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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.

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Full Length Research Paper

Microbiological evaluation of raw milk and coalho cheese commercialised in the semi-arid region of Pernambuco, Brazil

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The aim of this study was to evaluate the microbiological quality of raw milk and coalho cheese commercialised in the Afrânio, Petrolina and Santa Maria da Boa Vista municipalities. The rural production of dairy products contributes significantly to the economy of these municipalities. We analysed 30 samples of raw milk and 30 samples of coalho cheeses. The microbiological quality was assessed by analysing total and thermotolerant coliforms, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* spp. High counts of the coliforms were detected in 86.7% of the raw milk samples analysed, and 80% of the samples had thermotolerant coliform counts above the levels permitted by legislation. *E. coli* was found in 66.7% of the samples, whereas 6.7% was contaminated with *Salmonella* spp. The total and thermotolerant coliform counts in coalho cheeses samples were 93.3 and 90%, respectively, which exceeded the standards permitted by law. In total, 80% were contaminated with *E. coli* and 20% with *Salmonella* spp. *Listeria* spp. was not detected in either the raw milk or the coalho cheese samples. Therefore, of the 60 samples analysed, 85% were not within the standards required by legislation. The implementation of good manufacturing practices is not only necessary to ensure the quality of products offered to consumers but also important for sustainability and food safety.

Key words: Dairy, coliforms, *Listeria* spp., *Salmonella* spp., food safety.

INTRODUCTION

The consumption of raw milk is common in the Pernambuco State, and coalho cheese, a common product found in the Northeast, is produced from raw milk. In Brazil, the industrial production of cheese is only conducted using pasteurised milk. If raw milk is used, a ripening period of a minimum of 60 days is required

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Abbreviations: MPN, most probable number; **LST**, lauryl sulphate tryptose; **EC**, *Escherichia coli* broth; **EMB**, eosin methylene blue; **TSA**, trypticase soy agar; **IMViC**, indole-methyl red-Voges Proskauer-citrate; **TSA-YE**, trypticase soy agar with 0.6% yeast extract; **TSI**, agar triple sugar iron; **LIA**, lysine iron agar; **CFU**, colony forming units; **PCR**, polymerase chain reaction; **LAB**, lactic acid bacteria; **PCA**, principal component analysis.

(Brazil, 1996). Nonetheless, in the majority of cases, especially in the semi-arid region, this practice does not occur.

Coalho cheese is a popular product that is part of the culture and of great social importance in this region. However, there is no standardisation for the elaboration process. It is a common practice to use raw milk, which presents a risk to the health of consumers. Most cheese is manufactured either in farmstead cheeses or in small cheese factories (Escobar et al., 2001). Several studies (Santos et al., 1995; Nassu et al., 2000; Bastos et al., 2001) that investigated the microbiological quality of coalho cheese, in other states, reported an increase in the occurrence of pathogenic microorganisms and high microorganism counts. In Pernambuco State, a semi-arid region, there is no report on the microbiota pathogen and hygiene indicator microorganisms in the product.

The quality and safety of raw milk can be evaluated by assessing hygiene indicator microorganisms. Total and thermotolerant coliforms are used as hygienic parameters for cheese production, as they indicate the conditions of raw milk obtaining and storage, and inadequate handling during the manufacturing process. These microorganisms are usually associated with foodborne diseases and outbreaks, as recorded by official health organizations (Carmo et al., 2002).

The raw milk produced in Brazil is known for its poor microbiological quality and chemical residues (Nero et al., 2004). Thus, this product and its derivatives are potential carriers of several pathogenic microorganisms, such as food borne pathogens, which include *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* (Nero et al., 2004; Denny et al., 2008; Costa Sobrinho et al., 2012). Food borne pathogens in raw milk pose a major public health threat and cause millions of illnesses each year (Oliver et al., 2005; Enurah et al., 2013).

Considering the high rates of raw milk consumption by this population and the great socioeconomic importance of the coalho cheese, the limited information regarding the microbiological quality of these products warrants further investigation. The overall objective of this study was to evaluate the microbiological quality of commercialised raw milk and coalho cheese in the cities of Afrânio, Petrolina and Santa Maria da Boa Vista, which are located in semi-arid region of Pernambuco State.

MATERIALS AND METHODS

Raw milk and coalho cheese sampling

The samples were collected from the semi-arid region, in the municipalities of Afrânio (latitude S08°30'54"; longitude W41°00'18"), Petrolina (latitude S09°23'55"; longitude W40°30'03") and Santa Maria da Boa Vista (latitude S08°48'28"; longitude W39°49'32"), in the State of Pernambuco, Brazil. Samples were obtained from retail stores, small markets, supermarkets, bakeries and free markets in the aforementioned cities between the months of January and July 2010. The samples were kept in sterile bags (Whirl-Pak; Nasco, Fort Atkinson, WI) in a cool box that was stored at 4°C until delivery to the laboratory for microbiological analysis.

Microbiological analysis

Hygiene indicators were assessed for each sample (30 samples raw milk and 30 samples cheese). An aliquot of raw milk (25 g or mL) or coalho cheese (25 mL or g) was aseptically transferred into 225 mL of 1% peptone water (Acumedia) and homogenised for 2 min in a stomacher (Mayo Homogenius HG 400, São Paulo, Brazil). The concentration of total and thermotolerant coliforms was evaluated using the most probable number (MPN) test, which was carried out as previously described by Kornacki and Johnson (2001). Dilutions were performed in a series of three tubes containing lauryl sulphate tryptose (LST) broth (Himedia). After incubation at 37°C for 24 to 48 h, the tubes with gas production (bubble in the inverted Durham tube) were considered positive. The sample was then transferred to tubes containing Brilliant Green broth (Himedia), and incubated at 37°C for 24 to 48 h for confirmation of total coliforms. Tubes containing *E. coli* (EC) broth (Himedia) were incubated in a circulating water bath at 44.5°C for 24 to 48 h to confirm the presence of thermotolerant coliforms. For confirmation of *E. coli,* positive tubes with gas production were streaked on Eosin methylene blue (EMB) agar (Merck) and were incubated at 37°C for 24 h. Typical colonies, Gram and oxidase-negative, were streaked onto the inclined surface of trypticase soy agar (TSA) (Himedia) and were incubated at 37°C for 24 h for the biochemical test, Indole-Methyl red-Voges Proskauer-Citrate (IMViC).

Detection of the *L. monocytogenes* was conducted as previously described by Hitchins (2003), 25 g or 25 mL of each sample was homogenised in 225 mL of *Listeria* Enrichment broth and incubated at 30°C for 24 h. After pre-enrichment, 0.1 mL was transferred into a tube containing 10 mL of Fraser broth and incubated at 35°C for 24-48 h until a blackening of the media was observed. The tubes with darkening were then streaked onto Palcam and Oxford Agar (Oxoid) and incubated at 35°C for 24 to 48 h.

Suspected *Listeria* spp. colonies were streaked onto plates with TSA containing 0.6% yeast extract (TSA-YE; Himedia) and incubated at 30°C for 24-48 h. Proper biochemical tests, which included assessing the production of catalase, fermentation of carbohydrates (dextrose, xylose, sorbitol and rhamnose), hemolysis on sheep blood agar and motility at 25°C, were then conducted. The DNA of the microorganisms was double extracted, which is the technical term for the extraction of the bacterial DNA (Greco et al., 2008). For PCR analysis, the reaction was carried out in a final volume of 25 µL containing 0.5 µL Taq DNA polymerase (2.5 U, Promega, Milan, Italy), 2.5 µL buffer 1×, 1.0 µL dNTP, 1.0 µL of each primer, 1.0 µL $MgCl_2$ (2 mM) and 4 µL of extracted DNA. In total, 2 pairs of primers were used for multiplex PCR. A primer pair was used to identify *Listeria* spp. from 16S rRNA: Li454-472F (5' CAA GGA TAA GAG TAA CTG C 3') (forward) and Li438-1451R (5' AGG TTG ACC CTA CCG ACT TC 3') (reverse), which amplified DNA fragments with an expected size of 1003 bp. A primer pair was used to identify *Listeria* spp. from the gene *iap* (*invasion-associated protein*) of *L. monocytogenes*: Lm486-505F (5' ACA AGC TGC ACC TGT TGC AG 3') (forward) and Lm1060-1079R (5' GAA CCT TGA TTA GCA TTC GT 3') (reverse), which amplified DNA fragments with an expected size of 593 bp. The amplification was carried out using the following protocol: template DNA was denatured for 3 min at 95°C, then denatured at 95°C for 60 s, and annealed at 53°C for 2 s for 30 cycles. Primer extension was carried out at 72°C for 4 min. Gel runs were carried out for 1 h at 100 V, then stained with ethidium bromide and observed under ultraviolet light.

For *Salmonella spp*. detection, the methodology described by ISO 6579 (2007), 25 g or 25 mL of each sample were homogenised with buffered peptone water and incubated at 37°C for 18±2 h in a shaker. After pre-enrichment, 0.1 mL was transferred to a tube containing 10 ml of Rappaport-Vassiliadis broth and incubated at 42°C for 24 h.

In addition, 10 mL of each sample was transferred into a tube containing tetrathionate broth and incubated at 35°C for 24 h. After incubation, both broths were streaked onto bismuth sulphite (Himedia), Hektoen Enteric (Himedia) and xylose lysine desoxycholate (Himedia) agar plates, incubated at 35°C for 24 h. Suspected *Salmonella* spp. colonies were streaked on plates of TSA. Next, proper biochemical tests were conducted, which included triple sugar iron agar (TSI; Himedia), lysine iron agar (LIA; Himedia), urease, indole, motility and phenylalanine; confirmation was assessed using somatic and flagellar polyvalent antisera (Probac, SP, Brazil).

In PCR analysis, extracted DNA was analysed for the presence of the *SDIA* gene, which has an expected size of 274 bp. The 21 µl reaction mix contained the following: 4 µl of DNA, 1.0 µl of SdiA1 primer (5'-AAT ATC CAC GCT TCG TAC-3'), 1.0 µl of SdiA2 primer (5'-GTA AAA CGA GGT GGA GCA G-3'), 1,0 µl of $MgCl_2$ (2 mM), 1.0 µl of dNTPs, 2.5 µl of Taq buffer, 0.3 µl (2,5 U) of the enzyme, Taq DNA polymerase (2.5 U, Promega, Milan, Italy), and 14.2 µl of Milli-Q water.

The amplification was carried out using the following protocol: template DNA was denatured for 2 min at 94°C, denatured at 94°C for 30 s, and annealed at 60°C for 60 s for 30 cycles. Primer extension occurred at 72°C for 90 s. The tubes were then incubated for 4 min at 72°C for the final extension, which was repeated for 25 cycles. The PCR results were visualised on a 1.5% agarose gel stained with ethidium bromide under ultraviolet light.

Data analysis

Fisher's exact test was performed to assess the frequency of pathogenic microorganism (*E. coli, Salmonella* and *Listeria*) detected in the raw milk and coalho cheese from each of the municipalities investigated in this study. A *P* value less than 0.05 was considered significant. The software used for statistical analysis was the Biostat 2.0. The detection of microorganisms in the semi-arid region municipalities was analysed by principal component analysis (PCA), using the software XLSTAT 7.5.2 (Addinsoft, New York, NY, USA).

RESULTS AND DISCUSSION

Microbiological standards for health food legislation require that the numbers of thermotolerant coliforms do not exceed 4 MPN/mL in pasteurised milk (Brasil, 2001). Considering the microbiological criteria for pasteurized milk established by the Brazilian legislation (Brasil, 2001), 80% of samples (24 positive samples) presented with higher counts of thermotolerant coliforms (Table 1). The greatest contamination of milk, with 90% samples testing positive for thermotolerants coliforms, was found in the municipality of Santa Maria da Boa Vista and Afrânio.

The high counts of coliforms groups confirmed the poor hygienic conditions associated with the production and the quality of raw milk consumed in the semi-arid region. In other northeast states, high counts of coliforms groups were also reported (Maciel et al., 2008; Tebaldi et al., 2008; Alves et al., 2009). In accordance with the Normative Instruction n° 62 (Brasil, 2011), the marketing of raw milk for human consumption is prohibited; the milk must be pasteurised and have no pathogenic microorganisms.

In the samples of coalho cheese analysed (Table 2), 93.3% (28 positive samples) presented high counts of total and thermotolerant coliforms, and 90% (27 positive samples) had counts above the standards allowed by the legislation (Brasil, 2001), which is $<5.0 \times 10^2$ MPN/g of thermotolerant coliforms. In the municipalities of Petrolina, Afrânio and Santa Maria da Boa Vista, 80, 100 and 90% of samples, respectively, were not suitable for consumption.

Oliveira et al. (2010) and Duarte et al. (2005) also reported samples in disagreement with the microbiological standards required in the state of Pernambuco. Similar findings were also reported in other northeastern states (Leite et al., 2002; Feitosa et al., 2003; Alves et al., 2009). Throughout northeastern Brazil, coalho cheese is abundantly consumed. Moreover, the artisan production of coalho cheese, as well as the utilisation of raw milk, compromises product quality and consumer health.

E. coli was detected in 66.7% (20 positive samples) of raw milk samples. In the municipalities of Petrolina, Santa Maria da Boa Vista and Afrânio, *E. coli* was found in 50% (5 positive samples), 50% (5 positive samples) and 100% (10 positive samples), respectively.

In this study, the frequencies of detection for *E. coli* were significantly dif-ferent by Fisher's exact test (P= 0.03) in each of the municipalities. The raw milk from Afrânio had a great number of microorganisms detected as compared to the Petrolina and the Santa Maria da Boa Vista municipalities.

The presence of *E. coli* in coalho cheese was confirmed in eight, ten and nine samples obtained from the municipalities of Petrolina, Afrânio and Santa Maria da Boa Vista, respectively. There were no significant differences, which were evaluated by Fisher's exact test (P > 0.05), in the detection of this microorganism in the coalho cheese obtained from each of these municipalities.

The results from this study further demonstrated that *L. monocytogenes* was not present in either the raw milk or the coalho cheese. However, this microorganism has a low prevalence in the raw milk of Brazil. Several studies have reported the absence of *L. monocytogenes* in dairy products (Nero et al., 2004; Arcuri et al., 2006; Brito et al., 2008; Moraes et al., 2009; Costa Sobrinho et al., 2012).

The indigenous microbiota of Brazil, especially above 5 log CFU/mL, can interfere with survival or growth of *L. monocytogenes* in raw milk (Nero et al., 2008). The indigenous microbiota, lactic acid bacteria (LAB), are found in raw milk. LAB produce bacteriocins and antimicrobial peptides capable of combating microorganisms that can cause pathogenesis and food spoilage (O'Sullivan et al., 2002). Nisin and pediocin, which are bacteriocins, can reduce or inhibit the presence of *L. monocytogenes* in dairy products. Thus, these bacteriocins may potentially be utilised as a biopreservative in milk and its derivates (Muriana, 1996; Cleveland et al., 2001).

Salmonella spp. was detected in two samples of raw

Table 1. Counts of total coliforms, thermotolerant coliforms, *Escherichia coli*, *Listeria* spp. and *Salmonella* spp. in the raw milk commercialized in the municipalities in the semi-arid region of Pernambuco State.

Sample	Municipality	Total coliforms[1]	Thermotolerant coliforms[1]	*E. coli*[2]	*Listeria*[2]	*Salmonella*[2]
M1	Petrolina	1.36*	0.6	Aus	Aus	Aus
M2		0.95	0	Aus	Aus	Aus
M3		>3.38*	0	Aus	Aus	Aus
M4		1.63*	1.63*	Aus	Aus	Aus
M5		0.95	0.6	Aus	Aus	Aus
M6		>3.38*	2.66*	Pres	Aus	Aus
M7		>3.38*	1.30*	Pres	Aus	Aus
M8		>3.38*	>3.38*	Pres	Aus	Aus
M9		1.97*	1.97*	Pres	Aus	Aus
M10		2.66*	2.66*	Pres	Aus	Aus
M11	Afrânio	>3.38*	>3.38*	Pres	Aus	Aus
M12		>3.38*	2.32*	Pres	Aus	Aus
M13		>3.38*	2.18*	Pres	Aus	Aus
M14		0.95	0.60	Pres	Aus	Aus
M15		>3.38*	>3.38*	Pres	Aus	Aus
M16		2.38*	2.38*	Pres	Aus	Aus
M17		2.66*	1.97*	Pres	Aus	Aus
M18		1.97*	1.97*	Pres	Aus	Aus
M19		1.63*	1.63*	Pres	Aus	Aus
M20		0.95	0.95*	Pres	Aus	Aus
M21	Santa Maria da Boa Vista	>3.38*	2.66*	Pres	Aus	Pres
M22		2.38*	1.63*	Aus	Aus	Aus
M23		>3.38*	1.45*	Aus	Aus	Aus
M24		>3.38*	>3.38*	Pres	Aus	Pres
M25		2.38*	1.36*	Pres	Aus	Aus
M26		>3.38*	1.45*	Aus	Aus	Aus
M27		>3.38*	>3.38*	Pres	Aus	Aus
M28		>3.38*	0	Aus	Aus	Aus
M29		>3.38*	1.04*	Aus	Aus	Aus
M30		>3.38*	2.32*	Pres	Aus	Aus

*Samples above the level allowed for total coliforms: 1Log MPN /mL (Brasil, 1997); and thermotolerant coliforms: 0.6 Log NMP/mL (Brasil, 2001); [1]Log MPN/mL: most probable number; [2]Log CFU/mL: colony forming unit; Aus: absent; Pres: present.

milk in the municipality of Santa Maria da Boa Vista. In coalho cheese, six positive samples were confirmed by biochemical and agglutination tests, as well as by PCR. In Petrolina, Afrânio and Santa Maria da Boa Vista municipalities, one, two and three positive samples of *Salmonella* spp., respectively, were detected. There was no significant difference in the detection of this microorganism, in any of the municipalities, using the Fisher's exact test ($P > 0.05$). Because Brazilian legislation does not allow the presence of *Salmonella* spp. in 25 g of food (Brasil, 2001), these samples are considered unfit for consumption.

The absence or low frequency of *Salmonella* in raw milk has been previously demonstrated (Nero et al., 2004; Little et al., 2008; Moraes et al., 2009), thereby suggesting this product and its derivates are relatively safe. Nonetheless, there are numerous studies that have reported diseases, caused by *Salmonella*,nthat were associated with the consumption of raw milk or coalho cheese (Borges et al., 2003; Feitosa et al., 2003; Nadvorny et al., 2004; Duarte et al., 2005).

Similar to the study by Nero et al. (2004), negative and low detection rates for *Listeria* and *Salmonella,* respectively, were reported. These findings indicate that unacceptable levels of hygiene indicators are a more important risk factor than the presence of either *Salmonella* spp.

Table 2. Count of total coliforms, thermotolerant coliforms, *Escherichia coli*, *Listeria* spp. and *Salmonella* spp. in the coalho cheese commercialized in the municipalities in the semi-arid region of Pernambuco State.

Samples	Municipality	Total coliforms[1]	Thermotolerant coliforms[1]	*E. coli*[2]	*Listeria*[2]	*Salmonella*[2]
C1	Petrolina	>3.38*	>3.38*	Aus	Aus	Aus
C2		2.66	2.18	Aus	Aus	Aus
C3		>3.38*	>3.38*	Pres	Aus	Aus
C4		2.66	2.18	Pres	Aus	Aus
C5		>3.38*	>3.38*	Pres	Aus	Aus
C6		>3.38*	>3.38*	Pres	Aus	Aus
C7		>3.38*	>3.38*	Pres	Aus	Aus
C8		>3.38*	>3.38*	Pres	Aus	Aus
C9		>3.38*	>3.38*	Pres	Aus	Aus
C10		>3.38*	>3.38*	Pres	Aus	Pres
C11	Afrânio	>3.38*	>3.38*	Pres	Aus	Aus
C12		>3.38*	>3.38*	Pres	Aus	Pres
C13		>3.38*	3.04*	Pres	Aus	Aus
C14		>3.38*	3.04*	Pres	Aus	Aus
C15		>3.38*	3.04*	Pres	Aus	Aus
C16		>3.38*	3.04*	Pres	Aus	Aus
C17		>3.38*	>3.38*	Pres	Aus	Aus
C18		>3.38*	3.04*	Pres	Aus	Aus
C19		>3.38*	3.04*	Pres	Aus	Pres
C20		>3.38*	3.04*	Pres	Aus	Aus
C21	Santa Maria da Boa Vista	>3.38*	>3.38*	Pres	Aus	Pres
C22		>3.38*	>3.38*	Pres	Aus	Aus
C23		>3.38*	>3.38*	Pres	Aus	Aus
C24		>3.38*	3.04*	Pres	Aus	Pres
C25		>3.38*	3.04*	Pres	Aus	Aus
C26		>3.38*	>3.38*	Pres	Aus	Aus
C27		>3.38*	>3.38*	Pres	Aus	Aus
C28		3.04*	>3.38*	Pres	Aus	Pres
C29		3.04*	0.6	Aus	Aus	Aus
C30		>3.38*	>3.38*	Pres	Aus	Aus

*Samples above the level allowed for thermotolerant coliforms: 2.69 log NMP/g (Brasil, 2001); [1] Log MPN/mL: most probable number; [2]Log CFU/mL: colony forming unit; Aus: absent; Pres: present.

spp. or *L. monocytogenes* in milk produced by Brazilian small milk producers.

Of the 60 samples analysed, including both raw milk and coalho cheese, 85% were not within the standards set forth by the legislation and are therefore unfit for human consumption. Our results reflect the poor sanitary conditions during production handling, storing and/or marketing of these products, which compromise the quality, sustainability and food safety.

To discriminate the microorganisms found in the raw milk and the coalho cheese obtained from each of the municipalities investigated in this study, PCA was assessed based on frequency of detection. The first two components explained 100% of the total variance, PC1 and PC2 accounted for 58.51 and 41.49%, respectively, of the raw milk (Figure 1) and 64.73 and 35.27%, respectively, of the coalho cheese (Figure 2). The frequency of detection of microorganisms was markedly separated in the plane of the biplot. Santa Maria da Boa vista was characterised by the major frequency of detection to total coliforms and *Salmonella* in raw milk (Figure 1) and *Salmonella* in coalho cheese (Figure 2). In Afrânio, *E. coli* was detected at a higher frequency in both raw milk and coalho cheese. Out of the 3 municipalities, Petrolina had the lowest frequency of microorganism detection in the raw milk and in a sample

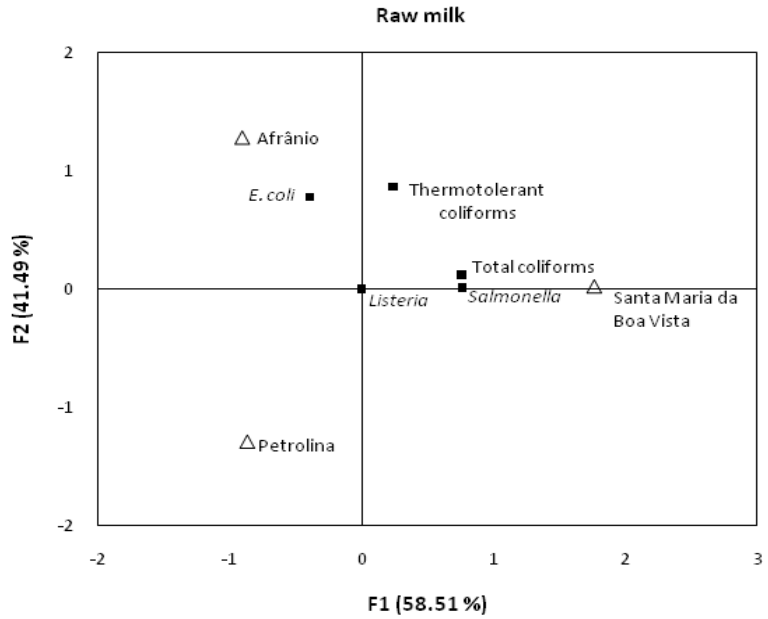

Figure 1. Principal component analysis (PCA) based on the frequency of detection of microorganisms in the raw milk obtained from the three municipalities in the semi-arid region of Pernambuco State.

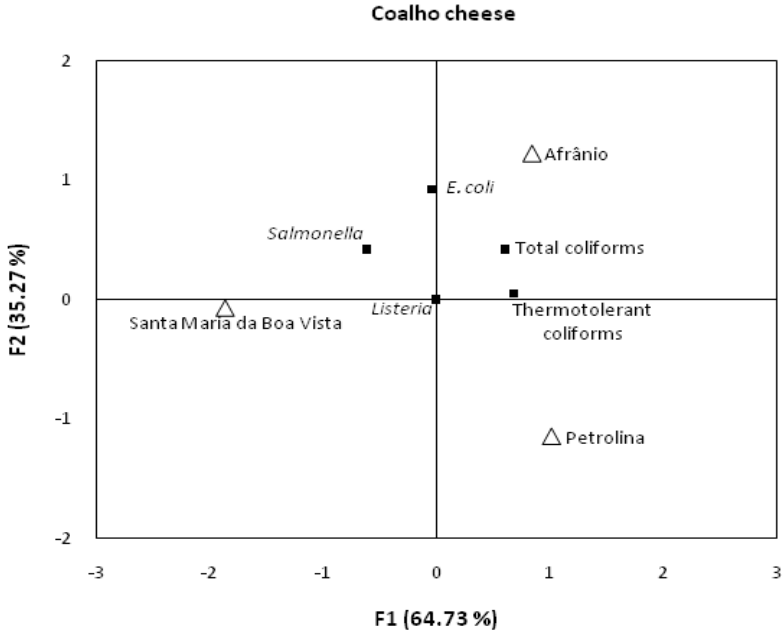

Figure 2. Principal component analysis (PCA) based on the frequency of detection of microorganisms in the coalho cheese obtained from the three municipalities in the semi-arid region of Pernambuco State.

of coalho cheese*, Salmonella* spp. was isolated.

Conclusion

The results indicate that the quality of raw milk and coalho cheese, produced in semi-arid regions by local farmers, was associated with absence of *L. monocytogenes*, a low incidence of *Salmonella,* but high levels of hygiene indicator microorganisms. Samples with high levels of hygiene indicators are not permitted by the Brazilian legislation and reveal inadequate conditions for

the production of raw milk and cheese. Therefore, it is recommended that hygiene practices be adhered to in the production environment, and during the milking, obtaining, storing, cheese making and marketing of these products. Furthermore, the training of Brazilian small milk producers and improved sanitary supervision is essential for the quality of milk and dairy products.

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Full Length Research Paper

Antimicrobial activity of methanolic extract and fractionated polysaccharide from *Loligo duvauceli* Orbingy 1848 and *Doryteuthis sibogae* Adam 1954 on human pathogenic microorganisms

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In the present study, *in vitro* antimicrobial activity of crude methanolic extract of squid body tissue and fractionated gladius polysaccharide of two squid (*Loligo duvauceli* and *Doryteuthis sibogae*) from Cuddalore and Mudasalodai (Southeast Coast of India) landing centre was evaluated. The antimicrobial activity was screened against nine species of clinically isolated human pathogenic bacteria namely *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Vibrio cholerae, V. parahaemolyticus, Staphylococcus aerues, Pseudomonas aeruginosa, Salmonella typhii* and *Shigella* sp. and four fungal strains such as *Candida* sp., *Rhizopus* sp., *Aspergillus flavus* and *A. fumigates*. Different concentrations such as 25, 50, 75 and 100% were prepared and tested against the microbial strains for their inhibitory activities, using the disc diffusion method. The minimum inhibitory concentration (MIC) of methanolic body tissue extract and fractionated gladius polysaccharide of squid ranged from 60 to 100 mg/ml. The results were discussed in the light of positive and negative control apart from the concentrations tested.

Key words: Antimicrobial activity, squid, gladius, minimum inhibitory concentration (MIC) and pathogenic microorganisms.

INTRODUCTION

The marine environment is a rich source of biologically active natural products of diverse structurally active natural products, many which have not been found in terrestrial sources. Antitumor, antiviral, antibacterial, anticoagulant, hemolytic, analgesic, cardio inhibitory, anticonvulsant, vasopressive and other active substances have been found in marine organisms and their surrounding environment. The marine organisms have had a major impact on the development of medical science. More recent studies on marine organisms have focused mainly on their application for the treatment of human diseases. Many marine chemicals often possess quite novel structures which lead to pronounced biologically activity and novel pharmacology (Lei and Zhou, 2001). A number of discovery efforts have yielded several bioactive metabolites which have been successfully developed by the pharmaceutical industry (Kong et al., 1994). From 1969 to 1999, approximately 300 patents on bioactive marine natural products were issued. From humble beginning, the number of compounds isolated from various marine organisms has virtually soared and now exceeds 10,000 (Marin Lit, 2002) with 100s of new compounds still being discover every year (Faulkner, 2000, 2002).

Molluscs are widely distributed throughout the world and have many representatives in the marine and

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estuarine ecosystems. Among the molluscs, cephalopods are very good sources of bioactive compounds. The fluid from the ink sac is found to have antibiotic effect and a pigment from the ink sac of the cuttlefish has been used in medicine, especially in homeopathy. Apart from the ink, the cuttlebone is also widely used as a home - made remedy for ear ache and skin diseases in India and China. In most of the publications concerning antimicrobial activity in molluscs, either single body components alone, like haemolymph and egg masses, or extracts of whole bodies have been tested for activity (Kubota et al., 1985). The presence of antimicrobial activity in molluscs has been reported from the mucus of the giant snail *Achantia folica* (Yamazaki, 1993), from the egg mass and purple fluid of the sea hare *Aplysia kurodai* and the body wall of the sea hare *Dolabella auricularia* (Iijima et al., 2003) and body tissue of six species of cephalopods (Ramasamy et al*.,* 2011). The antimicrobial activity of polysaccharides extracted from cephalopods such as *Sepia aculeata* and *Sepia brevimana* and heparin and heparin - like glycosaminoglycans (GAGs) from the cephalopod *Euprymna berryi* was reported against the human pathogenic microorganism (Shanmugam et al., 2008a; Shanmugam et al., 2008b). Further, these compounds are being extracted not only from the whole animal but also from the different body parts including the skeleton (internal shell of cephalopods), which showed many pharmacological properties and hence medicinal value (Shanmugam et al., 2008a).

Scientific interest for cephalopods is increased over the last century for, at least a couple of reason: i) their value as experimental animals for biomedical and behavioral research (Hochner, 2008; Castellanos-Martinez and Gestal, 2013); ii) their position in the world marked as a major fishery resource (Boyle and Redhouse, 2005; Castellanos-Martinez and Gestal, 2013). The present study has attempted to focus on the attention on the study of antimicrobial activity shown by the mehtanolic extract of body tissue and fractionated gladius polysaccharide from squid gladius from two squid species (*L. duvauceli* and *D. sibogae*).

MATERIALS AND METHODS

Sample collection and identification

Two species of squid such as *L. duvauceli* and *D. Sibogae*, were freshly collected from Cuddalore (Lat.11°42'N; Long.79°46'E) and Mudaslodai (Lat. 11°29'N; Long. 79°46'E) landing centre which is situated south east coast of India. The studies carried out by Shanmugam et al. (2002) have been of considerable help on developing the identification keys and description which in most cases have also been corroborated with examination of actual specimen.

Preparation of methanoloic extracts of body tissue

Squids were brought to laboratory; body tissues were removed, cut into small pieces and homogenized (REMI, RQ-127 A) and extracted with methanol (MeOH) at room temperature for 24-48 h (Ely et al., 2004). Then, the methanolic extract was centrifuged to collect the supernatant and concentrated under vacuum in a rotary evaporator (LARK, Model: VC-100A) at low temperature. The crude methanolic extract was assayed for antibacterial and antifungal activities using standard disc diffusion method.

Extraction of polysaccharide from squid gladius

The polysaccharide extract were obtained from the squid of *L. duvauceli* and *D. sibogae* by following the method of Okutani and Morikawa (1978). The air- dried shell powder was pulverized and washed with acetone. The powder was extracted with hot 10 mM EDTA solution and filtered (whatman No.1) with hyflosuper cel. Then, saturated barium hydroxide solution was added to the filtrate and allowed to stand over night. Then, the precipitate was filtered on a filter paper (Whatman. No.1) with hyflosuper cel and washed with distilled water. The precipitated was dissolved in 10 mM EDTA solution and was dialyzed against deionised water using 8 KDa cut-off dialysis membrane. The dialyzate solution present in the dialysis membrane was then freeze-dried and a pure white colored powder was obtained. This lyophilized powder was used for the antibacterial and antifungal activity.

Purification of polysaccharide using ion exchange resin

The crude polysaccharide was fractionated by ion - exchange chromatography on a column (2.5x15 cm) of Amberlite IRA - 900 forms in aqueous solution of 1% polysaccharide and was applied to the column and washed initially with water. Then, the sample was recovered by stepwise elution with 0.4 M NaCl and 0.8 M NaCl at the flow rate of 1 ml per minute. Both elutes were combined, dialysed and lyophilized (Nishino et al., 1989).

Gel chromatography

Gel filtration was done by using Sephadex G-50 (Sigma). Purified polysaccharide sample (Approximately 3g /10 ml 0.2 M-NaCl, was applied to the column; the eluting medium was 0.2 M-NaCl and the temperature was 4°C at a flow rate of 60 ml/h. Fractions (15 ml) were collected and the active fractions were pooled then dialyzed against distilled water and freeze-dried (Laurent et al., 1978).

Estimation of sugar content

Sugar (in crude) content was determined by phenol - sulphuric acid method (Dubois et al., 1956).

Elementary analysis

The element of C, H and N content was analyzed using elemental analyzer, Elementer Vario EIII, Carlo Erba-1180.

Microbial culture

Bacterial and fungal strains

Nine bacterial (*Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Vibrio cholerae, Vibio parahaemolyticus, Staphylococcus aerues, Pseudomonas aeruginosa, Salmonella typhii* and *Shigella* sp.) and four fungal (*Candida* sp., *Rhizopus* sp.,

Aspergillus flavus and *Aspergillus fumigatus*) strains were used for studying the antibacterial and antifungal activities of methanolic extract and fractionated gladius polysaccharide. These strains isolated from the HIV patients, were obtained from Raja Muthyiah Medical College Hospital, Annamalai University, Tamil Nadu, India. These are all human pathogens that have developed some resistance to common antibiotics particularly in the in the clinical environment.

Inoculum preparation for bacteria

Nutrient broth was prepared and sterilized in an autoclave at 15 lbs pressure for 15 min. All the nine bacterial strains were individually inoculated in the sterilized nutrient broth and incubated at 37°C for 24 h. Mueller Hinton Agar (MHA, Himedia) was prepared, sterilized in an autoclave at 15lbs pressure for 15 min and poured into sterile petridishes and incubated at 37°C for 24 h. The 24 h old bacterial broth cultures were inoculated in the Petri dishes by using a sterile cotton swab.

Inoculum preparation for fungi

Czapek dox (Hi-media) broth was prepared and sterilized in autoclave at 15 lbs pressure for 15 min. Four fungal strains were inoculated in the broth and incubated at 37°C for 72 h. The sterilized Czapek dox agar was poured into sterile petridishes and incubated at 37°C for three days. The 72 h old fungal broth cultures were inoculated in the petridishes using a sterile cotton swab.

Antimicrobial assay (disc diffusion method)

In vitro antibacterial and antifungal activity was determined following the method of El-Masry et al. (2000). Briefly, a suspension of each tested microorganism was carefully mixed in the tube containing bacterial and fungal inoculums and media for bacterial and fungal were plated separately, and the respective strains were cotton swabbed on Petri dishes. Sterile antimicrobial disc (Hi-media) was impregnated with 50 µl of crude methanolic extract and fractionated gladius polysaccharide of the four concentrations tested. Positive control discs contained 50 µl of tetracycline (1 mg/ml) and negative control 50 µl of methanol and water. The stocks for methanolic extracts and fractionated polysaccharide were prepared in the concentration of 100 mg/ml. These impregnated discs were allowed to dry at laminar air flow chamber for 3 h, and were placed at the respective bacterial and fungal plates and incubated at 37°C for 24 h for bacteria and 72 h for fungi. The diameter (mm) of the growth inhibition halos produced by the methanolic extracts and fractionated polysaccharide of squids were examined. Result was calculated by measuring the zone of inhibition in millimeters. All the tests were performed in triplicates.

Determination of the minimum inhibitory concentration (MIC)

The methanolic extract and fractionated gladius polysaccharide of squid which showed significant antimicrobial activity was selected for the determination of MIC followed by the method of Rajendran and Ramakrishnan (2009). A stock solution of 100 mg/ml was prepared and was serially diluted to obtain various ranges of concentrations between 20 and 100 mg/ml. 0.5 ml of each of the dilutions of different concentrations was transferred into sterile test tube containing 2.0ml of nutrient broth.

To the test tubes, 0.5 ml of test organism previously adjusted to a concentration of 105 cells/ml was then introduced. A set of test tubes containing broth alone was used as control. All the test tubes and control were then incubated at 37°C for 24 h. After the period of incubation, the tube containing the least concentration of extract showing no visible sign of growth was taken as the minimum inhibitory concentration.

Statistical analysis

Data on the inhibitory effect of methanolic extracts and fractionated gladius polysaccharide of squid was analyzed by one-way analysis of variance (ANOVA) using SPSS-16 version software followed by Duncan's multiple range test (DMRT). P values ≤0.05 were considered as significant.

RESULTS

Sugar content of fractionated gladius polysaccharide showed 42.7 and 48.1% in *L. duvauceli* and *D. sibogae* respectively. At the same time, chemical analysis that is C, H and N content of fractionated gladius polysaccharide showed 29.6, 6.3 and 1.2% and 31.8, 5.6 and 1.8% in *L. duvauceli* and *D. sibogae* respectively.

The results of antibacterial activity are presented in Table 1. The highest activities of 10 mm (inhibition zone) were recorded in 100% against *K. pneumoniae* and *E. coli* in *D. sibogae* and 5 mm against *P. aeruginosa* in *L. duvauceli* of fractionated squid polysaccharides. At the same time, the methanolic extracts showed the highest inhibition zone of 10 mm observed against *E. coli* in *D. sibogae* extract and against *V. cholerae* in *L. duvauceli* extract. The lowest inhibition zone of 4 mm was observed against *S. typhii* and *K. pnemoniae* in *L. duvauceli* and *D. sibogae* extract.

In 75%, maximum activities of 7 mm were observed against *S. aureus, K. pneumoniae* and *E. coli* in *D. sibogae* and 4 mm against *S. typhii* in *L. duvauceli* fractionated polysaccharide. The methanolic extract showed the maximum activity of 7 mm against *Shigella* sp. in *L. duvauceli* extract and 8 mm against *S. typhii* in *D. sibogae* extract. The minimum activity of 4 mm against *K. pneumonia* and *S. aureus* and *E. coli* in *L. duvauceli* and *D. sibogae* extract respectively was observed.

In 50%, 6 and 3 mm inhibition zone were recorded against *K. pneumonoiae* and *E. coli* and *P. aeruginosa* and *V. parahemolyticus* in *D. sibogae* and *L. duvauceli* respectively. Whereas the maximum activity of 6 mm was noticed against *V. cholerae* in *L. duvauceli* methanolic extract and 4 mm against *V. parahemolyticus* in *D. sibogae* methanolic extract. The minimum activity of 3 mm was recorded against *Shigella* sp. and *V. parahemolyticus* in both *L. duvauceli* and *D. sibogae* methanolic extracts.

In *L. duvauceli* (25%), 3 mm inhibition zone was observed against *V. parahemolyticus, P. aeruginosa* and *E. coli*. The activities were completely absent against *B. subtilis* and *V. cholerae.* In *D. sibogae*, it was 5 mm against *B. subtilis, P. aeruginosa*, *S. aureus* and *E. coli*, and 3 mm against *Shigella* sp., *S. typhii* and *V. parahemolyticus* and activity was absent against *V.*

Table 1. Antibacterial activity of methanolic extract and fractionated polysaccharides of squids.

Bacterial strain	Methanolic extract								Fractionated polysaccharide							
	L. duvauceli (%)				*D. sibogae* (%)				*L. duvauceli* (%)				*D. sibogae* (%)			
	25	50	75	100	25	50	75	100	25	50	75	100	25	50	75	100
B. subtilis	-	-	-	-	-	-	-	-	-	-	-	-	+	+	++	++
Shigella sp	+	+	++	++	-	+	+	++	-	+	+	+	+	+	+	++
P. aeroginosa	-	-	-	+	-	-	+	++	+	+	+	+	+	+	+	++
S. typhii	-	+	+	++	-	+	++	++	-	+	+	+	+	+	+	++
V. parahaemolyticus	+	+	+	++	+	+	++	++	+	+	+	+	+	+	+	++
V. cholerae	-	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-
S. aereus	+	+	++	++	+	+	+	++	-	-	+	+	+	+	+	+
K. pneumoniae	-	-	+	++	-	-	-	+	-	-	+	+	+	++	++	++
E. coli	-	-	-	+	-	+	+	++	-	+	+	+	+	++	++	++

-, No activity; +, weak activity (3-5 mm); ++, Good activity (6-10); +++, Very good activity (>10 mm) *The statistical significance: P values ≤0.05 (DMRT).

*cholera*e. In methanolic extracts it showed 3 mm inhibition zone against *Shigella* sp., *V. parahemolyticus* and *S. aureus* and *P. aeruginosa* in both *L. duvauceli* and *D. sibogae* extracts respectively.

In the case of control, no activity was recorded against any bacterial strain in the two species of squid studied.

All the results of antifungal activity are presented in Table 2. In the control, no activity was recorded for all the fungal strains in both *L. duvauceli* and *D. sibogae*. The maximum of 12 mm inhibition zone were recorded against *Rhizopus* sp. and *A. flavus* in *L. duvauceli* and 5 mm against *A. fumigatus* in 100% concentration. In 75%, 7 and 8 mm inhibition zones were observed against *Rhizopus* sp. and *A. flavus* respectively and *A. fumigatus* recorded an inhibition zone of 4 mm only whereas 6 mm inhibition zone were recorded in 50% against *Rhizopus* sp., 4 mm against *A. flavus* and 3 mm against *A. fumigatus*. In 25%, 5 mm inhibition zone were observed against *Rhizopus* sp., 3 mm against *A. flavus* and *A. fumigatus*. At the same time, the activity were completely absent against *Candida* sp.

In *D. sibogae*, the highest activity was observed (10 mm) against *A. fumigatus* in 100% and 4 mm against *Rhizopus* sp. In 75%, 7 and 4 mm inhibition zones were recorded against *A. fumigatus* and *Rhizopus* sp. and *A. fumigatus* respectively. 3 mm inhibition zone was recorded against *A. fumigatus* and *Rhizopus* sp. in 25%. The activity was absent against *Candida* sp. and *A. flavus*. But at the same time, the methanolic extracts showed no activity against all the fungal strains studied.

MIC of the active extract against the test organisms

The MIC of bacterial strains results are given in Table 3. MIC values of methanolic extracts of *L. duvaucel* against bacterial strains such as *Shigella* sp., *S. typhii, V. parahaemolyticus, V. cholerae,* and *S. aureus* were 100, 100, 100, 80 and 100 mg/ml respectively. In *D. sibogae,* the MIC for *P. aeruginosa, S. typhii, V. parahaemolyticus, S. aureus* and *E. coli* was recorded as 100, 80, 80, 80 and 100 mg/ml respectively whereas in fractionated polysaccharide of *D. sibogae*, the MIC for *S. subtilis, Shigella* sp.*,S. typhii, V. parahaemolyticus, K. pneumonia* and *E. coli* was recorded as 80, 100, 100, 100, 80 and 80mg/ml respectively. At the same time in *L. duvauceli*, the MIC for *E. coli* noticed was 100 mg/ml.

The MIC of fungal strains results are given in Table 4. In fractionated polysaccharide, the MIC values for fungal strains such as *A. flavus* and *Rhizopus* sp. Were 80 and 100 mg/ml in *L. duvauceli* and 60 and 100 mg/ml in *D. sibogae* respectively.

DISCUSSION

Our results clearly show (Tables 1, 2, 3 and 4) that the methanolic and fractionated polysaccharide extract exhibited appreciable antimicrobial activity against human pathogenic bacteria and fungi. The usage of antibiotic disc susceptibility tests or disc-diffusion assays has the ability to rapidly identify active metabolites and therefore is particularly useful in the initial screening for antimicrobial activity and as the means for following activity during chemical purification (Gunthorpe and Cameron, 1987).

Antibacterial activity varies with the bioactive compounds of different species and pathogenic bacterial strains. Diluting extract usually weakens their antimicrobial activity. For the first time attempts to study the antimicrobial activity in marine organisms were initiated around 1950s (Jensen et al., 1996). Since that time, a large number of marine organisms from a broad range of phyla have been screened for their antimicrobial activity (Rinhart et al., 1981).

Table 2. Antifungal activity of methanolic extract and fractionated polysaccharides of squids

Fungal strains	Methanolic extract								Fractionated polysaccharide							
	L. duvauceli (%)				*D. sibogae* (%)				*L. duvauceli* (%)				*D. sibogae* (%)			
	25	50	75	100	25	50	75	100	25	50	75	100	25	50	75	100
A. flavus	-	-	-	-	-	-	-	-	+	++	++	+++	-	-	-	-
Candida sp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. fumigatus	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
Rhizopus sp	-	-	-	-	-	-	-	-	+	+	+	+++	+	+	+	++

-, No activity; +, weak activity (3-5 mm); ++, Good activity (6-10); +++, Very good activity (>10 mm) *The statistical significance: P values ≤0.05 (DMRT).

Table 3. MIC of methanolic extracts and fractionated polysaccharides of squids against clinically isolated human pathogens.

Bacterial strains	Methanolic extract										Fractionated polysaccharide									
	L. duvauceli (mg/ml)					*D. sibogae* (mg/ml)					*L. duvauceli* (mg/ml)					*D. sibogae* (mg/ml)				
	100	80	60	40	20	100	80	60	40	20	100	80	60	40	20	100	80	60	40	20
B. subtilis	+	++	++	+++	+++	+	+	++	+++	+++	+	++	++	+++	+++	-	-	+	++	+++
Shigella sp	-	+	++	+++	+++	+	++	++	+++	+++	+	+	++	+++	+++	-	+	+	++	+++
P. aeroginosa	+	+	++	+++	+++	-	+	+	++	+++	+	+	+	++	+++	+	+	+	++	+++
S. typhii	-	+	+	++	+++	-	-	+	+	++	+	++	++	+++	+++	-	+	++	+++	+++
V. parahaemolyticus	-	+	++	++	+++	-	-	+	++	+++	+	++	++	+++	+++	-	+	+	++	+++
V. cholerae	-	-	++	++	+++	+	++	++	+++	+++	+	+	++	+++	+++	+	++	++	+++	+++
S. aereus	-	+	++	++	+++	-	-	+	++	+++	+	++	++	+++	+++	+	+	++	++	+++
K. pneumoniae	+	+	++	+++	+++	+	+	++	+++	+++	+	+	++	++	+++	-	-	+	++	+++
E. coli	+	++	++	+++	+++	-	+	+	++	+++	-	+	+	++	+++	-	-	+	++	+++

MIC concentration: -, No growth; +, Cloudy solution (slight growth); ++, turbid solution (strong growth); +++, highly turbid solution (dense growth).

Potent antibacterial activity in haemocytes and haemolymph has been detected in various molluscs (Anderson and Beaven, 2001). Antimicrobial peptides have been isolated and characterized from the haemocytes of *Mytilus edulis* (Charlet and Prem Anand).

In the present study, high antibacterial activity was found in *K. pneumoniae, S. aureus* and *E. coli* (*D. sibogae*) and highest antifungal activity was recorded against *Rhizopus* sp. and *A. flavus* (*L. duvauceli*). The activity of the gladius extracts was found to be high in 100% concentration than the other concentration. In general, as the concentration increased, activity also increased. Polysaccharide extracts of *D. sibogae* showed activity against 8 bacterial strains except *V. cholerae* but *L. duvauceli* showed activity against 7 bacterial strains except *V. cholerae* and *B. subtilis*. For antifungal activity, *D. sibogae* showed activity against 2 fungal strains (*A. fumigatus* and *Rhizopus* sp.) and *L. duvauceli* showed activity against 3 strains. Both extracts showed no activity against *Candida* sp.

In the study, wide spectral antibacterial and antifungal activity were recorded in almost all the extract which is the significant finding of this study. Acetone extracts of oyster *Pteria chinensis*, showed higher degree of inhibition zone against *K. pneumoniae* (5 mm), *Streptococcus pneumoniae* (4 mm), *Serratia marcescens* (4 mm) and *Proteus mirabilis* (4 mm) (Yang et al., 2005). Escapin isolated from sea hare *Aplysia californica,* was most sensitive to escapin, with a minimum inhibitory concentration (MIC) of 0.25μg/ml. The pathogenic species *S. aureus* and *P. aeruginosa* were the next most sensitive to escapin, with MIC values of 0.31 μg/ml. Bacillus strains showed the highest resistance to escapin, with MIC of 2.5 μg/ml (Vivek et al., 2002).

The substances extracted from squid (*L. duvauceli*) ink tested for antibacterial activity, showed large inhibition zones against *E. coli* followed by *Salmonella* spp., V*. cholerae* and *Staphylococcus* spp. (Patterson and Murugan, 2000). The moderate antibacterial and antifungal activity were also reported from the extracts of various bivalve molluscs (Prem Anand and Patterson Edward, 2002) and broad spectrum of antibacterial activity has been reported for aqueous ink extracts of the cephalopods *L. duvauceli* and *S. pharaonis* against nine human pathogens (Patterson and Murugan, 2000). Antibacterial activity of the *S. Pharaonis* ink showed maximum activity of 9 mm for *S. epidermidis* and all other pathogens (*P. arruginosa, E. coli, Citro bactor and K. pneumonia*) showed lower activity (Nithiya et al., 2011).

A peptide (containing 12 cysteine of about 6.2Kda MW)

Table 4. MIC of methanolic extracts and fractionated polysaccharides of squids against clinically isolated human pathogens.

Fungal strain	Fractionated polysaccharide									
	L. duvauceli (mg/ml)					*D. sibogae* (mg/ml)				
	100	80	60	40	20	100	80	60	40	20
A. flavus	-	-	+	+	++	-	-	-	+++	+++
Candida sp	+	++	++	+++	+++	+	+	++	+++	+++
A. fumigatus	+	++	++	++	+++	+	+	++	++	+++
Rhizopus sp	-	+	++	+++	+++	-	+	+	+++	+++

MIC concentration: -, No growth; +, Cloudy solution (slight growth); ++, turbid solution (strong growth); +++, highly turbid solution (dense growth).

from a blood mussel *M. edulis* showed antifungal activity against *N. crassa* (Anderson and Beaven, 2001); “Haminol- A” from the Hawaiian opisthobranch mollusc, *Philliopsis speciosa* showed 8 mm inhibition zone at /ml concentration against *Saccharomyces cerevisae* and no activity at all against *C. albicans;* “dolabelanin-B_2” from the sea hare *D. auricularai* showed antifungal activity (Adam and Charis, 2004). The secondary metabolites Lissoclinolide isolated from ascidians possesses antimicrobial activity (Jayaseeli et al., 2001). Likewise in the present investigation also, the polysaccharide extracted showed potent antifungal activity against pathogenic fungi.

In 100% concentration, the highest inhibition zone of 12 mm was observed against *K. pneumonia* and *S. aureus* in methanolic extract of whole body tissue and *S. aureus* alone in EDTA extract from cuttlebone. The lowest inhibition zone of 8 mm was observed against *V. parahaemolyticus* and *Salmonella* sp. in the methanolic extract of whole body tissue and *V. parahaemolyticus* alone in EDTA extract from cuttlebone (Vairamani et al., 2012).

Only very few studies have been carried out on the antimicrobial activity of the internal shell of cephalopods. But many such studies are available for the extracts from the whole body tissue whose results could be compared with that of the present study. When studied, the antibacterial activity of *Donax modesta, Circe scripta* and *G. pectinatum* against nine pathogenic bacteria such as *S. aureus, K. pneumoniae, S. typhii, E. coli, B. subtilis, P. vulgaris, P. mirabilis, V. cholerae* and *S. flexeri* reported broad spectrum antibacterial activity for the water and heptane extracts (Jeyaseeli et al., 2001). 50 and 100 µl concentration of methanolic extract from body tissue of *S. brevimana* extract showed maximum zone (17 and 19 mm respectively) of inhibition against gram negative bacteria *K. pneumonia* (Mohanraju et al., 2013). In 100% concentration, the highest inhibition zone of 17 mm was observed against *E. coli* in whole body parts of *Octopus dolfusi*, 15 mm against *V. parahaemolyticus* in *Octopus aegina* and the lowest inhibition zone of against *S. pneumonia, E. coli, S. aureus, Streptococcus* sp. in *O. dolfusi* and *O. aegina* extract respectively (Monolisha et al., 2013).

The fact that some of the polysaccharide extracts showed high antibacterial and antifungal activity against human pathogens indicates that polysaccharide is at least not solely responsible for the antimicrobial activity detected.

Conclusion

In the present study, squids were collected from the Mudasalodai and cuddalore landing centre, southeast coast of India and showed potential antimicrobial activity against human pathogenic bacterial and fungal strains. Conceptually, it is clear that the marine ecosystem offers a huge potential as a natural based bioactive compounds. Fractionated products isolated from marine organisms have served as a source of drugs and starting material for the synthesis of useful drugs. In addition, because of the differences in the environmental conditions, marine organisms can evolve new or unusual biochemical entity having biological activity. So, it is believed that the studies of new and unique compounds derived from the marine organisms well continued to increase our basic knowledge with respect to pharmacology and medicine. We found potent antibacterial and antifungal activity against human pathogenic strains. This finding is very significant and may pave way for the discovery of new potent drugs against these dangerous pathogens.

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Full Length Research Paper

Spectrum of dermatophytoses in Jaipur, India

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In order to determine the extent and causative agent of ringworm infections in Jaipur area, a study was done in outdoor patient Department of Skin, SMS Hospital, Jaipur. Out of 196 diagnosed cases, 148 were found to be positive by KOH examination while culture positive cases were 160. *Trichophyton rubrum* was the most common etiological type reported from 53 cases followed by *Trichophyton mentagrophytes* (23 cases). *Trichophyton verrucosum, Trichophyton ferrugineum, Trichophyton concentricum, Trichophyton megninii, Microsporum canis, Microsporum audouinii, Microsporum fulvum* were reported for the first time in Jaipur. *Scopulariopsis* sp., *Paceilomyces* sp. and *Curvularia* sp. were also reported for the first time from human skin in Jaipur. Tinea corporis was the most common clinical type reported from all age groups. Tinea cruris was the second most common clinical type, followed by tinea capitis, tinea manuum, tinea unguium, etc. These infections were observed more frequently in the age group of 21-30 (26 %), followed by 31-40 (18.8%) and 11-20 (16.3%). Males (75.5%) were more infected than females (24.5%).

Key words: Dermatophytoses, ring worm, tinea infection, *Trichophyton*, *Microsporum*.

INTRODUCTION

Infections caused by fungi in humans and animals are common throughout the world. Dermatophytoses pose a serious concern to the sociologically backward and economically poor population of India. Dermatophytoses are superficial infections of keratinised tissue caused by organism of three genera of fungi known as dermatophyton (Bhadauria et al., 2001). The dermatophytes represent more than 40 closely related species classified in three genera: *Microsporum*, *Trichophyton* and *Epidermophyton*. Only a few of these species are responsible for most human fungal infection (Wagner and Sohnle, 1995). Skin infection due to dermatophytes has become a significant health problem affecting children, adolescent and adults. Recently, there has been an increase in the incidence of fungal infections in developing countries. This may be the result of frequent usage of antibiotics, environmental condition, immunosuppressive drugs and various conditions, like organ transplantation, lymphomas, leukemia and human immunodeficiency virus (Petmy et al., 2004).

In the present day living conditions, human and animal (domesticated) live their lives in close proximity to one another and are housed under the same roof or sleep on a common floor. In such a situation, skin and other infections are contracted easily and are perpetually multiplied. Fungal etiological infections in human are frequently observed during those seasons of the year when the environmental temperature and relative humidity are high (Karmakar et al., 1995). Jaipur has got a dry climate and in summer, the temperature exceeds even 46°C with high humidity during monsoon season. These climatic conditions favour the incidence of the

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fungi and consequently the disease.

The aim of this investigation was to find out unexplored species of dermatophytes and other related fungi and also to determine the pattern of dermatophytoses in Jaipur.

MATERIALS AND METHODS

Collection of infected skin scraping

In order to determine the extent and causative agent of ringworm infection in Jaipur area a study was done for six months period from April 2006 to September 2006. The collection of infected material was done in Dermatology department, SMS hospital at Jaipur. First of all, the infected area was cleaned with the help of 70% ethyl alcohol swabs and then, skin was scraped with the help of sterilized scalpel from peripheral area of the lesion. The scraping was collected in sterilized paper folders or in sterilized plastic bags. In the case of tinea capitis, the hairs or stump of broken hair were plucked from the affected area with sterilized forceps. In the case of onychomycosis or tinea unguium, the nail scraping or part of nails were removed with the help of sterilized scalpel or blade.

KOH mounting

The preliminary examination of the material was done immediately. For this, small portions of these scraping were taken and mounted in 10% potassium hydroxide (KOH) solution and examined after 5 min under microscope for the presence of fungal hyphae and/or arthrospores.

General characters

The following additional points were also recorded: Sex (male or female), age of patient, nature of infection, body part involved, profession and surrounding history of the patients, date of collection of scraping etc. for para-clinical data.

Culture and maintenance

Sabouraud's dextrose chloramphenicol agar (Himedia) medium was used for the isolation, purification and maintenance of dermatophytes.

Identification of fungi

The mycological identification was based on macroscopic and microscopic examination of the culture isolates. The macroscopic examination of dermatophytes was characterized by duration of growth, surface morphology and pigment production on the reverse. Strains were identified by their morphological and physiological characteristics according to the procedure described by Conant et al. (1959) and Forbes et al. (2002).

Identification characteristics

Trichophyton rubrum

Colonial types vary from white downy to pink granules; reverse yellow when colony is young; however, wine red color commonly develops with age. Microconidia usually teardrop, most commonly borne along sides of the hyphae, macroconidia are smooth and pencil shaped.

Trichophyton mentagrophytes

White granular and fluffy colony growth, reverse buff to reddish brown. Spiral hyphae commonly seen, microconidia most commonly borne in grapelike clusters, macroconidia are thin walled, smooth, club-shaped and multiseptate.

Microsporum gypseum

Cimmamon- colored powered colony, reverse light tan, macroconidia multiseptate thick walled, rough, elliptical. Microconidia few or absent.

Microsporum canis

Colony usually membranous with feather periphery center of colony white to buff over orange-yellow; lemon yellow or yellow-orange apron and reverse. Macroconidia thick walled, spindle shaped, multiseptate, rough walled, some with curved tip. Microconidia rarely seen.

Microsporum audouinii

Downy white to salmon-pink colony, reverse tan to salmon pink. Sterile hyphae, terminal chlamydospores favic chandeliers and pectinate bodies, macroconidia rarely seen-bizarre shaped if seen, microconidia rare or absent.

Microsporum fulvum

Colonies are fast growing, flat, suede-like, tawny-buffy to pinkish-buff in colour and frequently have a fluffy white advancing edge. A dark red undersurface is occasionally seen otherwise it is colourless to yellow brown. Macroconidia abundant, thin walled, elongated, ellipsoidal closely resemble those of *M. gypseum* except that they are longer and more bullet shaped with 3-6 septa.

Trichophyton verrucosum

Glabrous to velvety white colonies, rugal folds with tendency to skin into agar surface. Macroconidia extremely rare, but form characteristic "rat tail" types when seen; many chlamydospores are seen in chains particularly when colony is incubated at 37°C. Microconidia are rare, tear shaped.

Trichophyton violaceum

Port wine to deep violet colony, may be heaped or flat with waxy-glabrous surface; pigment may be lost on subculture. Mycelium branched tortuous hyphae that are sterile, Chlamydospores commonly aligned in chains.

Trichophyton tonsurans

White, tan to yellow or rust, suede like to powdery, wrinkled with

heaped or sunken center, reverse yellow to tan to rust red. Microconidia are teardrop or club shaped with flat bottoms, vary in size but usually large to dermatophytes. Macroconidia are rare and balloon forms are found when present.

Trichophyton schoenleinii

Irregularly headed, smooth white to cream colony with radiating grooves, reverse white, hyphae usually sterile, many antler type hyphae is seen.

Trichophyton megninii

The colony is cottony to velvety and white at first, but later become pale rose to violet. A non-diffusible red pigment is seen on the reverse side of the culture. Macroconidia and microconidia are produced more abundantly on trypticase dextrose agar.

Trichophyton concentricum

The colony is heaped, deeply folded, glabrous and white at first, but becomes deep brown in the center with cream colored somewhat powdery periphery, the pigment in the agar becomes deep amber in color. This fungus is macroscopically and microscopically identical with many strains of *T. schoenleinii.*

Epidermatophyton floccosum

Center of colony tends to be folded and is khaki green, periphery is yellow, reverse yellowish brown with observable folds. Macroconidia large, smooth wall, multiseptate, clavate and borne singly or in clusters of two or three, microconidia not formed by this species.

Candida albicans

White to cream colored, smooth, glabrous and yeast like in appearance. Microscopic morphology shows spherical to subspherical budding yeast like cells or blastoconidia.

RESULTS

The data presented in Table 1 shows prevalence of various clinical types and etiological agents of ringworm infections. It may be noted that *T. rubrum* is the most predominant etiological agent reported from 53 cases (32.1%). It was isolated from all clinical types except tinea versicolor (which was found to be negative in culture test). The other important species isolated are *T. mentagrophytes* 23 (14.3%), *T. schoenleinii* 6 (3.7%), *T. verrucosum* 4 (2.5%), *T. violaceum* 10 (6.2%), *T. ferrugineum* 1 (0.6%), *T. tonsurans* 9 (5.6%), *T. concentricum* 2 (1.2%), *T. megninii* 2 (1.2%), *M. gypseum* 5 (5.6%), *M. canis* 8 (5%), *M. audouinii* 1 (0.6%), *Microsporum* sp. 1 (0.6%), *Epidermophyton floccosum* 4 (2.5%) and *M. fulvum* 1 (0.6%) (Table 1).

T. verrucosum, *T. ferrugineum, T. concentricum, T. megninii, M. canis, M. audouinii, Curvularia* are reported for the first time in Jaipur district Rajasthan. Other related keratinophilic fungi like *Scopulariopsis* sp., *Paecilomyces* sp. are also reported for the first time in Rajasthan.

In the present investigation, tinea corporis is found to be the most prevalent disease. Out of 196 dignosed cases tinea corporis is observed in 69 patients (35.2%). The second most common clinical type is tinea cruris reported in 44 patient (22.4), followed by tinea capitis (11.2%), tinea manuum (7.1%), tinea unguium (6.6%), tinea pedis (6.6%), tinea corporis + cruris (5.1%), tinea versicolor (1.0%) and tinea manuum + corporis (0.5%) (Table 1).

It is evident from the results (Table 2) that tinea infections are more common in the age group of 21-30 (26.0%) followed by 31-40 (18.8%), 11-20 (16.3%). Tinea corporis is the most commonly observed infection in 21-30 and 31-40 age groups. Incidence of tinea infection in higher age groups was found to be less.

Tinea infections are more dominant in males (75.5%) rather than females (24.5%) for all age groups except infant-10 year age group where the percentage of female (7.65%) patients is higher than male patients (5.61%).

Among all clinical types, tinea capitis is more frequently reported in females (7.1%) than males (4.0%). Professional background sometimes plays an important role in the development as well as the spread of disease as observed in the present study. Data incorporated in Table 3 shows that Employers (60 cases) are more commonly infected with this disease followed by students (59 cases) and housewives/house girls (23 cases).

DISCUSSION

The dermatophytes are among the commonest infectious agents of man and no persons or geographic areas are free of them. In the present study, *T. rubrum* was found to be the predominant dermatophytic species in Jaipur (32.1%). Bhadauria et al. (2001) reported 34% incidence of *T. rubrum* in Jaipur area during 1999-2001. Tinea corporis was the most common clinical type of infection followed by tinea capitis, tinea pedis and tinea manuum. A review of available literature (Gupta and Shome, 1959; Gupta et al., 1993; Narayanan and Sareesh, 2011; Hanumanthappa et al., 2012) about dermatophytoses occurring in India confirmed beyond doubt that *T. rubrum* was the most prevalent species. The common occurrence of this species in various part of the country may be due to its greater adaptability to survive in varying climatic, populated and unhealthy conditions besides more susceptible individuals with poor nutrition. The second most common etiological agent was *T. mentagrophytes* isolated from 23 cases (14.3%). This is in general agreement with the earlier reports (Bhadauria et al., 2001; Maraki and Tselentis, 1998). Sharma et al.,

Table 1. Prevalence of various clinical types and etiological agents of ringworm infections at Jaipur.

Clinical diagnosis	Tinea corporis	Tinea capitis	Tinea cruris	Tinea pedis	Tinea manum	Tinea barbae	Tinea unguium	Tinea versicolor	Tinea corporis + cruris	Tinea manum + corporis	Total no. percentage (%)
1. No. of cases examined	69	22	44	13	14	8	13	2	10	1	196
2. No. of cases positive by microscopy	51	18	31	10	10	7	11	0	9	1	148
3. No. of cases positive by culture	57	17	36	10	10	7	12	-	10	1	160
4. No. of cases negative by culture	12	5	8	3	4	1	1	2	-	-	36
Species											
1. *T. rubrum*	18	3	13	4	3	3	3	-	5	1	53 (33.1%)
2. *T. mentagrophytes*	12	1	6	2	-	1	-	-	1	-	23 (14.3%)
3. *T. verrucosum*	1	1	2	-	-	-	-	-	-	-	4 (2.5%)
4. *T. schoenleinii*	3	2	1	-	-	-	-	-	-	-	6 (3.7%)
5. *T. violaceum*	3	4	-	1	-	-	2	-	-	-	10 (6.2%)
6. *T. ferrugineum*	-	-	-	-	-	-	1	-	-	-	1 (0.6%)
7. *T. megninii*	1	-	1	-	-	-	-	-	-	-	2 (1.2%)
8. *T. tonsurans*	6	1	2	-	-	-	-	-	-	-	9 (5.6%)
9. *T. concentricum*	1	-	-	-	-	-	-	-	1	-	2 (1.2%)
10. *M. gypseum*	1	1	1	-	-	1	1	-	-	-	5 (3.1%)
11. *M. canis*	2	-	-	-	4	1	-	-	1	-	8 (5%)
12. *M. audouinii*	-	1	-	-	-	-	-	-	-	-	1 (0.6)
13. *Microsporum* sp.	-	1	-	-	-	-	-	-	-	-	1 (0.6%)
14. *E. floccosum*	-	-	1	-	2	-	1	-	-	-	4 (2.5%)
15. *M. fulvum*	-	-	-	-	-	1	-	-	-	-	1 (0.6%)
16. *C. albicans*	1	-	2	-	1	-	2	-	1	-	7 (4.3%)
17. *Candida* sp.	-	-	2	-	-	-	1	-	-	-	3 (1.8%)
18. *G. reessii*	1	-	-	2	-	-	-	-	1	-	4 (2.5%)
19. *Curvularia* sp.	1	-	3	-	-	-	-	-	-	-	4 (2.5%)
20. *Scopulariopsis* sp.	-	-	-	-	-	-	1	--	-	-	1 (0.6%)
21. *Paecilomyces* sp.	-	-	1	-	-	-	-	-	-	-	1 (0.6%)
22. *B. dermatitidis*	1	-	-	-	-	-	-	-	-	-	1 (0.6%)
23. *T. simii*	2	-	-	1	-	-	-	-	-	-	3 (1.8%)
24. *Torulopsis* sp.	1	1	-	-	-	-	-	-	-	-	2 (1.2%)
25. *A. fumigatus*	-	1	-	-	-	-	-	-	-	-	1 (0.6%)
26. *C. tropicum*	2	-	1	-	-	-	-	-	-	-	3 (1.8%)
Total	57 (35.6%)	17 (10.6%)	36 (22.5%)	10 (6.2%)	10 (6.2%)	7 (4.3%)	12 (7.5%)	(0%) -	10 (6.2%)	1 (0.6%)	160 (100%)

Table 2. Clinical analysis of 196 cases of dermatophytoses in relation to age and sex.

Clinical types	Infant-10 Years		11-20 Years		21-30 Years		31-40 Years		41-50 Years		51-60 Years		61 & above		Total Males		Total Females	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	No.	%	No.	%
Tinea corporis	5	1	6	3	16	4	8	6	8	2	6	-	4	-	53	27.0	16	8.1
Tinea capitis	4	12	4	2	-	-	-	-	-	-	-	-	-	-	8	4.1	14	7.1
Tinea cruris	-	-	6	1	15	-	7	-	8	1	5	1	-	-	41	20.9	3	1.5
Tinea pedis	1	1	1	1	3	-	1	1	2	-	1	-	1	-	10	5.1	3	1.5
Tinea manuum	1	-	3	-	2	1	4	3	-	-	-	-	-	-	10	5.1	4	2.0
Tinea unguium	-	1	1	2	2	1	2	-	1	1	2	-	-	-	8	4.1	5	2.5
Tinea manuum + corporis	-	-	-	-	-	-	-	-	1	-	-	-	-	-	1	0.5	-	-
Tinea barbae	-	-	1	-	1	-	3	-	2	0	1	-	-	-	8	4.1	-	-
Tinea corporis + cruris	-	-	-	-	6	-	1	-	1	1	1	-	-	-	9	4.6	1	0.5
Tinea versicolor	-	-	-	1	-	-	-	1	-	-	-	-	-	-	-	-	2	1.0
Total percentage	11 5.6	15 7.6	22 11.2	10 5.1	45 22.9	6 3.1	26 13.2	11 5.6	23 11.7	5 2.5	16 8.1	1 0.5	5 2.5	- -	148	75.5	48	24.5

M = Male, F = female, percentage = %.

Table 3. Types of clinical infection and professional background of the patients.

Occupation	Tinea corporis	Tinea capitis	Tinea cruris	Tinea pedis	Tinea manuum	Tinea unguium	Tinea manuum + corporis	Tinea barbae	Tinea corporis + cruris	Tinea versicolor	Total	
											No.	Percentage
Students	13	21	12	4	4	2	-	1	1	1	59	30.1
Employees (clerk, technicians and peons)	23	-	13	3	4	4	1	5	7	-	60	30.6
Housewives	10	1	1	2	4	3	-	-	1	1	23	11.7
Businessmen	10	-	8	1	-	1	-	1	-	-	21	10.7
Farmers	4	-	3	3	2	2	-	1	1	-	16	8.2
Miscellaneous	8	-	6	-	-	-	-	-	-	-	14	7.1
Teachers	1	-	1	-	-	1	-	-	-	-	3	1.5
Total	69	22	44	13	14	13	1	8	10	2	196	100
Percentage	35.2	11.2	22.4	6.6	7.1	6.6	0.5	4.1	5.1	1.0	100	100

(2012) carried out a survey of dermatophytosis in Sitapura and Sanganer area, Jaipur. Tinea corporis was found to be the most common infection type followed by tinea cruris and tinea capitis.

During the present investigation, *T. verrucosum*, *T. ferrugineum, T. concentricum, T. megninii, M. canis, M. audouinii, M. fulvum* were reported for the first time in Jaipur district, Rajasthan. *T. verrucosum* is a zoophilic strain, usually isolated from animal. Rajpal et al. (2005) reported *T. verrucosum* from a 25 year old female. This zoophilic fungal infection occurs through occupational exposure to animal.

Other related keratinophilic fungi like *Scopulariopsis* sp. *Paecilomyces* sp., *Curvularia* sp. are also reported for the first time from human beings in Rajasthan.

In the present work, tinea corporis is the most common clinical type and the next most common is tinea cruris. Sharma et al. (1983) reported 34.48% incidence of tinea corporis, followed by 31.37% tinea cruris and 17.58% tinea capitis. *T. rubrum* (49%) was most common etiological agent followed by *T. mentagrophytes* (31.3%). Patwardhan and Dave (1999) studied 175 cases of dermatophytoses in and around Aurangabad. Tinea corporis (24.57%) was found to be most common clinical type followed by tinea cruris(22.28%). *Trichophyton rubrum* was most common etiological agent. Similar reports were given by various workers (Rajpal et al., 2005; Patwardhen and Dave, 1996). The higher rate of the prevalence of tinea corporis could be attributed to the fact that certain body parts are exposed more frequently than others like the hands and once it gets in contact with other body parts, it helps in the spread of the infection.

Once the disease is established, it can be transmitted to any person who comes in contact with the patient through his/her belongings like clothes, towel, soap, etc. Family members who are more frequently in touch with the patient have a greater change to contract the disease. In the present survey, it was recorded that some time, two or more members of the same family acquire tinea corporis, tinea cruris and tinea capitis infections.

During the present investigation, tinea infections are more frequently observed in the age group of 21-30 (26.0%), followed by 31-40 (18.8%). Sharma et al. (1983) had observed a majority of tinea infections in the age group of 21-30 year in Jaipur during 1980-81. According to Gokhale et al. (1999), the most commonly affected age group was 21-40 year (60.3%) in Pune. Same report was also given by Sen and Rasul (2006) during the study of dermatophytosis in Assam. During clinicomycological study of dermatophytoses in Bijapur, Karnataka. Peerapur et al. (2004) reported higher incidence in 21-30 years age group. Males were more frequently infected as compared to females. The higher incidence in young males could be due to greater physical activity and increased sweating.

The incidence of ringworm infections is found to be higher in males (75.5%) as compared to females (24.5%). This fact agrees with the reports and observations of Sharma et al. (1983). Karmakar et al. (1995) studied 250 cases of dermatophytes in a desert district of western Rajasthan. Incidence of dermatophytes was found to be 8.60% with tinea cruris 34.4% as the major clinical type followed by tinea corporis 24%. Male preponderance was observed (M : F = 2:1).

In the present study, the male prevalence is higher in all tinea infections except in the case of tinea capitis where the number of female patients (14) is higher than males (8). Kumar et al. (1996) studied 72 patients of tinea capitis between 1992 and 1994. The majority of the patients were children (94%), boys and girls being equal. This may be due to the lifestyle. Girls have longer and thicker hair than boys and usually wash it twice a week or on alternate days. In summer, due to sweat, the moisture in the hair also increases which provides a platform for the growth of dermatophytes. This is the reasons of higher incidence of tinea capitis in females in this age group. Sentamilselvi et al. (2004) observed tinea infection three times more frequently in male than female patients. Bhavsar et al. (2012) reported that males are infected more than females with a ratio of 2.14:1 during studies of superficial mycoses with clinical mycological profile in tertiary care hospital in Ahmedabad, Gujarat.

The lesser incidence of tinea infection in females may be due to: (i) social restrictions and personal inhibitions on the part of females which prevents them from reporting to hospitals and (ii) confinement of the majority of females to their house thereby minimising the chance of their coming into contact with infected persons outside the confines of their homes.

The reason for a high infection among employees could be public interaction, travelling, frequent handling of business articles or files and papers, which move from place to place, and hand to hand.

The above study clearly reveal the fact that person who have more responsibilities and outdoor activities are more prone to such infections, further, persons leading more of an indoor life or with lesser mobility or living away from thickly populated areas have lesser chances of getting such infections. Personal hygiene together with individual susceptibility to infection are also important factors for the incidence of these infections.

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Full Length Research Paper

Genetic characterization of H5N1 avian influenza viruses isolated from pet bird and chickens from live bird market in Bali and Bekasi (Indonesia), 2011

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Indonesia has endemic status of H5N1 and the largest number of human cases of H5N1. Sources of human infections include mostly intensive contact with H5N1 virus or the environment contaminated by this virus. This study shows that feaces from pet bird victims and chickens were sold in live bird market (LBM) suspected to be the source of infection for human H5N1 in Bali and Bekasi in 2011. This study focused on the virus' genetic character that originated from these areas, especially HA1, M, NS1 and PB2 genes. The results show that Bali and Bekasi viruses have similar genetic characters with human H5N1 viruses in Indonesia. The viruses have multiple basic amino acids in hemagglutinin that showed highly pathogenic, specific motif amino acid in the Matrix protein and ESEV motif on NS1. The viruses have no mutations in PB2 gene. The findings support that most cases of human H5N1 infection are as a result of exposure of birds to H5N1 virus.

Key words: Genetic character, H5N1, birds, LBM, source.

INTRODUCTION

Since identified in 2003, the H5N1 avian influenza virus in Indonesia has been circulating for almost 9 years (Dharmayanti et al., 2004; Wiyono et al., 2004). Various strategic measures to control and eradicate the disease have been done, but until now the virus is still circulating and even has become endemic throughout the year 2012. As at March 2012, there were 187 people confirmed infected due to AI/H5N1 virus and 155 are fatal cases (WHO, 2012).

Vaccination, bio-security and depopulation were some strategics measurenments taken by the Indonesian Government to control avian influenza disease. The strategies resulted in the reduction of the number of H5N1 cases in the field. The decreasing number of H5N1 cases in poultry sector was not followed by H5N1cases in human.

Cases of H5N1 still occur sporadically in some areas. Most of the source of human H5N1 infection in Indonesia is not yet known with certainty, even the highest human cases of H5N1 reported no history of contact with poultry or birds. But Aditama et al. (2012), in their findings, showed that most cases of AI/H5N1 infection were as a result of exposure to zoonotic sources of virus.

Data on the characters and genetic information related to dead victims caused by H5N1 virus infections of birds' origin in Indonesia are still limited. Continuous exposure to H5N1 virus by humans will increase the possibility of influenza pandemic in humans. Avian virus can adapt more efficiently in human through reassortment with other influenza strains in humans (Webster et al., 1992; Taubenbarger et al., 2005).

Several H5N1 infection cases were family clusters; however, it can be stated that virus transmission from human to human is still very limited (Ungehusak et al., 2005).

Viral transmission inter human is not yet proven, thus

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Table 1. The specimens origin during our surveillance on human animal interface in 2011.

H5N1 human case	Date of collected samples	District	Location	Type of sample	Number of sample	RT-PCR of positive for H5N1	Positive for viral isolation	Remark
Female, 31 y.o	3 March 2011	Bekasi, West Java	Live Bird Market	Chicken	21	11	2	100 m from victim house
				Environment	1	1	0	
			Near victim house	Pet Bird	61	14	0	
				Goose	3	0	0	
Female, 5 y.o; Male 10 y.o	13-15 October 2011	Bangli, Bali	Near victim house	Pet Bird (cendet bird like)	2	1	1	Victims kept the bird in their house
			Chicken collector	Chicken	24	0	0	
				Muscovy Duck	1	0	0	
				Duck	5	1	1	

most cases in humans occur due to virus spread from infected birds (World Health Organization 2005a). In this study, we reported the molecular character of human H5N1 viruses isolated from birds in Indonesia.

MATERIALS AND METHODS

Samples collection and virus isolation

We isolated viruses from cloacal swab samples collected from birds or chickens near H5N1 victim houses in Bekasi (West Java) and Bangli (Bali) in 2011 (WHO, 2011). Table 1 showed that the specimens collected from animal/environment side related with H5N1 human cases infection. H5N1 viruses from Bali were A/Bird/Bali1/2011 collected from feaces of the pet bird which has been kept by victims and A/Duck/Bali/2011 virus isolated from ducks and chicken located near houses of victims. The ducks look healthy and have no clinical sign. The other isolates (A/Chicken/West Java/Bks9/2011 and A/Chicken/West Java/Bks12/2011) were collected from LBM near the houses of victims in Bekasi (Tabel 1).

Sterile cotton-tipped swabs were used for sampling and were subsequently stored in viral transport medium. The transport medium consisted of Dulbecco's Modified Eagle Medium (DMEM) with 1000 IU penicillin and streptomycin. The samples were immediately transported to the laboratory after collection and were stored at -70°C. A 1000 µl sample in transport medium was homogenized by vortex and centrifuged with the speed of 2500-3000 rpm. The supernatants were then inoculated in embryonated specific pathogen free (SPF) eggs of 9-11 old days. The allantoic fluid was extracted using QIAmp RNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The extracted RNA was tested for H5 subtype by RT-PCR using H5-155F and H5-690R primers (Lee et al., 2001). Thermo cycling was performed in ABI 9700 and 2700 PCR machines.

DNA sequencing and visualization of 3D-protein prediction

To amplify full length HA, the primer of Senne et al. (1996) was used to amplify HA1 region, and HA2 was modified using H5-155F (Lee et al., 2001); NS890 primers were published by Hoffman et al. (2001). For NA gene amplification, primer of Komadina (2006, personal communication) was used, while for the amplification of M and NS genes, primer of Hoffman et al. (2001) was used. The primers for amplifying PB2 gene were desinged by the authors. PCR products were separated in 1% agarose by electrophoresis and the amplicon was excised and purified using QIAquick gel purification kit (Qiagen). The sequencing method used was direct sequencing using Cycle sequencing kit (BigDye Terminator version 3.1; Applied Biosystem) on Genetyx Analyzer 3130 (Applied Biosystems, USA). The nucleotide sequencing data obtained in this study were analyzed together with the genetic data available in the avian influenza database (NCBI) based on each gene. The production of multiple alignments of each gene and residue analysis were carried out by using BioEdit version 7 (http://www.mbio.ncsu.edu/BioEdit). Phylogenetic trees were generated by neighbor-joining bootstrap analysis (1,000 replicates), using the Tamura-Nei algorithm in MEGA version 4 (Http: //www.megasoftware.net). All of the viruses used in this study have been submitted to GenBank (www.ncbi.nlm.nih.gov) with accession number (yet to be submitted to NCBI).

RESULTS

The phylogenetic of HA gene (Figure 1) showed that Bekasi 2011 (Bks9/2011; Bks12/2012) and Bali 2011 (Bali1/2011; Bali9/2011) viruses (Bali1/2011; Bali9/2012) were in the same group with other Indonesian H5N1 viruses isolated in

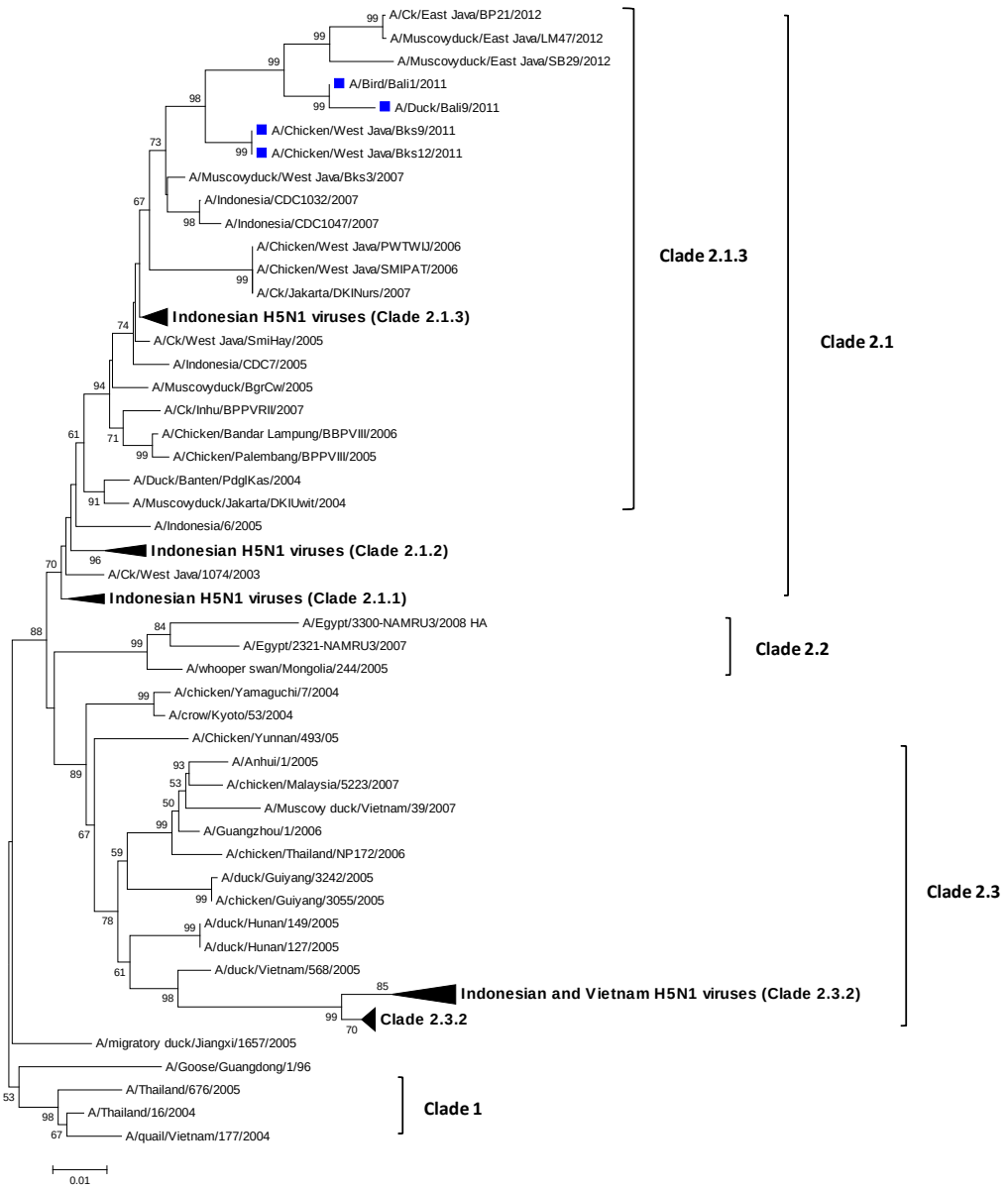

Figure 1. Phylogenetic of HA gen of H5N1 Viruses. Figure 3) Phylogenetic of M1, Figure 4) Phylogenetic of PB2; The viruses used in this study showed by blue color and in one group with Indonesian Human H5N1 viruses.

2011. Bekasi 2011 and Bali 2011 viruses belong to clades 2.1.3. which are the predominant clades in Indonesia. The genetic characteristic of Bekasi 2011 and Bali 2011 in terms of HA protein had amino acid QSG at positions 222, 223, 224, respectively (Figure 2). The condition showed that the viruses still recognize the 2,3 *avian receptor*. The cleavage site of HA protein sequences has PQRESRRKKR//G. Bekasi and Bali isolates have no significant differences with the majority of H5N1 avian influenza virus from Indonesia. Another substitution such as amino acid at position 53 is replaced with amino acid R and K amino acid (R→K). Substitutions are similar to the human H5N1 viruses A/Indonesia/CDC1047/2007, A/Indonesia/CDC370/2006, A/Indonesia/CDC390/2006). If Bali 2011 viruses are compared with previous viruses from Bali and other Indonesian AI viruses, there are differences at positions 183, 184 and 189. But the substitution of amino acids at positions 183 (D→N), 184 (A→G) and 189 (R→M) is similar to the Bekasi 2011 viruses.

Dharmayanti et al. (2011a) showed that human AI/H5N1 virus isolated from birds has a genetic similarity with Indonesian human AI/H5N1viruses and has specific character with the Matrix protein from birds collected from AI/H5N1 cases in humans. In this study (Table 2) showed that most of Indonesian H5N1 human viruses, Bekasi/2011

Table 2. Amino acid specific on Matrix protein belong to H5N1 Bali and Bekasi viruses.

Virus	M1 amino acid position				M2 amino acid position				
	37	95	137	249	8	18	20	27	50
West Java/Bks9/2011	A	K	A	H	Y	K	I	A	F
West Java/Bks12/2011	A	K	A	H	Y	K	I	A	F
Bali/Bangli1/2011	A	K	A	H	Y	K	I	A	F
Bali/Bangli9/2011	A	K	A	H	Y	K	I	A	F
Indonesian H5N1 Indonesia origin from birds/poultry	T	R	T	Q	C	R	S	V	C
Most of Indonesian H5N1 human viruses	A	K	A	H	Y	K	I	A	F
Indonesian H5N1 viruses (2011)	A	K	A	H	Y	K	I	A	F

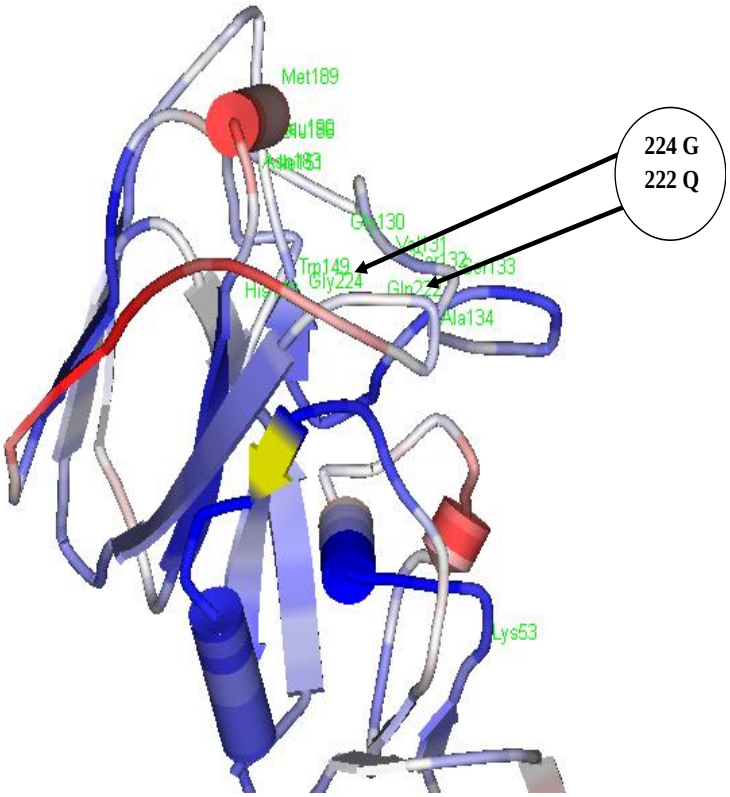

Figure 2. Prediction of three Dimension of HA protein of Bali virus. The viruses still recognize avian receptor. The template highest homology is obtained by using the BLAST search (DS server). HA1 template of H5N1 in this study is 21BX_A that is equal to 96%.

and Bali/2011 viruses have specific amino acid substitutions in the Matrix protein. This result had same result with Dharmayanti et al (2011a) findings. Phylogenetic analysis of M1 (Figure 3) showed that Bekasi and Bali viruses were in one group with H5N1 2011 viruses group; human H5N1 viruses and also avian viruses were isolated from human H5N1 in 2007 (Bks2/2007).

In the analysis of the NS protein level, there were substitutions in R44K; M79K and has a PDZ binding motif as ESEV (avian origin). This means there is a significant mutation at amino acid position 79 where Methionine (M) replaced the basic amino acid lysine (K) and had the possibility of affecting binding protein of the virus. Phylogenetic analysis of NS1 also showed that H5N1 Bekasi and Bali viruses are in one group with human H5N1 viruses and avian viruses.

Analysis of the PB2 protein showed that there is no mutation at position E627 and D701 amino acids. The phylogenetic of PB2 gene showed that the viruses used in this study close relationship with Indonesian human H5N1 viruses (Figure 4).

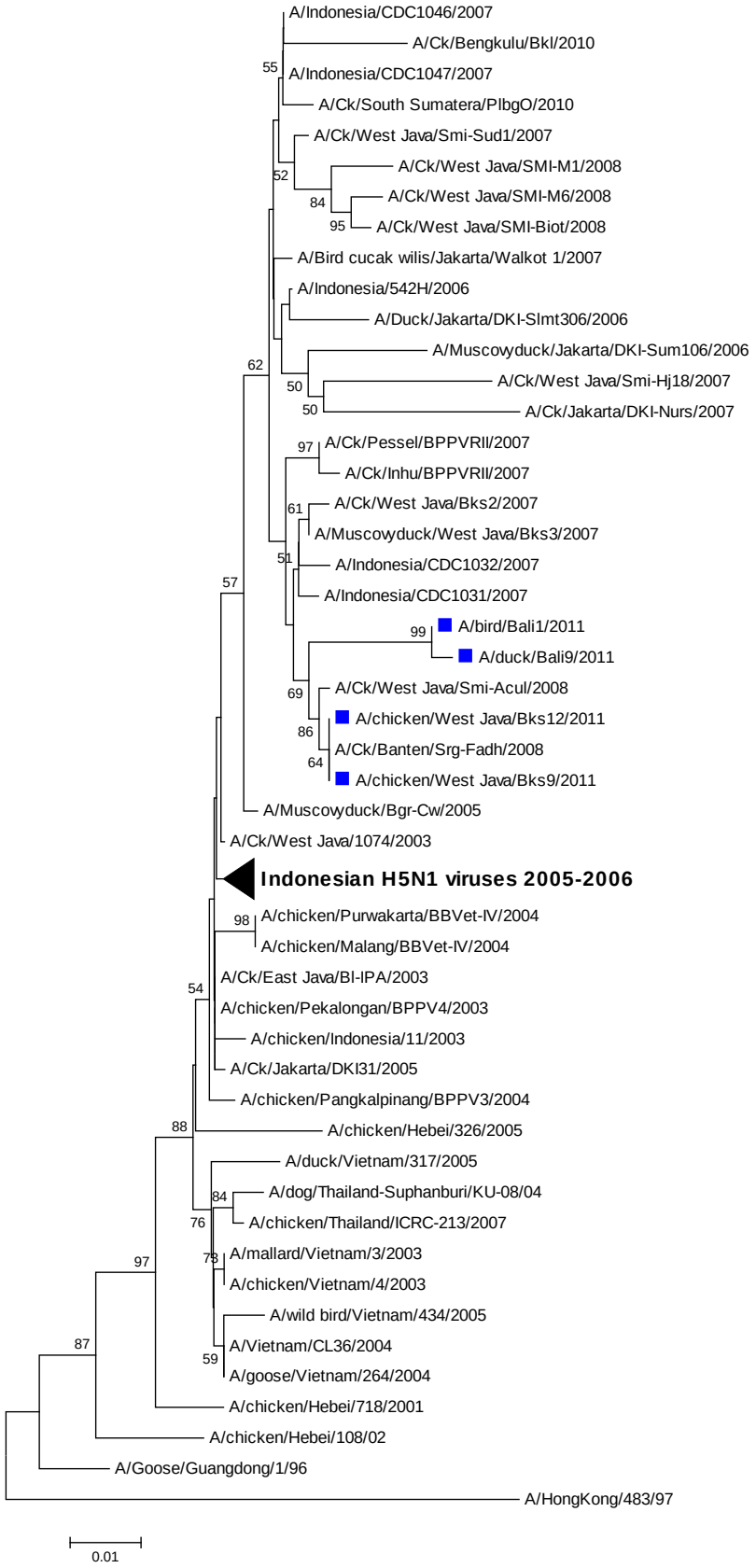

Figure 3. Phylogenetic of M1 of H5N1 viruses.

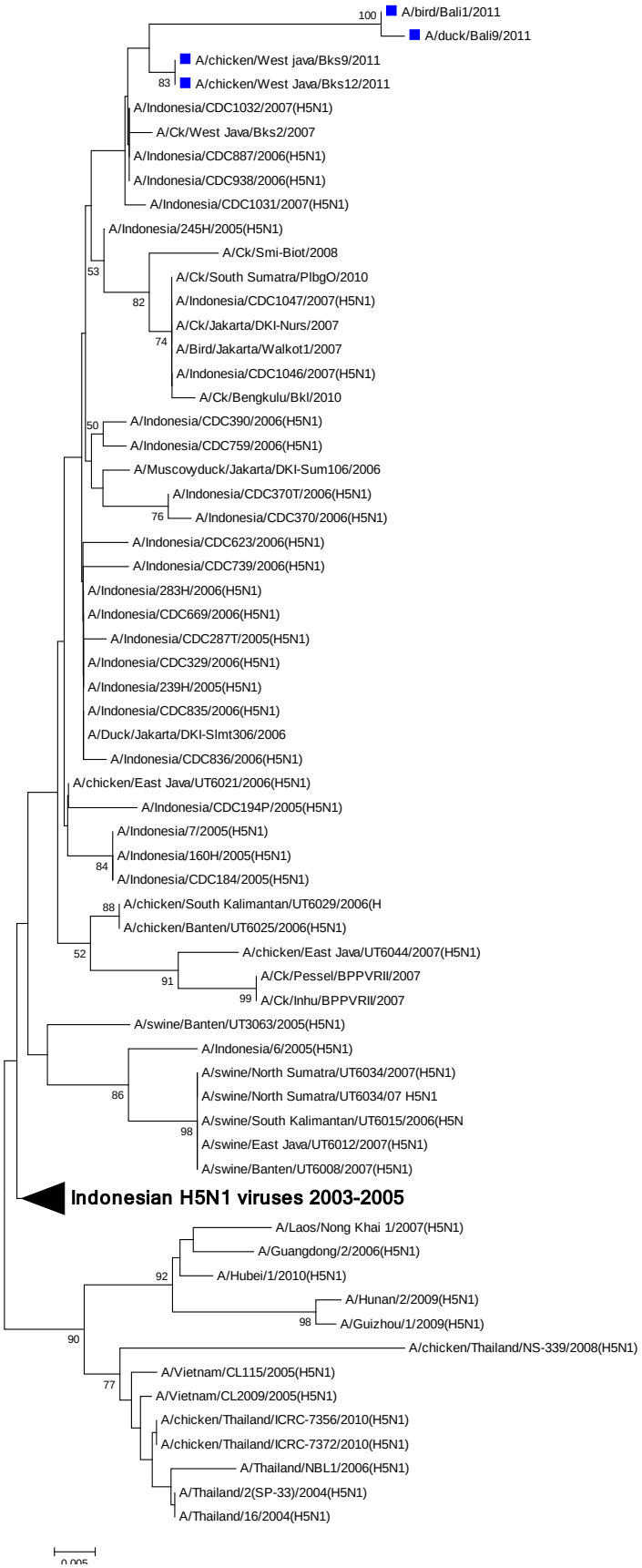

Figure 4. Phylogenetic of PB2 gene of H5N1 viruses.

DISCUSSION

We did a surveillance and collected specimen from birds/environment of victims' house to identify the risk factor and source of the viruses that infected human, especially in Bekasi 2011 and Bali 2011. We got four isolates of H5N1 viruses: two viruses from Bekasi and two isolates from Bali. The genetic characteristic showed that there is no mutation on 182 and 192 of HA protein. Mutations at positions 182 and 192 that change HA protein of H5N1 avian receptor are known to recognize human receptors. Such mutations are Asn182Lys and Gln192Arg that change the specificity of the receptor, because these two residues play a role in stabilizing the interaction between sugar and the bond sialic acid α2, 6 (Yamada et al., 2006). Herfst et al. (2012), in their study, also showed that four amino acid substitutions in the hemagglutinin (HA) protein and one in the polymerase complex protein basic polymarase 2 (PB2) in A/H5N1 influenza virus can have ability to do airborne transmission between mammals without adapting to an intermadiate host; and furthermore can become a risky influenza pandemic. Bekasi 2011 and Bali 2011 viruses used in this study have Asn and Gln at positions 182 and 192 respectively so that the bond is still not able to recognize α2, 6. In addition to the isolates, avian receptors were still recognized because they have residue 222 (Q) and 224 (G) (Stevens et al., 2006). Gao et al. (2009) also showed that substitution of amino acid in the HA protein (Thr160Ala) resulting in the loss of glycosilation at 156-160 was responsible for binding to sialylated glycans and was critical for H5N1 virus transmission in guinea pigs.

Bali and Bali viruses have 7 glycosylation sites; this number is a normal amount which is owned by Indonesia H5N1 virus; so it does not have or increase the number of glycosylation sites. H5N1 viruses which have decreasing number of glycosylation sites in Indonesia have been reported previously by Dharmayanti et al (Dharmayanti et al., 2011b). Mutation causes decrease in the effectiveness of vaccines used when challenged with H5N1 virus that has seven glycosylation sites.

Matrix protein of Bekasi and Bali isolates has a specific character in protein M1 and M2 as well as other H5N1 viruses that were isolated from humans infected with H5N1 (Dharmayanti et al., 2011a). Bekasi and Bali isolates showed a deletion of amino acids at amino acid positions, 80-84. Deletion of amino acids at positions 80-84 in the NS1 protein increases the virulence of H5N1 virus (Long et al., 2008). Dharmayanti et al. (2011a) also showed that most isolates of Indonesia have these deletions. The C termini of NS1 protein of the viruses have consensus PDZ domain ligand (PL); which means a protein-protein recognition modules that recognize this protein and are bound to the C-termini are on the NS1 protein residues, 227-230 (Obenauer et al., 2006). Result of the study showed that Bekasi and Bali viruses have ESEV motif, indicating that Bekasi and Bali isolates were of avian origin. ESEV or EPEV motif was found in the origin of avian H5N1 virus (Obenauer et al., 2006). H5N1 viruses isolated from birds in H5N1 human cases have a close genetic relationship at the protein level of HA, NS, M1, M2 and PB2 of H5N1 human viruses. Our study showed that amino acid in the PB2 gene contains Glu at 627 amino acid posiition. This means there is no substitution in PB2 gene. Substitution Glu627Lys (E627K) has been associated with increased virus replication in mammalian cells (Subbarao et al., 1993). Substitution of amino acid asparagine (Asn) at 701 posiition in PB2 protein was a prerequiste for virus transmission to guinea pigs. The genetic character from the isolates used in this study has high similarity with each other and general Indonesian H5N1 viruses in 2011. Our findings show that most of H5N1 human infections were as a result of exposure of birds to H5N1 virus.

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Full Length Research Paper

Incidence rate of *Staphylococcus aureus* and *Streptococcus agalactiae* in subclinical mastitis at smallholder dairy cattle farms in Hawassa, Ethiopia

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A longitudinal study was undertaken from December 2009 to February 2010 at Hawassa town, Ethiopia, in smallholder dairy farms to identify the role of *Staphylococcus aureus* and *Streptococcus agalactiae* in causation of subclinical mastitis and also to assess the role of selected risk factors in the transmission of these pathogens. A total of seven farms were selected. The farms were first screened for subclinical mastitis by California Mastitis Test (CMT) and those free of the disease were monitored. Each farm was visited at intervals of two weeks and during each visit CMT was conducted. A milk sample was aseptically collected from quarters that were CMT positive (a CMT score of greater than or equal to one). Milk samples were cultured, and *S. aureus* and *S. agalactiae* were isolated. A cow found positive in CMT in the first test and had *S. aureus* and *S. agalactiae* was then excluded and was not subsequently tested and sampled. The average subclinical mastitis incidence rate due to *S. aureus* and *S. agalactiae* was found to be 21.84 ± 0.06 Sd per 100 cow- months at risk. Out of 165 CMT positive milk samples cultured for isolation of *S. aureus* and *S. agalactiae,* 88 (53.32%) yielded *S. aureus* and 30 (18.17%) had *S. agalactiae.* Co-infection by *S. aureus* and *S. agalactiae* was found in 14 (8.48%) of CMT positive milk samples. Generally, 104 CMT positive milk samples (63.03%) were due to *S. aureus* and *S. agalactiae.* Out of the 12 questions to the milking practice and other contagious mastitis control measures, only two were practiced by all farms: milking mastitic cows last and treating all cases of clinical mastitis. This study reveals that *S. aureus* and *S. agalactiae* were the major causes of subclinical mastitis and mastitis control strategies in those farms, and possibly other local dairies which have to focus on these pathogens.

Key words: Contagious mastitis, Hawassa, Subclinical mastitis**,** *Staphylococcus aureus, Streptococcus agalactiae.*

INTRODUCTION

In most Western countries it is now possible to reduce the incidence of subclincal mastitis using udder health monitoring programs by setting a regulatory limit for bulk milk somatic cell count (BMSCC) (Barkema et al., 1998). Most smallholder dairy farmers in Africa including Ethiopia are generally aware of clinical mastitis (Almaw et al., 2008; Kivaria et al., 2006) because of the signs exhibited by the cow. However, farmers' awareness of

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subclinical mastitis is very low. According to Almaw et al. (2008) none of the interviewed Ethiopian farmers knew and screened their cows for subclinical mastitis except seeking veterinarian assistance whenever their cows got sick. Generally, smallholder dairy production in North-western Ethiopia is characterized by hand milking and poor sanitary milking practices (Tassew and Seifu, 2009). This practice could facilitate the spread of contagious mastitis pathogens. Several studies have been conducted in Ethiopia and elsewhere to isolate pathogens from subclinical mastitis (Getahun et al., 2008; GIanneechini et al., 2002; Moret-Stalder et al., 2009). Contagious mastitis tends to be sub-clinical in nature. The focus on mastitis prevention and control programs has to differ between regions and should be farm specific based on the existing situations. The new infection trend of *S. aureus* and *S. agalactiae* in the absence of adequate control measure has not been studied in Ethiopia. The implementation of effective specific control program could result in the eradication of *S. agalactiae* and substantial reduction in the incidence of *S. aureus* subclinical mastitis. This study was undertaken to estimate the incidence rate of *S. aureus* and *S. agalactiae* in subclinical mastitis under the smallholder dairy production system where milking is almost always by hand.

MATERIALS AND METHODS

Farm selection

The study was conducted in Hawassa town, the capital city of Southern Nations Nationalities and People Regional State of Ethiopia from December 2009 to February 2010. A total of seven smallholder dairy farms were selected; one government owned and six private. The farms were coded as A, B, C, D, E, F and G dairy farms. The herd size ranged from 5 to 19 lactating dairy cows.

Study animals

All animals included in the study were cows producing milk at various stages of lactation. The majority were crossbreds (Holstein-Zebu) of different blood level. Only two farms had pure local breeds (Zebu). Farm D had one and F had five local zebu breeds. All the cows were hand milked and milked two times a day in the morning and evening. The animals were kept indoor, the whole day and fed roughage *ad-libtum* with nug cake supplement. Milk yield was between 4 to 20 L per day in high grade exotic breeds and 1.5 to 5 L per day in both local and low grade cross breeds.

Study design and sampling

A longitudinal study was conducted to determine the incidence of subclinical bovine mastitis due to *S. aureus* and *S. agalactiae*. The farms were first screened for subclinical mastitis using California Mastitis test (CMT). The CMT was scored 0, T, 1, 2, and 3 and was interpreted as negative, trace, weak positive, distinct positive and strong positive in that order. For the purpose of this study a score of greater than or equal to one was taken as positive. CMT was conducted according to Quinn et al. (1999). From CMT positive quarters milk sample was collected aseptically according to the National Mastitis Council (NMC, 1990) for isolation of *S. aureus* and *S. agalactiae.* Those free of the disease were monitored at intervals of two weeks for a total of six visits. This was based on the assumption that subclinically infected cows remain so at least for two weeks so that there will not be missing of cows that became sick and recovered during the interval period. A cow with a positive CMT test and had *S. aureus* or *S. agalactiae* was excluded in the next visit and was not tested and sampled for the second time to avoid persistent infection. However, cows with CMT positive but culture negative were reexamined for both CMT and bacteriology until positive for *S. aureus* or *S. agalactiae*. A cow found positive for *S. aureus or S. agalactiae* was considered at risk for *S. agalactiae* or vice versa and was monitored until found *S. agalactiae* or *S. aureus* positive or end of the study period.

Data collection

Factors that were thought to have potential association with contagious mastitis pathogens (*S. aureus* and *S. agalactiae*) and udder health problems were recorded. Twelve check lists were prepared to collect data on milking practice and other contagious mastitis control measures. The check list itmes were hand wash before milking, udder wash before milking, towel usage, milking mastitic cows last, dry cow therapy, pre and post milking teat dipping and culling of chronic mastitic cows.

California mastitis test

The California Mastitis Test was carried out as screening test to detect subclinical mastitis. A squirt of milk, about 2 ml from each quarter was placed in each of four shallow cups in the CMT paddle. An equal amount of the commercial reagent was added to each cup. A gentle circular motion was applied to the mixtures in horizontal plane for 5 s and then the reaction was interpreted as described in study and sampling part of the paper.

Milk sample collection

Milk sample collection and storage was carried out following procedures recommended by NMC (1990) and Quinn et al. (1999). Quarter milk was collected from CMT positive cows only. The teat orifice was cleaned using cotton soaked in 70% ethyl alcohol and 5 to 10 ml of milk was collected in to sterile test tubes for bacteriological examination. During collection the test tube was held nearly horizontal to prevent contamination by dirt droppings. The sample was transported immediately to Hawassa University, Faculty of Veterinary Medicine, Microbiology Laboratory using ice box. Samples were processed immediately without storage.

Staphylococcus aureus and *Streptococcus agalactiae* isolation

Isolation of *S. aureus* and *S. agalactiae* from CMT positive milk samples was performed following standard procedures described by NMC (1990) and Quinn et al. (1999). One loopful from each milk sample was inoculated on to blood agar base enriched with 7% sheep blood. Blood agar plates were incubated at 37°C for 24 to 48 h. Each plate was examined for growth, morphology and hemolytic characteristics, Gram stain reaction and catalase tests. *Staphylococci* were identified based on catalase test, growth

Table 1. Incidence rate of subclinical bovine mastitis at cow level in smallholder farms at Hawassa, Ethiopia from December 2009 to February 2010.

Farm	Number of cows attended	Total number of cases based on culture[a]	Period of observation (month)	Contribution to cow-month at risk	Incidence rate per 100 cow-months risk
A	19	13	3	43	30
B	9	3	3	25.5	11.8
C	15	8	3	41.5	19.5
D	6	3	3	16.5	18.2
E	6	3	3	16.5	18.2
F	8	5	3	19.5	25.6
G	6	4	3	13.5	29.6
Total	69	39		176	21.84 ± 0.06sd[b] (average)

[a]*S. aureus* and/or *S. agalactiae;* [b]*sd:* standard deviation.

characteristics on Manitol salt agar and coagulase test. Coagulase positive *Staphylococcus* species (that is, *S. aureus, S. intermedius* and *S. hyicus*) were identified on the basis of acetoin production from glucose (Voges Proskauer test). *Staphylococcus aureus* is acetoin positive where as *S. intermidius* and *S. hycus* do not produce acetoin. In addition *S. hyicus* does not produce haemolysis on sheep blood agar. Isolates presumptively identified as *Streptococci* were characterized according to CAMP reaction, catalase test, and hydrolysis of esculin. A CAMP test positive *S.agalactiae* was differentiated from *S. uberis* which is also CAMP positive by production of dark brown colony on esculin blood agar (Edwards's medium) indicating esculin hydrolysis.

Data analysis

Chi square analysis was used to compare the incidence rate of subclincal mastitis between farms. The incidence rate (IR) of subclinical mastitis due to *S. aureus* and *S. agalactiae* (combined) at cow level was calculated according to the formula given in Thrusfield (2005).

$$IR = \frac{\text{Number of new cases of disease that occur in a population during a particular period of time}}{\text{The sum of overall individuals of the length of time at risk of developing disease}}$$

RESULTS

Incidence rate of subclinical mastitis caused by *Staphylococcus aureus* and *Streptococcus agalactiae*

In seven farms, a total of 69 lactating cows were monitored for the period of three months for the incidence rate of subclinical mastitis. At cow level out of 69 lactating cows 39 had subclinical mastitis caused by *S. aureus* and *S. agalactiae*. The average incidence rate was 21.84 ± 0.06 Sd per 100 cow-months. The highest subclinical incidence rate was observed on farm A (30 per 100 cow-months at risk) and the lowest was at farm B (11.8 per 100 cow-months at risk) (Table 1) and the difference was statistically significant (χ^2 (1) = 7.4667 Pr = 0.006).

Regarding the infection rate of *S. aureus* and *S. agalactiae,* there was no a continuous increase or decrease during visit period (Table 2). *S. aureus* was more prevalent than *S. agalactiae.* In farm A new infection by *S. agalactiae* ranging from 6.7 to 23% throughout all checking times was seen. And in all farms except D (*S. agalactiae* positive in the final check up) and E, which were positive for *S. agalactiae* in the previous check up, new infection by *S. agalactiae,* was seen at least in the next check up.

Bacteriology

A total of 165 CMT positive milk samples were cultured for isolation of *S. aureus* and *S. agalactiae.* Of these 88 (53.32%) yield *S .aureus* and 30 (18.17%) *S. agalactiae* (Table 3). Growth due to other bacteria was observed in 46 (27.90%) CMT positive samples but these were not further isolated. Out of 165 CMT positive milk samples, 104 (63.03%) were due to the contagious pathogens of *S. aureus* and *S. agalactiae.* Co-infection by *S. aureus* and *S. agalactiae* was seen in 14 (8.48%) samples (Table 3).

Milking practice and other contagious mastitis control measures

Of the 12 check lists which are considered important in contagious mastitis control, most of them were not in use by the farms studied (Table 4). All dairy workers used tap water to clean milking equipment, wash their hands and cows udder. All farms practiced hand milking where none of the milkers in all farms use soap to wash their hands (Table 4). The association of these milking practices with the occurrence of *S. aureus* and *S. agalactiae* were not tested statistically.

DISCUSSION

This study reveals that the majority of subclinical cases

Table 2 . Percentage of newly infected cows with *S. aureus* or *S. agalactiae* pathogens at the time of herd check-up.

Farm	Farm check up											
	1		2		3		4		5		6	
	S. aureus	*S. agalactiae*	*S. aureus*	*S. agalactiae*	*S. aureus*	*S. agalactiae*	*S. aureus*	*S. agalactiae*	*S. aureus*	*S. agalactiae*	*S. aureus*	*S. agalactiae*
A	5.3	10.5	16.7	11.8	0	6.7	13.3	7.1	0	23	15.4	10
B	11.1	0	0	0	0	0	0	11.1	0	12.5	12.5	0
C	13.3	0	0	0	7.7	0	8.3	0	11.1	6.7	25	14.3
D	0	0	0	0	0	0	16.7	0	20	0	25	16.7
E	0	0	0	0	0	0	16.7	0	0	16.7	16.7	0
F	0	12.5	12.5	28.6	14.3	0	0	0	16.7	20	0	25
G	16.7	0	20	16.7	0	20	0	25	0	0	25	0

[a]*S. aureus.*

Table 3. Frequency of *S. aureus* and *S. agalactiae* isolates in CMT positive sample in subclinical bovine mastitis in smallholder dairy farms at Hawassa, Ethiopia.

Isolate	Number	Percentage (%)
S. aureus	74	44.84
S. agalactiae	16	9.70
S. aureus and *S. agalactiae* (mixed infection)	14	8.48
Other bacateia (not identified)	46	27.90
No growth	15	9.09
Total	165	100

Table 4. Milking practices and other contagious mastitis control measures in selected farms at Hawassa, Ethiopia.

Number	Risk factor	Number of farms practicing	Percentage (%)	Farm
1	Hand wash before milking	2	28.57	A, B
2	Hand wash with soap	0	0	
3	Disinfect hand after washing	0	0	
4	Udder wash before milking	2	28.57	A, B
5	Use of towel for teat drying	1	14.28	B
6	Use of individual towel	0	0	
7	Pre-milking teat dipping	0	0	
8	Post milking teat dipping	1	14.28	B
9	Mastitic cow milked last	7	100	All
10	Dry cow therapy	0	0	
11	Culling of chronic mastitic cows	1	14.28	A
12	Treat clinical cases	7	100	All

(63.02% of CMT positive samples) were due to contagious pathogens (*S. aureus* and *S. agalactiae*) and the dominant pathogen was *S. aureus* (53.32%). This might be related to poor milking and contagious mastitis control practice seen in the studied farms. In the absence of hygienic milking practice contagious mastitis pathogens either from infected cow or milkers hand can easily spread. In cross-sectional studies of subclinical mastitis various isolation rates of *S. aureus* have been reported (Abera et al., 2010; Gianneechini et al., 2002; Moret-stalder et al., 2009). However, studies indicating the transmission rates are not common. In Germany, Sommerhäuser and his colleagues (2003) evaluated the spread of *S. aureus* in a six herds after implementing six

point control measure including strip cup testing, udder cleaning before milking using individual paper towel, post-milking teat disinfection, proper milking technique, culling and dry cow therapy. At the beginning of their study the intramammary infection rate for *S. aureus* was 24.2 to 27.1% in three herds and 4.2 to 11.9% in the other three herds. At the end of the study (Sommerhäuser et al., 2003) there was no new infection and persistent infection was observed only in one herd (1.2% of the cows) suggesting the control measures were effectively controlling the transmission of contagious mastitis. However, their study also indicated that there was dynamicity in the occurrence of *S. aureus* infection as herds which were negative in the previous check up showed 9.1% *S. aureus* new infection in the subsequent check up, in agreement with the present study. Unlike the German study, in the present study there was not a control measure in place except treating clinical cases and culling and hence the rate of new infection was bound to increase. The chance of a cow getting new *S. aureus* and *S. agalactiae* infection in a month per 100 lactating cows was found to be 21.84%. In Tanzania intervention trials were studied for a period of one year in smallholder dairy farming involving a total of 160 smallholder dairy farms with 247 lactating cows (Karimuribo et al., 2006). These studies were aimed to evaluate the effectiveness of two mastitis control practices: a single antibiotic infusion during lactation, and hypochlorite post-milking teat dipping. The result was intramammary antibiotics significantly reduced the proportion of bacteriologically positive quarters in the short-term (14 days post-infusion) but teat dipping had no detectable effect on bacteriological infection and CMT positive quarters. In the present study 30 of 165 CMT positive milk samples were having *S. agalactiae*. *S. agalactiae* an obligate parasite of the bovine mammary gland and which is susceptible to treatment with a variety of antibiotics and can be eradicated from a herd. Keefe (1997) in his review on *S. agalactiae* concluded that protocols for therapy of all infected animals in a herd were generally successful in eradicating the pathogen from the herd, especially if they are followed up with good udder hygiene techniques.

In conclusion the major causes of sub-clinical mastitis in smallholder dairy farms at Hawassa, Ethiopia were *S. aureus* and *S. agalactiae*; *S. aureus* being the dominant pathogen. This may be related to the absence of a lack of control measures for contagious mastitis pathogens observed in this study.

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Full Length Research Paper

Microbial dynamics as influenced by concentrate manure and inorganic fertilizer in alluvium soil of Varanasi, India

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A field experiment was conducted during rabi season, 2009-2010 at Agricultural Research Farm, BHU, Varanasi, on alluvial soils to determine the effects of concentrate organic manure (wellgrow formulations) with levels of inorganic fertilizers. The results reveal that the higher soil microbial population was seen with the application of 100% NPK + 300 kg wellgrow soil ha^{-1}. Soil enzymes varied with the production systems. The urease, phosphatase and dehydrogenase activities were higher in wellgrow dose application with recommended dose of NPK. The soil enzymes and microbial population (bacteria, fungi and actinomycetes) were very responsive to organic manure application, but their levels and activities were not reflected in wheat crop under alluvial soils. Enzymatic activities were positively and significantly correlated with content of organic carbon.

Key words: Dehydrogenase, phosphatase, urease, bacteria, fungi, actinomycetes, wellgrow.

INTRODUCTION

The role that microbial activity play in ecosystem processes is significant because approximately 80 to 90% of soil processes are mediated by microorganisms (Nannipieri and Badalucco, 2003). Soil microbial population are the driving force that regulate soil processes such as organic matter decomposition and nutrient cycling, it is imperative to have a better understanding of the factors that regulate its size, activity and structure (Masto et al., 2006).

Soils containing a high microbial diversity are characteristic of a healthy soil-plant relationship, whereas those with low microbial diversity are characterized as an unhealthy soil that often hardly responds to environmental changes (Tejada et al., 2011). Soil enzymatic activities can be used as an index of soil fertility and microbial functional diversity (Nannipieri et al., 2002; Maurya et al., 2011) in catalyzing several biochemical reactions which are necessary for the life processes of soil micro-organisms, organic wastes decomposition, organic matter formation and nutrients cycling (Tabatabai, 1994).

The microbial population dynamics is governed by interactions between plant type, climate and management practices. In addition, the soil microbial biomass in soil system responds more quickly to management practices than organic matter and is often used as an indi-

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cator of soil quality and health (Ge et al., 2010). The addition of organics and management practices greatly influence the microbial populations which is expected to cause changes in the soil enzymatic activities.

Incorporation of organic manures influenced soil enzymatic activity either because of the composition of the added materials themselves or because they increased micro-bial activity of the soil (DeForest et al., 2012). Improve-ment of the soil structure due to FYM application leads to a better environment for root development (Dejene and Lemlem, 2012) . FYM also improves soil water holding capacity (Tadesse et al., 2013). The fact that the use of organic fertilizers maintains soil health has raised interests in organic farming (Khan et al., 2010). The microbiological and biochemical conditions of a soil can serve as a marker of the soil status and is closely linked to its natural soil fertility.

Addition of the organic fraction stimulates the natural soil micro organisms and reac-tivates the biogeochemical cycles (Watts et al., 2010). Microbial population can also be increased through rhizosphere inoculation of bio-agents. Soil enzymatic activity is responsible for farming stable organic molecules that contributes to the per-manence of the soil ecosystem. Urease and phosphatase are two important enzymes involved in the N and P cycles, respectively (Badiane et al., 2001). Combined use of organic manures improved the microbial load of the soil rather than single organic manure application (Krishnakumar et al., 2005). The objective of this study was to evaluate the microbial population and enzymatic activities as influenced by manure and inorganic fertilizer in alluvium soil.

MATERIALS AND METHODS

Wellgrow is a plant product formulation in grain and in powder forms produced by an Indian Tobacco Company (ITC). In the case of wellgrow soil (certified organic input), organic manure (powder) from plant products with better nutritional value from non-timber forest product enhances efficiency of nitrogenous fertilizers and acts as a good nutritional media for the growth of bio-fertilizers and bio-pesticides to increase their performance (Table 1). Wellgrow grain is pelleted organic manure. It is also a certified organic input from plant products originating from non-timber forest product enhancing efficiency of nitrogenous fertilizers, improving soil fertility and crop vigor, especially targeted towards cereal crop (Meena et al., 2013)

Site description and field experiment

This study was conducted at the Agricultural Research Farm, Institute of Agricultural Sciences, BHU, Varanasi (25° 18′ N latitude, 83° 03′ E longitude and 128.93 m above MSL) The weekly mean maximum and minimum temperature during the experimentation ranged from 15.1 to 42.3°C and 7.1 to 29.7°C, respectively. Soil samples were collected from the experimental field and analyzed for phyco-chemical and biological properties. Some of the initial soil properties (0 to 15 cm) are present in Table 2.

Experimental design and treatments

The field experiment was laid out in a randomized block design with three replications having a plot size of 4 x 3.35 m^2 experiment consisting of nine treatments of wellgrow formulations and different levels of recommended dose of fertilizers (120:60:60 kg ha^{-1}); viz. (i) 100% NPK (e.g. nitrogen, phosphorous and potassium) (control), (ii) 50% NPK + 300 kg wellgrow soil ha^{-1}, (iii) 50% NPK + 300 kg wellgrow grain ha^{-1}, (iv) 75% NPK + 200 kg wellgrow soil ha^{-1}, (v) 75% NPK + 200 kg wellgrow grain ha^{-1}, (vi) 100% NPK + 200 kg wellgrow soil ha^{-1}, (vii) 100% NPK + 200 kg wellgrow grain ha^{-1}, (viii) 100% NPK + 300 kg wellgrow soil ha^{-1} and (ix) 100% NPK + 300 kg wellgrow grain ha^{-1}. Recommended doses of phosphorous and potassium was applied as basal doses before sowing of wheat through di-ammonium phosphate and muriate of potash. Nitrogen was applied through urea in three equal splits at basal, tillering and flowering stages of wheat. Wheat variety HUW-234 used as a test crop.

Soil sampling for chemical and microbial analysis and preparation

Initial soil samples were collected in August 2009 prior to the start of the experiment. After harvesting of rice, soil samples were taken from the surface layer (0 to 15 cm) of nine treatments with three replications, second soil sampling at the time of flowering stage of wheat and third soil sampling was done after the harvest of wheat crop in May, 2010. The moist soil samples were sieved (2 mm) after removing plant material and roots. Half of the soil samples were air-dried and stored at room temperature until chemical analysis. All chemical results are means of triplicate analyses and are expressed on an oven-dry basis. The rest of the sieved soil (2 mm) was immediately transferred to the laboratory for microbiological analysis. Soil samples were kept at 4°C in plastic bags for a few days to stabilize the microbiological activity disturbed during soil sampling and handling, and then analysed.

Microbiological analyses

Total bacteria, fungi and actinomycetes were estimated by the serial dilution and plating technique as described by Rolf and Bakken (1987). Total bacterial counts specific medium [1.0 g Di-potassium phosphate (K_2HPO_4), 0.2 g magnesium sulfate ($MgSO_4$), 0.1 g calcium chloride ($CaCl_2$), 0.1 g sodium chloride (NaCl), trace amount of ferric chloride ($FeCl_3$), 0.5 g potassium nitrate (KNO_3), 1.0 g aspargine, 0.1 g mannitol, 15 g agar-agar and 1000 mL distilled water], actinomycetes specific medium [1.0 g Dextrose, 0.10 g Di-potassium phosphate (K_2HPO_4), 0.10 g sodium nitrate, 0.10 g magnesium sulfate ($MgSO_4$), 15 g agar and 1000 mL distilled water] and fungal specific medium [10 g glucose, 5.0 g peptone, 1.0 g di-potassium phosphate (K_2HPO_4), 0.5 g magne-sium sulfate ($MgSO_4$), 15 g agar and 1000 mL distilled water], streptomycin @ 30 mg mL^{-1} was added to melted medium after it has cooled to 50°C, then prepared and plated on the glass Petri dish. 0.1 mL from 10^1 to 10^5 dilutions was poured on the surface of Petri dish with the help of a micropipette and was evenly spread with the help of a sterile spreader and incubated at 30±2°C. Colony count was carried out daily up to 10 days.

Soil dehydrogenase activity was estimated by reducing

Table 1. Characteristics of wellgrow soil and grain.

Parameter	Wellgrow soil	Wellgrow grain
	Range	
Organic carbon (%)	20-25	18-20
Total nitrogen (%)	1.6 -2.6	1.3 -1.4
Phosphorus (as P_2O_5) (%)	0.25-1.2	1.1-1.2
Potash (as K_2O) (%)	0.89-1.47	1.3-1.4
C/N ratio	10-16:1	13-15:1
Colour	Brown	Black
Moisture (%)	9 -10	8.2-8.4

2,3,5- triphenyltetrazolium chloride (Casida et al., 1964). Five grams of soil sample were mixed with 50 mg of $CaCO_3$ and 1 mL of 3% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC) and incubated for 24 h at 37 ± 1°C. Dehydrogenase enzyme converts TTC to 2,3,5-triphenylformazan (TPF). The TPF formed was extracted with acetone (3 × 15 mL), the extracts were filtered through Whatman No. 42 and absorption was measured at 485 nm with a spectrophotometer (Analytik Jena, Germany).

Urease activity was measured following the method of Tabatabai and Bremner (1969). Five grams of soil were incubated with 5 mL of 0.05 M THAM buffer (pH 9.0) and 1 mL of 0.2% of urea solution at 37 ± 1°C for 2 h. Excess urea was extracted with KCl-PMA solution and estimated colorimetrically at 527 nm.

Alkaline phosphatase activity was assayed using 1 g of soil (wet equivalent), 4 mL of 0.1 M modified universal buffer (pH 11 for alkaline phosphatase) and 1 mL of 25 m M p-nitrophenyl phosphate (Tabatabai and Bremner, 1969). After incubation for 1 h at 37 ± 1°C, the enzyme reaction was stopped by adding 4 mL of 0.5 M NaOH and 1 mL of 0.5 M $CaCl_2$ to prevent dispersion of humic substances. After centrifugation at 4000 rpm for 10 min, the absorbance was measured in the supernatant at 400 nm; enzyme activity was expressed as mg p-nitrophenol g^{-1} soil h^{-1}.

Statistical analysis

Data were assessed by Duncan's multiple range tests (Duncan, 1955) with a probability, P = 0.05. Least significant difference (LSD) between the mean values was evaluated by a one-way analysis of variance by using SPSS version 10.0.

RESULTS AND DISCUSSION

Microbial population of bacteria, fungi and actinomycetes

Table 3 indicates that in the experiment, the microbial population was significantly increased in all the treatments. In the data of bacterial population before sowing of wheat crop, maximum bacterial population (58×10^5 g^{-1} soil) was observed with 100% NPK with + 200 kg wellgrow grain ha^{-1}, it may be due to the residual effect of rice crop at flowering stage of wheat, bacterial population was significantly influenced by wellgrow formulation with doses of inorganic fertilization and significant increase in population of bacteria over all other treatments (viz. control, 50, 75% NPK with wellgrow levels). Maximum population of bacteria was recorded with 100% NPK + 300 kg wellgrow grain/soil ha^{-1}. At harvest, lower bacterial population was observed in comparison with flowering stage of wheat, but at harvest, the maximum bacterial population was also observed in T_9 (68×10^5 g^{-1} soil) treatment (Table 3). This treatment significantly increased over all the treatments except T_8 ($66 \times 10^5 g^{-1}$ soil), at harvest decreasing bacterial population which was due to decrease in organic carbon. This finding is in accordance with the finding of Watts et al. (2010). This clearly revealed that organic material significantly increases the bacterial population. The soil microbial population in 100% NPK +300 kg wellgrow grain ha^{-1}, soil microbial biomass has been used as an index of soil fertility which depends on nutrient fluxes (Krishnakumar et al., 2005; Meena et al., 2013).

Fungi population increased with advancement of growth stages of crop with all treatments. Before sowing of wheat crop maximum fungal population was observed (50×10^4 g^{-1} soils). At flowering stage of wheat, highest population of fungi was registered (70×10^4 g^{-1} soil) with 100% NPK + 300 kg wellgrow soil ha^{-1} followed by 100% NPK + 300 kg wellgrow grain ha^{-1} (67×10^4 g^{-1} soil) (Table 3). Maximum fungal population was observed at flowering stage as compared to at harvest of wheat. Application of 100% NPK + 300 kg wellgrow soil ha^{-1} significantly increased in terms of percent over control which was 33%. At harvest, fungal population was maximum in T_9 (58×10^4 g^{-1} soil) which showed significant superiority over all the treatments and at par with T_8 (100% NPK + 300 kg wellgrow soil ha^{-1}). Fungal population decreased at harvest due to lack of availability of nutrients and organic matter as compared to flowering stage of wheat. Fungi population increased with advancement growth stages of crop. It might be possible that increased total root biomass with the passage of time, might be instrumental to supporting higher fungi population. Similar results have been recently described in a study on wheat, in which total bacteria and fungi were evaluated (Vujanovic et al., 2012; Nedunchezhiyan et al., 2013).

Actinomycetes population varied significantly with application of concentrate manure. Before sowing of crop, highest population of actinomycetes was registered with 100% NPK + 200 kg wellgrow grain ha^{-1} (57×10^4 g^{-1} soil), this treatment is at par with (T_8 and T_9). This finding is in accordance with the findings of Zak et al. (2011). At flowering stage of wheat, significantly superior actinomycetes population was recorded with 100% NPK + 300 kg wellgrow soil ha^{-1}, followed by treatment having 100% NPK+ 200 kg wellgrow grain/soil formulations at harvest of crop population of actinomycetes decreased, significant maximum actinomycetes population was registered

Table 2. Initial soil Biochemical properties of experimental site (0-15 cm).

Properties	Value	Properties	Value
pH (soil:water, 1:2.5)	7.40	Bacteria (cfu×10^5 g^{-1} soil)	27
EC (dS m^{-1})	0.27	Fungi (cfu×10^4 g^{-1} soil)	20
Organic C (g kg^{-1} soil)	2.4	Actinomycetes (cfu×10^4 g^{-1} soil)	16
Available nitrogen (mg kg^{-1} soil)	88	Dehydrogenase (µg TPF g^{-1} soil day^{-1})	59
Available phosphorous (mg kg^{-1} soil)	5.65	Urease (µg UH g^{-1} soil h^{-1})	206
Available potassium (mg kg^{-1} soil)	57	Alkaline Phosphatase (µg PNP g^{-1} soil h^{-1})	37

Table 3. Soil microbial population (bacteria, fungi and actinomycetes) at different growth stages (cfu g^{-1} of soil) of wheat as influenced by concentrate manure and inorganic fertilizers.

Treatment	Bacteria (cfu×10^5 g^{-1} soil)			Mean	Fungi (cfu×10^4 g^{-1} soil)			Mean	Actinomycetes (cfu×10^4 g^{-1} soil)			Mean
	S_1	S_2	S_3		S_1	S_2	S_3		S_1	S_2	S_3	
T1	42e	60^{c}	51^{f}	51	35^{d}	47^{d}	36^{e}	39	37f	48^{e}	44^{fg}	43
T2	46d	62^{bc}	53^{ef}	54	37^{cd}	49^{d}	40^{d}	42	39e	54^{cde}	47^{ef}	47
T3	45^{d}	66^{abc}	56^{de}	56	36^{d}	49^{d}	35^{e}	40	37^{ef}	50^{de}	43^{g}	43
T4	48^{cd}	66^{abc}	54^{ef}	56	38^{cd}	52^{d}	41^{d}	44	43^{d}	55^{cde}	49^{de}	49
T5	49^{c}	72^{ab}	59^{c}	60	39^{c}	53^{d}	44^{c}	45	47^{c}	59^{bcd}	52^{cd}	53
T6	53^{b}	74^{ab}	64^{b}	64	44^{b}	60^{c}	48^{b}	51	50^{a}	62^{abc}	57^{b}	56
T7	58^{a}	70^{abc}	59^{bcd}	62	48^{a}	63^{bc}	50^{b}	54	57^{a}	63^{abc}	55^{bc}	58
T8	56^{a}	76^{a}	66^{ab}	66	47^{a}	70^{a}	56^{a}	58	56^{a}	70^{a}	62^{a}	63
T9	57^{a}	76^{a}	68^{a}	67	50^{a}	67^{ab}	58^{a}	58	56^{a}	67^{ab}	62^{a}	62
Mean	50	69	59	-	42	57	45		47	59	52	
LSD (P=0.05)	2.8	10.90	3.14		3.0	6.31	3.07		3.2	9.17	3.50	

T_1: 100% NPK (120:60:60 kg ha^{-1}), T_2: 50% NPK + 300 kg wellgrow soil ha^{-1}, T_3: 50% NPK + 300 kg wellgrow grain ha^{-1}, T_4: 75% NPK + 200 kg wellgrow soil ha^{-1},T_5: 75% NPK + 200 kg wellgrow grain ha^{-1}, T_6: 100% NPK + 200 kg wellgrow soil ha^{-1}, T_7: 100% NPK + 200 kg wellgrow grain ha^{-1}, T_8: 100% NPK + 300 kg wellgrow soil ha^{-1}, T_9: 100% NPK + 300 kg wellgrow grain ha^{-1}. S_1: Before sowing, S_2: flowering stage, S_3: after harvest.

registered (62 × 10^4 g^{-1} soil) with 100% NPK + 300 kg wellgrow grain/soil formulations. This is consistent with the finding of Bohme et al. (2005) who reported that microbial biomass was greater in soil after the application of farmyard manure.

Soil enzymatic activities

Soil enzyme activity is an indirect indication of the activities of microbes which is directly correlated with soil microbial dynamics. Enzyme activity in the soil environment is considered to be a major contributor of overall soil microbial activity (Burns et al., 2013).

In the present investigation, significantly increased enzyme activity of urease, dehydrogenase and alkaline phosphatase was noticed due to application of wellgrow soil, wellgrow grain and nutrient levels (Table 4). Due to the effects of external disturbance on their activity, enzymes can serve as sensitive indicators of soil quality (Dick et al., 1994; Nedunchezhiyan et al., 2013; Meena et al., 2013).

Urease activity

Urease is an important enzyme responsible for the hydrolysis of urea fertilizer applied to the soil, NH_3 and CO_2 with the concomitant rise in soil pH (Byrnes and Amberger, 1989). Before sowing of wheat crop, maximum urease activates registered 100% NPK + 200 kg wellgrow grain ha^{-1} (320 µg UH g^{-1} soil hr^{-1}), this treatment was followed by 100% NPK + 300 kg wellgrow soil ha^{-1} and 100% NPK + 300 kg wellgrow grain ha^{-1} (306 and 316 µg UH g^{-1} soil hr^{-1}, respectively). At flowering stage of wheat, highest urease activities was registered with 100% NPK + 300 kg wellgrow soil (327 µg UH g^{-1} soil hr^{-1}) followed by 100% NPK + 300 kg wellgrow grain ha^{-1} (324 µg UH g^{-1} soil hr^{-1}). This could be attributed to their higher N content and faster decomposition and release

Table 4. Soil enzymes activities [urease (UA) dehydrogenase (DHA) and alkaline phosphatase (APA)] at different growth stages of wheat in the study soils as influenced by concentrate manure and inorganic fertilizers.

Treatment	UA (µg UH g^{-1}soil h-^{1})			Mean	DHA (µg TPF g^{-1} soil 24 h^{1})			Mean	APA (µg p-NP g^{-1} soil h^{-1})			Mean
	S_1	S_2	S_3		S_1	S_2	S_3		S_1	S_2	S_3	
T1	213^{d}	257^{e}	224^{e}	231	191^{f}	151^{e}	126^{c}	156	26^{f}	46e	35^{e}	36
T2	243^{cd}	266^{d}	237^{d}	249	124^{e}	154^{e}	139^{c}	139	39^{e}	53de	43^{d}	45
T3	251^{c}	269^{cd}	237^{d}	252	118^{e}	172^{d}	140^{c}	143	38^{e}	55cd	46^{cd}	46
T4	271^{bc}	275^{c}	245^{c}	264	136^{d}	178^{d}	147^{c}	154	49^{d}	62c	48^{c}	53
T5	274^{bc}	275^{c}	247^{c}	265	143^{cd}	218^{c}	146^{c}	169	54^{d}	59cd	50^{c}	54
T6	289^{ab}	290^{b}	256^{b}	278	162^{bc}	285^{b}	215^{b}	221	64^{c}	69b	57^{b}	63
T7	320^{a}	296^{b}	257^{b}	291	157^{a}	291^{b}	225^{b}	224	86^{a}	72b	57^{b}	72
T8	306^{a}	327a	264^{a}	299	159^{ab}	295^{b}	252^{a}	235	77^{b}	84a	70^{a}	77
T9	316^{a}	324^{a}	261^{ab}	300	138^{ab}	313^{a}	225^{b}	225	82^{ab}	83a	70^{a}	78
Mean	276	287	248	-	148	229	179	-	57	65	53	-
LSD (P=0.05)	30.0	6.39	5.22		8.6	13.84	22.50		7.2	7.03	4.50	

T_1: 100% NPK (120:60:60 kg ha^{-1}), T_2: 50% NPK + 300 kg wellgrow soil ha^{-1}, T_3: 50% NPK + 300 kg wellgrow grain ha^{-1}, T_4: 75% NPK + 200 kg wellgrow soil ha^{-1},T_5: 75% NPK + 200 kg wellgrow grain ha^{-1}, T_6: 100% NPK + 200 kg wellgrow soil ha^{-1}, T_7: 100% NPK + 200 kg wellgrow grain ha^{-1}, T_8: 100% NPK + 300 kg wellgrow soil ha^{-1}, T_9: 100% NPK + 300 kg wellgrow grain ha^{-1}. S_1: Before sowing, S_2: flowering stage; S_3 after harvest.

Table 5. Correlations of organic carbon with enzyme activities in wheat at flowering and harvest of crop as influenced by concentrate organic manure and inorganic fertilization.

Soil properties		Urease		Dehydrogenase		Phosphatase		Organic carbon	
		S2	S3	S2	S3	S2	S3	S2	S3
Urease	S_2	-	0.890**	0.893**	0.913**	0.932**	0.952**	0.511**	0.901**
	S_3		-	0.910**	0.881**	0.900**	0.925**	0.423*	0.859**
Dehydrogenase	S_2			-	0.932**	0.895**	0.902**	0.464*	0.873**
	S_3				-	0.889**	0.875**	0.447*	0.825**
Phosphatase	S_2					-	0.931**	0.635**	0.919**
	S_3						-	0.496**	0.918**
Organic carbon	S_2							-	0.561**
	S_3								-

S_2: Flowering stage, S_3 after harvest; **Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).

of NH_4-N (Saha et al., 2008; Meena *et al.,* 2013)

Soil enzymes regulate the transformation process of elements required for plant growth in soil (Burns, 1982). Crop growth stages also influenced the urease activity. Under field conditions, urease activity was highest at flowering stage but under greenhouse conditions, the activity was more pronounced at tillering stages (Watts et al., 2010).

At harvest, lower urease activity in comparison with at flowering stage of wheat, maximum urease activity was registered (264 µg UH g^{-1} soil hr^{-1}) with treatment 100% NPK + 300 kg wellgrow soil ha^{-1} and at par with 100% NPK + 300 kg wellgrow grain soil ha^{-1} (261 µg UH g^{-1} soil hr^{-1}), the lower activity of urease at harvest of crop could be related to lower microbial biomass and decreasing content of soil organic carbon. The correlation between urease and organic carbon at flowering and at harvest stages (r = 0.511 and r = 0.901, respectively) were positively significant (Table 5). Maestre et al. (2011) reported a decrease in the urease activity with addition of

inorganic N whereas crop residues and organic manure additions increased it. Enzyme activities of soils are usually correlated with their organic carbon and available N contents (Ndubuisi-Nnaji et al., 2011). Higher levels oforganic carbon stimulate microbial activity, and therefore enzyme synthesis.

Dehydrogenase activity

Dehydrogenase is an enzyme that occurs in all intact viable microbial cells. These soil enzymes function as a measurement of the metabolic state of soil microorganisms by relating it to the presence of viable microorganisms and their oxidative capacity. Therefore, dehydrogenase can be used as a measure of microbial respiration and a reliable index of microbial activity in soil (Tejada et al., 2011). Data on dehydrogenase activities before sowing of crop maximum observed with 100% NPK + 300 kg wellgrow grain ha^{-1} (188 µg TPF g^{-1} soil day^{-1}) followed by T_8 and T_7 may be due to higher organic matter content and relatively higher organic carbon (Wlodarczyk et al., 2002). Similar trend was also observed in flowering stage of wheat and maximum dehydrogenase activities was registered (313 µg TPF g^{-1} soil day^{-1}) with 100% NPK + 300 kg wellgrow grain ha^{-1} at flowering stage of wheat. Crop growth stage also greatly impacted dehydrogenase activity. Dehydrogenase activity measured during flowering stage was almost double that measured before sowing of the crop (Table 4). The higher dehydrogenase activity after addition of concentrate manure could be due to increased microbial activity, which is known to stimulate the dehydrogenase activity (Watts et al., 2010). At harvest of wheat, lower urease activity was seen in comparison with at flowering stage of crop, highest dehydrogenase activity was registered as 252 µg TPF g^{-1} soil day^{-1} with treatment 100% NPK + 300 kg wellgrow soil ha^{-1} and it is at par with T_9, T_7 and T_6 (225, 225 and 215 µg TPF g^{-1} soil day^{-1} respectively), the increase in activity during the flowering stage compared well with harvest stage, suggesting that greater microbial biomass occurred with a change in growth stage. These results suggest that changes in the size of microbial populations and respiratory activity occurred in response to the increase in available substrate. In addition, an increase in available substrate corresponds to more readily available C and N pools, which were most likely disproportionally enhanced as a result of manure addition.

This was confirmed by the significant positive correlation between dehydrogenase and organic carbon at flowering and harvest (r = 0.464, 0.873, respectively) (Table 5). In the present study, lowest dehydrogenase activity measured after harvest can be attributed to oxidation status of the soil as water was drained at maturity.

Alkaline phosphatase activity

Alkaline phosphatase is an enzyme of great agronomic value because it hydrolyses compounds of organic phosphorus and transforms them into different forms of inorganic phosphorus that are assimilated by plants (Maestre et al., 2011). Data on alkaline phosphatase tend to be lowest in the control treatment before sowing, flowering and at harvest of crop (Table 4), and highest (86 µg p-NP g^{-1} soil h^{-1}) with the application of 100% NPK + 200 kg wellgrow grain ha^{-1} before sowing of crop. At flowering stage, maximum alkaline phosphatase activates (83 µg p-NP g^{-1} soil h^{-1}) 100% NPK + 300 kg wellgrow grain ha^{-1}. Sriramachandrasekharan and Ravichandran (2011) reported that the addition of organic substances to the soil served as a carbon source that enhanced microbial biomass and phosphatase activity, showing that these enzymes are of microbiological origin and crop growth stage also significantly influenced soil enzyme activities (Bohem et al., 2005).

This hypothesis is also supported by the alkaline phosphatase activity and organic carbon highly significant and positively correlated at flowering and harvest stages (r = 0.635 and r = 0.919, respectively), at the 0.01 level (2-tailed). The importance of organic carbon in nutrient cycling was evident, the enzyme activity quantified in the present study showed positive correlation with organic carbon. This indicates that organic material significantly increases the enzymatic activity in soil (Table 5). Several studies have observed inverse relationships between inorganic P availability and phosphatase activity although this depends on initial bio-available P (DeForest et al., 2012).

Conclusions

Integrated use of organic manure (wellgrow) and inorganic fertilizers improved the enzymatic activities as well as microbial population of bacterial, fungal and actinomycetes with the best application at 100% NPK + 300 kg wellgrow grain/soil ha^{-1}. Decomposition of organic matter and recycling of carbon have substantial effect on the activity of enzyme evolved in mineralization of nutrients. Soil enzymes significantly contribute to soil health. The activities of urease, dehydrogenase and phosphatase were significantly influenced by the crop growth stages. Enzymatic activity increased up to flowering stage of wheat and declined thereafter. Hence judicious application of 100% NPK + 300 kg wellgrow grain/soil ha^{-1} emerged as the best treatment for both flowering and harvest stage for enzymatic activity as well as microbial population. Manure together with reduced

dose of nutrient levels not only improved crop growth but also significantly buildup nutrient in the soil; it also maintained a balanced enzymatic activity with a lesser pollution potential then high dose of nutritional levels.

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Full Length Research Paper

Incidence of keratinophilic fungi in areas of Raipur City, Chhattisgarh region, India

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In an endeavor to search for some wild isolates of keratinophilic fungi from the Raipur City of Chhattisgarh, 24 soil samples from different regions of the city were collected and screened using hair baiting technique. A total of six species which belong to two genera were isolated. They are *Chrysosporium keratinophilum* (27.78%), *Chrysosporium pannicola* (5.56%), *Chrysosporium tropicum* (13.89%), *Chrysosporium* sp. (close to *Chrysosporium pannicola*) (11.11%), *Chrysosporium* sp. (16.67%) and *Microsporum gypseum* (25%). The *Chrysosporium* species prevalent in this region are able to tolerate temperature of about 40°C and all isolates belong to moderate individual risk group.

Key words: Keratinophilic fungi, Raipur, *Chrysosporium*, *Microsporum*.

INTRODUCTION

Soil is the main reservoir of different types of fungi and some of them are pathogenic to both humans and animals. Soil is also a good source of keratinophilic fungi and the probability of the incidence of such fungi increases manifold if it is rich in keratinous materials (Marchisio, 2000). Keratinophilic fungi, also known as dermatophytes, are mostly pathogens of humans and other animals but also have the ability to live saprotrophically. Every keratinophilic fungus has the potential to cause infection and tissue invasions (Simpanya, 2000).

In recent past, many investigations have been carried out to find the distribution pattern of the keratinophilic fungi in many parts of India (Jain and Sharma, 2011; Singh et al., 2009; Sharma et al., 2008; Anbu et al., 2004; Deshmukh, 2002, 2004; Ghosh and Bhatt, 2000; Kaul and Sumbali, 1997). Barring a few reports, this area of Chhattisgarh remained unexplored for the occurrence of keratinophilic fungi (Khanam et al., 2002). Hence, it was felt significant to screen soil of different areas of Raipur City, in order to evaluate the extent and presence of keratinophilic fungi in the environments. The study was undertaken at two potential sites viz. poultry farm and hair-dumping/garbage area and the results obtained are reported.

MATERIALS AND METHODS

Soil samples were collected from the superficial layer at a depth of 3-6 cm of four poultry farms and six hair-dumping/garbage sites from different areas in and around Raipur city. The soil samples were placed in sterile polythene bags, brought to the laboratory and stored overnight at 4°C. Approximately 18 to 20 g of soil from each sample were placed in 90 mm sterile Petri plates in five replicates. Short (1-2 cm length) sterilized defatted human hair fragments were scattered on the surface of soil for baiting. The plates were moistened with an antibiotic solution containing cycloheximide (0.5 mg/ml) and chloramphenicol (0.05 mg/ml). The plates were incubated at room temperature (26±2°C) for a period of 4 to 6 weeks and remoistened with sterile deionized water periodically. The plates were examined daily under a stereoscopic binocular microscope and if growth was observed then the baits were selected at random from each Petri plate and transferred to plates containing Sabouraud Dextrose Agar (SDA) medium supplemented with cycloheximide (0.5 mg/ml) and chloramphenicol (0.05 mg/ml). The SDA plates were incubated at room temperature (26±2°C) for

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Table 1. Distribution of soil sample examined.

Site	Name	Soil sample type		Positive		Positive (%)
		F	H/G	F	H/G	
I	Nandanvan	3	2	1	1	40
II	Krishak Nagar	4	3	1	1	42.86
III	Sarona	3	3	2	1	33.33
IV	Raipura	3	3	1	1	33.33
Overall total		13	11	5	4	37.5

F- Poultry farm; H/G- hair dumping/garbage site.

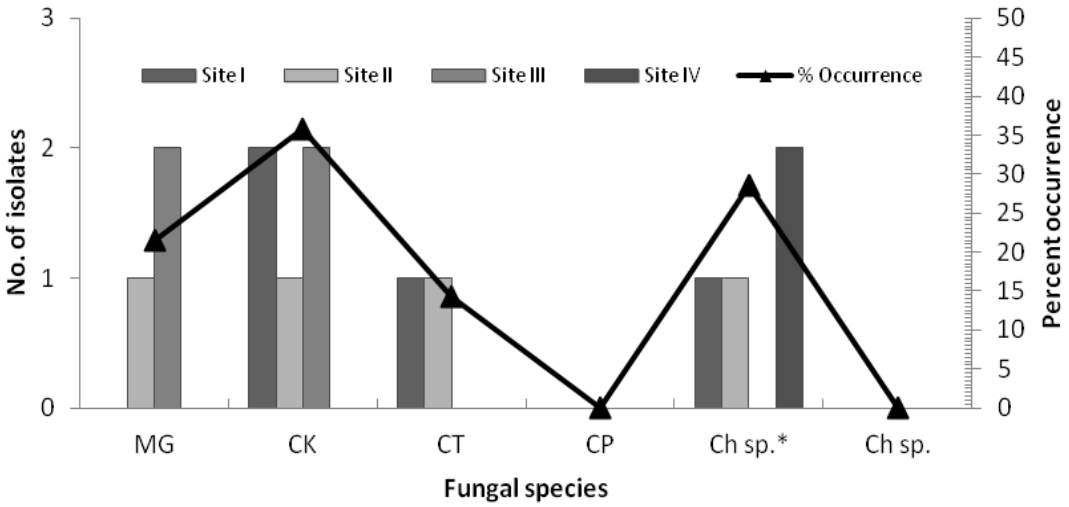


Figure 1. Distribution of fungi in poultry farm soil (MG- *M. gypseum*, CK- *C. keratinophylum*, CT- *C. tropicum*, CP- *C. pannicola,* Ch- *Chrysosporium*).

further examination.

Fresh developed colonies were examined and identified following the key proposed by Chabasse (1988), von Oorschot (1980) and Carmichael (1962). The identification of isolates was further confirmed by NFCCI (Pune) and IMTECH (Chandigarh).

RESULTS

A total of 24 soil samples were examined from poultry farm and hair-dumping/garbage sites. Out of them nine samples (37.5%) were positive for keratinophilic fungi (Table 1).

Distribution of keratinophilic fungi in poultry farm soil

Distribution of keratinophilic fungi in poultry farm soils around Raipur is presented in Figure 1. A total of 14 colonies of keratinophilic fungi were isolated from five soils samples. Four species belonging to two genera were observed in poultry farm soil. Only one dermatophyte, *Microsporum gypseum* (21.43%) was observed and among them closely related species *Chrysosporium keratinophilum, Chrysosporium tropicum* and *Chrysosporium* sp.* (close to *Chrysosporium pannicola*) were present. Occurrence of *C*. *keratinophilum* (35.71%) was highest followed by *Chrysosporium* sp.* (28.57%) and *C. tropicum* (14.29%) (Figure 1). Other than keratinophilic fungi, different species of the fungal genera viz., *Aspergillus*, *Penicillium*, *Fusarium* and *Rhizopus* were also observed.

Distribution of keratinophilic fungi in hair-dumping/garbage soils

Occurrence of keratinophilic fungi from hair-dumping/-garbage sites is given in Figure 2. Out of four soil samples examined, 22 colonies of keratinophilic fungi were isolated. *Microsporum gypseum* (21.43%) the only dermatophyte and four other closely related species of *Chrysosporium* were isolated. Most commonly observed species was *Chrysosporium* sp. (27.27%) followed by *C.*

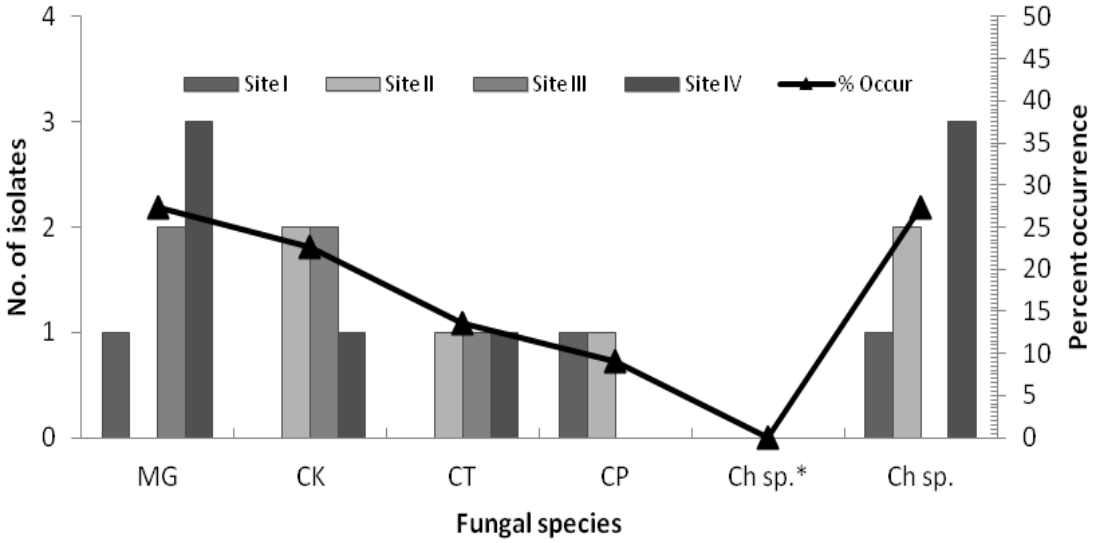


Figure 2. Distribution of fungi in hair dumping/garbage soil.

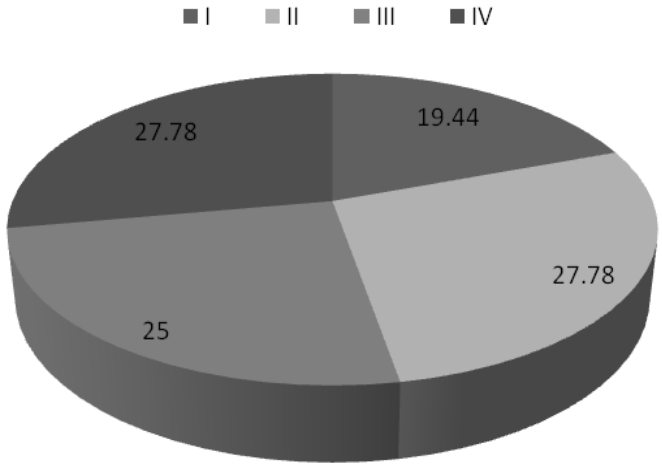


Figure 3. Site-wise percent distribution of keratinophilic fungi.

keratinophilum (22.73%), *C. tropicum* (13.64%) and *C. pannicola* (9.09%) (Figure 2). Other than keratinophilic fungi different species of the fungal genera viz. *Aspergillus*, *Penicillium* and *Fusarium* were observed.

Distribution of keratinophilic fungi at different areas

The percent distribution of keratinophilic fungi in different sites is depicted in Figure 3. Site II (27.78%) and site IV (27.78%) had the highest percentage of keratinophilic fungi, followed by sites III (25%) and I (19.44%), respectively. The species diversity was highest in sites I and II (6 sp./site, each) followed by sites IV (5 sp./site) and III (3 sp./site) respectively. Overall in this region *C. keratinophilum* (27.78%) was the most prevalent species among the areas studied followed by *M. gypseum* (25%), *Chrysosporium* sp. (16.67%), *C. tropicum* (13.89%), *Chrysosporium* sp.* (11.11%) and *C. pannicola* (5.56%) (Figure 4).

DISCUSSION

Keratinophilic fungi are very closely related to dermatophytes, having the ability to cause infection. They parasitize hard keratin tissues and can also invade and degrade them. Thus, they are keratinophilic and keratinolytic in nature. Soils enriched with keratin substrate like hairs, feathers, horn and hoofs, skin etc. are good

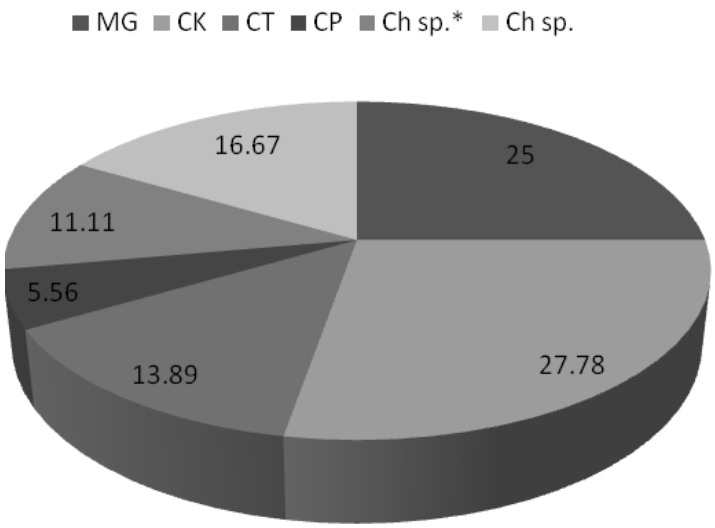


Figure 4. Percent distribution of keratinophilic fungi in areas of Raipur City.

reservoir of dermatophytes as well as keratinophilic fungi (Marchisio, 2000; Ajello, 1974). Dermatophytes are classified into three genera namely *Epidermophyton*, *Microsporum* and *Trichophyton* (Summerbell, 2000). Other than this, closely related species includes 100 genera like *Chrysosporium*, *Malbranchea*, *Geomyces*, *Scopulariopsis*, *Doratomyces* and many others having keratinolytic ability (Blyskal, 2009). Kushwaha (2000) reviewed the presence of 47 species, a few of them with uncertain positions, among the genus *Chrysosporium* throughout the globe.

In the present study, the only dermatophyte isolated was *M. gypseum* (25%), the most common species reported amongst the dermatophytes. It is one of the most prevalent soil borne species and a major causative agent of infection to human and animals (Simpanya, 2000). Among the closely related species the occurrence of *C. keratinophilum* (27.78%), *C. tropicum* (13.89%) and *C. pannicola* (5.56%) were noteworthy in this region. These species are thermotolerant and can grow considerably at 35 to 40°C; the exceptional one is *C. tropicum* which can withstand temperature upto 45°C (Ellis et al., 2007; van Oorschot, 1980). The high occurrence of keratinophilic fungi especially *Chrysosporium* sp. in the soil of Raipur region support the fact that these species are able to withstand the high temperature of this region which is about 45 ± 2°C in summer.

According to World Health Organization (Laboratory Biosafety Manual, 2004) infective microorganisms are classified into four risk groups viz. RG - 1 to 4. It is based on pathogenicity of the organism, modes of transmission and host range of the organism. RG-1 having no or low individual and community risk whereas RG-2 have the moderate individual risk and low community risk. RG-3 shows high individual risk but low community risk and RG-4 have high individual and community risk. Following the classification criteria of WHO, Australia, Canada, European Union (EU), USA and other nations has placed *Microsporum* sp. in RG-2 (American Biological Safety Association, 2013).

Other than *M. gypseum*, all isolated *Chrysosporium* sp. were geophilic and keratinolytic, often recovered from skin and nail scrapings of feet. Some of the *Chrysosporium* sp. closely resembles true deramatophytes like *Trichophyton* and *Microsporum* (van Oorschot, 1980). Based on pathogenicity, *Chrysosporium* sp. has been placed in RG-2 (Table 2) (Ellis et al., 2007).

The prevalence of fungal infections in the region as reported routinely in district hospital and Medical College of Raipur showed that 66.7% infective patients belong to urban areas and 33.3% from rural areas. Superficial fungal infection by various species of *Trichophyton* was reported to be highest (89%) followed by *Microsporum* sp. (11%). The reported disease types most prevalent in this area are tinea corporis (44.5%) followed by tinea pedis (22.3%).

Among other types of tineas, tinea capitis, tinea cruris and tinea manuum have also been reported occasionally in this region (Pradhan, 2012). In any region, tinea corporis and tinea pedis are the most common types of infection and all types of dermatophytes have the potential to cause tinea corporis. Tinea pedis is possibly caused by *Trichophyton rubrum*, *Trichophyton mentagrophytes* var. *interdigitale* and *Epidermophyton floccosum* (Degreef, 2008). Apart from these anthropophilic dermatophytes mentioned above the present study revealed that this part of India has good presence of geophilic and keratinophilic *Chrysosporium* and *Microsporium* species, which could add up to the increase in dermatophytic infection in this region.

Table 2. Species wise characteristics and pathogenicity.

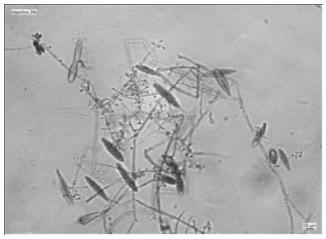
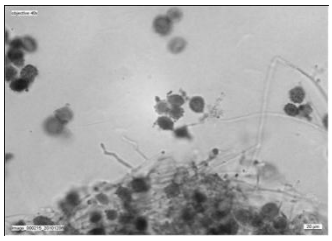
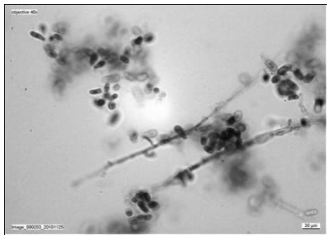
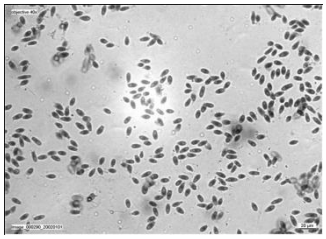
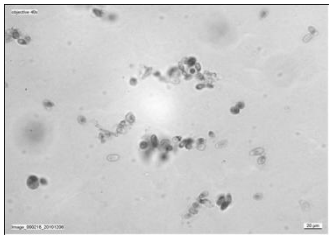
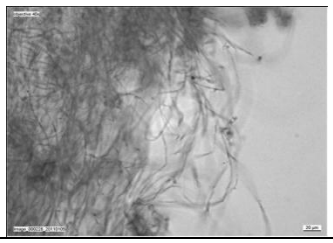
S/N	Species	Brief characteristics & Pathogenicity
1.	*Microsporum gypseum* 	Geophilic; Macroconidia abundant, symmetrical, thin-walled, ellipsoidal, verrucose, four- to six-celled, clavate-shaped Microconidia also present RG-2 organism
2.	*Chrysosporium keratinophilum* 	Geophilic; Conidia numerous, single-celled, smooth-walled or echinate, thick-walled, obovoid to clavate shaped RG-2 organism
3.	*Chrysosporium pannicola* 	Geophilic; Sub-hyaline, single-celled, fairly thick-walled, obovoid to clavate, initially smooth-walled, chinulate at maturity; Chlamydospores absent. RG-2 organism
4.	*Chrysosporium tropicum* 	Geophilic; Numerous conidia, hyaline, single-celled, slightly thick-walled, clavate to pyriform, smooth surface; Macroconidia absent RG-2 organism
5.	*Chrysosporium* sp. (close to *C. pannicola*) 	Geophilic; RG-2 organism

Table 2. Contd.

	Chrysosporium sp.	
6.		Geophilic; RG-2 organism

(Scale - 20 µm), Fungal photographs were taken in Leica DM 1000 microscope with microphotographic attachment.

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Full Length Research Paper

Biofilm formation by *Acinetobacter baumannii* isolated from medical devices at the intensive care unit of the University Hospital of Tlemcen (Algeria)

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***Acinetobacter baumannii* is an opportunistic pathogen responsible for nosocomial infections due to biofilm formation on the surface of implantable medical devices. Thirty (30) strains of *A. baumannii* were isolated from medical devices and tested for their ability to form a biofilm. The factors that may influence this process, such as the hydrophobicity of the bacterial wall, temperature, duration of implantation and the nature of the medical device, were also investigated. Strains were able to form a biofilm; however this process was more substantial at 30°C than at 37°C and was maximal after 96 h of incubation. Strains seem to adhere better to silicone and latex than to polyvinylchloride (PVC) and no apparent relationship was found between hydrophobicity and biofilm formation.**

Key words: *Acinetobacter baumannii*, biofilm, medical devices.

INTRODUCTION

Biofilm is a microbially derived sessile community which is characterized by cells that are irreversibly attached to a substratum or interface with each other, and embedded in a matrix of self-produced extracellular polymeric substances (Lee et al., 2008). Biofilm formation has been linked to the survival of pathogenic bacteria in the hospital environment and has been connected to infections associated with indwelling medical devices (Martí et al., 2011). The widespread use of medical devices has caused a great advance in the management of many diseases. Indwelling medical devices are being increasingly used for the treatment of functional deficits in numerous medical fields (Abd El-Baky, 2012). Despite the considerable success achieved with new material devices, these abiotic surfaces are susceptible to bacterial colonization which creates an important public health problem (Treter and Macedo, 2011). The temporary implantation of a vascular catheter, a urinary catheter or an endotracheal tube can become a site for bacterial adhesion and infection (Espinasse et al., 2010). More than 60% of hospital-acquired infections worldwide are due to bacteria forming biofilms on medical devices (Lichter et al., 2009; Treter and Macedo, 2011). The medical consequence of these devices-related infections can be life threatening and may lead to device removal. In such a situation, the management of these devices can be a difficult and costly affair (Singh et al., 2011).

Although medical devices may differ widely in design

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and use of characteristics, specific factors determine susceptibility of a device to microbial contamination and biofilm formation. For example, duration of use, flow rate and composition of the medium in or on the device, conditioning films on the device, and device material construction all may influence biofilm formation (Donlan, 2001). In fact, in vitro studies have shown that the most significant factors influencing biofilm formation on the surface of a synthetic implant are: its structure and hydrophibicity and the species of bacteria involved (von Eiff et al., 2005). Biofims on indwelling medical devices may be composed of gram positive or gram negative microorganisms (Linski et al., 2009). These micro-organisms may originate from the patient's skin or mucous membranes during implantation. Sometimes, the pathogens may also be acquired from the hands of the surgical or clinical staff (Kokare et al., 2009). *Acinetobacter baumannii* is one of the common bacteria associated with biofilms on indwelling medical devices causing bacteremia, urinary tract infections, secondary meningitis, and pneumonia (King et al., 2009; McQueary and Actis, 2011). Some of the challenges in the prevention and treatment of infections caused by this opportunistic pathogen are its remarkable widespread resistance to different antibiotics and its ability to persist in nosocomial environments (Tomaras et al., 2003). In fact *A. baumannii* can survive on fingertips and inanimate objects such as glass, plastic and other environmental surfaces, even after exposure to dry conditions (desiccation, nutrient starvation and antimicrobial treatments), during extended periods of time, and the environment can be a transmission route in some outbreaks (Tomaras et al., 2003; Gaddy and Actis, 2009; Espinal et al., 2012). The ability of *Acinetobacter* strains to adhere to surfaces is an important mechanism in the pathogenicity of these bacteria. Although the adhesion ability is determined by specific factors, such as adhesins, and non-specific factors, such as hydrophobicity and cellular surface electrical discharge, it varies among strains (Costa et al., 2006)**.**

With regard to the role of *A. baumannii* in medical device-related infections, the purpose of this paper is to study for the first time in Algeria, the ability of clinical *A. baumannii* isolated from medical devices to form biofilm. The factors influencing this process such as cell surface hydrophobicity (CSH), temperature and time of implanttation of medical devices were investigated. The nature of the medical device surface was also studied by comparing the adhesion ability of strains on three different biomaterials (silicone, latex and polyvinylchloride) used in medical device manufacturing.

MATERIALS AND METHODS

Bacterial strains

A total of 30 strains of *A. baumannii* were studied. Isolates were collected from urinary catheters, endotracheal tubes and central venous catheters in the intensive care unit of the University Hospital of Tlemcen (Algeria). The strains were identified by macroscopic, microscopic, and biochemical tests, including an oxidase test and using the API 20 NE system (bioMerieux SA, Lyon, France).The capacity of strains to grow at 41 and 44°C was also used for identification of *A. baumannii*.

Antibiotic resistance

Antimicrobial susceptibility testing was performed for 10 different therapeutically relevant antibiotics using the standard protocol for diffusion of antimicrobial agents on Mueller-Hinton agar as described in National Committee for Clinical Laboratory Standards NCCLS guidelines (CLSI, 2003). The antibiotics tested included: ticarcilin, ticarcillin/clavulanic acid, ceftazidim, imipenem, gentamicin, amikacin, tobramycin, ciprofloxacin, trimethoprim/sulfamethoxazole and colistin.

Quantification and kinetics of biofilm formation to polystyrene

The biofilm formation was performed in 96-well plates according to the procedure of O'Toole and Kolter (1998). Its growth was determined in Luria Bertoni broth using an initial OD_{600} of 0.01 and incubated at 37 and 30°C for 24 h without shaking. Two wells were left uninoculated and for use as negative controls. The biofilm was stained with 0.5% crystal violet (w/v) for 20 min at room temperature and the wells were washed to remove the unbound crystal violet. Biofilm formation was finally quantified at 570 nm after solubilisation in 95% ethanol. The bacterial isolates were considered to be positive for biofilm formation when the readings obtained were at least twice greater than the negative control. The strain *A. baumannii* (ATCC 19606) was used as a positive control.

The kinetics of Biofilm formation was performed by extending the incubation time to 6 days.

Hydrophobicity assays

The hydrophobicity of the bacterial wall was evaluated with the MATH protocol (Rosenberg, 1984) using hexadecane as a solvent. *A. baumannii* strains were grown in 50 ml of Luria Bertani (LB) and incubated for 18 h at 37°C. The cells were recuperated by centrifugation (5000 rpm for 15 min). The pellet obtained was then washed after two successive centrifugations with PBS (Phosphate Buffered Saline pH 7.1) and suspended in the same buffer at an initial optical density (OD_i) between 0.8 and 1 at 600 nm. A volume of 0.3 ml of each solvent was added to 1.8 ml of bacterial suspension and the whole is vortexed for 2 min. After 20 min settling, the optical density (OD_f) of the aqueous phase was measured at 600 nm and the percentage of adhesion to solvent was then calculated using the following equation: $CSH\% = [(OD_i - OD_f)/ OD_i] \times 100$

Adhesion to silicone, latex and polyvinylchloride

Samples of sections 1 cm^2 from each support were introduced into tubes containing 5 ml of an *A. baumannii* bacterial suspension adjusted to an OD_{600} of 0.1 and incubated at 37°C for 24 h. The supports were recuperated, thoroughly washed with sterile distilled water and placed in 5 ml of PBS (pH 7.1). Sonication was performed three times using the WiseClean WUC-D06H ultrasound for 5 min, and interrupted at regular intervals by vortexes of 20 s. A series of dilutions 1/10 to 1/100000 was performed for each sample, and plated on nutrient agar. After 24 h of incubation, the

Table 1. Distribution of strains according to the medical device, implants duration and antibiotic resistance pattern.

Strain	Medical devices	Duration of medical devices implatation (days)	Antibiotic resistance
AB1	Central venous catheter	10	TIC TCC CAZ AK TM SXT CIP
AB2	Central venous catheter	8	TIC TCC CAZ AK TM SXT CIP
AB3	Urinary catheter	6	TIC TCC CAZ GN AK TM SXT CIP
AB4	Central venous catheter	8	TIC TCC CAZ IMP GN AK SXT CIP
AB5	Central Venous catheter	10	TIC TCC CAZ GN AK SXT CIP
AB6	Endotracheal tube	17	TIC TCC CAZ IMP GN AK SXT CIP
AB7	Endotracheal tube	15	TIC TCC CAZ IMP GN AK SXT CIP
AB8	Urinary catheter	4	TIC TCC CAZ IMP GN AK SXT CIP
AB9	Urinary catheter	5	TIC TCC CAZ GN SXT
AB10	Endotracheal tube	20	TIC TCC CAZ IMP GN AK SXT CIP
AB11	Urinary catheter	5	TIC TCC CAZ IMP GN AK SXT CIP
AB12	Endotracheal tube	17	TIC TCC CAZ IMP AK SXT CIP
AB13	Central venous catheter	12	TIC TCC CAZ GN AK TM SXT CIP
AB14	Urinary catheter	6	TIC TCC CAZ IMP GN AK SXT CIP
AB15	Central venous catheter	10	TIC TCC CAZ GN AK SXT CIP
AB16	Urinary catheter	11	TIC TCC CAZ GN AK SXT CIP TM
AB17	Urinary catheter	6	TIC TCC CAZ AK SXT
AB18	Endotracheal tube	18	TIC TCC CAZ IMP AK SXT CIP
AB19	Urinary catheter	4	TIC TCC CAZ SXT CIP
AB20	Urinary catheter	6	TIC TCC CAZ GN AK SXT CIP TM
AB21	Endotracheal tube	10	TIC TCC CAZ IMP GN AK TM SXT CIP
AB22	Central venous catheter	15	TIC TCC CAZ AK GN TM SXT CIP
AB23	Endotracheal tube	21	TIC TCC CAZ IMP AK SXT CIP
AB24	Endotracheal tube	12	TIC TCC CAZ IMP GN AK SXT CIP
AB25	Urinary catheter	7	TIC TCC CAZ GN SXT
AB26	Endotracheal tube	20	TIC TCC CAZ GN AK SXT CIP
AB27	Endotracheal tube	7	TIC TCC CAZ IMP AK SXT CIP
AB28	Urinary catheter	9	TIC TCC CAZ IMP AK SXT CIP
AB29	Central venous catheter	13	TIC TCC CAZ IMP AK SXT CIP
AB30	Endotracheal tube	19	TIC TCC CAZ GN AK SXT CIP TM

AB, A. baumannii; TIC, ticarcillin; TCC, ticarcillin/clavulanic acid; CAZ, ceftazidim; IMP, imipenem, TM, tobramycin; GN, gentamycin; AK, amikacin; Cip, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole.

colonies were counted at 37°C. The experiments were carried out in triplicate.

RESULTS

Characterization of the strains

An endotracheal tube, urinary catheters and central venous catheters used in patients catheterized between 4 and 21 days were collected. Eleven (11) strains were isolated from the endotracheal tube, 10 from urinary catheters and nine from central venous catheters (Table 1). The antimicrobial profiles showed that all isolates were resistant to ticarcillin, ticarcillin/clavulanic acid, Ceftazidim and trimethoprim/sulfamethoxazole. Twenty-seven (27) isolates were resistant to ciprofloxacin, 27 to amikacin, 20 to Gentamycin, 15 to imipenem, 10 to tobramycin and finally all isolates were susceptible to colistin.

Biofilm formation

A set of 30 *A. baumannii* strains were tested for their ability to form a biofilm by crystal violet staining. All strains had ability to form biofilm, however this process was more significant at 30°C than at 37°C, with values of OD_{570} (mean ± SD) varying between 0.63 ± 0.07 and 1.36 ± 0.1 and between 0.18 ± 0.06 and 1.23 ± 0.09, respectively (Figure 1).

The results of the kinetics of biofilm formation showed that a maximum OD value was reached after 72 h of incubation. However, a decrease in the density of the biofilm was noted after 96 h of incubation (Figure 2).

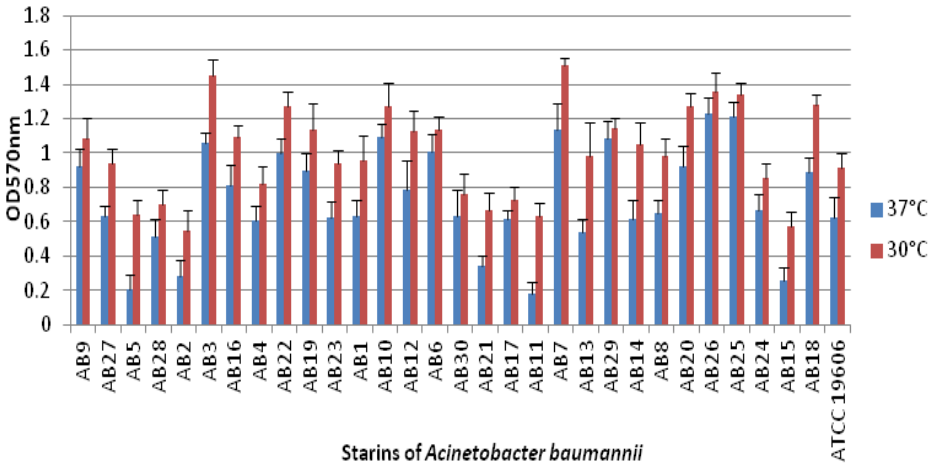

Figure 1. Quantification of biofilm formed by Strains of *A. baumannii* in Microplate at 30 and 37°C. Values are means ± SD of three independent experiments.

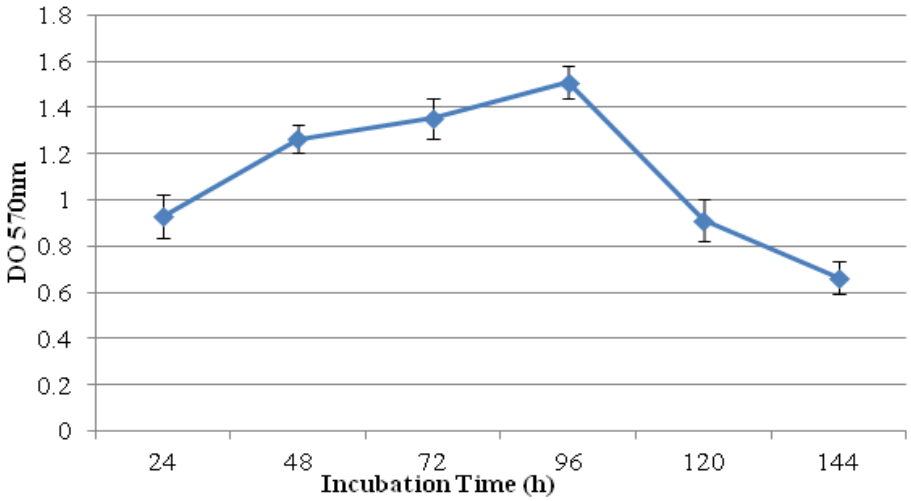

Figure 2. Kinetics of biofilm formation of *A. baumannii*.

Cell surface hydrophobicity

The study results of the hydrophobicity of *A. baumannii* strains indicated that out of the 30 strains tested, more than half, that is 18 (60%) of them, were hydrophilic with a percentage that varies between 1.25 and 15%. Moderately hydrophobic strains amounted to 7 (23%) with a percentage between 21.4 and 33.76%, and finally only 5 (17%) strains were hydrophobic with a percentage ranging from 42.5 to 90, 13% (Figure 3).

Adhesion to biomaterial

The strains AB25, AB29 and AB3 were selected for use in this study. They were chosen because of their high capacity to form a biofilm and their different hydrophobicity characters. The results of the adhesion of strains to the three different surfaces indicated that all strains had the same behavior, exhibiting a greater adhesion to silicone which was closely followed by latex. Finally, polyvinylchloride (PVC) comes last with a much lower adhesion compared to the two other surfaces (Figure 4).

DISCUSSION

During the last decades, *A. baumannii* has emerged globally as an important nosocomial pathogen that gives rise to outbreaks of colonization and infection of critically ill, hospitalized patients (de Breij et al., 2009). One reason for the emergence of this pathogen may be its persistence in hospital wards, in particular in the intensive

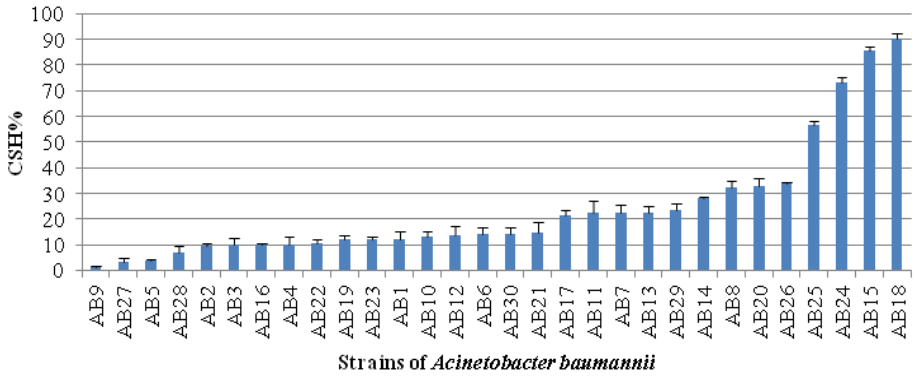

Figure 3. Cell surface hydrophobicity of *A. baumannii* strains. CSH was determined based on the difference of the OD of bacterial before and after adsorption to hexadecane, weak (0 - 20%), moderate (21 - 50%) and strong CSH > 40%.

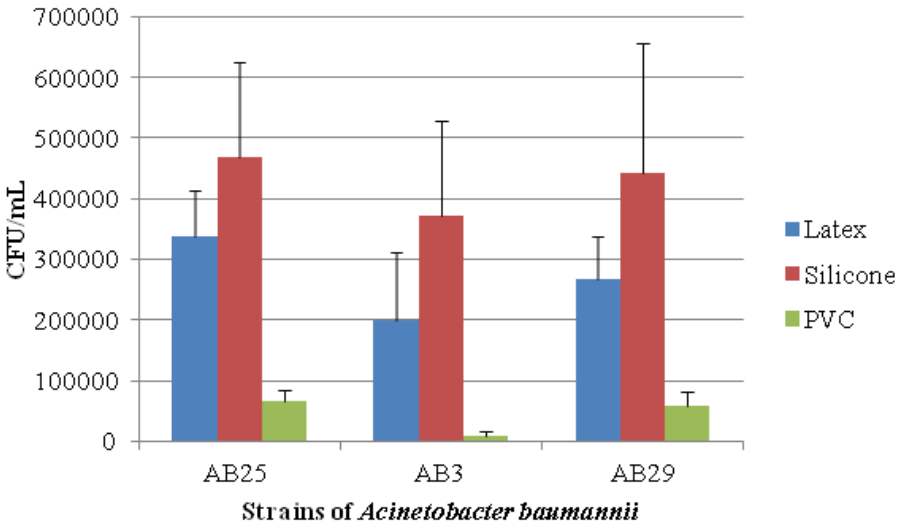

Figure 4. *A. baumanii* biofilm formation (CFU/ml) on Latex, Silicone and PVC for 24h of incubation.

care unit (Martí et al., 2011). Infections of hospitalized patients with Acinetobacter spp., often preceded by colonization, are frequently associated with invasive procedures and implantable medical devices. This process may be facilitated by the ability of a strain to form a biofilm (Wroblewska et al., 2008). It has become apparent that biofilm formation is a common trait of *A. baumannii* clinical isolates (McQueary and Actis, 2011). Several studies have demonstrated a high propensity among *A. baumannii* clinical isolates to form biofilms and a significant association of biofilms with multiple drug resistance and device-associated infections (Sechi et al., 2004; Rao et al., 2008; Rodríguez-Baño et al., 2008). In this study all *A. baumannii* strains were isolated from the endotracheal tube, urinary and central venous catheters at the intensive care unit of the University Hospital of Tlemcen formed biofilms, however this process is found to be more significant at 30 than at 37°C. This fact could explain the observed persistence of the members of the *A. baumannii* group in the inanimate hospital environment (Martí et al., 2011). Espinal et al. (2012) showed a relationship between the biofilm formation and the survival of *A. baumannii* clinical isolates, confirming the fact that isolates which produce biofilms survive longer than their non-biofilm forming counterparts on dry surfaces. The ability of *A. baumannii* to persist in nosocomial environments was also attributed to its widespread resistance to different antibiotics. In fact, the resistance profile revealed a remarkable resistance to most of the antibiotic agents tested, along with a significant susceptibility to colistin.

The duration of the device implantation significantly influences the biofilm formation (Domka et al., 2007). The kinetics of biofilm formation established in our study

showed that for up to 96 h of incubation, strains of *A. baumannii* adhere strongly and continuously. This first period corresponds to significant biofilm formation and the second to biofilm dispersion, with a release of bacterial cells into the culture medium (Djeribi et al., 2012). These observations suggest that the duration of implantation of the medical device must be reduced while it can go up to 21 days in our Intensive Care Unit, as shown in Table 1. Indeed, the risk of catheter-related infection is mainly connected with the time during which the catheter remains inserted. It is estimated that the risk increases by 5% each day (Mączyńska et al., 2010).

Cell surface hydrophobicity (CSH) of *A. baumannii* strains is also known to be associated with pathogenicity, bacterial adhesion and biofilm formation (Costa et al., 2006; Pour et al., 2011). Accordingly, the hydrophobicity of the isolates was evaluated by determining the affinity of cells to hexadecane (Rosenberg, 1984). The results obtained from the MATH method revealed that the majority of *A. baumannii* strains isolated from a hydrophobic medical device surface showed a hydrophilic character which contradicts several studies which admitted that the hydrophobic cells tend to adhere to a hydrophobic substrate, while the hydrophilic cells tend to adhere to a hydrophilic substrate (Costa et al., 2006; Djeribi et al., 2013). It is suggested that there is a positive correlation between the degree of bacterial hydrophobicity and the adhesion to abiotic surfaces (Costa et al., 2006; Pour et al., 2011). However, in this study, no apparent relationship was detected between hydrophobicity and biofilm formation, as the most hydrophilic strain AB9 formed a similar biofilm to the most hydrophobic one AB18. According to McQueary and Actis (2011), cell hydrophobicity is not a good predictor of the properties of *A. baumannii* biofilms, in contrast to other bacterial pathogens such as *Neisseria meningitides* and *Stenotrophomonas maltophilia*, which both display a direct correlation between surface hydrophobicity and biofilm formation on glass and plastic, respectively (Yi et al., 2004; Di Bonaventura et al., 2008; McQueary and Actis, 2011).

Another important factor influencing biofilm formation is the type of catheter and the chemical composition of the material it is made of (Mączyńska et al., 2010; Espinal et al., 2012). In this study, tests for adhesion to latex, silicone and PVC surfaces were carried out as these materials are used in the fabrication of implantable medical devices. The strains showed less adhesion to PVC as compared to silicone and latex. These results are in accordance with other studies performed by many laboratories where it has been reported that microbial adherence to biomaterials occurs in the following order: latex > silicone > PVC > Teflon > Polyurethane > stainless steel > titanium (Abd El-Baky, 2012). Dwornicz et al. (2003) found that *Enteroccocus feacalis* adheres highly to silicone and siliconized latex than to PVC. However, Maczynska et al. (2010) demonstrated that PVC was the biomaterial that was best colonized by *klebsiella* strains. These contradictions can be explained by the fact that various bacteria species probably prefer a certain chemical composition of the biomaterial to which their adhesion is the strongest (Mączyńska et al., 2010). Indeed, the material surface characteristics play an important role in bacterial adhesion. These characteristics include the material's surface-charge, the hydrophobicity and the surface roughness or physical configuration (Katsikogianni and Missirlis, 2004).

Conclusion

This paper show the great ability of *A. baumannii* strains to form a biofilm as well as the difference in the intensity of biofilm on three different biomaterials used in the manufacture of medical devices demonstrating the influence of chemical composition of the biomaterial on biofilm formation. Factors that may influence this process have also been demonstrated, as this can help in preventing diseases associated with infections caused by implanted medical devices. On the other hand, a better understanding of biofilm formation by *A. baumannii* is needed in order to provide new strategies to minimize the susceptibility of the device surface to colonization by this opportunistic pathogen.

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Full Length Research paper

Prevalence of antimicrobial resistant pathotypes of *Escherichia coli* in beef cattle and slaughterhouse premise

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The study was undertaken to assess distribution of antimicrobial resistant and virulent genes in 72 *Escherichia coli* isolated from beef cattle and slaughter house. All the isolates were subjected for detection of virulent genes viz. *stx1* and *stx2* of shigatoxic *E. coli* (STEC), *Lth* of Enterotoxigenic *E. coli* (ETEC); antimicrobial resistant genes *tetA, tetB, tetC* for tetracycline and *strA, strB* for streptomycin by PCR assay. The *Stx1* and *Stx2* genes could be detected in 9.72 and 15.28% of the *E. coli* isolates, respectively. None of the isolates harbored both *Stx1* and *Stx2* genes in combination. However, 1.38 and 2.77% of the isolates, respectively possessed combinations of *Stx1* and *Lth* and *Stx2* and *Lth*. ETEC was the predominant pathotype isolated (31.94%) as compared to STEC (25%). Isolates exhibited maximum sensitivity to antimicrobials cephotaxim, ciprofloxacin, norfloxacin and cefriaxone, and resistance to gentamicin, cloxacillin and ampicillin. Tetracycline and streptomycin resistant genes were detected in 17 (23.61%) and 19 (26.38%) *E. coli* isolates, respectively. *tetB and strA+strB* were predominant genes harbored whereas *tetC* gene was not detected in any of the isolate. Observations of the present study conclude that *E. coli* isolates harboring drug resistant genes could be widely distributed in beef cattle and slaughter house environment. It may create a pool of transferable resistance genes for other pathogens in simulated environment. Since slaughtering and meat handling operations at majority of municipal slaughterhouses in India are unhygienic producing very conducive environment for foodborne environmental pathogens, this study suggests the need for effective implementation of good hygienic practices (GHP) in slaughterhouses to reduce the risks of contamination with antimicrobial-resistant bacteria.

Key words: *Escherichia coli,* shigatoxic *E. coli* (STEC), beef, drug resistant genes.

INTRODUCTION

Although, *Escherichia coli (E. coli)* are normal inhabitant of gastrointestinal tract of all animals including human, some of the pathotypes causes potential public health hazards. Role of animals in harboring virulent *E. coli* is well established as several virulent strains are recorded from different animals and birds (Nataro and Kaper, 1998). Shigatoxigenic *E. coli* (STEC) strains are of special importance notably, O157: H7 being recognized as emerging foodborne pathogen extensively transmitted through consumption of contaminated beef (Gyles, 2007).

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STEC strains can cause severe clinical manifestations ranging from mild to bloody diarrhea and in rare cases post-infection hemolytic-uremic syndrome (Zheng et al., 2008). Enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of acute infectious diarrhea and death in children living in developing countries. ETEC is also a major cause of traveler's diarrhea and the organism is regularly imported to the developed world (Qadri et al., 2005). ETEC strains colonize the surface of the small bowel mucosa and elaborate their enterotoxins heat-labile (LT) and heat-stable (ST), giving rise to a net secretory state.

Carcass easily contracts such infectious agents from slaughter house environment if meat hygiene practices are compromised. Majority of the slaughter houses in developing parts of world do not meet the international standards and floor slaughtering is a common practice. Water used for dressing and cleaning operations is also another risk factor in production of safe and wholesome meat to the consumer. In Indian context, small ruminants are slaughtered and dressed on overhead rails, but cattle (males) slaughtering is commonly done on floor at majority of the municipal slaughter houses except few modern abattoirs.

STEC is recognized as important pathogen of public health significance and beef cattle are reservoir. The major virulence factor of STEC is *stx*, the potent cytotoxin that leads to death and many other symptoms in patients infected with Enterohemorrhagic E. coli (EHEC). The *Stx* family contains two major, immunologically non-cross-reactive groups called *Stx1* and *Stx2*. A single EHEC strain may express *Stx1* only, *Stx2* only, or both toxins or even multiple forms of *Stx2* (Nataro and Kaper, 1998).

India has huge cattle population; however information on occurrence of STEC in them is very scanty. Bacterial resistance to antimicrobial agents is alarming since they develop resistance through the acquisition of new genetic material from other resistant organisms. This *horizontal evolution* may occur between strains of the same species or between different bacterial species or genera (Tenover, 2006). In view of these facts, purpose of this investigation was to assess the prevalence of STEC and ETEC in cattle feces, beef, slaughter house floor and to study the antimicrobial resistance (AMR) pattern of *E. coli* isolates phenotypically and genotypically.

MATERIALS AND METHODS

Sampling (n=150)

Sampling of cattle feces, meat and slaughter house floors were done aseptically at municipal abattoir in Mumbai and Pune cities of Maharashtra state, India during December to March, 2012. Sampling plan was designed to collect 150 samples from 50 subjects; 50 samples each of feces, meat and slaughter house floor. Fresh fecal sample was collected aseptically from healthy animal before slaughter, followed by swab of floor to be used for slaughter and meat sample immediately after slaughtering and dressing of the same animal. All the samples were placed in sterile specimen tubes on ice and transported to our laboratory for bacterial isolation within a same day of collection.

Culture procedure for isolation of *E. coli*

All the collected samples were subjected for enrichment in Enterobacteriaceae Enrichment (EE) broth (HiMedia Laboratories, Mumbai, India) in 1:10 dilution followed by selective plating on eosine methylene blue (EMB) agar and MUG Sorbitol agar (HiMedia Laboratories, Mumbai, India). Incubation was done at 37°C for 24 h. Candidate colonies were further streaked on nutrient agar and identified biochemically (Agarwal et al., 2003) and kept at 4°C in Brain Heart Infusion (BHI) broth until use. All the *E. coli* isolates were individually screened for the presence of virulent and drug resistant genes by polymerase chain reaction (PCR).

Antimicrobial susceptibility testing

Antibiotic sensitivity was determined by single disc diffusion method of Bauer et al. (1966) using Mueller Hinton agar (MHA). Bacteria were cultivated in Brain Heart Infusion Broth (BHI) and incubated for 12 h. Further, the bacterial suspension seeded onto MHA and plates were incubated at 37°C. The antibiogram was studies against 15 different antimicrobials commonly used in veterinary and human practices in Indian context viz. ampicillin (10 mcg/disc), ciprofloxacin (10 mcg/disc), cephalexin (30 mcg/disc), cloxacilin (10 mcg/disc), chlortetracycline (30 mcg/disc), ceftriaxone (10 mcg/disc), cephotaxime (10 mcg/disc), erythromycin (10 mcg/disc), enrofloxacin (10 mcg/disc), furazolidone (50 mcg/disc), gentamicin (10 mcg/disc), nalidixic acid (30 mcg/disc), norfloxacin (10 mcg/disc), streptomycin (10 mcg/disc), tetracycline (10 mcg/disc) (Table 3). The antimicrobial discs were procured from HiMedia Laboratories and results were interpreted according to the manufacturer's instruction.

Molecular detection

All the *E. coli* isolates were screened by PCR for detection of virulent pathotypes viz. STEC and ETEC and presence of drug resistant genes. Virulent genes viz. *Stx1* and *Stx2* of STEC and *Lth* of ETEC were targeted according to the methods of Nguyen et al. (2005) and Ratchtrachenchai et al. (2004). Genomic DNA was extracted by boiling and snap chilling method and PCR reaction was performed using oligonucleotide primers indicated in Table 1 in a thermal cycler (Eppendorf, Germany). For DNA extraction, 200 µl 24 h grown broth culture of *E. coli* isolates was centrifuged at 9500 rpm under low temperature (4°C) for 5 min. After discarding the supernatant, pellet was washed twice with sterile phosphate buffer (PBS). Finally, the pellet was resuspended in 100 µl PBS; boiled in water bath for 8 min and immediately snap chilled into ice. From snap chilled lysate, 3 µl supernatant was directly taken as DNA template for PCR. The reaction were performed in 25 µl volumes comprising of 12.5 µl KAPA master mix (KAPA Biosystems) with *Taq* polymerase, 0.5 µl each forwards and reverse primers, 3.0 µl template DNA and 8.5 µl nuclease free water (NFW).

Similarly, genomic confirmation of antimicrobial resistance viz. tetracycline and streptomycin targeting *tetA, tetB, tetC* and *strA, strB* genes was performed as per the methods of Lanz et al. (2003). The reaction was performed same as described earlier except use of 1.0 µl primers each and 7.5 µl NFW. The amplifications were performed as described in Table 1. The PCR products were cooled at 4°C and resolved by electrophoresis on 2% agarose gel in horizontal submarine electrophoresis apparatus. Each run contained a 100 bp DNA molecular weight marker (GeneRuler, Fermentas). The gels were stained with ethidium bromide

Table 1. Oligonucleotide sequences and PCR conditions used for detection of virulent and AMR genes.

Target genes	Nucleotide sequence	PCR Condition s			Product size (bp)	Reference
		Denaturation	Annealing	Extension		
30 cycles each						
stx1 (*vt1*)	(F) GAAGAGTCCGTGGGATTACG (R) AGCGATGCAGCTATTAATAA	94°C/20 s	55°C/20 s	72°C/10s	130	Nguyen et al. (2005)
stx2 *(vt2)*	(F) TTT ACG ATA GAC TTC TCG AC (R) CAC ATA TAA ATT ATT TCG CTC	94°C/60 s	48°C/60 s	72°C/2 min	228	
LTh	(F) AGC AGG TTT CCC ACC GGA TCA CCA (R) CGT GCT CAG ATT CTG GGT CTC	94°C/60 s	48°C/60 s	72°C/2 min	132	Ratchtrachenchai et al. (2004)
34 cycles each						
strA	(F) CCTGGTGATAACGGCAATTC (R) CCAATCGCAGATAGAAGGC	94°C/30 s	55°C/90 s	72°C/90 s	546	
strB	(F) ATCGTCAAGGGATTGAAACC (R) GGATCGTAGAACATATTGGC	94°C/30 s	55°C/90 s	72°C/90 s	509	
tetA	(F) GGCGGTCTTCTTCATCATGC (R)CGGCAGGCAGAGCAAGTAGA	94°C/30 s	64°C/90 s	72°C/90 s	502	Lanz et al. (2003)
tetB	(F) CATTAATAGGCGCATCGCTG (R) TGAAGGTCATCGATAGCAG	94°C/30 s	64°C/90 s	72°C/90 s	930	
tetC	(F)GCTGTAGGCATAGGCTTGGT (R)GCCGGAAGCGAGAAGAATCA	94°C/30 s	64°C/90 s	72°C/90 s	888	

(0.001 µg/ml), observed under UV light at a wavelength of 420 nm and images were captures through gel documentation system (Invitrogen).

RESULTS

The prevalence of *E. coli* recorded in the present study was 48% and of the 72 *E. coli* isolates, 38 (52.77%) were virulent pathotypes. *E. coli* could be isolated from 66, 62 and 16% fecal, beef and floor swab samples, respectively. Streaking on 4-Methylumbelliferyl-β-Glucuronide-Sorbitol (MUG) agar also revealed 14.67% pathogenic strains phenotypically. Pathogenic *E. coli* strains lack the sorbitol degrading ability within 48 h of incubation. It does not synthesize the enzyme glucuronidase and hence, there is no fluorescence production by these strains when MUG is present in the medium. Sorbitol degrading organisms produce pink to red colonies while sorbitol negative colonies are colorless. Isolation of *E. coli* from beef and slaughter house floor is indicator of unhygienic proces-

Table 2. Prevalence of tetracycline and streptomycin resistant *E. coli*

Isolates from	Number and per cent prevalence (%) against tetracycline					
	tetA	***tetB***	***tetC***	***tetA+tetB***	***tetA+tetC***	***tetB+tetC***
Feces (33)	2 (6.06)	9 (27.27)	0 (0.00)	1 (3.03)	0 (0.00)	0 (0.00)
Beef (31)	1 (3.22)	2 (6.45)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
S.H. Floor (8)	2 (25.00)	0 (0.00)	0 (0.00)	1 (12.50)	0 (0.00)	0 (0.00)
Overall prevalence (72)	5 (6.94)	11 (15.28)	0 (0.00)	2 (2.78)	0 (0.00)	0 (0.00)
Number and Per cent prevalence (%) against streptomycin						
	strA		***strB***		***strA+B***	
Feces (33)	2 (6.06)		1 (3.03)		6 (18.18)	
Beef (31)	2 (6.45)		1 (3.22)		5 (16.13)	
S.H. Floor (8)	1 (12.5)		0 (0.00)		1 (12.50)	
Overall prevalence (72)	5 (6.94)		2 (2.78)		12 (16.67)	
Number and Per cent prevalence (%) against tetracycline + streptomycin						
	tetA + strA,B	***tetB + strA,B***	***tetA,B + strA,B***		***StrA + tetA***	
Feces (33)	0 (0.00)	4 (12.12)	0 (0.00)		0 (0.00)	
Beef (31)	0 (0.00)	0 (0.00)	0 (0.00)		0 (0.00)	
S.H. Floor (8)	0 (0.00)	0 (0.00)	1 (12.50)		1 (12.50)	
Overall prevalence (72)	0 (0.00)	4 (5.55)	1 (1.39)		1 (1.39)	

S.H., Slaughter house.

sing operations and is important public health concern.

We screened all the *E. coli* isolates (n=72) for detection of virulent pathotypes targeting *Stx1* and *Stx2* genes for STEC and *Lth* gene for ETEC strains (Figure 1). Of the 72 strains, 9.72% carried *Stx1* and 15.28% carried *Stx2*. None of the isolates gave positive amplification for presence of both genes. However, 1.38 and 2.77% of the isolates possessed combinations of *Stx1* and *Lth* and *Stx2* and *Lth*, respectively. ETEC was the predominant pathotypes isolated (31.94%) as compared to STEC (25%).

All the *E. coli* isolates after biochemical confirmation were subjected for antimicrobial susceptibility testing and genomic detection for presence of virulent and drug resistant genes. *E. coli* isolates under study were highly sensitive to the cephotaxime (100%), ciprofloxacin (98.61%), ceftriaxone (95.83%), norfloxacin (91.66%) and enrofloxacin (83.33%). Moderate degree of sensitivity was recorded for cephalexin, furazolidone, erythromycin, chlortetracycline, streptomycin and gentamicin. However, maximum *E. coli* exhibited resistance to cloxacillin (97.22%), ampicillin (58.33%) and streptomycin (25%) (Table 3). All the STEC and ETEC strains were found to be resistant to one or more antimicrobials.

Out of 72 isolates, 30 (41.66%) *E. coli* were found to harbor drug resistant genes (Figure 2). Tetracycline and streptomycin resistant genes were detected in 17 (23.61%) and 19 (26.38%) *E. coli* isolates, respectively. *tetB* (15.28%) and combination of *strA*+*strB* (16.67%) genes were predominantly recorded in the study. *tetC* gene was not detected in any of the isolates. Sixteen (16) *E. coli* expressed phenotypic resistance to tetracycline by disc diffusion method; of them tetracycline resistant genes were detected in 13 (81.25%) isolates. Similarly, of the 18 streptomycin resistant *E. coli* confirmed by disc diffusion method, 14 (77.77%) harbored streptomycin resistant genes. Source wise distribution of drug resistant genes is shown in Table 2. Distribution of drug resistance and virulent genes in the isolated *E. coli* was random and both virulent and drug resistant genes were detected only in five isolates (Table 4).

DISCUSSION

Presence of *E. coli* in the cattle feces is obvious; however, its presence in the fresh beef and slaughter floor is not good indicator of food hygiene and safety. Prevalence rate was found to be more in the present study as recorded previously from different parts of the world in cattle feces and beef (Son et al., 1998; Elder et al., 2000; Rathore et al., 2010). In India, although STEC is not incriminated as a major cause of childhood diarrhea, however some of the reports on isolation of STEC including O157:H7 and non O157 strains from cattle feces, beef, diarrheal stool and water have highlighted the risk of this pathogen pose to human health (Dutta et al., 2000; Chatopadhyay et al., 2001; Khan et al., 2002; Dhanashree and Mallya, 2008). Dutta et al. (2000) conducted the surveillance study on STEC including O157:H7 in dairy cattle and pigs slaughtered at municipal slaughter house Calcutta and found STEC in 6.5 and 1.5% fecal samples, respectively from diarrheic and healthy cattle. Chattopadhyay et al. (2001) recorded

Table 3. Source wise Per cent sensitivity/resistance of *E. coli* isolates by disc diffusion methods (n=72).

Name of antibiotic with concentration (mcg/disc)	Fecal isolates (n= 33)			Beef isolates (n = 31)			Floor swab isolates (n= 8)			Overall pattern		
	S	MS	R	S	MS	R	S	MS	R	S	MS	R
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Ampicillin (A) (10)	00 (00)	7 (21.22)	26 (78.78)	00 (00)	19 (61.30)	12 (38.70)	00 (00)	4 (50)	4 (50)	0.00	30 (41.66)	42 (58.33)
Ciprofloxacin (CIP) (10)	32 (96.96)	1 (3.04)	00 (00)	31 (100)	00 (00)	00 (00)	8 (100)	00 (00)	00 (00)	71 (98.61)	1 (1.38)	0.00
Cephalexin (CN) (30)	1 (3.03)	31 (93.93)	1 (3.04)	2 (6.45)	28 (90.32)	1 (3.23)	1 (12.5)	5 (62.5)	2 (25)	4 (5.55)	64 (88.88)	4 (5.55)
Cloxacilin (COX) (10)	00 (00)	2 (6.07)	31 (93.93)	00 (00)	00 (00)	31 (100)	00 (00)	00 (00)	8 (100)	0.00	2 (2.77)	70 (97.22)
Chlortetracycline (CT) (30)	5 (15.15)	26 (78.78)	2 (6.07)	13 (41.94)	17 (54.83)	1 (3.23)	2 (25)	4 (50)	2 (25)	20 (27.77)	47 (65.27)	5 (6.94)
Ceftriaxone (CTR) (10)	30 (90.90)	3 (9.10)	00 (00)	31 (100)	00 (00)	00 (00)	8 (100)	00 (00)	00 (00)	69 (95.83)	3 (4.16)	0.00
Cephotaxime (CTX) (10)	33 (100)	00 (00)	00 (00)	31 (100)	00 (00)	00 (00)	8 (100)	00 (00)	00 (00)	72 (100)	0.00	0.00
Erythromycin (E) (10)	5 (15.15)	26 (78.78)	2 (6.07)	2 (6.46)	24 (77.42)	5 (16.12)	00 (00)	5 (63.5)	3 (36.5)	7 (9.72)	55 (76.38)	10 (13.88)
Enrofloxacin (EX) (10)	32 (96.96)	1 (3.04)	00 (00)	23 (74.19)	8 (25.81)	00 (00)	5 (63.5)	3 (36.5)	00 (00)	60 (83.33)	12 (16.66)	0.00
Furazolidine (FR) (50)	2 (6.07)	23 (69.69)	8 (24.24)	00 (00)	30 (96.78)	1 (3.22)	00 (00)	7 (87.5)	1 (12.5)	2 (2.77)	60 (83.33)	10 (13.88)
Gentamicin (GEN) (10)	14 (42.42)	18 (54.54)	1 (3.04)	16 (51.62)	15 (48.38)	00 (00)	3 (37.5)	5 (62.5)	00 (00)	33 (45.83)	38 (52.77)	1 (1.38)
Nalidixic acid (NA) (30)	10 (30.30)	21 (63.63)	2 (6.07)	13 (41.94)	9 (29.03)	9 (29.03)	4 (50)	3 (37.5)	1 (12.5)	27 (37.5)	33 (45.83)	12 (16.66)
Norfloxacin (NX) (10)	28 (84.85)	5 (15.15)	00 (00)	30 (96.77))	1 (2.23)	00 (00)	8 (100)	00 (00)	00 (00)	66 (91.66)	6 (8.33)	0.00
Streptomycin (S) (10)	4 (12.13)	22 (66.66)	7 (21.21)	6 (19.36)	14 (45.16)	11 (35.48)	3 (36.5)	5 (63.5)	00 (00)	13 (18.05)	41 (56.94)	18 (25)
Tetracycline (TE) (10)	5 (15.15)	17 (51.52)	11 (33.33)	3 (9.67)	25 (80.66)	3 (9.67)	00 (00)	6 (75)	2 (25)	8 (11.11)	48 (66.66)	16 (22.22)

S, sensitive; MS, moderately sensitive; R, resistant.

6.02% STEC prevalence in diarrheic cattle. STEC strains were also isolated from fresh fish, shell fish and meat sold in open market from Manglore (Kumar et al., 2001). Khan et al. (2002) studied virulence gene and molecular profiles of STEC isolates from human, beef and cattle feces in Calcutta, wherein different combinations of virulence genes were observed. The dominant combination of virulence factors present in the strains studied were *stx1* and *stx2* (44.5% of strains) and *stx1*, *stx2*, and *hlyA* (19% of strains) lacking in *eae.* Very limited studies are conducted on prevalence of STEC in food animals in Indian context; our findings highlighted the need to con-duct nationwide surveillance on occurrence of STEC in foods of animal origin. ETEC is a major cause of traveler's diarrhea and is being imported to the developed world (Qadri et al., 2005). It is a frequent cause of diarrhea in children ≤ 2 years of age (Wolf, 1997; Nataro and Kaper, 1998). The role of ETEC in animal diarrhea is not extensively studied. However, its higher prevalence recorded in beef cattle may increase the risk of spread this enteropathogen to human.

We have not undertaken serotyping studies for STEC and ETEC strains isolated but their presence in cattle feces, beef and slaughter house floor is confirm. It is practically impossible to control the excretion of STEC strains through cattle feces or any other food animal feces in countries like India where, a great degree of bio-geo-diversity exists. However, most significant approach to combat various issues related to meat safety is upgrading the standard of municipal slaughter Houses and application of good hygienic practices (GHP), good manufacturing practices (GMP) and HACCP in the entire meat production chain.

The phenotypic resistance in the absence of resistance genes may be due to other resistance mechanisms not involving resistance genes, such as point mutations (Gow et al., 2008). In a previous study, the majority of *E. coli* isolates from cattle carried *tet*A or *tet*B genes or a combination of these genes, and the *tet*B gene was more

Table 4. Comparative study of *E. coli* isolates characterized by antibiogram and PCR.

Isolate number	AMR pattern	Virulent genes detected	Drug resistant genes detected
01	A COX	*LTh, ,stx2*	-
02	A COX FR S TE	-	*strA, strB, tetB*
03	A COX FR S TE	-	*strA, strB, tetB*
04	A COX	*LTh, ,stx1*	-
05	A COX TE	*LTh*	*tetA, tetB*
06	A COX FR S TE	-	*strA, strB, tetB*
07	A COX FR S TE	-	*strA, strB, tetB*
08	A COX	*Stx2*	-
09	A COX E FR	-	-
10	A FR	*stx2*	-
11	A COX FR	*Lth*	*tetA*
12	A CN E GEN TE	-	*tetB*
13	A COX	-	-
14	COX FR	-	*strA, strB*
15	A COX	*LTh, stx2*	-
16	COX	*LTh*	-
17	COX CT NA TE	-	*strB*
18	A COX	*LTh*	-
19	A COX	*LTh*	*tetA*
20	A COX	*stx1*	-
21	A COX	-	-
22	A COX TE	-	*strA*
23	A COX S	-	*strA, strB*
24	COX CT NA TE	-	*tetB*
25	A COX TE	*LTh,*	*tetB*
26	A COX	*Stx2*	-
27	COX	*LTh*	-
28	A COX	*Stx1*	*strA*
29	COX	*LTh*	-
30	A COX	*LTh*	-
31	A COX S	*Stx2*	-
32	COX	*LTh*	-
33	A COX TE	-	*tetB*
34	COX	*Stx1*	-
35	A COX	-	-
36	COX	*LTh*	-
37	A COX S	-	*strA*
38	A COX	*Stx1*	-
39	COX	*LTh*	-
40	A COX	*Stx2*	-
41	A COX TE	-	*tetB*
42	A COX	*Stx2*	-
43	A COX	*LTh*	-
44	COX	-	-
45	COX CT NA TE	*LTh*	-
46	COX NA S	-	*tetB*
47	COX E	*Stx1*	-
48	COX NA S	-	*strA, strB*
49	COX NA S	-	-
50	A COX FR S	-	*strA, strB*
51	A COX E NA S	*LTh*	-
52	COX NA S	-	*strA, strB*

Table 4. Contd.

53	A COX NA S	-	*strA*
54	COX NA S	-	*strB*
55	COX NA	*LTh*	-
56	CN COX S	-	*strA, strB*
57	COX	*Stx2*	-
58	COX S	-	*strA, strB*
59	COX	*Stx2*	-
60	COX	-	-
61	A COX E	*LTh*	-
62	COX E TE	-	*tetA*
63	COX NA S	*Stx2*	-
64	A COX	-	-
65	COX	*LTh*	-
66	A CN COX CT TE	-	*tetA*
67	COX E FR	-	-
68	COX	*LTh*	-
69	A CN COX CT TE	-	*strA, tetA*
70	COX E	*Stx1*	-
71	A COX E	-	-
72	A COX NA	*LTh*	*strA, strB, tetA, tetB*

Isolate No. 1-33, fecal sample; 34-64, beef; 65-72, Slaughter house floor.

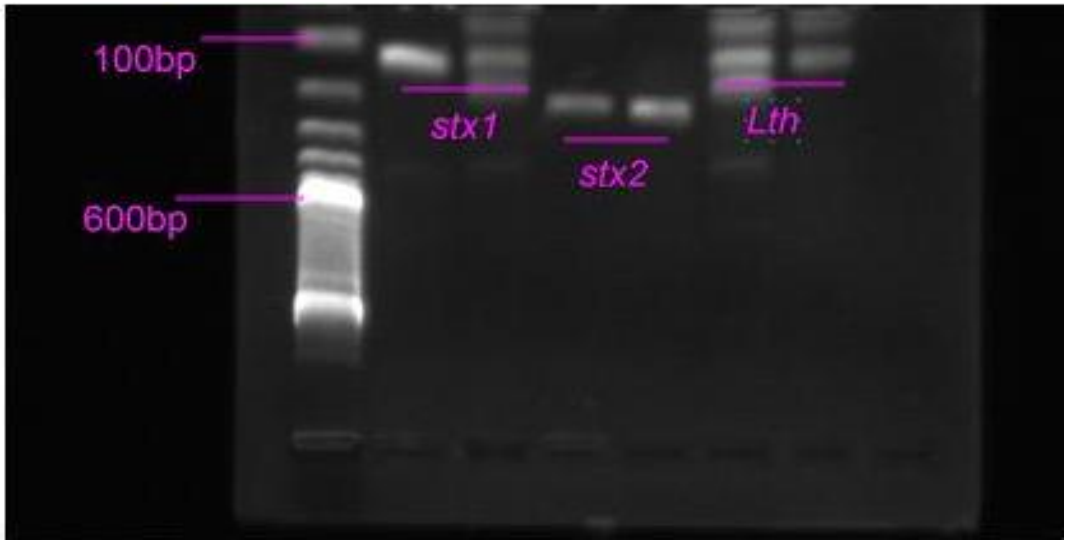

Figure 1. Detection of virulent by PCR. Lane 1, 100 bp ladder; lanes 2 and 3, *Stx1* gene (130 bp); lanes 4 and 5, *Stx1* gene (228 bp); lanes 6 and 7, *LTh* gene (132 bp).

common (Bryan et al., 2004; Gow et al., 2008). Bryan et al. (2004) studied frequency and distribution of tetracycline resistance genes in genetically diverse *E. coli* strains isolated from diverse human and animal sources.

The majority of *E. coli* isolates from cattle carried *tet*A or *tet*B genes or a combination of these genes, and the *tet*B gene was more common. Similar pattern of resistance was also observed by Gow et al. (2008). Our findings are in agreement with them but in contrast with those of Aslam et al. (2009) who recorded *tet*C as the most com-mon gene found in tetracycline resistant *E. coli* (21% of isolates) and was significantly associated with tetracycline resistant phenotypes. Studies of Skockova et al. (2012) recorded *tet*A and *tet*B as most frequently

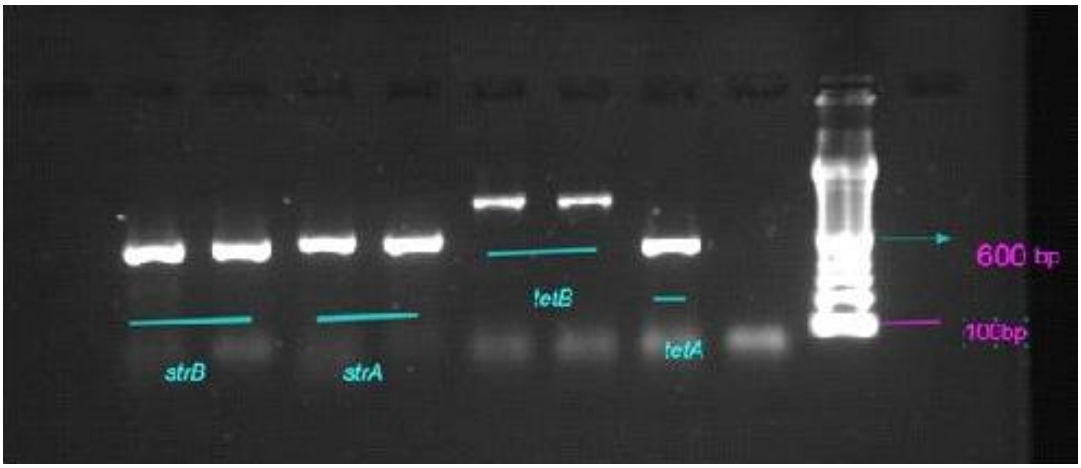

Figure 2. Detection of AMR genes. Lanes 1 and 2, *str B* gene (502 bp) lanes 3 and 4, *strA* gene (546 bp); lanes 5 and 6, *tet B* gene (930 bp); lanes 7, *tetA* gene (509 bp); lane 9, 100 bp.

detected genes in the *E. coli* isolates from raw cow's milk on the monitored farm. A significant shift in the distribution pattern of the *tet* genes was observed by them over a five-year period. Based on the data obtained, it was concluded that the *tet*A gene might easily spread in an environment under low selective pressure of tetracycline antibiotics while the *tet*B gene could serve as an indicator of high selection pressure of tetracycline antibiotics. Because only a small number of isolates were analyzed in this study, further studies are needed with more isolates from different sources and processing plants. That approach may provide the data necessary to explain the association of resistance genes with sample sources as suggested by Aslam *et al.* (2009).

Since human may obtain antimicrobial resistant (AMR) *E. coli* or resistance genes of animal origin directly via contact with animals, food of animal origin or the environment which may lead to bacterial infection with limited therapeutic options and an increased risk of treatment failure. Therefore, the use of antimicrobial agents regarded as critically or highly important for use in humans should be judicious or minimum in food animals, to preserve the efficiency of these antimicrobial agents for treatment of infection in humans (Hammerum and Heuer, 2009). In Indian context, municipal slaughterhouses are ill maintained and unhygienic practices often deteriorate the quality of meat produced. It also poses health risks to the consumers. Hygiene is directly proportional to the quality and safety of food. Bell, (1997) studied distribution and sources of microbial contamination on beef carcasses. Sites contaminated by direct fecal contact or contact with focally contaminated hides typically had aerobic plate count (APC) greater than log 4 cfu cm^{-2} accompanied by *E. coli* count exceeding log 2 cfu cm^{-2} as compared to sites not subjected to fecal contamination with APC lower than log 2 cfu cm^{-2} accompanied by the occasional detection of *E. coli* at level below log 1 cfu cm^{-2}.

Observations of the present study conclude that *E. coli* isolates harboring drug resistant genes could be widely distributed in slaughter house environments. It may create a pool of transferable resistance genes for other pathogens in simulated environment. Since slaughtering and meat handling operations at majority of municipal slaughterhouses in India are unhygienic which produce very conducive environment for foodborne environmental pathogens, this study suggests the need for effective implementation of Good Hygienic Practices (GHP) in slaughterhouses to reduce the risks of contamination with antimicrobial-resistant bacteria. This study further suggests the undertaking of surveillance on AMR and distribution of *E. coli* pathotypes in beef cattle and slaughterhouse environment on large scale.

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Full Length Research Paper

Antifungal agent production from a new marine *Bacillus pumilus*SMH101

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Twenty two (22) marine bacterial strains were isolated and tested to inhibit some plant and human pathogenic fungi; *Fusarium solani*, *Rhizoctonia solani, Aspergillus niger*, *Fusarium exosporium* and *Candida albicans*. The most potent marine bacterium was identified as *Bacillus pumilus*SMH101 on using 16S rRNA. The peptone water medium (PW) showed maximum antifungal activity. The Placket-Burman experimental design was applied and the optimum culture conditions were inoculum size (8.0 × 10^6 cfu/ml), temperature (25°C), incubation period (24 h) and pH value of 7.0. The trickle flow column was tested for propagating the antifungal production using luffa pulp and synthetic sponge as solid packing materials. The adsorbed *B. pumilus*SMH101 showed lower average fungal suppression (~ 40%) compared to the free bacterial cells (58.6%). Thin layer chromatography (TLC) was applied using a solvent system of dichloromethane: methanol: water (65:32:3 v/v). A single ultraviolet (UV) spot was obtained with a retardation factor (R_f) of 0.75. It analyzed using UV, infrared (IR) and mass spectrometry (MS) spectra and showed a molecular weight of 875 Da. Also, it showed a relatively low bio-toxicity (LC_{50} = 1072 ppm) and a broad antifungal spectrum with a bioactivity of 87, 80 and 70% against *F. solani*, *R. solani* and *C. albicans*, respectively, compared to some commercial antifungal drugs based on the active fluconazole compound which applied in a concentration of tenfold more than the used *B. pumilus*SMH101 antifungal agent concentration (0.05 mg/ml).

Key words: Antifungal, optimization, Placket-Burman, trickle flow column, *Bacillus pumilus*.

INTRODUCTION

In recent years, the incidence of invasive opportunistic fungal infections has been increasing due to increases in the number of immune-compromised patients (Soeta et al., 2009). Infections due to *Candida* species in immune-compromised patients are the most common; however, *Aspergillus* and other pathogenic fungi are also emerging as a threat to public health (Richardson, 2005). The mor-tality rate due to invasive aspergillosis has risen stea-dily with a 35.7% increase which caused significant mor-bidity and mortality during 1980 to 1997 (Rapp, 2004). Infecions due to *Fusarium* species are emerging hyalo- hypho-mycoses of immune-compromised patients and are asso-ciated with high mortality (Nucci and Anaissie, 2006). The dismal prognosis of *Fusarium* infection is the result of

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limited therapeutic options. Recent reports suggest that *Fusarium* species not only affect bone marrow transplant recipients and patients with haema-tological malignancies (Nucci et al., 2004), but also solid-organ transplant patients. In some centers, *Fusarium* species are the second most common cause of mold infection after *Aspergillus* species. The *Fusarium* species most frequently implicated in human infections include *Fusarium solani, Fusarium oxysporum* and *Fusarium moniliforme* (Lisboa et al., 2006).

Candida species belong to the normal micro-biota of an individual's mucosal oral cavity, gastrointestinal tract and vagina and are responsible for various clinical manifestations from muco-cutaneous overgrowth to blood-stream infections (Eggimann et al., 2003). Moreover, fungal infections have increased since the 1980s, especially in the large population of immune-compromised patients and/or those hospitalized with serious underlying diseases (Espinel-Ingroff et al*.,* 2009; Sardi et al*.,* 2013).

Recently, marine bacteria are being recognized as an important resource for many antimicrobial products, especially antifungal activities (Woo et al., 2002). Moreover several researchers extracted bioactive substances from different bacteria and actinomycetes, which exhibited biological activity against many pathogenic fungi; *Lactobacillus plantarum* (Storm et al*.,* 2002), *Chromobacterium violaceum* (Barreto et al., 2008), chitinolytic marine strains (Gohel et al., 2004), *Kitasatospora* sp. (Haesler et al*.,* 2008), *Streptomyces malaysiensis* (Li et al*.,* 2008), *Streptomyces* sp. (Park et al*.,* 2008). Also, some marine *Bacilli* were used for antifungal production such as *Bacillus firmus* (Ortega et al., 2009), *Bacillus megaterium* (Kong et al., 2010).

The application of statistically based experimental designs is a must to deal with the effect of different culture variables in the same time (Ooijkaas et al*.,* 1998). The Plackett-Burman designs are powerful tools for searching the most significant factors for antimicrobial production (Bie et al., 2005).

Immobilization techniques mostly applied for the production of secondary metabolites through cell entrapment and cell adsorption on solid supporters (Asanza et al*.,* 1997). The application of immobilized cells to study microbial processes is one of the main trends in modern biotechnology. In addition, cell immobilization shows many operational and economic advantages such as prolong metabolic activities, reuse of the biocatalyst, increase of cell concentration and preventing washing out of cells when high flow rates were applied (Gautam et al*.,* 2002).

In this study, a new marine *Bacillus* strain was isolated and identified using 16S rRNA as *Bacillus pumilus*SMH101. It was tested to produce antifungal agent acting against some plant and human pathogenic fungi. The optimization for antifungal production was carried out using Plackett-Burman experimental design as, the immobilization technique. A partial purification and characterization of the produced antifungal agent was carried out and the LC_{50} of the bioactive spot was determined using *Artemia salina* as a biomarker.

MATERIALS AND METHODS

Pathogenic fungi

The pathogenic *Aspergillus niger, Fusarium solani, Penicillium oxilacum***,** *Rhizoctonia solani* and *Candida albicans* ATCC 14053 were kindly provided from Dr. Eman AbdEl-Zaher, Tanta University, Egypt. The maintenance of these fungi except *C. albicans* was carried out using a modified Czapek Yeast Extract Agar (CYEA) medium, it Composed of (g/L): Sucrose, 20; $NaNO_3$, 2.0; K_2HPO_4, 1.0; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4$, 0.01; Yeast extract, 2.0. The pH was adjusted at 5.0 (Atlas, 2010). While, *C. albicans* was maintained using Sabouraud-dextrose agar (SDA) medium (British Pharmacopoeia) according to Sandven and Lassen (1999).

Isolation and purification of antifungal producer(s)

Seawater samples were collected from different locations in Alexandria shore line using 500 ml sterile blue screw-caped bottles according to the standard methods published by American Public Health Association (APHA, 1995). Serial dilutions were made using filtered sterilized seawater (from 10^{-2} to 10^{-6}). A portion (0.1 ml) from each diluted sample was spread on seawater nutrient agar plate medium (5 g peptone, 3 g beef extract, 20 g agar, 1000 ml seawater). Plates were incubated at 30°C for 24 h. A purification of the obtained bacterial colonies was carried out by streaking technique. The purified bacterial colonies were kept separately and tested to inhibit the pathogenic fungi; *A. niger, F. solani***,** *P. oxilacum, R. solani* and *C. albicans* ATCC 14053.

Bioactivity test

The bioactivity of each bacterial isolate against the pathogenic fungi was estimated as follows; a suspension of 24 h old bacterial culture (OD ~ 1.0) was centrifuged at 12000 rpm for 10 min. Then 5 ml of each bacterial supernatant was added to 100 ml (CYEA) medium and poured using 9 cm sterile Petri dishes (amended (CYEA) plates). 0.5 cm cylinders with 5 mm diameter were cut out from pre-activated pathogenic fungi using a sterile cork borer, and then transferred separately to the center of the amended (CYEA) plates and the control (un-amended (CYEA) plates); all plates were incubated at 28°C for a week. The suppression percentage was calculated as follows: (fungal growth diameter on un-amended (CYEA) plates in mm - fungal growth diameter on amended (CYEA) plates in mm)/ fungal growth diameter on un-amended (CYEA) plates in mm × 100% (APHA, 1995).

While, the bioactivity against *C. albicans* was carried out as follows: 5ml of each bacterial supernatant was added to 100 ml (SDA) medium and poured using 9 cm sterile Petri dishes (amended (SDA) plates). 100 µl of *C. albicans* suspension (OD ~ 1.0) was added to amended (SDA) and the control (un-amended (SDA) plates).

All plates were incubated at 30°C for 48 h. The suppression percentage was calculated as follows: (No. of colonies on un-amended (SDA) plates in cfu/ml - No. of colonies on amended agar plates in cfu/ml)/ No of colonies on un-amended (SDA) plates × 100% (APHA, 1995).

Molecular identification process

This process was carried out at National Research Institute, El-Doky, Cairo, Egypt.

DNA extraction

Total DNA content was extracted from overnight pure culture of the most bioactive marine bacterial isolate using Qiagen DNeasy kit (QIAGEN-Inc., Germany) and Genomic DNA purification kit (Promegal). The procedure was identical to that recommended by the manual instructions. The preparations were analyzed on a 0.7% agarose gel and then determined spectrophotometry (Sambrook et al., 1989).

Polymerase chain reaction (PCR)-amplification and sequencing of 16S rRNA gene

The amplification of the 16S rRNA *gene* was carried out by a PCR technique using the following primers: 5'-TCGAATTCGGATCCAGTITGATCCTGGCTC-OH-3' and 5'-TCGAAITCGGATCCAAGGAGGTGATCCAGCC-OH-3' according to Ausubel et al. (1999).

Nucleotide sequence and the accession number

The 16S rRNA gene of a pure culture of the most bioactive marine bacterial isolate generated in this study was sequenced and compared to the database presented at the GenBank.

Effect of different culture media on antifungal production

The antifungal agent production was carried out using five culture media, nutrient broth (NB) Oxide, king's medium B (Murray et al., 2003), a peptone water medium (PW) (Lab M Limited), it composed of (g/L): peptone (5.0), tryptone (5.0) NaCl (5.0). Two modified NB media were tested; the first composed of (g/L), NB, yeast extract (0.5), $NaNO_3$ (1.0), K_2HPO_4 (1.0), KCl (0.5), and $MgSO_4$ (0.5). While, the second modified medium was composed of (g/L); NB, K_2HPO_4 (11), KH_2PO_4 (5.5), $(NH_4)_2SO_4$ (1.2), $MgSO_4$ (0.4) and $CaCl_2$ (0.15). The pH of all these tested culture media was adjusted at 7.0. Erlenmeyer conical flasks containing 100 ml of each tested culture medium were inoculated with 1 ml (OD = 1.0) of pre-activated *B. pumilus* suspension. Then the flasks were incubated at 30°C for 48 h using a shaker incubator (150 rpm), aliquots of 5 ml of these cultures were taken, centrifuged at 12000 rpm for 20 min and used for measuring their bioactivity.

Optimization of antifungal production using the Plackett-Burman experimental design

Seven independent variables including three medium components; Tryptone, Peptone and NaCl, they were tested in three different concentrations 4, 5 and 6 g/l. In addition, four physiological conditions including; temperatures (25, 30 and 45°C), pH values (6, 7 and 8), inoculum sizes (8.0×10^6, 7.3×10^7 and 9.8×10^8 cfu/ml) and incubation periods (24, 48, and 72 h) were also tested. These seven independent variables were screened in nine combinations organized according to the Plackett-Burman design matrix. For each variable, a high (+) and low (-) level was tested. All trials were performed in triplicates and the averages of observed activities were treated as the response. The main effect of each variable was determined using the following equation:

$$E_{xj} = (\Sigma M_{i+} - \Sigma M_{i-})/N$$

Where, E_{xj} is the variable main effect, ΣM_{i+} and ΣM_{j-} are Σ of suppression percentages of fungal growth in trials where the independent variable (xi) was present in high and low levels, respectively, and N is the number of trials divided by 2. Verification of validity of the optimum medium compared to the basal medium and the Plackett-Burman reverse medium was applied. The *t*-test statistical analysis was performed for equal unpaired samples to determine the variable significance (Bie et al., 2005).

Application of a trickle flow column bioreactor for antifungal production

In this part of work a trickle flow column bioreactor connected with a peristaltic pump was used to perform antifungal agent production. It was composed of a glass column (60 cm long, 5 cm internal diameter and a total workable volume of 500 ml), it was also connected at the top with an air pump having a flow rate of 1.0 L/min through sterilized air filters. The column was separately packed with 2 to 5 g of the tested solid supporters (sponge and Luffa pulp particles). The packed column and its parts were sterilized using an autoclave for 30 min. Then the sterilized packed column was inoculated with 100 to 150 ml of the antifungal producer suspension (8.0×10^6 cfu/ml), and incubated at 25°C till a complete adsorption process of cells was observed. The optical density and pH were continuously detected in the effluent and the efficiency of the system was determined (El-Naggar et al., 2004).

Scanning electron micrograph

Scanning electron micrographs of the used solid supporters (sponge and luffa- pulp) with and without the adsorbed bacterial cells were captured at the Electron Microscopy Center, Faculty of Science; Alexandria University, Egypt.

Purification of antifungal agent using thin layer chromatography

Preparation of the plates

Glass plates (20 × 20 cm) were cleaned and air dried. About 40 g of silica gel G60 were suspended in 100ml of distilled water. The suspension was spread over the plates with a thickness of 0.1cm. The plates left for air dryness then activated by heating at 120°C for one hour. The R_f of the active components was determined using 100ml of different solvent systems preformed in vol/vol as follows: benzene:chloroform, 50:50; benzene:acetone, 90:10; benzene: ethyl-format: formic acid, 75:24:1; toluene: ethyl-format: formic acid, 50:40:10; toluene: chloroform:acetone, 40:25:35; chloroform: methanol, 50:50; dichloromethane : methyl alcohol: water, 50:30:20; dichloromethane: methanol: water, 65:32:3.

The obtained spots were separately scratched and dissolved in dimethylsulfooxide. Then Silica gel was removed by centrifugation at 5000 rpm for 15 min and then the antifungal activity of each extract against the tested pathogenic fungi was estimated as

mentioned before.

Bio-toxicity of the purified antifungal agent

The toxicity bioassay was carried out according to Meyer et al. (1982) using 24 h old neuplii of *Artemia salina* as a biomarker. Different concentrations of the purified agent (100, 200, 1000, 1500, 2500 and 5000 ppm) were made and distributed separately in triplicate using clean and dry glass vials (20 ml) then completed to a total volume of 10 ml/each using sterile seawater. Ten live neuplii of *A. salina* were transferred to each vial. The number of the viable biomarker was counted after 24 h of application. The mortality percentages and the half lethal concentration (LC_{50}) were determined using the probit analysis method (Reish et al., 1987).

A Comparison between the antifungal activity of the purified agent and some commercial antifungal drugs

The bioactivity of 50 ppm the purified antifungal component in comparison to the bioactivity of 500 ppm/each of three commercial antifungal drugs based on the active fluconazole compound (Diflucan, Flocoral and Fungimycin) was estimated using *A. niger, F. solani, P. oxilacum, R. solani* and *C. albicans*. The suppression growth % of the pathogenic fungi was detected compared to the control (untreated fungi).

Partial chemical characterization of the purified antifungal agent

The UV/Visible spectrum of the most active spot was determined using spectrophotometer UV-vis./Jenway 6800 and the dimethyl-sulfooxide solvent as a blank. The infrared spectrum of the antifungal agent was carried out using a Peak Find-Memory-27 spectrophotometer at the Microanalysis Center, Cairo University, Egypt. The Mass spectrum of the antifungal agent was subjected using DI Analysis Shimadzu Qp-2010plus/mass spectrophotometer at the Microanalysis Center, Cairo University, Egypt.

RESULTS

Isolation and molecular identification of the most potent marine antifungal producer

A preliminary analysis for antifungal activity of different marine bacterial strains isolated from the eastern harbor and the western harbor of Alexandria, Egypt was conducted using some plant and human pathogenic fungi. All strains were grown on nutrient broth medium and then screened to select the most potent marine bacterial isolate acting against at least three pathogenic fungi with an activity >30%. It was showed that the marine bacterial isolate coded MS12 was the most potent isolate acting against the tested plant and human pathogenic fungi, *F. solani, R. solani* and *C. albicans*, the antifungal activities were 50, 43 and 32%, respectively.

The obtained amplified PCR fragment (835 bp) was purified and detected using agarose gel electrophoresis. Then the obtained amplified 16S rRNA was sequenced and compared with the data presented in the Genbank using Blast search program. It was found that the bacterial isolate MS12 had a new genomic sequence which indicate the isolation of a new strain of *Bacillus pumilus*, it genetically identified as *Bacillus pumilus*SMH101 with a new association No.KF964031.

Effect of different culture media

Five different culture media were examined to obtain the highest antifungal activity from *B. pumilus*SMH101. It was found that the peptone water medium was the more effective medium tested compared to others. The percentage of the antifungal activity against *F. solani*, *R. solani* and *C. albicans* was 52.5, 47.1 and 34.9, respectively, (Table 1).

Application of Plackett-Burman design

The components *of B. pumilus*SMH101 culture medium in addition to the physiological conditions were optimized for a maximum antifungal activity against *F. solani, R. solani* and *C. albicans*; it was carried out using the Plackett-Burman experimental design (Table 2).

The main effect of the tested variable was presented as the difference between the fungal suppression % averages at both the high level (+) and the low level (-) of the examined variable (Figure 1). The obtained data of the main effect as well as the *t*-test values showed the physiological conditions; inoculum size, temperature and the incubation period must be adjusted at their low levels (8.0×10^6cfu/ml, 25°C and 24 h, respectively) to obtain more antifungal activities against *F. solani, R. solani* and *C. albicans*. Moreover, the inhibition of both *F. solani* and *R. solani* was maximized on the addition of the high level (6 g/L) of tryptone, while, the inhibition of *C. albicans* was maximized on adding the high level (6 g/L) of both peptone and NaCl.

Verification of Plackett-Burman experiment

In order to validate the obtained results and to evaluate the accuracy of the applied Plackett-Burman statistical design, a verification experiment was carried out in triplicates to predict the near optimum levels of independent variables. The data were examined and compared to the basal and anti-optimized medium. It was revealed that the average antifungal activity against *F. solani, R. solani* and *C. albicans* by *B. pumilus*SMH101 was increased by 1.5, 1.5 and 1.9 fold, respectively, when grow on the optimized medium (data not shown). The results indicated for inhibiting both *F. solani* and *R. solani* the

Table 1. The effect of different culture media on the antifungal activity (%) of *B. pumilus*MSH101 using some plant and human pathogenic fungi as indicators.

Culture Medium	[a]Antifungal activity (%) against		
	F. solani	***R. solani***	***C. albicans***
Nutrient broth (NB)	50	43	32
King's medium	38.0	29.8	18.0
Peptone Water	52.5	47.1	34.9
Modified-NB-1	41.1	38.7	23.0
Modified-NB-2	39.8	37.2	22.7

[a]Antifungal activity (%) = $\frac{\text{Control fungal diameter (mm) - Treated fungal diameter (mm)}}{\text{Control fungal diameter (mm)}} \times 100$

Table 2. The optimization of the antifungal production by *B. pumilus*SMH101 using the Plackett-Burman experimental design and its antifungal activities against some plant and human pathogenic fungi.

Trial no.	Independent variables 1							[a]Antifungal activity (%)		
	I. P (Days)	**I. S (ml)**	**Temp. (°C)**	**pH value**	**NaCl (gl^{-1})**	**Pep. (gL^{-1})**	**Try. (gL^{-1})**	***F. solani***	***R. solani***	***C. albicans***
1	-1	-1	-1	+1	+1	+1	-1	2.8	1.8	2.1
2	+1	-1	-1	-1	-1	+1	+1	64.3	58.6	36.2
3	-1	+1	-1	-1	+1	-1	+1	44.7	42.1	23.8
4	+1	+1	-1	+1	-1	-1	-1	54.3	50.6	27.5
5	-1	-1	+1	+1	-1	-1	+1	52.9	50.4	33.5
6	+1	-1	+1	-1	+1	-1	-1	34.2	29.4	22.8
7	-1	+1	+1	-1	-1	+1	-1	44.5	42.3	19.0
8	+1	+1	+1	+1	+11	+1	+1	2.9	2.4	2.3
9	0	0	0	0	0	0	0	52.5	47.2	34.9

[a]Antifungal activity (%) = $\frac{\text{Control fungal diameter (mm) - Treated fungal diameter (mm)} \times 100}{\text{Control fungal diameter (mm)}}$

culture should be formulated as follows (g/L): tryptone (6), peptone (5), NaCl (5), in addition, pH (7.0), inoculum size (8.0×10^6 cfu/ml), temperature (25°C) and incubation period of 24 h. While, for inhibiting *C. albicans* the formula should be as follows: (g/l): tryptone (5), peptone (6), NaCl (6), in addition, pH (8.0), inoculum size (8.0×10^6cfu/ml), temperature (25°C) and incubation period of 24 h.

Application of trickle flow column

A trickle flow column bioreactor connected with a peristaltic pump was used to perform antifungal agent production. The column was separately packed of the tested solid supporters (sponge and luffa pulp particles). At the end of the incubation period, the antifungal activity was determined and compared to free cells of *B. pumilus*SMH101. The adsorbed cells on sponge particles led to antifungal activities of 50.9, 45.4 and 29.2% against *F. solani*, *R. solani* and *C. albicans*, respectively. The adsorbed cells on luffa pulp particles led to antifungal activities of 47.2, 42.2 and 23.8%, respectively, (Table 3). Moreover, the development of the bacterial biofilms on these used solid supporters was investigated using scanning electron microscopy (Figure 2).

Bio-toxicity of the *B. pumilus*SMH101 purified extract using *A. salina* as a biomarker

The bio-toxicity of different concentrations (from 100 to 5000 ppm) of *B. pumilus*SMH101 purified extract were estimated using *A. salina* as a biomarker, then the mortality percent was calculated and presented using the probit analysis method. The results indicated the purified antifungal agent had a relatively low toxicity level (Table 4). The LC_{50} (the concentration at which 50% of the tested biomarker individuals die) of this agent was 1072 ppm and it was estimated from the best fit line obtained on using the probit analysis method.

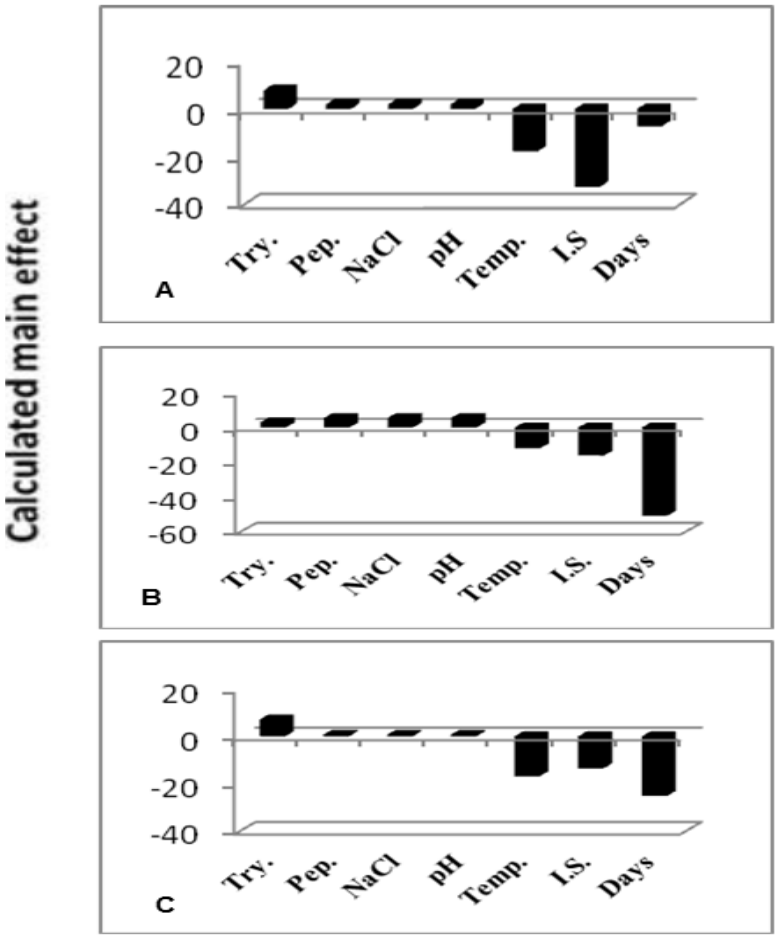

Figure 1. Elucidation of different cultured factors affecting the production of antifungal agent by *B. pumilus*SMH101 against *F. solani* (A), *C. albicans* (B) and *R. solani* (C), using the Placket Burman experimental design. Try. = tryptone, Pep = peptone, Temp. = temperature,

A comparison of the antifungal activity of the partially purified agent of *B. pumilus*SMH101 to some commercial antifungal drugs

The bioactivity of 50 ppm purified antifungal agent of the marine *B. pumilus*SMH101 was separately compared to 500 ppm of each commercial antifungal drug; this concentration is tenfold more than that of the produced antifungal agent of the marine *B. pumilus*SMH101. The data presented in Table 5 showed a broad antifungal spectrum of the purified agent of *B. pumilus*SMH101 with an average activity of 60% against the five tested human and plant pathogenic fungi compared to the tested commercial drugs based on the active fluconazole compound; Diflucan, Flocoral and Fungican; the obtained average inhibition percentage was 48, 49 and 54%, respectively. Moreover, the most inhibited fungus was *F. solani* (87%) followed by *R. solani* (80%) and *C. albicans* (70%) compared to the tested antifungal drugs; the average inhibition percent was 60, 55 and 70%, respectively. Moreover, the photographs in Figure (3) showed the inhibition percent obtained by the purified marine *B. pumilus*SMH101 agent (Figure 3-B) compared to the untreated fungi (control) (Figure 3-A).

Partial Characterization of the *B. pumilus*SMH101 purified antifungal agent

The chemical characterization presented in Figure (4) showed the UV-Vis, infra-red (IR), and Mass spectra of the purified antifungal agent. The UV spectrum of the compound (Figure 4-A) resulted in a single peak appeared at λ_{280}nm which proved the aromatic character of the compound. The IR spectrum showed seven absorption bands (Figure 4-B) the major five bands were

Table 3. Antifungal activity (%) of the adsorbed *B. pumilus*SMH101 cells against *F. solani*, *R. solani* and *C. albicans* using a glass trickle flow column and the optimized culture medium.

Supporter Packing material	[a]Antifungal activity (%) against			Average
	F. solani	*R. solani*	*C. albicans*	
Control (Free cells)	69.2	62.5	62.5	58.6
Sponge	50.9	45.4	29.2	41.8
Lufa pulp	47.2	42.3	23.8	37.8

[a]Antifungal activity (%) = Control fungal diameter (mm) - treated fungal diameter (mm) × 100
Control fungal diameter (mm).

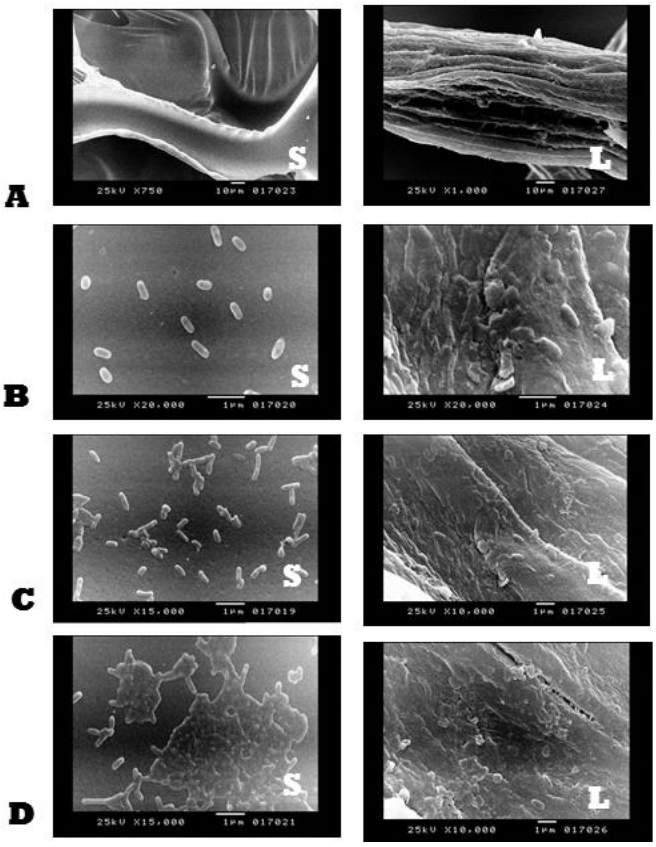

Figure 2. Micrographs show the development of the adsorbed B. pumilusSMH101 biofilm on sponge (S) and luffa pulp (L) as packing supporters of the used glass trickle flow column after 2 days (B), 4 days (C), and 6 days (D) compared to the un-inoculated supporters (control) (A).

explained as follows; the first band appeared at 3441 cm which indicated the presence of NH_2, OH or NH groups, the second band appeared at 2966.95 cm which indicated the presence of the aromatic C-H group, the third band appeared at 1638.23 cm which indicated the at 1457.92 cm which indicated the presence of aromatic ring in the compound.

The last band appeared at 1051.01 cm which indicated the presence of the ether linkage in this compound. Moreover, the obtained Mass spectrum of this compound (Figure 4-C) showed the appearance of a molecular ion peak at m/e = 888.40, while, the base ion peak was appeared at m/e =132.10. The molecular weight of this compound showed to be 875Da.

Table 4. The bio-toxicity of different concentrations of the purified antifungal agent of B. pumilusSMH101 using Artemia salina as a biomarker.

[a]concentration (ppm)	Log concentration	Mortality % after 24 h
100	2.00	10
500	2.7	18
1000	3.00	27
1500	3.18	57
2500	3.40	80
5000	3.70	97

[a]The value of LC50 was detected using the probit analysis, it was 1072 ppm.

DISCUSSION

Genus *Bacillus* has a long and distinguished history in the field of biotechnology. Since, members of this genus are used for the synthesis of a very wide range of important medical, agriculture, pharmaceutical and other industrial products including antibiotics, bacteriocins, enzymes, amino acids, sugars, surfactants and flavor enhancers (Anthony et al., 2009; Bhaskar et al., 2007; Lisboa et al., 2006; Parvathi et al., 2009; Ying et al., 2005).

Table 5. The antifungal activity (%) of the partially purified agent of *B. pumilus*SMH101 (0.05 mg/ml) compared to the antifungal activity (%) of some commercial antifungal drugs based on the active fluconazole compound (0.5 mg/ml) using different plant and human pathogenic fungi.

Types of antifungal agent	*Antifungal activity (%)					
	F. solani	*R. solani*	*C. albicans*	*A. niger*	*P. oxalaticum*	61
B. pumilus agent	87	80	70	23	44	48
Diflucan	55	51	78	26	28	49
Flucoral	57	52	80	27	29	51.8
Fungican	70	64	65	25	35	

*Antifungal activity (%) = $\frac{\text{Control fungal diameter (mm) - treated fungal diameter (mm)}}{\text{Control fungal diameter (mm)}} \times 100$

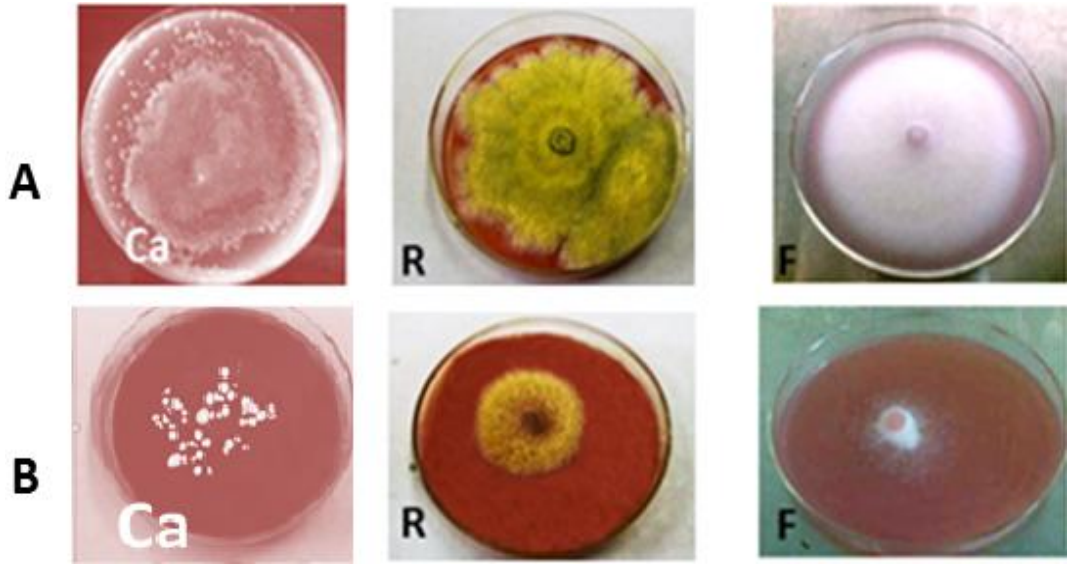

Figure 3. Photographs show the antifungal activities of the purified marine *B. pumilus*SMH101 agent (B) against *C. albicans* (Ca), *R. solani* (R) and *F. solani* (F) compared to the untreated fungi (control) (A).

Statistical experimental designs are powerful tools for an economic and a rapid search of the key factors from a multivariable system. It minimizes the error in determining the effect of these variables on the growth of the used microorganisms (Abou-Elela et al*.,* 2009; Xiong et al*.,* 2007). So, Plackett-Burman experimental design was used to optimize the components of the culture medium and reflect the relative importance of various environmental factors on the production of the antifungal agent by *B. pumilus*SMH101 in liquid cultures. It was found the optimum physiological conditions for antifungal agent production by *B. pumilus*SMH101 were achieved on applying the tested low levels; the temperature was 25°C, the inoculum size was 8.0×10^6 cfu/ml and the incubation period was 24 h, regardless the target pathogen. While the optimum culture components showed insignificant effect on the production of the antifungal agent by *B. pumilus*SMH101 and they varied according to the pathogenic target (Figure 1 and Table 2).

Cell immobilization shows many operational and economic advantages such as prolong metabolic activities, reuse of the biocatalyst, increase of cell concentration in preventing washing out of cells at high flow rates (Gautam et al*.,* 2002). However, the results obtained on using immobilized *B. pumilus*SMH101 cells (absorbed bacterial cells on solid supporters; luffa pulp and sponge) showed low antifungal activities against *F. solani*, *R. solani* and *C. albicans*. The average activity of the adsorbed cells was 37.8 and 41.8%, respectively, compared with the free bacterial cells of *B. pumilus*SMH101 58.6% (Table 3). Similar results were obtained by Freeman and Aharonowitz (1981) who developed a mild new method for the immobilization of the whole microbial cells. They found the yield of cepha losporins antibiotic

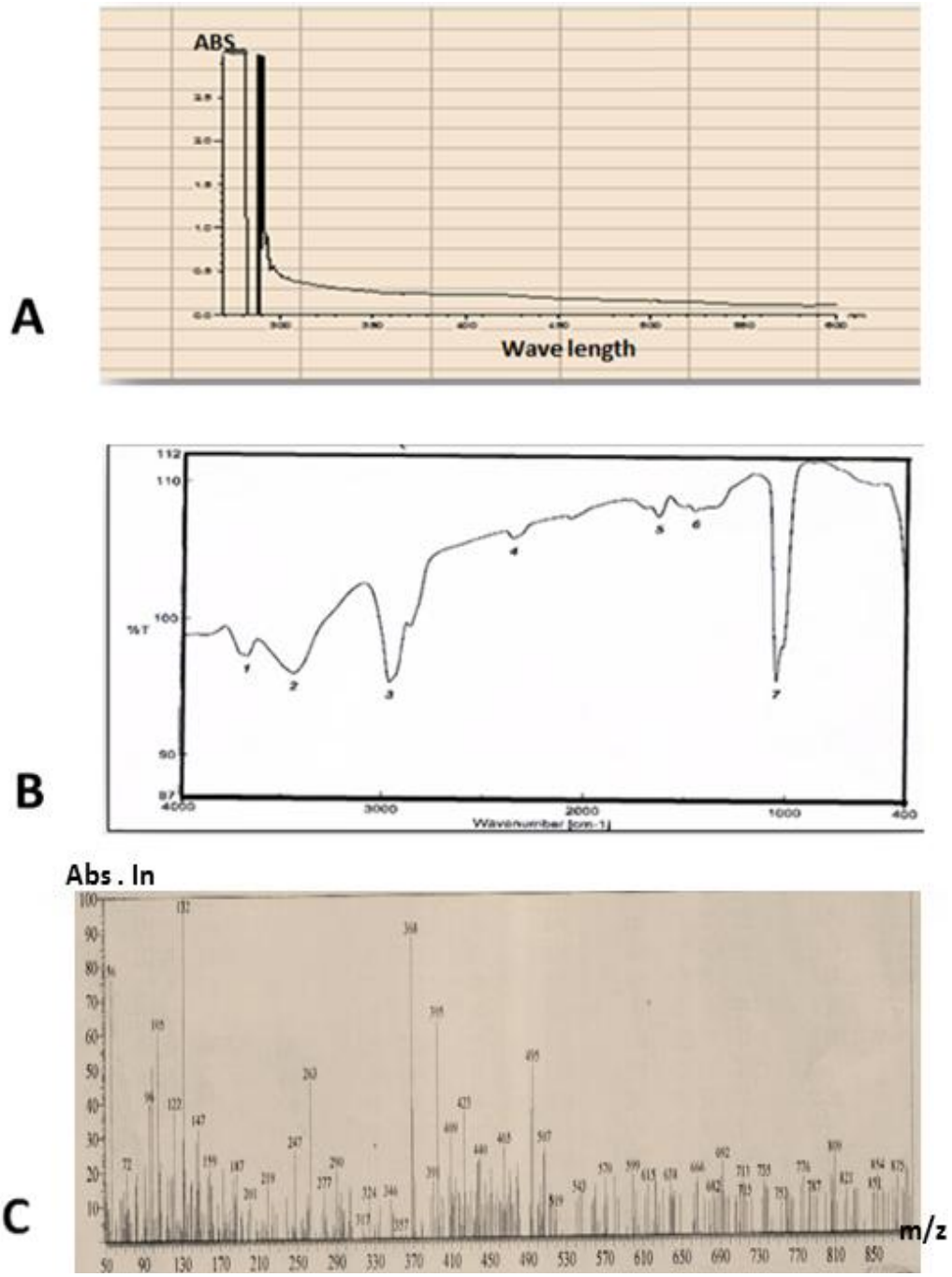

Figure 4. Some chemical characterization of the antifungal active component produced by *B. pumilus*SMH101 using dimethylsulfooxide (DMSO) as a blank. UV/Vis. Spectrum (A), IR spectrum (B) and Ms Spectrum (C).

production by the immobilized bac-terial cells using acrylamide monomers (direct poly-merization method) was significantly decreased com-pared to the free cells of *Streptomyces clavuligerus.* Moreover, Punita et al. (2007) used Immobilized *Acremonium chrysogenum* (mold) cells for cephalo-sporin-C production. It was found that cell growth rate of immobilized cells was reduced with about 39% of the growth rate of free cells. Contrary, Srinivasulu et al. (2003) studied the immobilization effect of *Streptomyces marinensis*NUV-5 using calcium alginate

for the production of neomycin. They reported the antibiotic productivity was enhanced with 32% on the use of the immobilized cells over the use of the conventional free-cell.

The fungicidal activity of another *B. pumilus* strain was investigated by Bottone and Peluso (2003). They found that a compound produced by *B. pumilus* inhibits *Mucor* and *Aspergillus* species through the inhibition of spore germination and aborted elongating hyphae. The molecular mass of this compound was determined by diffusion through dialysis membrane to be 500 to 3000 Da. These findings were agreed with the results obtained in this study; the photographs of the purified antifungal agent indicated the fungicidal action against *F. solani* and *R. solani* was carried out through inhibiting the hyphae elongation (Figure 3), the bioactivity was 87 and 80%, respectively, compared to the control (Table 5). Also, the determined molecular weight (875 Da) of this antifungal agent through MS-spectrometry was located between 500 and 3000 Da. Also, Aunpad and Na-Bangchang (2007) isolated *B. pumilus*WAPB4, it showed a remarkable antibacterial activity against methicillin-resistant *Staphylococcus aureus*MRSA, vancomycin-resistant *Enterococcus faecalis*VRE, and several Gram-positive bacteria. This Bacteriocin was designated as pumilicin-4 with a molecular mass of 1994.62 Da using a mass spectrometry.

Moreover, the obtained *B. pumilus*SMH101 antifungal agent found to have an antagonistic action against the tested *C. albicans* with a bioactivity of 70% compared to the control (Figure 3). Contrarily, Guo et al. (2009) used thymol (THY) which was found to have *in vitro* antifungal activity against 24 fluconazole (FLC)-resistant and 12 FLC-susceptible clinical isolates of *C. albicans* but no antagonistic action was observed.

In general, the results of the bio-toxicity test of the obtained purified antifungal agent of *B. pumilus*SMH101 showed a relatively low toxicity level where the LC_{50} was 1072 ppm which is much more than the concentration used in the application process of this study (Table 4). Dissimilarly, many authors worked on using *B. pumilus* in order to obtain bioactive secondary metabolites acting against different pathogenic fungi regardless the biotoxicity of these metabolites towards human or plant (Ghasemi et al., 2012; Munimbazi and Bullerman, 1997, 1998; Munimbazi and Bullerman, 1998). On the other hand, Yadav et al. (2007) purified a cytosolic protein from *E. coli* BL21; it demonstrated potent antifungal activity against pathogenic strains of *Aspergillus* species (*A. fumigatus, A. flavus, A. niger*) and *C. albicans* with MIC of 1.95 to 3.98 and 15.62 mg ml^{-1}, respectively, and it showed no cytotoxicity up to 1250 mg ml^{-1} *in vitro* toxicity tests, which is very high concentration compared to the LC_{50} of the antifungal agent of *B. pumilus*SMH101 (Table 4).

Finally, from the results of the comparative study it can concluded this produced safe antifungal agent from the marine *B. pumilus*SMH101 may act as a promising alternative tool for the treatment of some pathogenic fungi; *F. solani*, *R. solani* and *C. albicans* even on using a very low concentration (0.05 mg/ml) (Table 5), this is to face the increasing in the pathogenic fungus resistance towards the used commercial antifungal drugs especially those based on the active fluconazole compound. On the other hand, many investigations will be carried out in order to precisely identify the main active component in this produced antifungal agent.

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Characterization of *Lactobacillus* strains isolated from Algerian children faeces for their probiotic properties

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Lactic acid bacteria termed probiotics have preventive as well as curative effects on several types of diarrhoea of different aetiologies. The main objective of this study was to screen lactobacilli strains having probiotic traits, isolated from Algerian healthy children faeces on the purpose of using them further in children diarrheal illnesses. One hundred and twenty (120) lactic acid bacteria isolates were selected from faecal samples of healthy Algerian children aged between five and ten years. Gram positive rods and catalase negative bacteria (52 isolates) were screened, *in vitro*, for their probiotic potential properties including ability to survive in simulated gastro-intestinal conditions, adherence to Caco-2 cells and their antimicrobial activity. The results show that only five strains resisted in simulated gastric juice at pH 1.5 and pepsin. Four of them were resistant to simulated intestinal conditions at pH 8 and pancreatin and have a good adherence. In the end, three of them were retained as they display interesting probiotic profiles characterized by a strong antimicrobial effect against some intestinal pathogenic bacteria. They were identified by 16S rDNA sequencing as *Lactobacillus plantarum* F12, *Lactobacillus brevis* G6 and *Lactobacillus paracasei* B13.

Key words: Probiotics, *Lactobacillus*, selection criteria, human origin.

INTRODUCTION

The digestive tract of the newborn infant is almost sterile at the time of birth.However, it is rapidly colonized by diverse groups of microorganisms. First**,** the infant digestive tract colonization by vaginal mother lactobacilli flora is realized (Song et al., 1999). Then, this flora is rapidly replaced by a new flora composed of lactobacilli and other microorganisms originating from food and environment (Tannock et al., 1990). Among intestinal biota, lactobacilli and bifidobacteria are the important organisms recognized for their health and nutritional benefits as probiotics (Rosenfeldt et al., 2002; Kesarcodi-Watson et al., 2008). Probiotics are defined as “live microorganisms that when ingested in adequate amounts, confer beneficial effects to the host by improving its intestinal microbial balance (Schrezenmeir and Vrese, 2001). Furthermore, for being selected, the

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probiotic strains must overcomesome barriers and perform some functions including, for example, human origin for Human use, resistance to gastrointestinal conditions such as gastric acidity and bile toxicity, the ability of adherence to the host's epithelial cells, antimicrobial activity against pathogens and the possession of a health-promoting effects on the host (Dunne et al., 2001; FAO/WHO, 2001). Probiotic strains should also have good technological properties (Parvez et al., 2006) and be safe without virulence factors, harmful activities and transmissible antibiotic resistances (Salyers et al., 2004). A number of clinical studies have demonstrated the efficacy of lactic acid bacteria (LAB) probiotics, particularly *Lactobacillus* strains, in the treatment and/or prevention of diarrhoea (Fuller, 1991; Rosenfeldt et al., 2002; Servin, 2004; Nomoto, 2005; Guo et al., 2010). In Algeria, as in some developing countries, despite declining cases, infectious diarrhoeas remain a major cause for infant mortality. This is because of a poor hygiene, a bad nutrition and sometimes a lack access to health structures (UNICEF/WHO, 2009).

According to the joint report of FAO/WHO (2001), probiotics can be an important means to reduce diarrhoeas in developing countries. Though the efficacy of probiotics needs to be demonstrated and controlled by clinical trials, their primary preselecting is based on *in vitro* tests (Dunne et al., 2001; FAO/WHO, 2002). This work aims to collect faeces from Algerian children cohabiting diverse rural areas in Jijel city (Eastern Algeria). The lactic acid bacteria were isolated from these samples, and screened for their probiotic traits, in order to use them further in child nutrition, to prevent diarrhoeas. The characterization of the screened bacteria was based on the study of several criteria, that is, the tolerance to simulated human gastric and intestinal juice, the adherence to Caco-2 cells and antimicrobial activity against some intestinal pathogens. The choice of different remote rural areas of Jijel city to collect faeces samples is not trivial; the objective was to target a diversity of strains based on the variability of natural food bowls.

MATERIALS AND METHODS

Isolation and identification of lactic acid bacteria

In this study, the samples were recovered twice from healthy children faeces (five to ten years old). One gram of each faecal sample was mixed with nine ml of 0.9% of sterile peptone water, and decimal dilutions were prepared. Then, 0.1 ml of appropriate dilutions was spread on Man Rogosa Sharp (MRS) agar. After 48 to 72 h of plate incubation, the obtained bacterial colonies were purified several times on MRS agar. Preliminary identification of the isolated bacteria was carried out using microscopic observation, Gram staining, catalase reaction and API 50 CHL test strip (Biomerieux, France).

Survival ability under gastric conditions

The ability of bacteria to survive under gastric conditions was carried out according to (Huang and Adams, 2004) with slight modification. The simulated gastric juice was prepared (3 g/L of pepsin (EC 3.4.23.1, 7190 Merck) in 0.5% (w/v) NaCl), adjusted to pH1.5. Overnight cultures were prepared in MRS medium and 1 ml of each preculture strain was added to 9 ml of a simulated gastric juice and incubated at 37°C. One millilitre of culture was sampled at 0 and 2 h of incubation. The viable bacteria were determined by plating 100 µl of appropriate dilutions on MRS agar. The experiment was replicated three times.

Survival ability under intestinal conditions

The capacity of bacteria for surviving under intestinal conditions was carried out by using, simulated small intestinal juices (1 g/L pancreatin (EC 232-468-9, P7545 Sigma-Aldrich) in 0.5% NaCl), with and without 0.3% bile salts (Ox-gall, Sigma), and adjusted to pH 8. The total viable bacteria were determined, at 0 and 4h of incubation, as mentioned above (Huang and Adams, 2004).

In vitro adherence assay to Caco-2 cells

The ability of the strains to adhere to human cells was investigated in the Caco-2 intestinal cells following the procedure described by Sugimura et al. (2011) modified by Ren et al. (2012). Monolayers Caco-2 cells were grown in Dulbecco's Modified Eagle's Medium DMEM (Lonza BE12- 604F) supplemented with 10% heat-inactivated foetal bovine serum (Gibco 10270-106), penicillin (100 U ml^{-1}; Lonza) and streptomycin (100 mg ml^{-1}; Lonza), in 24-well (Cellstar 662- 160) tissue culture plates, at a concentration of 1.0 x 10^5 cells ml^{-1}. The medium of Cells were replaced by fresh unsupplemented DMEM for 1h prior to the adhesion assay and then rinsed three times with DMEM. The cells were incubated with approximately 10^9 CFU $ml^{-1}$of LAB isolates. After 2 h of incubation at 37°C under 5% CO_2 atmosphere, the monolayers were washed three times with sterile phosphate buffer saline (pH 7.4). The adherent bacteria were detached by repeated pipetting. The counting of adherent LAB cells was done by performing a series of dilutions and plating on MRS agar. The assay was repeated three times for every strain; counts were performed in duplicates. The percentage of adhesion (CFU/100 cells) was estimated using the formula (the adhered LAB cells/the Caco-2 cells x 100%). On the other hand, the adherent bacteria to Caco-2 cells were fixed with methanol for 30 min, Gram stained (0.5% crystal violet) and observed by microscopic examination under oil immersion (x 100 magnification).

Antimicrobial activity

The capacity of the strains for inhibiting a representative group of intestinal pathogens was determined using the well diffusion method described by Hechard et al. (1990).The tested pathogens were *Escherichia coli* O111*, Escherichia coli* O55*, Salmonella typhimurium*, *Listeria monocytogenes*, *Bacillus cereus* and *Escherichia coli* ATCC 29522. Twenty millilitres of MRS agar at 45°C were vigorously mixed with 200 µl of an overnight culture of the indicator strain and poured into Petri dishes. Supernatants were prepared by centrifugation of the culture at 8000 x *g* for 10 min, filter sterilized through a 0.22 µm pore membrane (Sartorius, Germany), added to a sterile solution of catalase (1000 U ml^{-1}, Sigma) to eliminate the possible inhibition effect of hydrogen peroxide and neutralized to pH 6.5 with NaOH mol^{-1}. Wells were made and 50 µl of overnight cultures and cell freesupernatants were placed in each well. Experiments were performed in three independent experiments, and each assay was performed in triplicate.

Table 1. Survival ability of lactic acid bacteriaisolates in simulated gastric conditions.

Lactobacillus strain	Code	Viable count (log CFU/ml) at time 0 and 2 h			
		Simulated gastric juice	0 h	2 h	Survival cells (%)
L. paracasei subsp *paracasei*	A20	pH 1.5	8.45 ± 0.04	3.49 ± 0.03	41.30
		pH 1.5 and pepsin		2.01 ± 0.13	23.78
L. plantarum	F12	pH 1.5	9.65 ± 0.11	6.81 ± 0.01	70.56
		pH 1.5 and pepsin		5.92 ± 0.07	61.34
L. paracasei	B13	pH 1.5	8.77 ± 0.02	5.56 ± 0.03	63.39
		pH 1.5 and pepsin		4.79 ± 0.01	54.61
L. brevis	G6	pH 1.5	7.89 ± 0.00	4.75 ± 0.04	60.20
		pH 1.5 and pepsin		3.75 ± 0.02	47.52
L. fermentum	H2	pH 1.5	8.80 ± 0.13	5.35 ± 0.32	60.79
		pH 1.5 and pepsin		4.23 ± 0.04	48.06
L. acidophilus	8*	pH 1.5	8.37 ± 0.03	2.04 ± 0.01	24.37
		pH 1.5 and pepsin		<1	/
Lactobacillus spp. (9 strains)		Low growth at pH 1.5			
Lactobacillus spp. (37 strains)		No growth at pH 1.5			

Log CFU ml^{-1} values express the mean ± standard deviation; each data point is the average of repeated measurements from 03 independently replicated experiments, n = 3. P <0.05.

Identification of the potent probiotic strains by 16S rDNA analysis

The strains showing the best probiotic profiles were identified by the 16S rDNA analysis as described by Diop et al., (2008). Genomic DNA from MRS broth cultures were extracted using the Wizard® genomic DNA purification kit (Promega, Madison, USA), and used as a template for the amplification of 16S rRNA genes by the polymerase chain reaction (PCR). The primer pair 16SPO and 16SP6 was used. PCR products were resolved by electrophoresis in 1% (w/v) agarose gels and visualized by ethidium bromide staining and purified with Microcon YM-100 kit (Bedford, MA, USA). The BigDye Terminator sequence was performed using the Vector NTI (Version 8) software package (BD Biosciences, San Jose, USA). Sequences were BLAST in GenBank database (www.ncbi.nlm.nih.gov) for species assignment.

Statistical analysis

The data were calculated with mean values, and standard deviations (mean ± SD) were determined from triplicate trials. Statistical significance of the results was evaluated by ANOVA (analysis of variance). Statistical significance was attributed to (P<0.05).

RESULTS

Isolation and identification of lactic acid bacteria strains

One hundred and twenty (120) of LAB were selected and purified from seven samples collected twice from children faeces. Fifty two strains of them were retained for further work. They were rods, Gram positive, catalase negative and the fermentation profile obtained by the API50 CHLsystem, showed that they belonged to the *Lactobacillus genus* (data no shown). They were closely related to *L. fermentum* (10 strains), *L. casei* (eight strains), *L.gasseri* (eight strains), *L. salivarius* (five strains), *L. plantarum* (five strains), *L. vaginalis* (four strains) *L.paracasei* subsp*paracasei* (four strains), *L. brevis* (two strains), *L. reuteri* (two strains), *L. acidophilus* (2 strains) and *L. ruminis* (two strains).

Survival ability under gastric conditions

In this study, the effect of pH 1.5 was investigated with and without pepsin (0.3%) to test the ability of LAB to survive in simulated conditions of gastric juice. The results showed that pH 1.5 without pepsin, was critical forthe majority of isolates after 2 h of incubation. In fact, thirty seven isolates among the fifty two sited above, weretotally inhibited and nine manifested a low rate of growth. However, only six isolates were characterized by a good viability in low pH conditions (pH 1.5). The tolerance to simulated gastric conditions test (pepsin, pH 1.5) showed that five isolates of them supported such conditions with different degrees of viability. The highest survival percentage was observed with *L. plantarum* F12 (61.34%), followed by*L. paracasei* B13 (54.61%), *L. fermentum* H2 (48.06%), *L. brevis* G6 (47.52%) and *L. paracasei* subsp *paracasei* A20 (23.78%) (Table 1). The addition of pepsin to acid conditions causes a significant loss in isolates viability compared to the low acidity alone.

Survival ability under intestinal conditions

The most resistant LAB to simulated gastric conditions (F12, B13, H2, A20 and G6), obtained in the previous test, were assayed for their ability to survive in simulated intestinal conditions at pH 8, in the presence of pancreatin, with and without 0.3% of bile. The obtained results showed a decrease in growth of all tested LAB, depending on the strain, in comparison with their culture in the same conditions but without bile (Table 2). Four LAB tested had a

Table 2. Survival ability of lactic acid bacteria isolates in simulated intestinal conditions.

Strain	Code	Viable count (log CFU ml^{-1})			
		Absence of bile		Presence of 0.3% of bile	
		0 h	4 h	0 h	4 h
L. paracasei subsp. *paracasei*	A20	8.35±0.02	7.43±0.10	8.35±0.03	2.90±0.12
L. plantarum	F12	9.60±0.06	9.19±0.00	9.60±0.03	9.56±0.06
L. paracasei	B13	8.83±0.04	9.65±0.32	8.83±0.12	7.01±0.05
L. brevis	G6	7.91±0.09	7.97±0.08	7.91±0.10	6.89±0.00
L. fermentum	H2	8.82±0.01	6.54±0.02	8.82±0.07	<1

Log CFU ml^{-1} values express the mean ± standard deviation; each data point is the average of repeated measurements from 03 independently replicated experiments, "n = 3. P < 0.05.

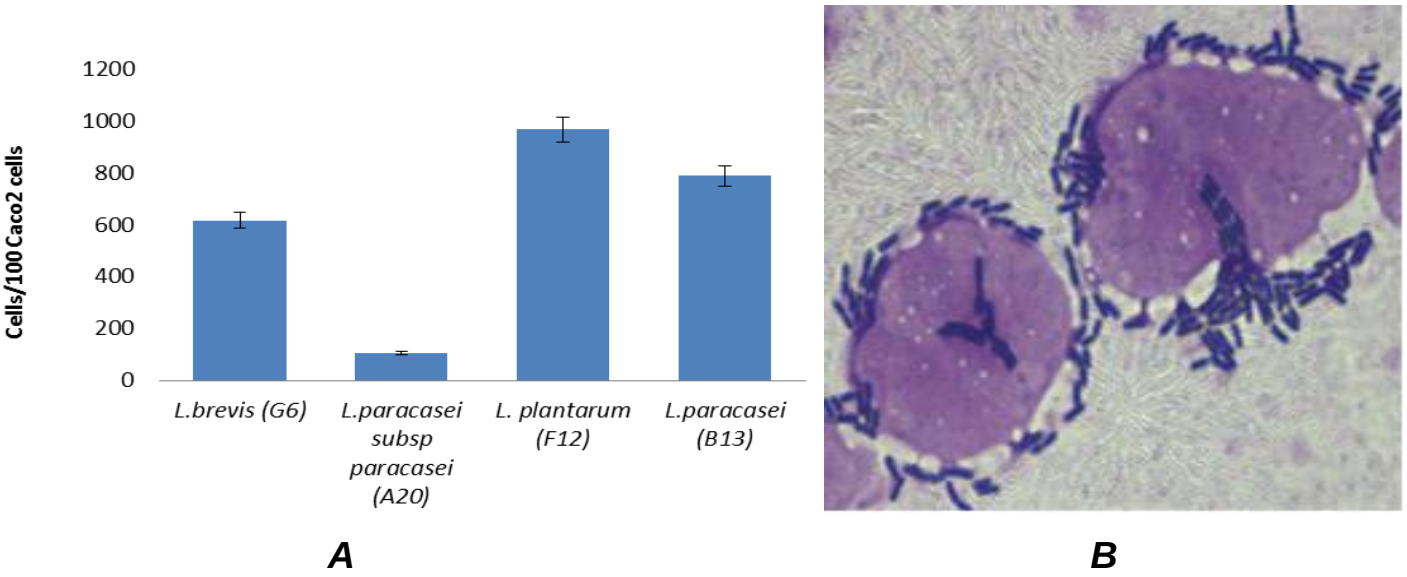

Figure 1. (A) Adhesion of Lactobacillus strains to Caco-2 cells after 2 h of incubation at 37°C in a 5% CO_2 atmosphere. **(B)** Adhesion image of L.plantarum (F12) to Caco-2 cells observed by microscope (x 100) after staining cells with crystal violet (0.5%). Values (cells/100 Caco-2 cells) express the mean ± standard deviation; each data point is the average of repeated measurements from 3 independently replicated experiments, "n = 3. P< 0.05.

good tolerance to simulated intestinal juice after 4 h of exposure. *L. plantarum* F12 was the most resistant strain, followed by *L. paracasei* B13, *L. brevis* G6 and *L. paracasei* subsp *paracasei* A20. The strain *L. fermentum* H2 was completely inhibited.

In vitro adherence assay to Caco-2 cells

L. plantarum F12 *L. paracasei* B13, *L. brevis* G6 and *L. paracasei* subsp *paracasei* A20 were tested for their adherence to Caco-2 cells as they displayed high tolerance rates tosimulated gastro-intestinal juice. The results showed a difference in the abilities of this LAB to adhere to Caco-2 cells. However, 03 of them exhibited a good adherence. These were: *L. paracasei* B13, *L. brevis* G6, and particularly, F12 *L. plantarum* strain which presented the highest adherenceto Caco-2 cells (Figure 1).

Antimicrobial activity

The strains *L. paracasei* (B13), *L. brevis* (G6) and *L. plantarum* (F12) which exhibited a good adherence to Coco-2 cells were tested for their antimicrobial activity.In this study, the overnight cultures of the three tested strains demonstrated a strong antagonist activity against pathogens but their neutralized supernatants at pH 6.5 and treated with catalase (NSC) were unable to inhibit any pathogenic Gram-negative microbe. Moreover, the NSC of three LAB isolates had a good antimicrobial activity against Gram-positive bacteria (Table 3).

Identification of the potential probiotic strains by 16S rDNA sequencing

Based on the results of our study, three strains were retained as they displayed a high potential probiotic profile. Their identification by API CH 50 system was confirmed by 16S rDNA sequencing. BLAST analysis of their corresponding 16S rDNA sequences, in GenBank database, showed that they were closely related to *L. plantarum* (F12), *L. brevis* (G6) and *L.paracasei* (B13) (Table 4).

Table 3. Antimicrobial activity of lactic acid bacteriaisolates against bacterial pathogens.

Indicator bacteria	*L. plantarum* F12		*L. brevis* G6		*L. paracasei* B13	
	Overnight	Supernatant	Overnight	Supernatant	Overnight	Supernatant
Escherichia coli ATCC 29522	++	–	++	–	+++	–
Escherichia coli O11	+++	–	+	–	+++	–
Escherichia coli O51	+++	–	++	–	+++	–
Salmonella typhimurium	++	–	++	–	+++	–
Listeria monocytogenes	+++	++	+++	+++	+++	++
Bacillus cereus	++	++	++	++	+++	++

All indicators bacteria were tested for inhibition of growth microbes' growth inhibition . Tested overnight cultures and supernatants were treated with catalase and neutralized to pH 6.5. Pathogen growth inhibition was determined by measuring inhibition zones . No inhibition (–); diameter between 0 and 3mm (weak, +); diameter between 3 and 6 mm (good,++) and diameter larger than 6 mm (strong,+++). Each data point is the average of repeated measurements from 3 independently replicated experiments, n = 3. P< 0.05.

Table 4. Comparison between API and 16S rDNA identification of the potential probiotic strains.

Isolate	API CHL identification	Percent of identification	Identification by 16S rDNA		
			Identification	Sequence number access	Percent of match
CWBI\B-1515 (B13)	*Lactobacillus paracasei*	99.3	*Lactobacillus paracasei*	JQ436724	98% *L.paracasei* DQ462440*
CWBI/B-1542 (G6)	*Lactobacillus brevis*	91.3	*Lactobacillus brevis*	JQ436725	98% *L.brevis* APO12167*
CWBI/B-1543 (F12)	*Lactobacillus plantarum*	97.2	*Lactobacillus plantarum*	JQ436723	95% *L.plantarum*KF171888*

* Strains from GeneBank used for comparison with our strains.

DISCUSSION

The initial screening of strains using *in vitro* methods remains a useful preliminary step in the detection of probiotic candidates, despite the difficulties encountered to characterize reliable probiotic strains in this way (Campana et al., 2012). In this work, fifty two isolates were selected among one hundred twenty LAB, screened from faecal samples of healthy children aged between five and ten years. The selected bacteria were rods, Gram positive, catalase negative and non-motile. Their identification according to API test revealed its appurtenance to *Lactobacillus* genus. They were tested, then, for their probiotic properties. In fact, human origin, resistance to simulated GI conditions, adherence to Caco-2 cells and antimicrobial activity against pathogens are the *in vitro*, tests suggested for preselction of the probiotic candidates in human use (FAO/WHO, 2002).

During fasting, the stomach pH can drop as low as 1.5 (Draser et al., 1969) and can dramatically affect bacterial growth. In the present study, among fifty two strains only six showed interesting viability at pH1.5 after 2 h of exposure. Previous reports indicated that pH (1.5-2) is the lethal acidity value for LAB isolated from human GI tract (Lankaputhra and Shah, 1995; Khalil et al., 2007; Xiaodong et al., 2009; Kirtzalidou et al., 2011). In the stomach, in addition to low pH, the probiotics are, also, subjected to the stress of proteolytic enzyme, pepsin (Holzapfel et al., 1998). In the current study, the most resistant LAB (6 isolates) to pH 1.5 were tested for their tolerance to simulated gastric juice. At pH 1.5 with 0.3% pepsin, only five isolates were able to survive. The high survival rates to these conditions observed with the strains: *L. plantarum* F12, *L. paracasei* B13, *L. brevis* G6, *L. fermentum* H2 and *L. paracasei* subsp *paracase*i A20. This makes them, may be, able to transit the stomach and to reach the intestinal level.

One of the roles of bile acids is the inhibition of the proliferation of bacteria in the upper part of the digestive system. The mean intestinal bile concentration is believed to be 0.3% w/v, in addition, the transit time is suggested to be 4 h (Parasad et al., 1998). In this study, the most resistant strains (F12, B13, H2, A20 and G6) to simulated gastric juice were tested for their ability to survive in simulated intestinal juice. Four of the tested LAB had a good tolerance to simulated intestinal juice after 4 h of exposure and their growth decreased, in comparison with their culture in the same conditions but without bile. These findings were conforming to those of Haller et al. (2001), Vamanu and Vamanu (2010). The most resistant of them was the strain *L. plantarum* F12 followed by *L. paracasei* B13, *L. brevis* G6 and *L. paracasei* subsp *paracasei* A20. The strain *L. fermentum* H2 was completely inhibited.

The ability of probiotic bacteria to adhere to the intestinal epithelium is prerequisite for probiotic microorganisms

to be effective (Collado et al., 2005; Xiaodong et al., 2009). It was reported that adhered probiotic cells interact with the immunomodulatory cells of mucosal immune system, such as leucocytes, in order to stimulate their phagocytic activity against pathogens (Azacarate-Peril et al., 2009). In our study, four strains were tested for their adherence to Caco-2 cells as they showed high tolerance to simulated gastro-intestinal conditions. There was a significant difference ($P<0.05$) in their ability to adhere to Caco-2 cells, however, three of them exhibited a good adherence. They were *L. paracasei* B13, *L. brevis* G6 and particularly, the strain *L. plantarum* F12 which presented the highest adherence to Caco-2 cells, compared to that of *L. rhamnosus* GG reported by other studies (Thornton, 1996; Ren et al., 2012). The lowest adherence was observed with the strain *L. paracasei* subsp *paracasei* A20. Previous studies concluded that some *Lactobacillus* strains of human origin have good adherence to Caco-2 cells (Dunne et al., 2001; Campana et al., 2012). In our study, the high adherence of the strains B13, G6, and F12to Caco-2 cells suggests their ability to colonize the human intestinal epithelium tissue and to be in competition with pathogens. They were tested for their antagonistic effect against a representtative group of intestinal pathogens bacteria.

Antibacterial activity is vital for the successful colonization of lactobacilli in the intestinal mucosa as they provide a barrier effect and defence against pathogens (Vaughan et al., 1999)**.** According to several studies, the antimicrobial activity of LAB may be due to a number of factors such as decreased pH levels, competition for substrates, production of H_2O_2 and bactericidal or bacteriostatic substances, including bacteriocins (Servin, 2004). In our study, the overnight culture of the strains *L. plantarum* F12, *L. brevis* G6, *L. paracasei* B13 demonstrated a strong antagonist activity against pathogens. Moreover, their supernatants neutralized andtreated with catalase were unable to inhibit any pathogenic Gram-negativetested. This suggests that antagonist activity of our LAB against Gram-negative rods is due to the combined action of low pH, organic acids and H_2O_2 or to one of them.

Moreover, we found that the NSC of the three tested strains has a good antimicrobial activity on Gram-positive bacteria, *Listeria monocytogenes* and *Bacillus cereus.* This is probably due to substances supposed bacteriocins. Our results go in accordance with the data reported by Dortu and Thonart (2009), where the authors reported that no bacteriocin produced by lactic acid bacteria with activity against Gram-negative bacteria has been described.

Owing to the wall structure of Gram negative bacteria, the bacteriocins can't cross the outer membrane to reach the inner membrane, the site of their activity. Similar results were obtained with the strains belonging to *L. paracasei*, *L. plantarum* and *L. brevis* (Vaughan et al., 1999; Tham et al., 2011; Rishi et al., 2011; Ren et al., 2012; Russo et al., 2012).

The identification of probiotic strains by 16S rDNA sequencing was recommended by FAO/WHO (2002). In our study, the identification by API system, of the three potential screened probiotic strains, was confirmed by16S rDNA sequences analysis using BLAST analysis. They were *L. brevis* (G6) *L. paracasei* (B13) and *L. plantarum* (F12).These strains were registered in the GenBank database under access numbers respectively JQ436725, JQ436724 and JQ436723.

Conclusion

The faeces of healthy children living in Algerian rural areas seem to be a good source for isolating potential probiotic strains**.** In this study, we have selected three strains of *Lactobacillus* with high potential probiotic; *L. plantarum, L.brevis and L. paracasei.* All the isolate strains belong to the dominant lactobacilli of intestinal populations of healthy people and are usually recognized for their probiotics traits. Furthermore, their strong antimicrobial activity against the tested intestinal pathogens suggests their use in the prevention of bacterial childhood diarrhoea. However, further investigations, *in vitro,* and, *in vivo,* are needed to study their potential health benefits and their technological properties.

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Short Communication

Unique mutations existing in open reading frame 7 (*ORF7*) of non-porcine transmissible gastroenteritis virus (TGEV) strains isolated in China

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Open reading frame (*ORF7*) of transmissible gastroenteritis virus (TGEV) plays important functions in regulating virus virulence. In this study, the *ORF7s* were cloned from non-porcine TGEV isolates (HYM-09, HYM-09-1 and HYM-09-2), respectively. Sequence analyses showed that there existed some new unique mutations in the three non-porcine TGEV *ORF7s*, and which had relative farther genetic relationship and evolution distance to *ORF7s* of other TGEV strains. The study can serve as a foundation for further insight into the virulence change or mutation trend of TGEVs.

Key words: Non-porcine transmissible gastroenteritis virus (TGEV), open reading frame (*ORF7*), phylogenetic analysis, homology modeling.

INTRODUCTION

Transmissible gastroenteritis (TGE) is an acute highly contagious disease of pig, and the occurrences of TGE have become more sporadic. Wild and domestic carnivores (dog, cat, fox and mink) are suggested as potential subclinical carriers of transmissible gastroenteritis virus (TGEV), serving as reservoirs between seasonal epidemics (Saif and Sestak, 2006). In recent years, virulence changes of TGEV have drawn researcher's attention to analyze the virulence-associated genes of TGEV. The open reading frame 7 (*ORF7*) of TGEV is a virulence gene that can regulate host cell defenses and extend the period of virus dissemination (Cruz et al., 2011). In addition, the *ORF7* sequence is relatively conservative and can be immunoprecipitable with porcine hyperimmuned anti-TGEV serum (Tung et al., 1992; Yin et al., 2005). Since *ORF7* is a virulence-related gene and nucleotide change in *ORF7* might influence TGEV virulence, it is necessary to isolate the *ORF7* from non-porcine TGEV because these non-porcine hosts are important transmission media of TGEV and may induce changes in virulence of TGEV. The aim of the present study was to generate new information with respect to TGEV strains isolated from non-porcine hosts.

MATERIALS AND METHODS

Three TGEV strains (HYM-09, HYM-09-1 and HYM-09-2) were all isolated from non-porcine animals, respectively (Man et al., 2011, 2012). Total RNA was isolated from purified virus pellets using SDS-Protease K according to the reference (Sambrook and Russell, 2001). A pair of sense (5'-ACGAGATGCTCGTC-3') and antisense primer (5'-ACCAGTTTTAGACATCGG-3') were designed based on corresponding conservative region of *ORF7* from reference strain (Purdue, GenBank: DQ811789) and contained the full-length ORF. RT-PCR products of approximate 0.25 kb were amplified by PCR and cloned into pMD18-T vector. The *ORF7* nucleotide

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Table 1. Summary of unique mutations in non-porcine TGEV isolates.

TGEV strain	species name	Strain-specific single-point mutations in non-porcine TGEV *ORF7* sequence	TGEV ORF7 three-dimensional structure
HYM-09	Dog	12nt (C to T); 32nt (A to T); 72nt (G to T); 73nt (A to C); 100nt (A to T);	Similar to 2GSM.B (1-76AA)
HYM-09-1	Cat	15nt (C to G); 31nt (C to T); 49nt (T to A); 68nt (A to T); 88nt (A to T); 111nt (G to C); 136nt (G to T); 158nt (A to T); 230nt (A to C);	Similar to 1L9H.B (1-74AA)
HYM-09-2	Fox	11nt (C to G); 38nt (A to G); 57nt (A to C); 73nt (A to G); 95nt (T to A); 137nt (G to T); 163nt (A to T); 199nt (G to C); 213nt (A to T); 214nt (G to T);	Similar to 2GSM.B (1-76AA)

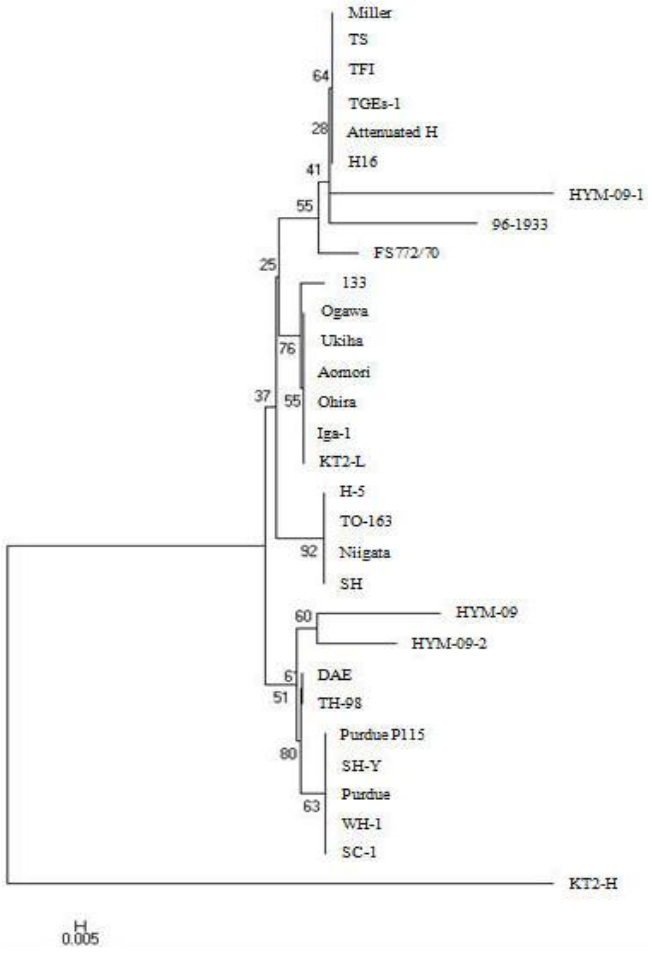

Figure 1. Phylogenetic tree of TGEV strains based on the amino acid sequences of ORF7. The tree was obtained by bootstrap analysis with the neighbor-joining method (boostrapping is 1000). H16 (China, FJ755618); TH-98 (China, AY337931); SC-1 (China, DQ437507); SC-Y (China, DQ437506); WH-1 (China, HQ462571); TGEs-1 (China, GQ914800); Attenuated H (China, EU074218); TS (China, DQ201447); HYM-09 (China, JN709474); HYM-09-1 (China, JN709475); HYM-09-2 (China, JN709476); DAE (Korea, EF100897); 133 (Korea, EF100896); KT2-H (Korea, EF100895); KT2-L (Korea, EF100894); Aomori (Japan, AB115409); Iga-1 (Japan, AB115408); Ohira (Japan, AB115407); Ogawa (Japan, AB115406); Ukiha (Japan, AB115405); Miller (Japan, AB115411); Niigata (Japan, AB115404); H-5 (Japan, AB115403); SH (Japan, AB115402); TO-163 (Japan, AB115401); 96-1933 (UK, AF104420); TFI (UK, Z35758); FS772/70 (UK, Y00542); Purdue (USA, DQ811789); Purdue P115 (USA, DQ811788).

sequences of HYM-09, HYM-09-1 and HYM-09-2 were determined by TaKaRa Biotechnology (Dalian) Co., Ltd. The complete nucleotide sequences of HYM-09, HYM-09-1 and HYM-09-2 *ORF7s* had been deposited in the GenBank Database and were assigned accession numbers JN709474, JN709475 and JN709476, respectively. From the deduced amino acid sequences of the non-porcine ORF7s, the theoretical isoelectric point (pI) and molecular weight (Mw) were computed using the compute pI/Mw tool (http://us.expasy.org/tools/pi_tool.html). Moreover, we constructed a phylogenetic tree using MEGA5.05 software and three-dimensional (3D) structure analyses of the TGEV ORF7s were also carried out using CPHmodels-3.0 Server (http://www.cbs.dtu.dk/services/CPHmodels/).

RESULTS AND DISCUSSION

Sequence analyses indicated that all the *ORF7s* of HYM-09, HYM-09-1 and HYM-09-2 consisted of 237 nucleotides coding for a basic protein of 78 amino acids. The pI/Mw of HYM-09, HYM-09-1 and HYM-09-2 ORF7 are 11.31/9128.48, 7.81/9129.43 and 9.84/8970.19, respectively. Interestingly, we found the pI value of HYM-09-1 ORF7 was lower than others (usually more than 9.5), which might be caused by new mutation sites in the HYM-09-1 *ORF7*.

Sequence analysis and comparison revealed that the three non-porcine TGEV *ORF7* DNA sequences shared over 90% identity with that of other TGEV strains and no deletion or insertion events were detected. The sequence homology of the three non-porcine *ORF7* showed lower than most of other TGEV strains. There were some new unique point mutations in the non-porcine TGEV *ORF7* sequences which did not exist in the other isolates used here, and most of changes were A/T and C/T substitutions (Table 1).

To evaluate the evolutionary relationships and possible virulence changes of the non-porcine TGEVs with other strains, a phylogenetic tree was constructed using MEGA5.05 software on the basis of the ORF7 amino acid sequences from 30 TGEV strains isolated in various parts of the world (Figure 1). The ORF7s from different strains were divided into three subgroups basically. One group contained TS, HYM-09-1, H16, Miller, 96-1933, Attenuated H, 133, Ukiha, Ogawa, TGES-1, Aomori, Ohira, Iga-1, KT2-L, H5, TO-163, Niigata, Sh, TFI and FS772/70 strain; The second group consisted of WH-1,

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HYM-09 ORF7: 1-LVLFHAVFITVLILLLIGRLQLLSRLLLNHSFILKTVNDFNILYRSLAGTRLLKVLLRVI-60
2GSM.B:      1-IHWLDGFILVIIAAITIFVTLLILYAVWRFHEKRNKVPARFTHNSPLE---IAWTIVPIV-60

HYM-09 ORF7: 61-FLVLLRFCCYRLLVTL-76
2GSM.B:      61-ILVAIGAFSLPVLFNQ-76
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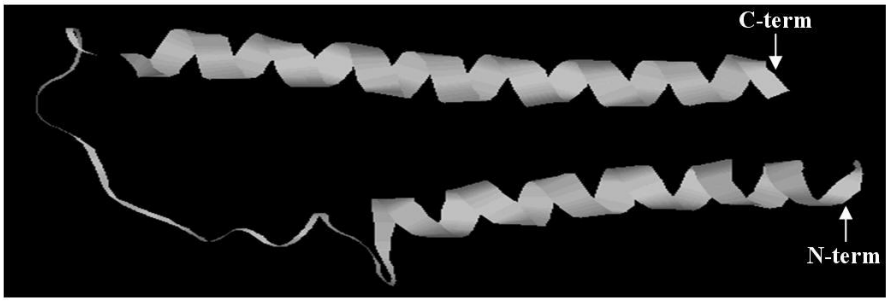

a

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HYM-09-1 ORF7: 1-VLLDAVFIIVLTLLQIGRLQFLERLLLIHSFNLKTLDDFNILYMSLAETRFLKVVLRLIF-60
1L9H.B:        1-SMLAAYMFLLIMLGFPINFLTL--YVTVQHKKLRTPLNYILLNLAVADLFMVFGGFTTTL-60

HYM-09-1 ORF7: 61-LVLLG-------FCCYLLLVI-81
1L9H.B:         61-YTSLHGYFVFGPTGCNLEGFF-81
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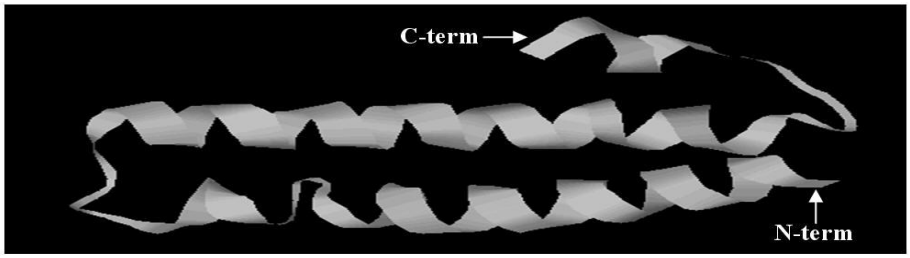

b

Figure 2. Homology modeling of the non-porcine TGEV ORF7s (1-76AA). a: Homology modeling of ORF7 (1-76AA) of HYM-09 isolate based on the crystal structure of the 2GSM.B (Chain B, Catalytic Core (Subunits I and II) of Cytochrome C Oxidase from RhodobacterSphaeroides). b: Homology modeling of ORF7 (1-76AA) of HYM-09-1 isolate based on the 1L9H.B (Chain B, crystal structure of Bovine Rhodopsin). Diagram comparing the relative position of the amino acid residues of TGEV ORF7s with that of the corresponding protein ortholog; In the 76AA of ORF7, some differences were also found in the exact amount of amino acids and orientation on 3D structures, e.g., in comparison with HYM-09-1 isolate, the 3D structures of the other most TGEV ORF7 (similar to 2GSM.B) had one longer random coils (containing 7-8AA) at middle positions 29-36AA and did not contain other structure in the C-terminal region of ORF7. The three-dimensional structures of ORF7 partial sequences were constructed by CPHmodels-3.0 Server.

Purdue, Purdue P115, SC-1, SC-Y, DAE, HYM-09, HYM-09-2 and TH-98. The KT2-H isolate was separated from the other TGEV strains and formed an independent group (Figure 1). HYM-09, HYM-09-2 and HYM-09-1 had relative farther genetic relationship with most TGEVs as far as the evolutionary distance was concerned, which implies that the non-porcine TGEV strains should be a better virus model to study the TGEV for understanding possible virus variation.

The structure analyses showed that 3D structures of the most TGEV ORF7s (1-76AA) by homology modeling were similar to that of 2GSM.B. However, the 3D structures of HYM-09-1, DAE, Niigata and 96-1933 (1-76AA) were similar to that of 1L9H.B (Figure 2). The causes of 3D structure differences came from missense mutations in the *ORF7*. Our 3D structure analyses may provide a basis for further studying the relationship between structure and virulence of TGEV.

Conclusion

In summary, this study indicates that there exist unique mutations in the *ORF7* of the three non-porcine TGEV isolates. Interestingly, the 3D structure of HYM-09-1 ORF7 is different from that of other TGEV isolates of China, and its pI value is also lower than other strains. Our study establishes the primary foundation for further insight into the TGEV *ORF7*.

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Short Communication

Assessment of the microbiological quality of *koozh*, a fermented millet beverage

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***Koozh* is a fermented beverage made with millet flour and rice, and consumed by ethnic communities in Tamil Nadu, India. Six street vended samples were assessed for the total bacterial count (TBC), lactic acid bacteria (LAB) count, yeast-mould count (YMC), coliforms at 35°C and pathogens. The *koozh* pH ranged from 4.3 to 4.9 with high titratable acidity. Although no *Staphaylococcus* sp. and *Listeria* sp. were found, high colony counts of *Clostridia* sp., *Salmonella* sp. and *Shigella* sp. were present in some samples. The LAB was dominant as compared to TBC, YMC and coliforms. Pathogens were detected, indicating contamination following processing in the traditional fermented food.**

Key word: Fermented millet, street food, lactic acid bacteria, pathogens, microbiological quality, contamination.

INTRODUCTION

Millets are important minor cereals in tropical and subtropical regions and India is the largest producer. Many traditional fermented products are made from millets both in African and Asian countries. *Koozh* (Tamil term for porridge) is a ready-to-eat (RTE) food/energy beverage either made from finger millet- *Eleusine coracana* (*Kezhvaragu*) or pearl millet- *Pennisetum glaucum* (*Kampu*), broken rice flour (*noyee*) (Kumar et al., 2010).

Koozh is prepared in an outdoor traditional kitchen common in rural India, using traditional methods that have been followed for generations. A flow chart of the typical process is given in Figure 1. The millet flour is made into slurry with water by hand-mixing on the first day and left to ferment overnight (12-15 h); on the second day, broken rice (20% by weight of millet) is cooked in excess water, into which the fermented (12-15 h) millet slurry is mixed, stirred and cooked to make a thick porridge called *noyee*.

The fermentation of this porridge overnight (24 h) results in *kali*, a semi solid porridge to which the required amount of potable water is added (1:6 w/v) and hand-mixed with salt to prepare *koozh*. The product may be further mixed with buttermilk (diluted curd a local preparation of yoghurt) to give a thin porridge consistency. The end product has a characteristic fermented flavour as a result of the microbial succession, which develops complex flavours. While *kali* has a shelf life of approximately one week at room temperature (25-30°C), *koozh* has low shelf life and is usually consumed within about 12 h of preparation. It is prepared in homes and offered in temples during special festivals. It is also sold in the streets or by mobile food vendors and consumed by daily-wage earners of Tamil Nadu, India.

Koozh is unique in that it is fermented twice- once before cooking and again after cooking, its preparation last for two days. Traditionally, *koozh* is considered nutritious and health promoting, but there is little scientific documentation on its nutritive or microbial composition, other than some studies on the nutritional advantages of fermented millets - finger millet (Antony and Chandra, 1998) and pearl millet (Kheterpaul and Chauhan, 1994).

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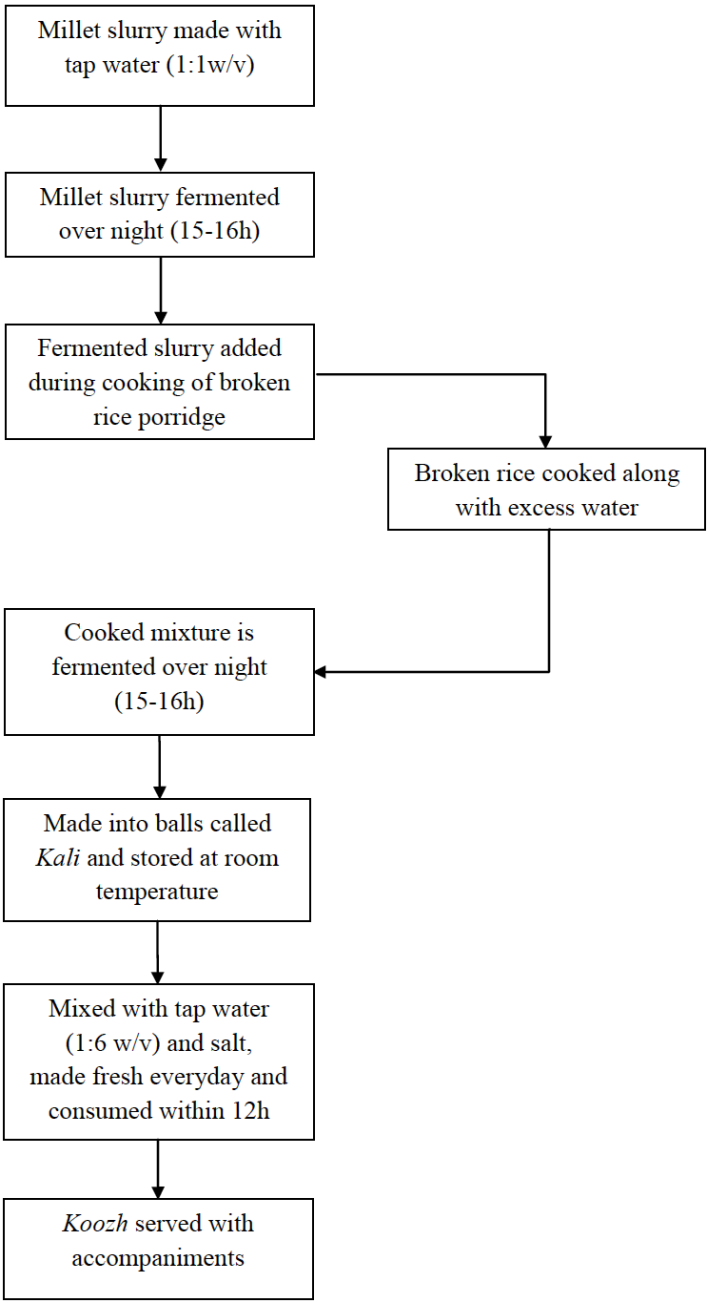

Figure 1. Indigenous preparation of *koozh* from finger millet- *Eleusine coracana* (*Kezhvaragu*) or pearl millet - *Pennisetum glaucum* (*Kampu*).

Geetha and Kalaichelvan (2013) had reported the microbial succession and biochemical changes in *koozh* made from finger millet-ragi, pearl millet-cumbu, sorghum and maize. The second fermentation after cooking of the millet in *koozh* preparation makes it an excellent source of live bacteria, while its storage and service may allow contamination. Hence, this study was undertaken to assess the microbiological safety of *koozh* sold as street food.

MATERIALS AND METHODS

Sample collection and its microbial load

Finger and pearl millet *koozh* were collected in 250-mL autoclaved, wide-mouthed, screw-capped plastic containers from market places in Salem district (Sa) and Chennai district (Ch), Tamil Nadu, India and immediately transported to the laboratory in insulated food containers with ice packs and analyzed. The finger millet *koozh* samples were collected from street vendors in different Chennai suburbs and tested on the same day according to Cappuccino and

Table 1. pH and total load of bacteria, lactic acid bacteria and yeast present in *koozh*.

Type of *koozh*	Samples and collection location	pH	Log cfu g^{-1}			
			TBC	LAB		Yeast
				MRS	M17	
			Mean ± SD[a]	Mean ± SD[a]	Mean ± SD[a]	Mean ± SD[a]
Kampu (pearl millet) *koozh*	S_1-Sa	4.91±0.15	8.56±0.38	10.81±0.60	8.32±0.39	8.85±0.89
Kezhvaragu (finger millet) *koozh*	S_2-Sa	4.48±0.02	5.95±0.62	8.96±0.72	7.88±0.42	8.47±0.91
	S_3-Ch	4.33±0.47	8.10±0.37	8.88±0.13	8.03±0.98	8.42±0.38
	S_4-Ch	4.56±0.55	7.99±0.99	7.96±0.18	7.84±0.89	5.57±0.65
	S_5-Ch	4.51±0.41	5.72±0.39	8.68±0.47	7.89±0.91	6.22±0.35
	S_6-Ch	4.68±0.12	7.12±0.49	8.85±0.10	7.91±0.49	7.99±0.13

a: Standard deviation from the mean of three independent estimation; Sa: Salem, Tamil Nadu, India; Ch: Chennai, Tamil Nadu, India.

Sherman (1996) Harrigan and McCance (1998). The total bacterial count (TBC), lactic acid bacteria count (LAB), yeast-mould count (YMC), coliforms at 35°C and pathogens was carried out for all samples. The microbial quality of the *koozh* was assessed based on the norms specified for RTE foods by the Health Protection Agency (HPA 2009).

Determination of pH

The sample pH was determined using AP-1 plus pH meter (Susima Technologies, Chennai). The titratable acidity was estimated in *koozh* filtrate by titration with 0.1 N sodium hydroxide to the end point with phenolphthalein indicator (AOAC, 2000).

Statistical analysis

The microbial count values were tested using paired student's *t* test and the correlation between variables was also determined using GraphPad 6 (SanDiego,Ca, USA) software. The calculated *r* values are interpreted.

RESULTS AND DISCUSSION

pH

The pH of *koozh* ranged from 4.3 to 4.9 (Table 1) with acidity ranging from 0.16 to 0.35%, in all samples. This may pose a problem because some samples with pH over 4.5 may allow spoilage or growth of pathogenic microbes. This observation is similar to other millet-fermented products reported in literature: an alcoholic Himalayan beverage from finger millet called *Kodo ko jaanr*, with pH ranging from 3.7 to 4.5 (Thapa and Tamang, 2004); the Northern Ghana's *Koko* sour water from pearl millet with a pH of 3.9 ± 0.1 (Lei and Jakobsen, 2004); a fermented mixture of millet and sorghum flour, called *bushera* of Uganda with 3.7 ± 0.1 pH, and *bushera* made only with sorghum with a pH range of 4.0-4.5 (Muyanja et al., 2003).

Bacteria, LAB and yeast-mould counts

In all *koozh* samples, LAB were found to be dominant and yeast-mould counts were comparatively lower. LAB counts on MRS showed significant differences ($p \leq 0.05$) with TBC and counts on M17 and yeast counts. The LAB counts on MRS showed a very strong correlation with counts on M17 ($r = 0.9396$) as both are selective media used for LAB enumeration. Yeast counts were strongly correlated with LAB count on MRS ($r = 0.7205$) and moderately correlated with counts on M17 ($r = 0.6261$). Co-metabolism between yeast and LAB may exist, where the bacteria provide the acid environment, which selects the growth of yeast, that in turn; provide vitamins and other growth factors to the bacteria (Steinkraus, 1996).

The pH of samples correlated strongly with LAB counts on MRS ($r = 0.7250$) apparently due to the acid production by LAB, and correlated moderately with counts on M17 ($r = 0.5950$), while with yeast the correlation was low ($r = 0.2327$). Moulds were absent in all samples tested. Thus, it can be concluded that LAB and yeasts mediate *koozh* fermentation.

Bacterial pathogen (hazard) classification

The isolated pathogens were *Bacillus cereus*, *Clostridium* sp., *Enterobacteria* sp., *Salmonella* sp. and *Shigella* sp. Coliforms at 35°C were found in all samples (Figure 2) and the count was positively correlated with TBC ($r = 0.7216$) and are probably from contamination of the water added and the preparation involving hand-mixing. Their presence is associated with poor hygiene, and therefore, indicates a potential health risk. The presence of coliforms has been reported in other traditional fermented products due to its survival in acidic environment (Sterinkraus, 1996).

Coliforms had moderate correlation with pH ($r = 0.5521$) and a negative correlation with yeast ($r = -0.4992$) indicating inhibitory action of the microbes on coliforms. Simango (1995) compared the contamination in a fermented cereal gruel (*Mahewu*) with that of a non fermented thick maize meal pap (*Sadza*) in Zimbabwe. After storing for 4 h, both foods were found to have 50%

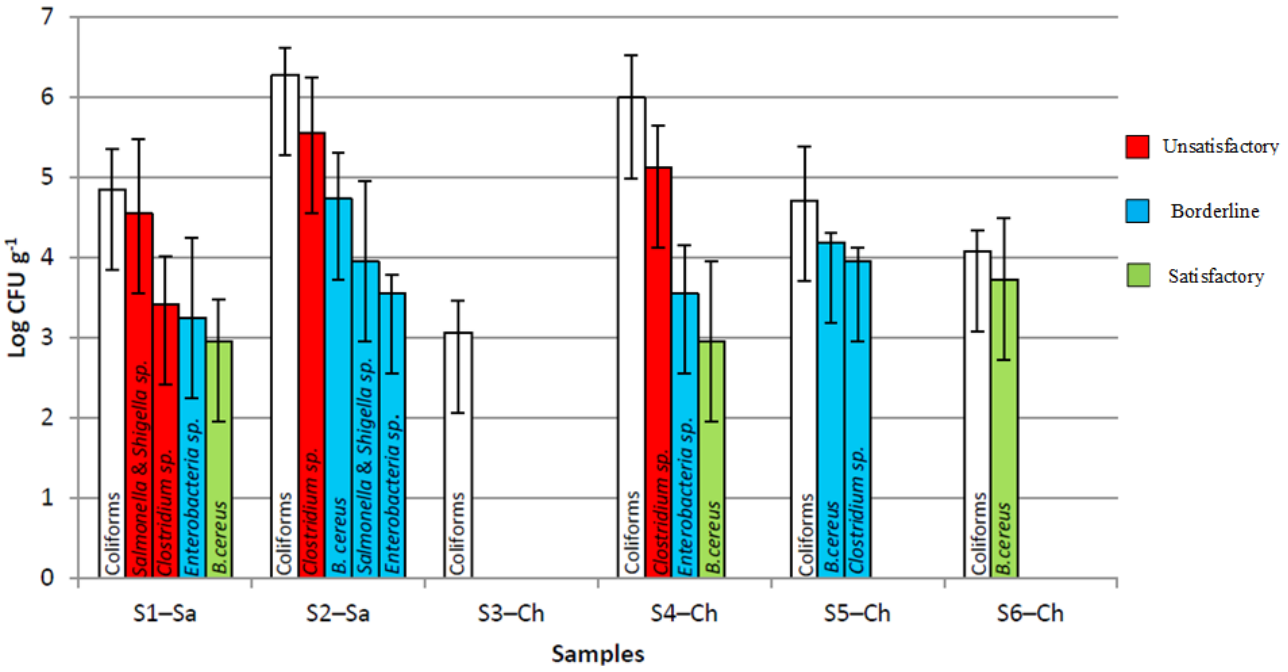

Figure 2. Pathogens present in *koozh* samples classified according to HPA (2009). Sa: Salem, Tamil Nadu, India; Ch: Chennai, Tamil Nadu, India.

Escherichia coli contamination but after 24 h no *E. coli* was found in the *Mahewu*. Simango and Rukure (1991) stated that this is due to further fermentation that leads to the inhibition of these contaminants during longer storage. Traditionally fermented laboratory-prepared sorghum bread had coliform in the first fermentation, but the microbes were not detected in the second fermentation (Gassem, 1999). In *bushera*, coliforms population decreased due to high acidity resulting from the metabolism of LAB (Muyanja et al., 2003).

The hazard classification based on pathogen count according to HPA (2009) for RTE is represented in Figure 2. The presence of *Clostridium* sp., *Salmonella* sp. and *Shigella* sp. in high numbers made three samples unsatisfactory. In one of these *koozh* samples pH was exceeded 4.5.

Other pathogens like *Listeria* sp. and *Staphylococcus* sp. were not detected in any of the samples. Sample S_3-Ch alone met the microbial stan-dards, indicating safe and hygienic handling of the pro-duct. It was also the product with the lowest pH. Two samples (S_5-Ch and S_6-Ch) with *B. cereus* and *Clostridum* sp. at low levels were acceptable.

Studies with other fermented foods show that pathogens are inhibited during the fermentation. Pathogens such as enterotoxigenic *E. coli*, *Shigella flexneri, Salmonella typhimurium*, *B. cereus*, and *Campylobacter jejuni*, when inoculated were adversely affected during the fermentation of sorghum (Kingamkono et al., 1994). The fermentation of finger millet provides antimicrobial activity against *S. typhirmurium and E. coli* even after 48 h of fermentation (Antony and Chandra, 1998). The inhibitory effect in fermentation is not only because of lactic acid but other factors like other organic acids, fermentation process, pH and temperature.

Presence of *Clostridium* in heat-treated foods would be due to inadequate cooking or post-processing contamination (HPA, 2009). *B. cereus* survives on processing equipments by germinating prior to sanitization. Handling, storage, or processing can be sources of contamination. Bacteria attaches to surfaces of food equipments, made in polystyrene, hydroxyapatite, glass, rubber and stainless steel might transmit pathogens to food (Mafu et al., 2011). The major contamination is through unclean water used for dilution and hand-mixing. Direct contaminations from unhygenic environment are predominant in market places and in tropical regions were these locations attract house-flies. *Koozh* and accompaniments were found exposed to dust and pathogens; serving utensils were not cleaned properly due to lack of safe running water. All these factors resulted in post process microbial contamination.

Conclusion

The microbiological quality of *koozh* traditionally considered healthy for its nutritional content, and sold as a street food varies widely. Lactic acid bacteria and yeasts were present in significant numbers. Hazardous levels of pathogens in 3 of 6 samples indicate unhygienic handling, despite the large numbers of LAB. Safety awareness

programmes targeting producers are imperative to eliminate such contamination and ensure safe food.

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Short Communication

Detection of extended spectrum beta lactamases in typhoidal salmonellae by phenotypic methods

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Extended spectrum beta lactamase (ESBL) producing members of the family Enterobacteriaceae are a major health problem in the hospitals and community. These enzymes confer bacterial resistance to penicillins, first, second and third generation cephalosporins and aztreonam. The prevalence of ESBLs varies from organism to organism and is increasing day by day. The study was designed to test typhoidal salmonellae for production of ESBL. One hundred and fifty eight (158) isolates of typhoidal salmonellae; *Salmonella* Typhi (n=126), *Salmonella* Paratyphi A (n=26) and *Salmonella* Paratyphi B (n=6) were collected from different hospitals of Lahore and Gujranwala. The isolates were identified morphologically, biochemically (API-20E) and serologically (BD Difco, USA). ESBL production has been tested by three methods; CLSI screening method, double disk diffusion synergistic method and CLSI phenotypic confirmatory method. CLSI screening method detected twenty two (22) strains as ESBL producers. However, when tested by the CLSI confirmatory method and by disk diffusion synergistic method, none proved to be ESBL producer. Based on our study, we concluded that the extended spectrum beta lactamase enzyme does not exist in tested clinical isolates of typhoidal salmonellae, however the isolates suspected to be ESBL by phenotypic methods must be subjected further for molecular analysis.

Key words: ESBL, typhoidal salmonellae, isolate.

INTRODUCTION

Salmonella enterica subspecies *enterica* is an important genus of the family Enterobacteriaceae. The genus Salmonella was named after an American microbiologist Daniel Elmer Salmon. *Salmonella* Typhi was isolated by Gaffky in Germany in 1884 and in 1886 by Salmon and Smith in United States (Winn et al., 2006).

Extended spectrum beta lactamases (ESBL) are enzymes that mediate resistance to the extended-spectrum cephalosporins; ceftazidime, cefotaxime, and ceftriaxone and monobactams like aztreonam but do not affect cephamycins. These ESBL enzymes also affect carbapenems; imipenem and meropenem (Coque et al., 2008) and are commonly inhibited by beta lactamase-inhibitors such as clavulinic acid (Coque et al., 2008), sulbactam and tazobactam. Plasmids responsible for ESBL production tend to be large (80 Kb or more in size) and carry resistance against several antimicrobials that are aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol and trimethoprim-sulfamethoxazole (Coque et al., 2008; Nathisuwan et al., 2001). This fact imparts an important limitation in the design of treatment alternatives (Jacoby and Medeiros, 1991).

ESBL enzyme production by *Salmonella* spp. was first detected in 1988 (Hammami et al., 1991). The prevalence

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Abbreviations: API 20E, Analytical profile index 20 Enterobactericeae; **ATCC,** American Type Culture Collection; **CLSI,** Clinical Laboratory Standards Institute; **ESBL,** extended spectrum beta lastamase; **MDR,** multi drug resistant.

of ESBL producing salmonellae in Kuwait is 17.6% out of which *S.* Typhi strains are 4%, while this prevalence is 15.5% in UAE and 5.3% of them are *S.* Typhi (Rotimi et al., 2008). A strain of *S.* Typhi producing CTX-M type of ESBL enzyme was reported from Germany in August 2009 (Pfeifer et al., 2009). Same year, there was another report of ESBL producing *Salmonella enterica* from Iran (Hamidian et al., 2009).

The objective of the present study was to screen the isolates of typhoidal salmonellae for ESBL production. The present study is focused on isolates from our local population to see the frequency of ESBLs in these isolates using phenotypic methods, that is, CLSI screening method, double disk diffusion synergistic method, and CLSI phenotypic confirmatory method.

MATERIALS AND METHODS

Bacterial strains

One hundred and fifty eight clinical isolates of typhoidal salmonellae were obtained from different hospitals of Lahore and Gujranwala; Sheikh Zayed Medical Complex Lahore (n=50), Fatima Memorial Hospital Lahore (n=25), Combined Military Hospital Lahore (n=22), Services Hospital Lahore (n=15), Ittefaq Hospital Lahore (n=14), Shaukat Khanum Memorial Cancer Hospital and Research Centre Lahore (n=12), and Fazil Memorial Hospital Gujranwala (n=20) from 2007 to 2011. The study was conducted in the Department of Microbiology, University of Health Sciences, Lahore.

BioMerieux, France, API-20E and BD Difco, USA antisera were used. Oxoid disks; Ceftazidime (30µg), Ceftriaxone (30µg), Aztreonam (30µg), Cefotaxime (30µg), Co-amoxiclav (clavulanate 10 µg + amoxicillin 20 µg), and Ceftazidime + Clavulanic acid (30 + 10 µg) were used.

The isolates were received on Nutrient agar slopes and subcultured on Nutrient agar. Isolates were identified by standard morphological and biochemical characteristics. Gram staining, Catalase and Oxidase tests were performed. Identification of isolates was done by API 20E (BioMerieux, France) and confirmed by antisera (BD Difco, USA). Antimicrobial susceptibility pattern was performed by Kirby Bauer disc diffusion method using Oxoid disks; Ceftazidime (30µg), Ceftriaxone (30µg), Aztreonam (30µg), Cefotaxime (30µg), Co-amoxiclav (clavulanate 10 µg + amoxicillin 20 µg), and Ceftazidime + Clavulanic acid (30 + 10 µg).

Method

For the detection of ESBL, three methods: CLSI screening method, CLSI phenotypic confirmatory method and double disk diffusion synergistic method were used.

CLSI screening method

Ceftazidime, ceftriaxone and aztreonam disks were placed on a MHA plate at appropriate distance. The plates were incubated aerobically overnight (18-24 hours/35°C). The strains showing ≤ 22 mm zone of inhibition around ceftazidime, ≤ 25 mm around ceftriaxone and ≤ 27 mm around aztreonam disks were suspected to be ESBL producers.

CLSI phenotypic confirmatory method

Strains that were suspected to be ESBL producer by screening method were confirmed for enzyme production by phenotypic confirmatory disk diffusion method.

Ceftazidime disk without clavulanic acid and ceftazidime with clavulanic acid combination disk were placed on the same plate. The plates with disks were incubated aerobically overnight (18-24 hours/35°C). The isolates showing an increase in zone size of 5 mm or more around ceftazidime with clavulanic acid as compared to ceftazidime alone were confirmed to be ESBL producer. No enhancement of zone indicates ESBL non-producer isolates (Cockerill et al., 2013).

Double disk diffusion synergistic method

A co-amoxiclav disk was placed in the center of MHA plate and ceftazidime, cefotaxime, ceftriaxone, and aztreonam, were placed at 20 mm distance, center to center, from co-amoxiclav disk. Plates were incubated aerobically overnight (18-24 hours/35°C). The isolates which showed an increase in zone of inhibition greater than 5 mm on co-amoxiclav side of the disk compared to the results seen on the side without co-amoxiclav were confirmed as ESBL producer. Whereas, no enhancement of zone on co-amoxiclav disk side indicates ESBL non-producer isolates (Jarlier et al., 1988).

RESULTS

Out of 158 isolates of typhoidal salmonellae i.e., *S.* Typhi (n=126), *S.* Paratyphi A (n=26) and *S.* Paratyphi B (n=6). No ESBL was detected. Initially 14% isolates were suspected to be ESBL producers when screened by initial screening disk diffusion method. These isolates were finally confirmed to be non-ESBL producers when tested with the disk diffusion phenotypic confirmatory method and by double disk diffusion synergistic method.

DISCUSSION

Among our 158 isolates of typhoidal salmonellae, no ESBL was detected. In another comparative study of screening methods for ESBL detection was done on 38 isolates comprising of 30 reference strains, that is, *E. coli* and *K. pneumoniae* already defined ESBL producers by Isoelectric focusing and DNA sequencing, and eight laboratory isolates not confirmed to be ESBL producer, no reduced susceptibility pattern to extended spectrum cephalosporins and aztreonam was seen, according to this study, only 52% ESBL producing isolates were detected by disk diffusion screening method (Vercauteren et al., 1997). In another reported study, 55% of ESBL producing isolates were detected by disk diffusion screening method (Goyal et al., 2008). Ceftazidime proved to be the best single indicator antibiotic for ESBL detection, however none of the isolates when subjected to molecular analysis produced any type of ESBL, that is, TEM-4, TEM 12, SHV-2, SHV-3 and SHV-5 (Vercauteren et al., 1997). Standard disk diffusion susceptibility test detected only 48% of ESBL producing reference strains (Vercauteren et al., 1997). Thus in agreement with other studies, it is evident that an additional testing method is

required for the confirmation of ESBL (Jacoby and Han, 1996; Katsanis et al., 1994; Thomson and Sanders, 1992). Several possibilities for these false negative results are known; among them one possibility is that TEM-type ESBLs with single amino acid substitutions have only low level oxyimino beta lactam activity (Jacoby, 1994). Other ESBL enzymes have relatively greater oxyimino beta lactam activity but lack intrinsic extended spectrum enzyme efficiency (Bush and Singer, 1989). In our study, 22 isolates were suspected to be ESBL producer by initial screening method, but were confirmed by phenotypic confirmatory disk diffusion method as recommended by CLSI (2013), to be non-ESBL producer. In another study described by Goyal et al. (2008) frequency of ESBL, detected by disk potentiation method, was 64.5%. Phenotypic confirmatory method cannot detect the ESBL in those bacterial isolates which also produce other classes of beta lactamases, AmpCs and TEMs. The probability for this false negative result could be masking effect of enzymes on inhibitory effect of clavulanic acid (Srisangkaew and Vorachit, 2004). According to Datta et al. (2004), 90% ESBL producers were detected correctly by disk potentiation method.

These 22 isolates were also confirmed by another phenotypic method described by Jalier et al. (1988). According to another study, this double disk synergy method, described by Jalier et al. (1988) detected 95% of isolates to be confirmed ESBL by CLSI phenotypic method (Goyal et al., 2008).

In our study and current settings, we only had the facility of phenotypic methods however these 22 isolates if subjected to molecular analysis could be confirmed to be ESBL producer or non producer.

ESBL producers also have the capability to take up quinolone resistance genes along with them, therefore ESBL producer become resistant to fluoroquinolones as well, which restricts the choice of treatment for typhoid patients (Cattoir et al., 2007).

The frequency of ESBL enzyme in clinical isolates of typhoidal salmonellae is reported as zero percent in our study. The possibility that ESBL producing typhoidal salmonellae have not been detected in study is because there are no recommendations by CLSI for ESBL detection methods in typhoidal salmonellae.

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