1N1)pdm09, spread rapidly around the world, prompting the World Health Organization (WHO) to declare the first influenza pandemic of the 21st century on June 11, 2009 (Enserink and Cohen, 2009). However, this virus was not detected in the African continent till lately, notably in West Africa (Nzussouo et al., 2012). Senegal was probably one of the last West African countries where the virus was detected, despite the well-established network for influenza virus monitoring. Indeed the pandemic virus was not detected in Senegal until the first week of 2010, months after being reported by other West African countries (e.g. Ivory Coast, Cape Verde, etc.). The analysis of the circulation profile of the new virus showed differences as compared to seasonal influenza viruses, a situation which has significantly altered the seasonality of influenza in Senegal in the following years (Niang et al., 2012).

MATERIALS AND METHODS

A prospective observational study was conducted during 2009,

2010 and 2011. Senegal, a Western Africa country, has a well-established surveillance system with several Influenza sentinel sites located in urban, sub-urban and rural. At each sentinel site trained physicians identified all influenza-like illness (ILI) cases presenting at the clinics from Monday to Friday. An ILI cases were identified as an outpatient presenting with sudden onset of fever ($\geq 38^{\circ}\text{C}$) and cough or sore throat accompanied or not by myalgia, prostration, headache or malaise, with the onset of symptoms occurring within the previous three days. A standardized form was used to collect demographic and clinical information from the enrolled patients.

Nasal-pharyngeal and oral-pharyngeal swabs were collected from all enrolled ILI cases, placed in 2 ml cryovials containing viral transport medium (Universal Transport Medium, COPAN Diagnostics Inc., Murrieta, CA, USA) and stored at 4°C on site. Upon arrival at the laboratory the specimens were processed immediately for virus detection, identification and characterization.

RNA was extracted from 200 μl of each sample using the QIAamp Viral RNA kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. One step real-time RT-PCR was performed using the ABI 7500 platform according to the CDC protocol for the identification of influenza A(H1 and H3) and B viruses (courtesy of the Centers for Disease Control, Atlanta) during the pandemic episode. Influenza A positives samples are run in a second real time RT-PCR for the subtyping using primers targeting haemagglutinin genes of seasonal (H1 and H3) viruses and A(H1N1)pdm09 (CDC, 2009a).

The weekly rainfall data over the 3 years were collected from the National Meteorological Department of Senegal with the aim to

Table 1. Detection rates of influenza virus by type and subtype in Senegal during the years 2009, 2010 and 2011.

Influenza virus	Years (tested samples number)			
	2009 (936)	2010 (1328)	2011 (922)	Total (3186)
Virus number (%)	274 (29.3%)	443 (33.4%)	246 (26.7%)	963 (30.2%)
Total/an	0	76 (5.7%)	109 (11.8%)	185 (5.8%)
Inf B	274 (29.3%)	367 (27.6%)	137 (14.9%)	778 (24.4%)
Inf A	3 (0.3%)	0	0	3 (0.1%)
H1N1	271 (28.9%)	22 (1.7%)	81 (8.8%)	374 (11.6%)
H3N2	0	345 (26%)	56 (6.1%)	401 (12.6%)
H1N1pmd09				

analyze the behavior of the different influenza viruses with respect to rainfall.

RESULTS

During this study period 3186 samples from patients with ILI were analyzed (Table 1): 936 (29.4%) during 2009, 1328 (41.7%) during 2010 and 922 (28.9%) during 2011. Patient ages ranged from 1 month to 96 years with a mean age of 3 years. 63% of the ILI cases during this period are children between 0 and 5 years age. The male to female ratio was 1:1 (1512 females versus 1674 males).

Of the 3186 samples analyzed, 963 (30.2%) were positive for influenza virus using the real-time RT-PCR method (Table 1). Among these positive, 778 (80.8%) were influenza A, and 185 (19.2%) were influenza B. Of the influenza A positives, 3 (0.4%) were seasonal H1N1, 374 (48.1%) were H3N2 and 401 (51.5%) were H1N1pdm09. The three H1N1 are detected in 2009. The pandemic virus was detected only from 2010 in Senegal (no case detected in 2009).

With regard to the temporal distribution of influenza positives samples during the 3 years of surveillance, we observed different profiles (Figure 1). For the year 2009 (Figure 1A) no influenza virus was detected until week 20 from which the first H3N2 cases were detected. The H3N2 subtype was the major influenza virus detected during this year with a peak around week 27 (beginning of July). The virus continued to circulate at a significant level until week 43 and then gradually disappeared. The end of year 2009 is marked by three cases of seasonal H1N1 (detected at weeks 51 and 52). If we consider the rainfall curve, we note that significant virus circulation coincides with the onset of rains.

The year 2010 (Figure 1B) began with the first cases of the pandemic virus in Senegal (with 2 cases at the week 1). H1N1pdm09 cases increase gradually and reach a peak around the weeks 6, 7 and 8 and disappear at the week 12. The influenza B type appeared at the week 17 with a low circulation level during the following weeks and disappeared at the week 46. Influenza B virus circulates

mainly during the raining period. The pandemic virus reappeared at week 38 and circulated at a low level until week 45. This reappearance coincided with the end of the rainfall. Few cases of H3N2 are detected during this period between weeks 32 and 44.

For 2011 (Figure 1C), only the pandemic virus circulated between weeks 5 and 27 and disappeared. The influenza B type circulation started at week 31 and reached a peak around weeks 37, 38 and 39. During these weeks, it co-circulated with the H3N2 subtype which attained a circulation peak at week 42. Circulation peaks of these two viruses are preceded by rainfall peak recorded at week 34. The pandemic virus reappeared at the week 37 and circulated at a very low level when compared with the others (Flu B and H3N2 subtype) until week 43.

DISCUSSION

In Senegal, the pandemic began in the first week of year 2010 and rapidly reached peak detection during weeks 6, 7 and 8. It is well known that influenza epidemics occur annually worldwide, and display a seasonal pattern in temperate areas, with marked peaks in the winter (typically December-April in the Northern Hemisphere and June-September in the Southern Hemisphere) (Viboud et al., 2006). Tropical and subtropical regions are also subjected to seasonal oscillations in influenza incidence, which have been linked to rainy seasons (Shek and Lee, 2003), even if the seasonal pattern was generally less pronounced than in temperate areas (Viboud et al., 2006). In Senegal, a sub-Saharan country, the seasonality of influenza epidemics has been well defined after 16 years of regular influenza monitoring (Niang et al., 2012).

Indeed there is a clear peak of influenza cases around August and September (in the middle of the rainy season which occurs from July to October) and a very low level of detection for the rest of the year. The first pandemic wave (March 2009) coincided with the end of the influenza season in the Northern Hemisphere and prior to the beginning of the influenza season in the Southern

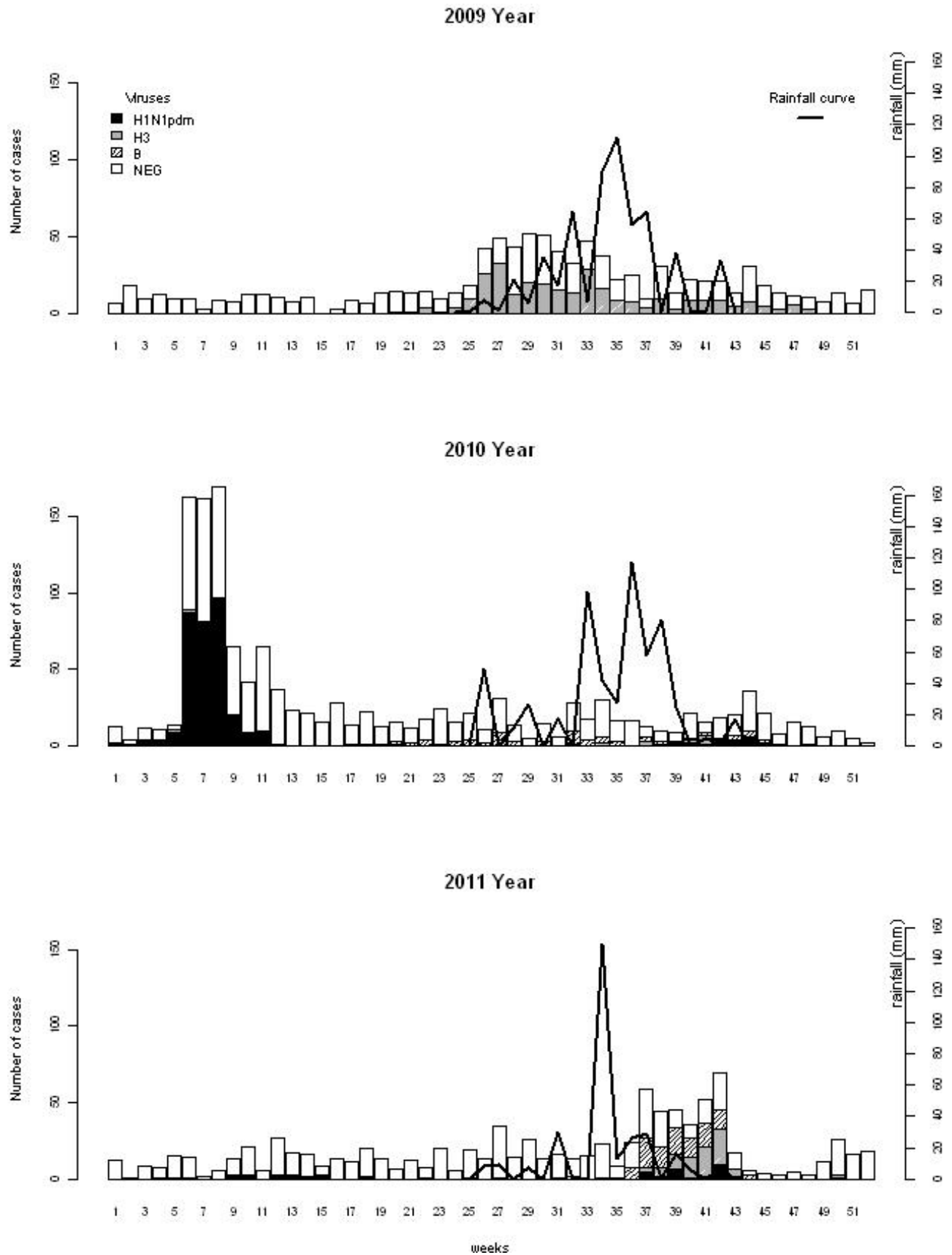


Figure 1. Distribution of weekly influenza positives and pluviometry in Senegal during years 2009, 2010 and 2011.

hemisphere. It would be anticipated that the pandemic virus would have sufficient time to reach Senegal for the regular influenza season; however, this did not occur. No cases of influenza A(H1N1)pdm09 were detected in Senegal during 2009. The 2009 influenza season in Senegal displayed a normal seasonal pattern with a peak between July and September which coincided with the rainy period.

The influenza A(H3N2) virus was the only circulating subtype during this period, although some seasonal A(H1N1) strains were detected at the end of the year (week 51 and 52). The A(H1N1)pdm09 arrived months after the end of the 2009 influenza season in the Senegal.

The reasons for the delayed circulation of A(H1N1)pdm09 in Senegal (and other West African countries) are not clear but unfavorable temperature and humidity conditions are indexed to explain some of the observed patterns (Lowen et al., 2008; Steel et al., 2010). However, this does not seem applicable to Senegal, as the conditions were favorable for seasonal influenza viruses. Although the international air transportation network was evoked by some authors in the spread of influenza viruses (Viboud et al., 2006), it would be inappropriate to use this argument to justify the delay in circulation of the pandemic virus in a city like Dakar (capital city of Senegal).

Considering the well-defined seasonal pattern of influenza circulation in Senegal (Mbayame et al., 2013), the arrival of the A(H1N1)pdm09 virus in January 2010, with the early seasonal peak detection (between January and February), was an atypical and yet unexplained event in the influenza seasonality in Senegal. Thus, for a complete description of the Senegal influenza season in 2010, it should be noted that the rainy season (typical peak influenza circulation) was marked by a disappearance of the pandemic virus and low level circulation of seasonal viruses (influenza B and A(H3N2)). The pandemic virus reappeared in early October, at the end of the typical rainy season. The A(H1N1)pdm09 virus continued to circulate the most in 2011, especially before the rainy season. During this rainy season, the pandemic virus co-circulated with seasonal viruses (influenza B and A(H3N2)), with a lower rate of detection.

With regards to the A(H1N1)pdm09 virus circulation and the annual rainfall distribution, we holistically observed a better circulation of the pandemic virus outside periods of heavy rain. The virus tended to slip away and to reappear at the end of rainy season. It should be noted that the rainy season in Senegal in 2011 started late, with a short duration (with a very low amount of rainfall), and certainly this would explain the circulation of the virus at this rate.

Thus it is clear that the pandemic virus has a seasonal flow profile significantly different from that of other seasonal viruses (the seasonal A(H1N1) having disappeared since the onset of the pandemic virus). To explain

this lag in the circulation of the pandemic virus, we cannot rely entirely on the weather and climate factors although their influence on the epidemiology of influenza viruses is very clear and undeniable (Altizer et al., 2006; Christopher, 2010).

The proposed theory of Edgar Hope-Simpson based on the solar radiation influence on the host susceptibility (depletion of the innate immune system) also cannot be either the argument that may explain this difference (Hope-Simpson, 1992). Indeed, in the Senegalese climatic context the solar radiation levels remained normal despite the cold winter period.

We believe that the intrinsic properties of the pandemic virus may have contributed to the difference in circulation patterns as compared to seasonal viruses, allowing the virus to circulate normally outside of the rainy season to such a high level.

It is now well known that the survival of an influenza virus is determined primarily by the characteristics of its outer casing, or envelope, which is composed of lipid compounds, suggesting that the lipid envelope encasing the virus remains intact longer when the air is sufficiently cold and dry (Polozov et al., 2008). With regards to its high capacity to circulate outside influenza season (rainy season), the pandemic virus seems to have less demand in terms of air humidity, and therefore is able to survive longer in the air as compared to seasonal viruses.

Conclusion

As the pandemic period had been well-described, the post period also need to be explored to collect epidemiologic and biologic information on respiratory viral infections. A better global surveillance would certainly aid in providing insights into understanding the factors contributing to the circulation, transmission and virulence of influenza viruses.

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REFERENCES

- Altizer S, Dobson A, Hosseini P, Hudson P, Pascual M and Rohani P (2006). Seasonality and the dynamics of infectious diseases. *Ecol. Lett.* 9:467-84.
- CDC (2009a). 2009 H1N1 flu: international situation update. Centers for Disease Control and Prevention, Atlanta, GA.
- Christopher F. (2010). The Effects of Weather and Climate on the Seasonality of Influenza: What We Know and What We Need to Know. *Geography Compass* 4/7:718-730, 10.1111/j.1749-8198.2010.00343.x
- Enserink M, Cohen J (2009). Virus of the year. The novel H1N1 influenza. *Science* 326:1607.
- Hope-Simpson RE (1992). The transmission of epidemic influenza. New York:Plenum Press.
- Lowen AC, Steel J, Mubareka S, Palese P (2008). High temperature (30 degrees C) blocks aerosol but not contact transmission of influenza virus. *J. Virol.* 82:5650-2.
- Neumann G, Noda T, Kawaoka Y (2009). Emergence and pandemic potential of swine-origin H1N1 influenza virus. *Nature* 459:931-939.
- New South Wales Public Health Network (2009). Outbreak of swine-origin influenza A (H1N1) virus infection - Mexico, March-April 2009. *Morbidity and Mortality Weekly Report* 58:467-470.
- Niang MN, Dosseh A, Ndiaye K, Sagna M, Gregory V, Goudiaby D, Hay A, Diop OM (2012). Sentinel surveillance for influenza in Senegal, 1996-2009. *J. Infect. Dis* 206 (Suppl 1):S129-S135.
- Nzussouo NT, Michalove J, Diop OM, Njouom R, Monteiro Mde L, Adje HK et al (2012). Delayed 2009 pandemic influenza A virus subtype H1N1 circulation in West Africa, May 2009-April 2010. *J. Infect. Dis.* 206 (Suppl 1):S101-S107.
- Polozov IV, Bezrukov L, Gawrisch K and Zimmerberg J (2008). Progressive ordering with decreasing temperature of the phospholipids of influenza virus. *Nat. Chem. Biol.* 4:248-255.
- Shek LP, Lee BW (2003). Epidemiology and seasonality of respiratory tract virus infections in the tropics. *Paediatr. Respir. Rev.* 4:105-11.
- Steel J, Palese P, Lowen AC (2010). Transmission of a 2009 pandemic influenza virus shows similar sensitivity to temperature and humidity as an H3N2 seasonal strain. *J. Virol.* 85:1400-1402.
- Viboud C, Alonso WJ, Simonsen L (2006). Influenza in tropical regions. *PLoS Med.* 3:e89.

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