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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

Detection of outbreak caused by multi-drug resistant *Acinetobacter baumannii* in Assiut University Hospitals

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There is mounting evidence that *Acinetobacter baumannii* has a naturally occurring carbapenemase gene intrinsic in this species. Presence of class 1 integrase gene in *Acinetobacter* isolates is a useful marker for causing outbreaks in hospitals and for being epidemic strains of *A. baumannii.* The goal of the present study was to detect the resistance and outbreak marker genes by multiplex polymerase chain reaction (PCR) (*blaOXA-51*-like gene and class 1 integrase gene). Also to detect the correlation between imipenem susceptibility and detection of *blaOXA-51*-like gene. For these purposes, 51 consecutive, non-duplicate, *A. baumanii* strains were isolated from various clinical and environmental specimens from the Intensive Care Units (ICUs) of Assiut University Hospitals, Egypt. All the isolates were identified by conventional standard methods. The antibiotic sensitivity pattern was determined by Kirby Bauer disc diffusion method. For imipenem, the minimum inhibitory concentrations (MICs) were determined using Epsilometer (E test). Multiplex PCR was performed for the detection of the *blaOXA-51*-like and Class I integrase genes. The *blaOXA-51*-like gene was detected in (95.8%) and (96.3%) in clinical and environmental isolates, respectively. Class I integrase gene was detected in (75%) and (70.3%) in clinical and environmental isolates, respectively with statistically significant difference (P value of clinical samples = 0.041 and P value of environmental samples =0.011). This means that these strains have metallo-beta-lactamase (MBL) gene (cause outbreak in hospital at any time). Also (67.35%) of *A. baumanii* isolates are imipenem sensitive and positive for *blaOXA-51*-like gene and this means that these isolates contain hidden metallo beta lactamase MBL gene.

Key words: *Acinetobacter baumanii*, *bla*OXA-51-like genes, Class I integrasegene, MBL gene.

INTRODUCTION

Acinetobacter baumannii is an important opportunistic pathogen responsible for severe nosocomial infections, especially in intensive-care-unit patients (Takagi et al., 2009). The majority of infections are of epidemic origin, and treatment has become difficult because many strains are resistant to a wide range of antibiotics, including broad-spectrum β-lactams, aminoglycosides, and fluoro-quinolones (Renu et al., 2010).

Carbapenems are the drugs of choice for *A. baumannii* infections and are often used as a last resort. However, decreased susceptibility to carbapenems has been recently observed worldwide (Peleg et al., 2008; Valenza et al.,

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2010).

There are several carbapenem resistance mechanisms described in *Acinetobacter* species (Peleg et al., 2008). Many carbapenem hydrolyzing beta- lactamases have been identified, amongst which are the metallo-beta-lactamases (MBLs). Most of the MBL-encoding genes reside on integrons and plasmids which in turn allows for the widespread dissemination of these genetic elements (Walsh et al., 2005; Perez et al., 2007).

Also, there is mounting evidence that *A. baumannii* has a naturally occurring carbapenemase gene intrinsic in this species. The first report of this gene described *blaOXA-51* (Brown and Amyes, 2005), but since then a large number of closely related variants have been found (*OXA* numbers 64, 65, 66, 67, 68, 69, 70, 71, 75, 76, 77, 83, 84, 86, 87, 88, 89, 91, 92, 94 and 95) and we have referred to them collectively as "*blaOXA-51*-like" genes (Brown and Amyes, 2006; He´ritier et al., 2005).

The *blaOXA-51*-like genes are unique to the species, and then their detection could provide a simple and convenient method of identifying *A. baumannii.* This method could more easily be carried out than current definitive methods, such as amplified rRNA gene restriction analysis and biochemical identification which is most commonly used (Vaneechoutte et al., 1995; Woodford et al., 2006).

In recent years, a novel mechanism of resistance gene dissemination among bacteria has been described (Stokes and Hall, 1989). This mechanism is based on the location of these genes on integrons. The majority of integrons belongs to class 1 and has been found predominantly in clinical isolates of Gram-negative bacteria, including *Acinetobacter* species (Martinez-Freijo et al., 1998). Presence of class 1 integrase gene in *Acinetobacter* isolates is a useful marker for causing outbreaks in hospitals and being epidemic strains of *A. baumannii* (Koeleman et al., 2001; Turton et al., 2005).

Identifying MBL carrying isolates has been challenging due to the emergence of carbapenem-susceptible MBL carrying organisms which may be missed in daily laboratory practice, compromising the sensitivity of detection methods. These carbapenem susceptible organisms with hidden MBL genes can spread unnoticed in hospitals if such isolates are reported as sensitive without screening for the presence of MBLs. The treatment of these organisms poses a serious therapeutic challenge as these strains are most often resistant to multiple drugs (Walsh et al., 2005).

The present study aimed to detect the*blaOXA-51*-like gene which can be carried out as part of a multiplex PCR, which detects both *blaOXA-51*-like gene (resistance gene) and class 1 integrase gene (marker for outbreak). Also to detect the correlation between imipenem susceptibility and detection of *bla OXA-51* like gene.

MATERIALS AND METHODS

Bacterial strains

A total of 51 consecutive, non-duplicate, *A. baumanii* strains were isolated from various clinical and environmental specimens from the ICUs of Assiut University Hospitals during period of February 2011 to February 2012. Regarding the clinical specimens, *A. baumanii* strains (24 strains) were isolated from urine (n= 5), sputum (n= 8), swabs from endotracheal tubes (n= 6), blood cultures (n=1), throat swabs (n=3) and wound swabs (n=1) that were submitted for bacteriological testing from patients admitted to the ICUs. A total of 27 isolates were obtained from environmental swabs from the ICUs. Swabs were taken from call bells, bedrails, and bedside tables, bedside equipments, commodes, doorknobs and faucet handles.

Identification of strains

Using MacConkey agar and Herellea agar (Dijkshoorn et al., 2005), also using simple biochemical reactions as oxidase test, nitrate test, growth on TSI, Citrate test, Urease test, motility test and growth at 44°C (Collee et al., 1996).

Biochemical identification of the isolates

Usingthe analytical profile index procedure (API 20NE system; bioMe´rieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was done for all isolates using commercially available discs (HiMedia, Mumbai, India) by Kirby Bauer disk diffusion method and interpreted as recommended by Clinical Laboratory Standards Institute (CLSI, 2010).

The following antimicrobial discs were used, Ampicillin (10 µg), Amoxacillin-Clavulanic acid (20-10 µg), Cefaclor (30 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), Ceftriaxone (30 µg), Amikacin (30 µg), Gentamicine (10 µg), Tobramycin (10µg), Netlimicin (30 µg), Tetracycline (30 µg), Ciprofloxacin (5 µg), Imipenem (10 µg), Chlo-ramphenicol (30 µg) andAztreonam (30 µg).

Determination of imipenem minimal inhibitory concentration (MIC) by IPM E-Test

AB Biodisk, Solna and Sweden E-test strips were placed over-culture streaked over Muller Hinton agar. After overnight incubation in incubator at 35°C, the MIC was read as intersect where the ellipse of growth inhibition intersects the strip. It was used at a cut-off point of ≥16 µg/ml to define imipenem resistance and a cut-off point of ≤ 4 µg/ml to define imipenem susceptibility (CLSI, 2006).

Multiplex PCR for detection of *blaOXA-51*-like gene & Class 1 intgrase gene (Turton et al., *2005)*: a- DNA extraction

The boiling method was used to extract the DNA from the bacteria (Vaneechoutte et al., 1995). Briefly, one colony of a pure culture was suspended in 50 µl of sterile water and heated at 100°C for 15 min. After centrifugation in a micro centrifuge (6,000 x *g* for 3 min), the supernatant was stored at -20°C for further use.

b-PCR Amplification and detection

This was carried out in 25 µl reaction volumes with 3 µl of extracted DNA, 12.5 pmol of each primer as shown in Table 1 and 1.5 U of Taq DNA polymerase in 1X PCR buffer containing 1.5 mM $MgCl_2$ (QIAGEN) and 200 µM of each deoxynucleoside triphosphate. Conditions for the multiplex PCR were as following: 94°C for 3 min,

Table 1. Primer sequences of *blaOXA-51*-like gene and Class 1 integrase gene (Koeleman et al., 2001; Woodford et al., 2006).

Primer	Sequence	Target gene	Amplicon size (bp)
OXA-51-likeF	5_-TAA TGC TTT GAT CGG CCT TG-3_	*blaOXA-51*-like	353
OXA-51-likeR	5_-TGG ATT GCA CTT CAT CTT GG-3_	*blaOXA-51*-like	
Int1F	5_-CAG TGG ACA TAA GCC TGT TC-3_	Class 1 integrase	160
Int1R	5_-CCC GAG GCA TAG ACT GTA-3	Class 1 integrase	

and then 35 cycles at 94°C for 45 s, at 57°C for 45 s, and at 72°C for 1 min, followed by a final extension at 72°C for 5 min. Amplified products from the isolates were analyzed by electrophoresis on 1.2% (w/v) agarose gels, stained with ethidium bromide. *A baumannii* ATCC 19606 was used as positive control.

Statistical analysis

All data were analyzed using the computerized statistical analysis (SPSS, version 16). Descriptive statistics was used. The P value <0.05 was considered statistically significant. The percent differrence of each antimicrobial agent versus Imipenem was calculated at 99% confidence interval.

RESULT

Fifty one strains of *Acinetobacter* sp. were isolated in infection control laboratory at Assiut University Hospitals from February 2011 to February 2012 .They comprised of two sets of isolates, The first set consisted of 24 isolates recovered from clinical samples, the second set consisted of 27 isolates isolated from environmental samples .

All *Acinetobacter* strains were described as Gram negative cocco-bacilli, non-motile, non-spore forming, capsulated, oxidase negative, not reduce nitrate to nitrite, not ferment sugar and citrate positive bacilli.

Acinetobacter grow on blood agar showing mucoid colonies, on MacConkey agar showed non-lactose fermenting colonies, on Herellea agar showed purple colonies. API20NE showed that these strains belong to *Acinetobacter baumannii/calcoaceticus* complex, and isolates identified as *Acinetobacter baumannii* by its ability to grow at 44°C.

Resistance of *A. baumannii* to pencillin derivatives, cephalosporines, monobactam (Aztronam) carbapenam (imipenem), quinolones (ciprofloxacin), tetracycline, aminoglycosides (netlimicin, tobramycin, gentamicin and amikacin) and chloramphenicol were 61.82, 61.8, 60.6, 31.24, 64.18, 25.2, 56.48 and 53.01%, respectively. Tetracycline and imipenem were the most active antimicrobial agent against *A. baumannii* (Table 2).

The susceptibility of *A. baumannii* to different antimicrobial agents was compared to imipenem. Imipenem resistant *A. baumannii* are not susceptible to penicillin derivative or cephlosporine, but these strains are susceptible to tetracycline (more active), chloamphenicol (moderate active) and aminoglycosides (less active) in descending manner. But susceptibility of *A. baumannii* to quinolones is variable (Figure 1).

Phenotypic detection of metallo-B-lactamase by IPM E-Test showed that (31.37%) of *A. baumannii* isolates of environmental and clinical samples contain metallo-β-Lactamase enzyme (MIC>16), while (68.6%) of the isolates show MIC below 4 µg/ml (Table 3).

Detection of *blaoxa-51*-like gene and Class I intgrase gene showed that (95.8%) and (96.3%) of *A. baumannii* isolated from clinical and environmental samples respectively gave positive result for *blaoxa-51*-like gene (intrinsic carbapenamase gene) while (75%) and (70.3%) of *A. baumannii* isolated from clinical and environmental samples respectively gave positive result for Class I intgrase gene (Table 4 and Figure 2).

Relation between imipenem susceptibility and detection of *blaOXA-51*-like gene showed that 67.5% of *A. baumannii* isolates are imipenem sensitive and positive for *blaOXA-51*-like gene and this means that these isolates contain hidden MBL gene (Table 5).

DISCUSSION

A. baumannii infections present a global medical challenge. The interest in this organism has been growing rapidly because of the emergence of multi-drug resistant strains (MDR), some of which are pan resistant to antimicrobial agents (Muthusamy and Boppe, 2012). In the present study the majority of *A. baumannii* isolates were MDR showing resistance to three or more classes of antibiotics. There has been a lot of debate concerning the definition of multidrug resistance (MDR). Renu et al. (2010) defined MDR as resistance to 4 or more classes of antimicrobials. Others defined MDR as resistance to two or more drugs or drug classes of therapeutic relevance (Navon-Venezia et al., 2005). Resistance against carbapenemsis, in itself, considered sufficient to define an isolate of *A. baumannii* as highly resistant (Poirel and Nordmann, 2006).

In our study, the results of antimicrobial susceptibility test shown resistance to penicillin derivatives (61.82%), Cephalosporine derivatives (61.82%), Quinolones (64.18%), Monobactam (60.6%), Aminoglycosides (56.48%) and Chloramphenicol (53.01%). The lowest rate of resistance

Table 2. Resistance patterns of *A. baumannii* to different antibiotics.

Sample	Resistance pattern (%)							
	B-lactam							
	Penicillin derivative	Cephalosporine	Monobacam aztronam	Carbapenam imipenam	Quinolone	Tetracyclines	Aminoglycosides	Chloramphenicol
Clinical sample	66.67	62.5	58.33	29.16	66.67	20.83	58.33	54.17
Environmental sample	56.97	61,11	62.96	33.33	62.96	29.6	54.63	51.85
Total main resistance	61.82	61.8	60.6	31.24	64.18	25.2	56.48	53.01

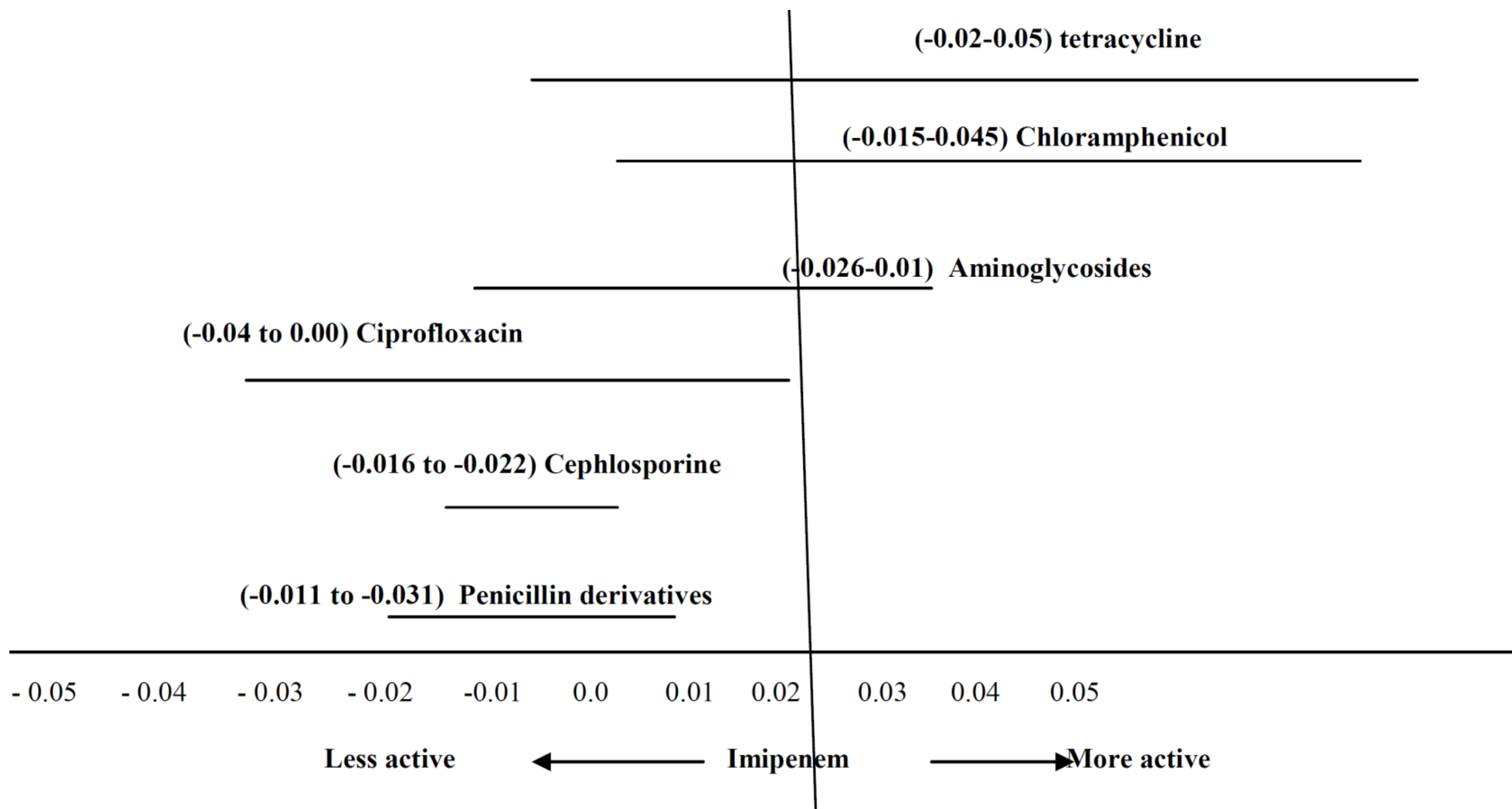

Figure 1. Forest representation of the 99% confidence interval comparing imipenem activity against other antimicrobials.

Table 3. Detection of Metallo-B-lactamase in *A. baumannii* by IPM E-test.

Sample		MIC (below 4 µg/ml)		MIC (16-256 µg/ml) MBL-producing strain	
Type	**Number of total isolate**	**Number of isolate**	**Percentage (%)**	**Number of isolate**	**Percentage (%)**
Clinical	24	17	70.83	7	29.16
Environmental	27	18	66.7	9	33.33
Total	51	35	68.6	16	31.37

Table 4. Detection of *blaoxa-51*-like gene & Class 1 integrase gene by Multiplex PCR

Result		*blaoxa-51*-like gene				Class I intgrase gene			
Sample		**Positive**		**Negative**		**Positive**		**Negative**	
Type	**No**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**
Clinical	24	23	95.8	1	4.17	18	75	6	25
Environmental	27	26	96.3	1	3.7	19	70.3	8	29.63
Total	51	49	96.1	2	3.9	37	72.5	22	27.5

P value of clinical samples = 0.041, P value of environmental samples = 0.011, P value of < 0.05 indicates significant results. No = number.

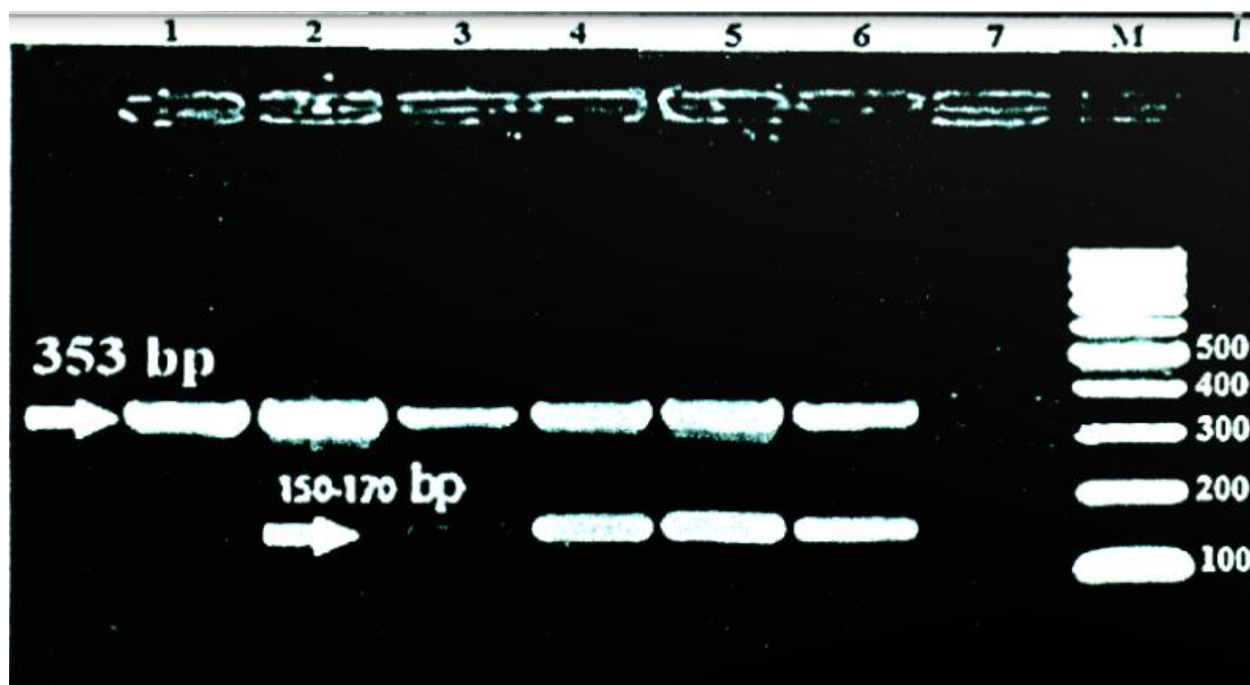

Figure 2. Multiplex PCR for detection of *blaOXA-51*-like gene and Class I integrase gene. Lane 1 to 5: positive results for *blaOXA-51* like gene. Lane 4 and 5: positive results for class I integrase gene. Lane 6: positive control- Lane 7: negative control -M: DNA marker (100 bp).

was tolmipenem (31.24%) and tetracyclines (25.2%). This agreed with Hashem et al. (2011) who showed that tetracycline was the most effective antimicrobial agent against *A. baumannii* derivatives and cephalosporins were the least active agents against *Acinetobacter* when compared with Impinem. Similar results reported by Hanaa et al. (2010) who found that sensitivity of *Acinetobacter* to imipenem. The findings of the present study confirm that penicillin was 64.2% while susceptibility to penicillin derivatives and cephalosporines was 0%.

Table 5. Relation between Imipenem Susceptibility & Detection of *blaOXA-51*-like gene (intrinsic carbapenamse gene).

Sample		Imipenem susceptible and positive blaOXA-51-like gene(Hidden Metallo-B-lactamase)		ImipenemResistant and positive blaOXA-51-like gene(Expressed Metallo-B-lactamse)	
Type	**Number**	**Number**	**Percentage**	**Number**	**Percentage**
Clinical	23	16	70	7	30
Environmental	26	17	65	9	35
Total	49	33	67.5	16	32.5

The predominant *Acinetobacter* sp. in clinical settings are the members of the *A. calcoaceticus-baumannii* complex which are multi drug resistant and are responsible for causing outbreaks. Carbapenem resistance in *A. calcoaceticus-baumannii* complex is very high and is predominantly due to carbapenemase production, metallo-β-lactamases, oxacillinases, mobile genetic elements, and reduced expression of outer membrane proteins (Limansky et al., 2002; Poirel et al., 2003; and Anil et al., 2011).

E-test results were showed that (34/51 or 66.67%) of *Acintobactersp* isolates were *imipenem* susceptibile (MIC below 4 µg/ml), while (17/51 or 33.33%) were imipenem resistant (MIC above 8 µg/ml). This agreed with (Livermore, 2002) who found that the high levels of imipenem MIC (16-256 mg/L) observed in these *A. baumannii* isolates suggested the presence of a metallo-β-lactamase (MBL) or an oxacillinase, since these carbapenemases were considered the major mechanism of carbapenem resistance in these organisms.

The resistance of *A. baumannii* to antimicrobial agents is mediated by all of the major resistance mechanisms that are known to occur in bacteria. β-Lactamases are the most diverse group of enzymes that are associated with resistance, and more than 50 different enzymes, have been identified so far in *A. baumannii*. OXA-51-like carbapenemases are class D β-lactamases which are intrinsic to *A. baumannii* and confer resistance to carbapenems (Turton et al., 2006a; Brown et al., 2005).

In this study, (96%) of *A. baumannii* were showed band of *blaOXA-51*-like genes. This agreed with Turton et al. (2006b) who found that all *A. baumannii* gave a band in the blaOXA-51-like PCR, but they remain alert to the possibility of non-detection of some variants. A further potential problem is that these genes are sometimes associated with *ISAba1*, which may render them mobile. We currently encounter also results of Hanna et al. (2010) that showed that detection of *blaOXA-51*-like genes is the most specific, simple and reliable method for detection of *A. baumannii* as carbapenamse gene is intrinsic to this species.

Among imipenem-susceptible and resistant *A. baumannii* which were screened by PCR for different β-lactamases. The *blaOXA-51*-like gene was the only one detected, even in imipenem-susceptible strain (Takagi et al., 2009). It has been reported that among *A. baumannii* isolates with *blaOXA-51*-like as sole carbapenemase gene, imipenem and/or meropenem resistance was associated only with isolates in which *ISAba1* was upstream of *blaOXA-51*-like, suggests that *ISAba1* is providing the promoter for this gene (Turton et al., 2006a).

In this study, (72.5%) of *A. baumannii* showed positive bands for class I integrase gene (gene responsible for outbreaks in hospitals). The analysis of *A. baumannii* strains with known epidemic behavior demonstrates that early identification of epidemic strains may be possible by detection of integrons or multiple antibiotic resistances. The integrase gene PCR identified almost 75% of the epidemic *A. baumannii* strains. Multiple antibiotic resistances, defined as resistance to five or more antibiotics, showed good correlation with the presence of integrons and epidemic behavior of the strains.

This result agree with (Dijkshoorn et al., 1996) who showed that strains may vary considerably in their epidemiological potential, and those strains that have been known to spread widely and rapidly among hospitalized patients have been designated epidemic *A. baumannii* strains. Antibiotic resistance has been shown to be one of the factors which can influence the nosocomial dissemination of *A. baumannii.* Few reports credit outbreak control to reduced prescribing of broad spectrum antibiotics, such as fluoroquinolones or carbapenems (Villegas and Hartstein, 2003).

Among imipenem-susceptible and resistant *A. baumannii* which were screened by PCR for different β-lactamases. The *blaOXA-51*-like gene was the only one detected, even in imipenem-susceptible strain (Takagi et al., 2009). It has been reported that among *A. baumannii* isolates with *blaOXA-51*-like as sole carbapenemase gene, imipenem and/or meropenem resistance was associated only with isolates in which ISAba1 was upstream of *blaOXA-51*-like, suggests that *ISAba1* is providing the promoter for this gene (Turton et al., 2006a).

Conflict of Interests

The author(s) have not declared any conflict of interests.

Conclusion

The detection of *bla OXA-51*-like gene is the most specific method for detection of *Acinetobacter baumannii* carbapenamase gene which is intrinsic in this species. Also de-

tection of class I integrase gene is very important in the rapid epidemiologic investigation of an outbreak.

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Review

Human papilloma virus and cervical neoplasia in HIV positive women: A non systematic review

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Human papilloma-virus (HPV) infection confers 85-90% of the attributable risk for the development of cervical dysplasia. Worldwide and in particular in Nigeria, HPV 16 has been shown as the most prevalent HPV type and it also contributes more to the development of invasive squamous cell carcinoma. Studies have also shown that the prevalence of HPV is higher among HIV-positive women than among HIV-negative women of all age groups. HIV-positive women also have a higher incidence of squamous intra-epithelial lesion (SIL) and invasive cervical cancer. Progression to cervical cancer is also more rapid amongst these patients and often refractory to treatment with high incidence rates. Current screening recommendations for HIV-positive women are accessible and developed in rich countries. The best strategy for screening infected women in poorer nations where human immunodeficiency virus (HIV) is rampant remains uncertain and challenging.

Key words: Human papilloma-virus (HPV), human immunodeficiency virus (HIV), cervical dysplasia, invasive squamous cell carcinoma.

INTRODUCTION

Historical perspective

Historically, Papillomavirus has co-evolved with vertebrates. Virtually all vertebrate species have warts. This has been described for thousands of years. In the beginning of the current century, Cuffo established the viral etiology of human warts (Papillomas) when he used cell-free extracts from wart tissue as an innoculum for man-to-man transmission experiments (Stoler, 2000). In 1933, Shoppe first described Papillomavirus in cotton-nail rabbits (Stoler, 2000).

In 1973, zur Hausen proposed the concept of viral oncogenesis in the development of cervical cancer and in 1977 the same author indicated the possible role of Human Papillomavirus (HPV) in the development of squamous cell carcinoma of the uterine cervix (Ruud et

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al., 2004). In the late 1980s, the development of a technology to test for the presence of HPV DNA in cellular specimen in conjunction with multidisciplinary collaborative efforts, made possible the establishment of a definitive etiological role for HPV in cervical cancer (Bosch et al., 2002).

THE HUMAN PAPILLOMAVIRUS

Viruses from Papilomaviridae family have been classified as members of the Papova super-family. Its name was given by taking the first two letters of the major genera: Papilloma, Polyoma and Simian Vacuolating Viruses, respectively. All members of Papilomaviridae family are small, double-stranded DNA viruses that replicate in the nucleus and have icosahedral protein capsules that form non-enveloped virions. They are biologically distinct from the Simian Virus 40 (SV40) and polyoma viruses. They have 55nm capsids.

The HPV genome can be divided into an early region, a late region and a non-coding long control or upstream regulatory region (URR). The early region encodes for proteins that are expressed before the onset of viral DNA replication, while the late region encodes viral capsid proteins. The early and late regions have several open reading frames (ORF) resulting in translation of functional proteins. Through gene splicing ORF encode for all viral gene products. The early region ORF is expressed early in the viral life cycle and they include: E1, E2, E4, E5, E6 and E7. The E1 encodes a protein that maintains the viral genome. E2 is involved in the transcriptional regulation and control of genes E6 and E7. The E4 gene encodes a protein that finally breaks-off the cytoplasmic keratin networks, resulting in koilocytic cells in the upper layers of the epithelium. The E5 gene encodes a protein which boosts the mitogenic responses of the epithelial host cells to stimulate replication of the virus. E6 and E7 encode multifunctional proteins that control proliferation and transformation, and they are the only open reading frames that are conserved and expressed in all HPV-associated pathologies. These pathologies include the full spectrum from low grade lesions with no neoplastic potential to high grade invasive cancers. L2 and L1 encode major and minor capsid protein of the virus, respectively (Stoler, 2000).

HPV-mediated carcinogenesis

Infection with HPV is an early event in the multistep process of cervical carcinogenesis. In benign and low grade lesions, the HPV genome is maintained in an episomal state (free from the nucleus). With the progression of cervical intraepithelial neoplasm (CIN), HPV is often found integrated in the genome of the host cell. This integration process disrupts E2 region and function, leading to over-expression of E6 and E7 (Stoler, 2000). The proteins encoded by E6 and E7 are high-risk oncoproteins. HPV E6 interacts with p53, interfering in this way with its functions. p53 mediates cell cycle arrest during the G1 phase in order to allow for DNA-repair, but also activation of apoptosis to eliminate cells with damaged DNA. Interaction of E6 leads to p53 dysfunction, thus impairing the ability to block the cell cycle when errors develop. Independent of its effects on p53, E6 also activates telomerase, a ribonucleoprotein complex that catalizes the synthesis of telomere repeat sequences, thereby preventing telomere shortening and leading to cell immortalization (Stoler, 2000).

EPIDEMIOLOGY OF HUMAN PAPILLOMAVIRUS

For decades, the epidemiological profile of women with cervical cancer was recognized as suggestive of a sexually-transmitted process. Several agents were implicated as causative, agents such as syphilis, gonorrhea and type 2 herpes simplex virus (Bosch et al., 2002). However, by the beginning of the current century, sufficient evidence, which included a large and consistent amount of studies, showed beyond all reasonable doubt strong and specific associations, relating HPV infection to cervical cancer (Bosch et al., 2002).

Other studies have also shown that HPV infection preceded the development of cervical cancer by several years (Bosch et al., 2002). Determinants of clinical progression include persistence of infection, involvement of high risk types, high viral load, integration of viral DNA and several other potential risk factors (Cuoltee et al., 2005). Co-factors, which are now viewed as surrogates of HPV exposure, include low socio-economic status, young age at sexual debut, high parity, high numbers of lifetime sexual partners, smoking and use of oral contraceptives, as well as any combinations of the described above (Bosch et al., 2002).

GEOGRAPHIC DIVERSITY IN HPV GENOTYPE DISTRIBUTION

Two and half percent of all cancers in the developed world are associated with HPV, while 7.8% of all cancers in the developing world are associated with HPV (Ruud et al., 2004). HPV genotypes can be divided into mucosotropic types, which are found in the mucous epithelium of the oropharynx and anogenital tracts, and cutanous types, which predominantly infect the skin (Ruud et al., 2004). More than 35 genotypes have been shown to infect mucosal surfaces, and at least 18 of them have been associated with cervical cancer. HPV genotypes differ widely in their geographic distribution. In sub-Saharan Africa, where two-thirds of the world’s HIV infected people live, it has been shown that HPV types vary by

both country and HIV status, and they differ significantly from types seen in other regions of the world.

To investigate the geographic variations in the distribution of HPV types, Bosch and fellow researchers (1995) obtained more than 1000 specimens from sequential patients with invasive cancer. These specimens were stored frozen at 32 hospitals in 22 countries. Polymerase chain reaction- (PCR)-based assays were used to detect the different HPV types. According the results, HPV DNA was detected in 93% of the tumors. HPV 16 was presented in 50% of the specimens, HPV 18- in 14%, HPV 45- in 8%, and HPV 31- in 5%, respectively. HPV 16 was the most predominant type in all the countries except Indonesia, where HPV 18 was more common. These authors also discovered significant geographic variation in the prevalence of some less common viral types with a clustering of HPV 45 apparent in Western Africa, while HPV 39 and HPV59 were almost confined to Central and South America. They also showed that HPV 16 predominated in squamous cell tumors (51%), while HPV 18 predominated in adenocarcinomas (56%) and in adenosquamous tumors (39%).

In a similar study, carried out by Sanjose et al. (2010) on HPV genotype attribution in invasive cervical cancer, a retrospective cross-sectional worldwide study, paraffin embedded samples of histologically confirmed cases of invasive cervical cancer were collected from 38 countries in Europe, North America, Central and South America, Africa and Oceania. HPV has been detected by PCR with SPF-10 broad spectrum primers, followed by DNA enzyme immunoassay and genotyping with a reverse hybridization line probe assay. According the data obtained, the most common HPV types identified were types 16, 18, 31, 33, 35, 45, 52 and 58. HPV types 16 and 18 were detected in 71% of invasive cervical cancers, while types 16, 18 and 45 were detected in in 94% of cervical adenocarcinomas. Also, related to HPV, types 16, 18 or 45 invasive cervical cancers have been presented in women at a younger mean age than in those, related to other HPV types (Sanjose et al., 2010).

In order to study the relative carcinogenicity of HPV types in Nigeria, as well as to estimate the vaccine preventable portion of invasive cervical cancer (ICC), Okolo et al. (2010) compared HPV type prevalence among 932 women from the general population of Ibadan with that among a series of 75 ICC cases diagnosed in the same city. According to the results obtained, 26.3% of the women were HPV-positive, and among them the prevalence of HPV 35 and 16 has been equally frequent. In ICC patients, however, HPV 16 predominated strongly (67.6%) with the next most common types being 18, 35, 45 and 56 in descending order. It was concluded that in Nigeria, as elsewhere, women infected with HPV 16 and 18 are at a higher risk of developing ICC than those infected with other high risk types and that current HPV 16/18 vaccine have enormous potential to reduce cervical cancer in Nigeria.

Other studies, carried out in other West African countries like Gambia, equally showed HPV 16 to be the most prevalent HPV type, probably strongly associated with squamous intraepithelial lesion (Wall et al., 2005). Similar studies, carried out in Dakar, Senegal, have also indicated HPV 16 (2.4%) and HPV 58 (1.6%) to be the most frequent HPV types in this population, as well as to be the most strongly associated with the risk of high grade squamous intraepithelial lesion and cancer. These data suggest that in addition to HPV type 16, HPV type 58 should be also considered in the strategic planning of vaccination against cervical cancer in this geographic region (Xi et al., 2003).

HPV and HIV

It is estimated that 33 million people around the world are living with human immunodeficiency syndrome and acquired immune deficiency syndrome (HIV/AIDS). The burden of this epidemic resides largely in sub-Saharan Africa, which in 2007 accounted for 67% of all people living with HIV and 70% of all AIDS deaths (World Health Organisation HIV burden available at http://www.searo.who.int/Linkfiles). In Nigeria about 4 million people living with HIV/AIDS have been estimated (Anorlu et al., 2007). Cervical intra-epithelial neoplasia is considered an HIV-related condition, while invasive carcinoma of the cervix is an AIDS defining disease (Paintomowitz and Michelow, 2010). Several studies have demonstrated a higher prevalence of HPV in HIV-positive women, when compared with HIV-negative women (Paintomowitz and Michelow, 2010), probably due to the fact that both HIV and HPV are both sexually-transmitted diseases. In HIV-negative women with competent immune systems, most of the infections are cleared spontaneously because of a cell mediated immune response regulated by CD4+ lymphocytes (World Health Organisation HIV burden available at http://www.searo.who.int/Linkfiles). HIV co-infected individuals are at a higher risk of persistent HPV infection largely due to their impaired ability to clear HPV and are thus at an increased risk to develop cervical dysplasia and cancer (Firnhaber et al., 2009).

In 2005, in a study of HPV and cervical cytology in infected and non-infected with HIV Rwandan women, Singh and co-authors carried out an observational prospective cohort study on 710 HIV-positive and 226 HIV-negative Rwandan women. According these authors, the prevalence of HPV was higher in HIV-positive than in HIV-negative women in all age groups. Among HIV infected women, 69% have been positive for greater than one HPV type, 46% - for a carcinogenic HPV type, and 10% - for HPV16, respectively. HPV prevalence peaked at 75% in HIV-positive women aged 25-34 years and declined with age to 37.5% in those greater than 55 years old. Among the study population, certain HPV types (11,

39, 43, 51 and 59) occurred more frequently in HIV-positive women (Baay et al., 2004).

In New York, USA, Sun et al. (1985) performed a study of HPV infection among HIV-positive women, and the results have shown that HIV-sero-positive women were more likely than HIV-sero-negative women to have HPV DNA of any type detected (60% versus 36%), HPV type 16 and 18 were also more common among HIV-positive than among HIV-negative women- (27 vs. 17% and 24 vs. 9%), respectively. HIV co-infection has also been found to have an impact on HPV genotype distribution (Baay et al., 2004). According to many studies in this direction, HIV-positive women harbor a broad diversity of both high and low risk HPV genotypes, usually with a high frequency of oncogenic types such as types 16, 18, 33, 35, 52 and 59 (Sahasrabuddhe et al., 2007).

HIV-positive women with low CD4+ lymphocytes counts have had the highest prevalence of HPV infection and have also shown higher detection rates of mixed HPV types. They were also at greater risk of persistent cervical HPV infection (Strickler et al., 2005). Plasma RNA levels have been found to be strongly associated with HPV incidence than with HPV persistence (Strickler et al., 2005).

To determine the possible role of cellular immune-deficiency as a co-factor in the genesis of genital neoplasia, in Hannover, Germany, Petry et al. (1994) examined 48 HIV infected women and 52 allograft recipients periodically during a 3-years period. Colposcopy, cytology and HPV DNA genotyping were performed at each visit. Each cervical lesion was matched prospectively with 2 lesions from immuno-competent controls. According to the authors, low grade lesions among patients progressed more often than among controls. Also, recurrent lesions after destructive treatments were seen more frequently among patients than among controls. Patients with counts of CD4+ lymphocytes less than 400 cells/mm^3 or immuno-suppression for more than 3 years have also been found to suffer from progressive lesions. However, in study of squamous intraepithelial lesions among HIV-sero-positive women from July 1993 to June 1994 in Italy, Sopracordevole et al. (1996) discovered no significant difference in the CD4+ lymphocytes count between women with and without squamous intra-epithelial lesion (SIL), as well as no relationship between those counts and the severity of SIL. Similar studies, performed by Cardillo et al. (2001) among 108 HIV infected women, showed that there was no apparent difference between the counts of CD4+ white blood cells from women with low grade lesions and those with high grade lesions. However, the HIV viral load was significantly higher in patients with cytologic abnormalities than in these with negative Papanicolaou (Pap) smears. It was therefore concluded that the degree of immune-suppression may contribute to the development of intra-epithelial lesions in HIV-positive women, but once the lesion is established, disease progression may not be affected by CD4+ lymphocytes counts (Cardillo et al., 2001).

THE ROLE OF HIGHLY ACTIVE ANTI-RETROVIRAL THERAPY (HAART)

The introduction of HAART in the late nineties led to dramatic improvement of clinical outcomes and life expectations for people living with HIV/AIDS. It also gave hope that improved immunological status would lead to better clearance of HPV infection in HIV-positive women, just as occurs in other opportunistic AIDS-associated infections (Bratcher and Sahasrabuddhe, 2010). Increasing number of HIV-infected women are now accessing life prolonging HAART in developing countries. Data regarding the impact of HAART on reducing incidence and progression or facilitating the regression of HPV infection and cervical abnormalities is largely inconsistent (Bratcher and Sahasrabuddhe, 2010). This inconsistency may be due to the study designs, carried out in the past (prospective or retrospective cohorts, or record linkage studies) screening and diagnostic protocols, duration and type of HAART use, recruitment and referral strategies and definition of screening test, as well as disease positivity (Bratcher and Sahasrabuddhe, 2010).

In a study, carried out by Heard et al. (1998) in France, for determination of the outcome of SIL in HIV infected women initiating triple combination antiretroviral therapy, 49 women were examined prior to and after a median of 5 months treatment. It was discovered that the prevalence of SIL decreased from 69 to 53% during follow-up. Among 13 women who initially presented with high grade SIL, conversion to lower grade was observed in 2 women and a full regression to normalcy - in one woman, respectively. Cytology also returned to normalcy in 9 out of 21 women, initially presented with a low grade SIL. These results suggested that HAART may result in reduced prevalence of cervical SIL despite none clearance of HPV infection. Similar studies on the regression of cervical intra-epithelial neoplasia (CIN) in HIV infected women on anti-retroviral therapy (ART) also showed that the risk of regression of CIN was twice as high in women receiving HAART as compared to women not receiving HAART (Heard et al., 2002). It was concluded that the positive impact of HAART on CIN regression may be associated with some restoration of specific immune reactivity. However, studies carried out in South Africa to determine HPV prevalence, viral load and precancerous lesions of the cervix in women initiating HAART therapy, showed that these women have a high prevalence of abnormal Pap smears and high risk HPV, thus emphasizing the need for locally relevant, rigorous screening protocols so that the benefits of HAART are not partially offset by an excess risk in cervical cancer (Moodley et al., 2009).

In Spain, Sierra et al. (2008) carried out a retrospective cohort study to evaluate the effect of HAART on HIV infected women with normal cytology and CD4+ lympho-

cytes counts above 350 cells /mm^3. The patients were divided into two groups: on HAART and not on HAART. Both groups were similar with respect to demographic characteristics except for HIV viral load and previous HAART inclusion. SIL has been diagnosed in 27 out of 90 (30%) patients in the HAART group and in 7 out of 37 (19%) in the non-HAART group, respectively. The actuarial probability of remaining free of SIL at 3 years was 70% in HAART group. It was therefore concluded that when patient's immunological status is above 350 CD4+ lymphocytes/mm^3, the HIV infected women, treated with HAART present a similar cervical SIL incidence to HIV infected women not on HAART (Sierra et al., 2008).

According another study, carried out to evaluate the effect of HAART on HPV clearance and cervical cytology, among HIV-positive women with cervical squamous intraepithelial lesions, HAART was associated with an increased likelihood of HPV clearance unlike in HIV-positive women with normal cytology or atypical squamous cells of undetermined significance (Paramsothy et al., 2009). Use of HAART was also not significantly associated with an increased likelihood of cervical cytologic regression or of cervical cytologic progression (Paramsothy et al., 2009).

HIV and squamous intraepithelial lesions (SIL)

HIV-positive women have been characterized with higher rates of squamous intraepithelial lesions as compared to those who are HIV-negative (Anorlu et al., 2007). In the study, performed by Anorlu et al. (2007) for determination the prevalence of abnormal cervical smears in HIV-positive Nigerian women in Lagos, the prevalence of SIL was found to be higher in HIV-positive than in HIV-negative. Also, higher grade SIL among HIV-positive than among HIV-negative subjects has been observed. There was no significant difference in the prevalence of inflammatory smears between the two categories (Anorlu et al., 2007).

It has been proposed that young women are more susceptible to cervical infection due to immaturity of the cervix, which could be explained with the fact that HPV has more access to the basal cells of the differentiating epithelium. Exposure to this virus before the stabilization of the transformation zone and maturation of the cervix could lead to an increased susceptibility to infection (Calore et al., 1998). In a study of cervical smears of 82 adolescent HIV-seropositive women (13-21years of age), Calore et al. (1998) found that 21 cases (25.6%) possessed characteristic features of HPV infection and SIL. Sixteen cases aged from 17 to 21 years (19.5%) had low grade SIL (LSIL), while five cases (6.1%) had high grade of SIL. There were no significant differences between the mean age of patients with LSIL and HSIL. Two cases have had atypical squamous cells of undetermined significance (ASCUS). It was therefore concluded that HIV-seropositive adolescents have probably a high risk of pre-neoplastic cervix lesions (25.6%), as well as high incidence of more aggressive lesions (6.1% of HSIL), in comparison with the general population of adolescents (Calore et al., 1998). Among HIV-infected women, HPV disease, as manifested by findings of SIL or cervical intraepithelial neoplasia (CIN), is influenced by HIV-induced immuno-suppression. Indeed HIV-positive women with severe immuno-suppression (defined as CD4+ lymphocyte counts below 200 x 10^6) are at greatest risk of CIN (Ferenzy et al., 2003). While the degree of immuno-suppression may contribute to the development of SIL in HIV-positive women, there appears to be no difference in CD4+ white blood cells counts between women with high and low grade lesions (Cardillo et al., 2001). These data suggest that once there is establishment of SIL, disease progression may not be affected by CD4+ lymphocytes counts alone. The converse may also be true for HIV infected women with high CD4+ cells counts. In a study for determination of the incidence of SILs in HIV-seropositive women with normal cytology data by baseline HPV DNA results, it was discovered that HIV-positive women with CD4+ lymphocytes counts higher than 500 x 10^6 have had similar incidence of SIL as those who were HIV-seronegative (Lehtovirta et al., 2003). It was therefore suggested that similar cervical cancer screening practices may be applicable to both groups, although the strategy would warrant evaluation in an appropriate clinical trial.

Studies, carried out confirmed the suggestion that HIV infected women with CIN experienced high recurrence rates after treatment (Foulot et al., 2008). Recurrence was also inversely related to CD4+ lymphocytes counts with the highest rates seen in women with values < 200 x10^6.

INVASIVE CERVICAL CANCER

According to case-control studies, case series and prevalence surveys, performed beyond all reasonable doubt, HPV DNA can be detected in adequate specimens of cervical cancer in 90-100% of cases, as compared to a prevalence of 5-20% in cervical specimen from women identified as suitable epidemiological controls (Bosch et al., 2002). This association has been recognized as causal in nature since the early 1990's and a claim that it is the first necessary cause of human cancer that has ever been identified has been made. This implies that in the absence of HPV DNA, cervical cancer does not develop (Bosch et al., 2002). HPV-associated malignancies have also been shown to be more common among the patients with HIV/AIDS (Morten et al., 2000). Studies have also shown that cervical cancer is more frequent in HIV infected women than their un-infected counterparts (Morten et al., 2000). Besides that, progression to cervical cancer in these individuals has been found to be more rapid and often more refractory to therapy with high recurrence

rates (Paintomowitz and Michelow, 2010). Cervical cancer has also been considered as an AIDS defining illness since 1993 and studies have also shown that cervical cancer develops several years earlier in people, infected with HIV/AIDS than in uninfected counterparts (Paintomowitz and Michelow, 2010).

In a retrospective review of 60 HIV-sero-positive and 776 HIV-sero-negative new cases of cervical carcinoma in South Africa, it was discovered that HIV–sero-positive patients, presented with invasive cervical cancer almost 10 years earlier than HIV-sero-negative patients. Even though, HIV-sero-positivity on its own did not appear to adversely affect the extent of disease at presentation, patients with CD4+ lymphocytes counts below 200/mm^3 are significantly more likely to have advanced –stage disease at initial diagnosis than with HIV-negative patients (Lomalisa et al., 2000).

THE LABORATORY DIAGNOSIS OF GENITAL HPV INFECTION

Specimen collection and transport

Superficial epithelial cells from the ectocervix are usually collected by scraping with a spatula. Cyto-brushes and Dacron swabs are also used to collect cells from the squamo-columnar junction (Cuoltee et al., 2005). The sensitivity of HPV detection is greater when a cyto-brush is used in the collection of samples than a Daccon swab (Peyton et al., 1998). After specimen collection, exfoliated cells are resuspended into appropriate transport medium used with DNA-based methodology for HPV detection.

Microscopy

Cervical cytology samples are usually viewed with microscope. This may reveal certain lesions such as koilocytes. Those are squamous cell, exhibiting perinuclear halo or clearing with increased density of surrounding cytoplasm. Hallmarks of productive HPV infection include nuclear atypia (enlargement), hyperchromasia, irregular membranes and double nucleation of intermediate and superficial cells (Cuoltee et al., 2005).

The sensitivity of conventional cytology smears in the detection of cervical lesions ranges from 29-56%. This is due to the fact that at best only 20% of the cells are smeared on a slide with the remaining 80% lost with the collection device (Ferenzy and Franco, 2001). Liquid-based thin layer cytology is a promising alternative that has been shown to improve the sensitivity of conventional cytology for detecting HSIL by 60% and providing an overall sensitivity of 80% (Ferenzy and Franco, 2001). Another advantage of liquid-based cytology medium for the collection of cervical specimen is the fact that multiple tests can be done with a single sample. This is very helpful when considering cases such as atypical squamous cells of undetermined significance (ASCUS), where HPV testing can be performed on the temporarily stored Pap specimen without the need for another follow-up visit (Peyton et al., 1998).

DIAGNOSTIC TESTS FOR HPV DETECTION

Nucleic acid hybridization methods

There are essentially three types of nucleic acid hybridization method formats used to detect HPV. These include hybridization signal amplification, target amplification methods and direct nucleic acid probe method.

Signal amplification DNA-based assays: Hybrid capture system

This test can detect low quantities of DNA by amplifying the detection signal without modifying the initial amount of nucleic acids contained in the samples (Coultee et al., 1997). There are two main types: the first generation hybrid capture tube test and the second generation Hybrid Capture II (HCII), which is the only type approved by the US Food and Drug Administration with an increased analytical sensitivity, but it is also a more efficient kit format (Cuoltee et al., 2005). Using these tests, exfoliated cervical cells are collected in a conical brush provided by the specimen collection kit and re-suspended in the specimen transport medium that can be kept at room temperature for up to 2 weeks.

Target amplification based assays: Polymerase chain reaction (PCR)

PCR is nowadays the gold standard test of HPV research. Type-specific PCR tests are not practical means of detecting HPV infections in clinical specimens due to the large number of types involved in genital disease. Due to the genetic polymorphisms of HPV, consensus PCR assays are now being employed to amplify in one reaction, the majority of known and novel anogenital HPV genotypes (Cuoltee et al., 2005). Subsequent typing can be accomplished on filters by hybridization with type-specific oligonucleotide probes, homogenous hybridization reactions with RNA probes, restriction fragment length polymorphisms or by DNA sequencing (Doorn et al., 2002).

Direct probe methods: Southern blotting

This method is the gold standard of HPV genomic analysis. Because formalin-catalysed DNA cross-linking with resulting DNA degradation makes it impossible to per-

form, this assay cannot be carried out on formalin preserved tissues (Hubbard, 2003).

Cervical cancer screening

The objective of cervical cancer screening is to prevent the occurrence of cervical cancer and death from it, by detecting promptly and treating precursor lesions of this malignacy. The most widely used screening approach is to detect high grade squamous intra-epithelial lesion (HGSIL) by conventional cytology, followed of the investigation of positive women by colposcopy and directed biopsy (Monsonego et al., 2004). In some parts of the world, such as the United States, the mortality from cervical cancer has been decreased by over 70% owing to the introduction of Papanicolaou (Pap) test. In these regions, pre-invasive lesions of the cervix are detected far more frequently than invasive cervical cancers (Saslow et al., 2002).

There is no single, agreed upon guideline for cervical screening in HIV patients (Paintomowitz and Michelow, 2010). According to the American Cancer Society (ACS), women between the ages of 21 and 30 years, infected with HIV, should be screened annually for cervical cancer, and every 2-3 years for women 30 years and above if three consecutive Pap tests are negative (Saslow et al., 2002). The Center for Disease Control and Prevention (CDC) recommend screening of HIV-positive women at six monthly intervals for the first year after an HIV diagnosis, followed by annual cervical smears if the results are normal (Paintomowitz and Michelow, 2010). The British HIV association recommends that HIV-positive women should do baseline colposcopy soon after diagnosis and cervical smears every year. The age range screened should be the same as for HIV-negative women (Browser et al., 2008). Some authors recommend that surveillance of these women should be based on the individual woman's risk for cervical intraepithelial neoplasia (CIN). Women who are not immune suppressed (CD4+>500/mm^3) and have only slightly increased risk of CIN, may be followed by annual or possibly semiannual Pap smears. Immuno-suppressed women (CD4+ <500/mm^3), and especially those with CD4+<200mm^3, whose risk for CIN might be the same as in the women from the general population, who have SIL on their Pap smear, should be subsequently subjected on colposcopy (Mark, 1999).

According other investigators, there are significant limitations to cytologic screening for identification of SIL in HIV-positive women, as compared to the general population, which has been proposed to be due to high frequency of occurrence of false negatives in HIV-positive women (Womack et al., 2000). Taking into consideration all that, colposcopy has been suggested to be performed routinely for HIV-positive women (Browser et al., 2008). Baseline colposcopy is also recommended for examination of the entire anogenital region, probably because of the increased vulval, vaginal and anal intraepithelial neoplasia (AIN) in HIV-positive women (Paintomowitz and Michelow, 2010). However, routine colposcopy for all HIV infected women is not supported by everyone. This procedure, however, should need personnel required to be carried out (Paintomowitz and Michelow, 2010), which would be difficult in many resource-poor settings due to the cost. In the presence of both CD4+ lymphocytes counts as alluded above and the results of HPV DNA test, appear to be useful indicators of the risk.

Due to the relative insensitivity of conventional cytology, frequent testing is required for optimal cancer protection, thus compromising cost efficiency. The most cost effective regimen is to use the most sensitive possible test at the longest possible interval, thus relieving the system of the cost of evaluation and treatment of large numbers of abnormal screening tests. In most cases, these tests represent low grade transient abnormalities, whose recognition adds greatly to cost without increasing the cancer protection (Monsonego et al., 2004). Studies have shown that the addition of HPV testing to the two cervical cytology smears obtained in the year after HIV diagnosis, together with subsequent modifying cytology screening intervals, based on the results, appears to be a cost-effective modification to current recommendations for annual cytology screening in HIV infected women (Goldie et al., 2001). However, according to other studies, HPV testing, although characterized by high sensitivity, may not be ideal due to the low specificity that results largely from a very high prevalence in non-diseased women (Womack et al., 2000). Some authors advocate that women who test negative for HPV and who have two negative initial Pap test results, could undergo annual cytology screening. However, those who are positive for high risk of HPV DNA, should have Pap tests every six months. This differs from the recommendations for HIV-negative women, in whom prolongation of screening interval to not less than three years is recommended if both cytology and HPV results are normal (Paintomowitz and Michelow, 2010). Further studies are required to refine appropriate screening protocols, intervals and follow-up algorithms in HIV-positive women (Paintomowitz and Michelow, 2010). The usefulness of HPV test as a screening method for cervical cancer in areas of high HPV prevalence would depend on local health resource availability, disease priorities and policies regarding clinical case management (Womack et al., 2000).

Treatment

The British society for colposcopy and cervical cytology recommends only lesions, which are cervical intraepithelial neoplasia (CIN2) and above, should be treated. Women who have lower grade lesions should be monitored by regular cytologic reviews, since these lesions may clear on their own (Browser et al., 2008). Once con-

firmed by tissue biopsy, high grade CIN can be treated by both ablative and excisional methods. Ablative methods include cryo-therapy and laser ablation, while excisional methods include cold knife, laser conization and loop electrosurgical excision (LEEP) (Paintomowitz and Michelow, 2010). Studies have also shown that in HIV infected women, CIN may recur despite multiple treatments and that chronic condylomatous changes are common (Frutcher et al., 1996). Intra-vaginal application of 5-Fluorouracil (5-FU) after standard surgery for high grade lesions can reduce recurrence rates of CIN in HIV-positive women (Maimam et al., 1999).

Invasive cervical cancer in HIV-infected patients remains a challenge due to the fact that the management of malignancy may further impact the patient's immune system (Moodley, 2007). Most HIV-positive patients with cervical cancer present with late stage disease (Moodley, 2007). The standard management of invasive cervical cancer in them is surgery, radiotherapy and chemotherapy, depending on the cancer stage (Paintomowitz and Michelow, 2010). However, women with early stage cervical cancer are managed by radical hysterectomy and lymph node dissection (Moodley, 2007).

CONCLUSIONS

HPV infection confers 85-90% of the attributable risk for the development of cervical dysplasia (Stoler, 2000). Worldwide and particularly in Nigeria, HPV 16 has been shown to be the most prevalent HPV type and it also contributes more to the development of invasive squamous cell carcinoma (Okolo et al., 2010). Studies have also shown that the prevalence of HPV is higher among HIV-positive women than HIV-negative women of all age groups (Singh et al., 2009). HIV-positive women also have a higher incidence of SIL, and invasive cervical cancer (Anorlu et al., 2007; Morten et al., 2000). Besides that, the progression to cervical cancer is more rapid amongst these patients and it is often refractory to treatment with high incidence rates (Paintomowitz and Michelow, 2010). Current screening recommendations for the HIV-positive women pertain largely to developed countries. However, the best strategy for screening of infected women in poor nations, in which HIV is rampant, remains uncertain and challenging (Paintomowitz and Michelow, 2010). Only lesions that are CIN2 and as was described above, treated and once confirmed on tissue biopsy, high grade lesions could be treated by both ablative and excisional methods. Invasive cervical cancer is currently managed by a combination of surgery, radiotherapy and chemotherapy (Moodley, 2007).

Conflict of interest

The authors declare that they have no conflict of interest.

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Full Length Research Paper

Chemical control of dry bubble disease induced by *Verticillium fungicola* [Preuss] Hassebr on white button mushroom, *Agaricus bisporous*

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Dry bubble disease induced by *Verticillium fungicola* has been observed as an important disease of white button mushroom (*Agaricus bisporus*) in India. The symptoms produced on well differentiated fruit body are localized light brown depressed spots. The adjacent spots coalesce together to form irregular blotches. If the host pathogen infection is established before differentiation, sclerodermoid fruiting bodies appear on casing surface. All the four fungitoxicants tested *in vitro* by poisoned food technique inhibited the growth of *V. fungicola* and *A. bisporus.* Carbendazim gave highest percent growth inhibition of pathogen and host followed by Thiophanate-methyl, Dithane Z-78 and Dithane M-45. In all the fungitoxicants when tested in bed condition, Carbendazim was observed to be most effective in reducing the disease incidence

Key words: *Agaricus bisporus, Verticillium fungicola,* dry bubble, carbendazim, thiophanate-methyl, dithane Z-78 and dithane M-45.

INTRODUCTION

White button mushroom is cultivated throughout the world, contributing about 40% of total world production of mushroom (Flegg, 1992). In India, white button mushroom is being cultivated in majority of the states both under seasonal and controlled conditions with an annual production of approximately 42,500 tons (Dandge, 2012). Haryana has become one of the leading states in white button mushroom production with 5312 tons/annum (Tiwari, 2004). Mushroom production is adversely affected by a large number of biotic and abiotic factors. Among the

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Table 1. Compost substrate without chicken manure.

Wheat straw compost		
Wheat straw	300	300
Calcium ammonium nitrate	09	09
Urea	03	03
Wheat bran	15	15
Murate of potash	03	03
Single super phosphate	03	03
Gypsum	30	30
Molasses	05	05

several biotic factors associated with reduction in yield of mushroom, fungal diseases significantly affect the mushroom production and yield.

Most common fungal diseases of white button mushroom are cobweb, dry/wet bubble, false truffle and green mould (Sharma, 1995). Of these diseases, dry bubble caused by *Verticillium fungicola* (Preuss) Hassebr is prevalent in all mushroom growing areas and has 25-50% incidence (Sharma, 1995). Dry bubble disease of white button mushroom causing brown spots was reported for the first time by Malthouse in 1901. He found a species of *Verticillium* associated with this disease. Two types of symptoms were observed. Initially, fungal growth appeared on the casing soil which later spread and turned grayish yellow. After that, light brown superficial spots appeared on the caps which finally coalesced to become large brown blotches. This disease is transmitted by contaminated compost, casing soil (Kumar et al., 2014), human beings and splash of water (Fekete, 1967; Cross and Jacobs, 1969). Mushrooms infected by *V. fungicola* shows typical thickening of stem, resulting in onion shaped fruiting bodies. However, the symptoms vary with the age of the mushroom and the stage of development at which the infection takes place. When mushrooms are infected by this fungus at an early stage, symptoms appear as small undifferentiated masses of tissue up to 2 cm diameter. Fruiting bodies are not properly formed and caps are partially differentiated. When infected at a later stage the stipes are distorted and have tilted caps. Infected mushroom show the presence of grey white mycelial growth and become discoloured and dry but do not rot. They show small pimple like outgrowth or brown grey spots (1-2 cm diameter) on the surface. Such spots often have a yellow or bluish grey halo around them.

In Haryana, the white button mushroom is being cultivated on compost prepared by long method in low cost mushroom houses under seasonal conditions. These factors coupled with poor sanitation and persistence of *V. fungicola* in soil pose a serious threat to the future of mushroom cultivation in Haryana.

MATERIALS AND METHODS

Glassware and equipment

Glassware used in the present study were of Borosil. Polythene bags (30 x 45 cm), polypropylene (7.50 x 30 cm) bags and 500 ml empty glucose bottles were used for spawn and inoculum preparation.

Chemicals

Standard analytical grade chemicals were used in the present study.

Sterilization of glassware

Glasswares were sterilized at 180°C for 2 h in a hot air oven.

Maintenance of culture

Pure cultures of *A. bisporus* and *V. fungicola* were maintained on PDA at 20±1°C.

Preparation of compost

Two methods of composting viz., long method of composting (LMC) and short method of composting (SMC) were followed: Six types of composts were used: Wheat straw compost (LMC); Wheat straw compost with chicken manure (LMC); Wheat straw compost (SMC); Wheat straw compost with chicken manure (SMC); Brassica straw compost with chicken manure (LMC); Brassica straw compost with chicken manure (SMC). These composts were prepared for conducting the experiment.

Compost preparation

Six types of compost were used. The compost was prepared with or without chicken manure in both methods. Wheat and *brassica* straw were spread separately on a pucca floor and wetted thoroughly with clean water for 48 h to attain 70-75% moisture content. Wheat bran was dry mixed, with chemical fertilizer, moistened with water, covered with polythene sheets and kept overnight to facilitate adsorption of chemical fertilizer on the bran. The mixture was evenly spread on wet wheat and *Brassica* straw, mixed and stacked to make a compact rectangular pile. Seven turnings were given to the pile using the turning schedule of 0, 6, 10, 13, 16, 19, 22, 25 and 28 days. At each turn, approximately 30 cm layer was separated from all the exposed surface of the pile and moistened, if necessary. The remaining pile was also dismantled and mixed well. The material was restacked in such a way that the outer portion of the previous pile was in the center of the new pile. Molasses was mixed at first turn, gypsum at the third. Two days after the last turn, the pile was dismantled and the contents were mixed thoroughly. The compost was checked for desirable characteristics, that is, dark brown colour, pH (7-8), absence of ammonia smell and appropriate moisture content (68-72%). The composition for LMC and SMC of the substrates are given in Table 1.

Spawning

Thorough spawning was done at 1% spawn before filling of compost in the polythene bags. In further studies, wheat straw compost with chicken manure prepared by LMC was used (Table 2).

Table 2. Compost substrate with chicken manure.

Wheat/Brassica straw compost		
Ingredient	**LMC (kg)**	**SMC (kg)**
Wheat/ *Brassica* straw	300	300
Chicken manure	100	130
Urea	08	04.00
Wheat bran	15	15
Murate of potash	03	-
Single super phosphate	03	-
Gypsum	30	30
Molasses	05	05

The compost was made separately.

Table 3. Disease appearance on cut-mushrooms.

Treatment	Number of fruit bodies	Number of fruit bodies infected			Infected percentage (%)		
		After hours			hours		
		24	48	72	24	48	72
Inoculated cut mushroom	10	0	6	10	0	60	100
Uninoculated cut mushroom	10	0	0	0	0	0	0

This table show pathogenicity test for dry bubble disease in white button mushroom caused by *Verticillium fungicola.*

Spawn run

After spawning, bags were covered with newspaper sheets sterilized with formalin (4%) and water was sprinkled to keep moist. Temperature was maintained 24 ± 2°C with relative humidity of 85-90%. After pin head initiation, temperature was lowered down to 16± 2°C and RH of 90%. Fresh air was circulated for 3-4 h daily during cropping.

Casing

Casing soil was prepared by mixing well decomposed (16-18 month old) farm yard manure and burnt rice husk (4:1 v/v). The casing mixture was disinfected with 4% formalin solution using 600 ml formalin (36%) diluted to 5 L for 100 kg of casing material. The formalin treated moistened casing material was kept covered with polythene sheets for at least 48 h followed by frequent turnings to evaporate formalin fumes. Before casing, the newspaper sheets were removed from the spawn impregnated compost and the surface was covered with disinfected casing material (4 cm) for uniform thickness.

Cropping

Adequate humidity (85-90%) was maintained inside the growing room by spraying water on the walls and the floor. Water was sprayed on the bags twice a day, very little or no ventilation was provided until the first appearance of pin heads. Thereafter, intermittent cross ventilation was given for a total 4-6 h per day. The mushrooms were harvested by gentle twisting of the fruit body. The depressions created in the casing layer were filled with fresh disinfected casing soil. The lower part of the pileus of harvested fruit bodies were trimmed off and yield was recorded.

Yield data and statistical analysis

The yield data was recorded for upto 45 days of cropping period. A daily record of the number of fruit bodies and their weight (g) per bag per treatment was maintained and the yield data was expressed as kg mushroom per 100 kg compost. The critical difference (CD at 5%) was calculated from the replicate data using factorial experiment and in common complete randomized design (CRD).

Isolation of pathogen

The diseased mushroom pileus showing typical symptoms of dry bubble disease were cleaned gently by wiping the outer surface with sterile cotton moistened with distilled sterilized water. Pieces of infected cut mushroom pileus were planted on PDA slants and incubated at 20±1°C. To suppress the bacterial contamination, the medium was amended with streptocyclin at a concentration of 50 ppm. The subculturing was done periodically at regular intervals during the course of present investigations (Table 3).

Identification

Pathogen was identified as *V. fungicola* (Preuss) Hassebr on the basis of culture, colour, microscopic studies and type of sporulation with the help of mycologist in the Department of Plant Pathology, CCS HAU, Hisar.

Pathogenicity (Koch postulates)

The pathogenicity was proved by placing actively growing mycelia agar bit (5 mm diameter) of the *V. fungicola* on cut healthy white button mushroom (*A. bisporus*) fruit bodies incubated at 20±1°C

Table 4. List of fungitoxicants and their active ingredient test against *V. fungicola*.

Test fungicides	Active ingredient	Group	Common name
Bavistin	50% WP (2-methoxy-carbamoyl-benzimidazole	Benzimidazole	Carbendazim
Mancozab	75% WP (Zinc manganous ethylene) bis thiocarbamate	-	Dithane M-45
Zineb	75% WP (Zinc ethylene) bis di thiocarbamate	-	Dithane Z-78
Topsin –M	50% WP (Thiophanate methyl)	Thiophanate	Thiophanate methyl

Table 5. Effect of different fungitoxicants on mycelial growth of *V. fungicola in vitro.*

Fungitoxicants	Colony diameter (mm)			Percent growth inhibition		
	Concentration (ppm)					
	1	10	100	1	10	100
Carbendazim	80.23	60.11	10.5	10.86	33.22	88.33
Thiophanate-Methyl	81.93	61.42	12.74	8.97	31.76	85.84
Dithane Z- 78	84.78	63.22	18.31	5.81	29.76	79.65
Dithane M- 45	88.01	72.41	30.35	1.33	19.55	66.28
Control	90.00	90.00	90.00	0.00	0.00	0.00
CD at 5%	1.53	1.23	0.832	1.65	1.23	1.46

and 85% humidity. The observations were recorded for disease appearance. Reisolated pathogen was compared with the original one. In the case of control, only agar bit was placed on the cut healthy fruit bodies.

Effect of different fungitoxicants on *V. fungicola* and *A. bisporus*

Four fungitoxicants namely Carbendazim, Mancozeb, Thiophanate-methyl and Zineb were evaluated at different concentations (1, 10 and 100 ppm) against *V. fungicola* and *A. bisporus* using poisoned food technique (Schmitz, 1930).

To evaluate the effectiveness of fungitoxicants in controlling dry bubble disease of white button mushroom, the fungitoxicants namely, Carbendazim. Dithane M-45, Dithane Z-78 and Thiophanate-methyl were used at 1, 10, 100 ppm concentrations *in vitro.* Treatment without any fungitoxicants served as control.
For this purpose, double strength fungitoxicants were added to the double strength PDA media to get the desired concentrations. The PDA amended with test fungitoxicant was poured in Petri-plates (20 ml/plate) (Table 4). After solidification, the poisoned medium was seeded with 5 mm mycelial agar bit of actively growing *V. fungicola* and *A bisporus* separately. The three replications of each treatment were kept (Table 5 and 6).

Observations were recorded at regular intervals for radial growth of *V. fungicola* till whole plate (in control) was covered with mycelial growth of this pathogen (14 days) of incubation at 20±1°C. Growth inhibition (%) was calculated with the growth of the test fungus in control (devoid of fungitoxicant).
Observations were recorded for radial growth of pathogen.

$$\text{Percent inhibition} = \frac{C-T}{C} \times 100$$

where C = Diameter of colony in the control; T = diameter of the colony in the treatment

Effect of different fungitoxicants on the development of dry bubble disease

The test fungitoxicants were mixed in compost to get desired concentration. In the case of Carbendazim and Thiophanate methyl, 400 mg each was dissolved in 4.0 L of water and sprayed on 20 kg of compost, spread over a clean polythene sheet. 800 mg each of Dithane M-45 and Dithane Z-78 was dissolved in 4.0 L of water sprayed over the 20 kg compost. After mixing the compost thoroughly, spawning with M140 of *A. bisporus* (1%) and *V. fungicola* (0.3%) was done and filled in polythene bags weighing 5.0 kg each, four replication of each treatment were kept. In the control, water without fungitoxicant was sprayed before spawning. The yield of mushroom was recorded upto 45 days and compared with control treatment. The different concentrations of different fungicides were used so as to see comparable results and which was cheaper than the control of the disease. The spawn of *A. bisporus* (1%) is necessary for better results and for the inoculum of *V. fungicola* (0.3%), if percent inoculum taken is more, the more disease will occur (Table 7).

RESULTS AND DISCUSSION

Isolation, purification and identification of dry bubble pathogen and pathogenicity

Isolation of pathogen was made on PDA from diseased sporophores suspected of having Verticillium infection. The pathogen cultures were further purified and incubited at 20±1°C. For morphological studies, pure culture was transferred in Petri dishes and incubated at desired temperature. The colony growth charactesrtics were recorded.

The colonies were white in appearance, under part of the plate was colorless to yellow and had scalloped

Table 6. Effect of different fungitoxicants on mycelial growth of *A. bisporus*.

Fungitoxicant	Colony diameter (mm)			Per cent growth inhibition		
	Concentration (ppm)					
	1	10	100	1	10	100
Carbendazim	88.80	83.32	58.17	1.33	7.42	47.75
Thiophanate-methyl	89.18	85.62	60.90	0.91	4.86	43.65
Dithane Z-78	89.40	87.24	52.74	0.66	3.06	40.89
Dithane M-45	89.70	88.50	64.19	0.33	1.66	38.72
Control	90.00	90.00	90.00	0.00	0.00	0.00
CD at 0.05%	NS	1.11	1.16	NS	1.42	2.10

Table 7. Efficacy of different fungitoxicants in controlling dry bubble disease on *Agaricus bisporus*.

Fungitoxicant	Concentration (ppm)	Spawn run (days)		Pin head initiation/first picking (days)		Yield (kg/100 kg compost)		Per cent in increase yield over control	
		First year	Second year	First year	Second year	First year	Second year	First year	Second year
Carbendazim	100	13	14	30/34	29/33	12.30	15.40	67.80	75.84
Thiophanate methyl	100	15	16	31/35	31/35	09.26	12.26	57.23	69.65
Dithane Z-78	200	17	17	32/36	32/36	07.10	10.13	44.22	63.27
Dithane M-45	200	18	19	34/38	34/38	06.54	09.67	39.75	60.80
Control		20	20	35/39	36/40	03.96	03.72		
CD at 5%						0.43	0.41		

edges .Old cultures developed light purple colouration starting from the center of the colony and spreading outwards. The colony characterctics of the pathogen isolated resembled the characters desired by the Nair and McCauley (1987), Calonje et al. (2000), Khanna et al. (2003) and Justyna et al. (2011). So it was concluded that our pathogen was *V. fungicola* after proving the Koch's postulates. The pure culture was maintained and used in further experiment.

Effect of different fungitoxicants on *Verticillium fungicola*

Four fungitoxicants (two systemic and two contact) viz, Carbendazim, Thiophanate –methyl, Dithane Z-78, and Dithane M-45 when screened *in vitro* by poisoned food technique and were found quite effective. Maximum percent growth inhibition was recorded in Carbendazim followed by Thiophanate–methyl, Dithane Z-78 and Dithane M-45, respectively. Our findings gets support from the reports of Sinden (1949), Hu and Dough (1965), Smith (1970), Fletcher (1971) and Gea et al. (1977, 2011, 2012) who observed that different isolate of *V. fungicola* were sensitive to Prochlorage Mn complex followed by Prochlorage + Carbendazim.

Effect of different fungitoxicants on *Agaricus bisporus*

Fungitoxicant have been reported to have some inhibition effect on the host (mushroom) though they are targeted at the pathogen. Thus, use of fungitoxicant may have bearing on the growth of mushroom being a fungus. Result of *in vitro* screening against *A. bisporus* revealed that 100 ppm concentration of Carbendazim inhibited growth of *A. bisporus* up to 47.75%, followed by Thiophanate –methyl (43.65%), Dithane Z-78 (40.89%) and Dithane M-45 (38.72%). The present results are in agreement with work done by Gandy (1985) who also reported that Carbendazim fungicides were less toxic to basidiomycetes than to other pathogen. Seth and Bharadwaj (1989) have shown that Benlate inhibited the growth of *A. bisporus* least followed by Bavistin during *in vitro* studies. Similarly, the present studies support the work of Dhar and Kapoor (1990) and Navarro et al. (2011) who stated that use of Bavistin can control the fungal pathogens and competitors of white button mushroom and it also had less inhibitory action on mushroom mycelium. Thus, it may be inferred that to minimize damage to *A. bisporus*, extra care should be taken in the selection of fungicides for application to manage the disease. Bhalla (1998) reported that the inhibition percent of *A. bisporus* by

Bavistin, Benlate, Sporogon, Dithane M-45 and Dithane Z-78 ranged from 18.18 to 100% at 50, 100, 200 and 500 ppm concentration.

Effect of different fungitoxicants on dry bubble disease

Bhatt and Singh (2002) reported Sporogon (0.075%) to be effective against V. fungicola. Maximum yield and number of fruit bodies were obtained by using Bavistin. Earlier, Bavistin has been tested against brown plaster mould and found to be effective (Dhar, 1978; Arora et al., 1990; Sharma, 1995). Bhatt (1992) reported that Bavistin at 100 ppm showed 40.50% increase yield. Efficacy of Bavistin against *Papulaspora byssina*, *Trichoderma viride*, *V. fungicola* and *Thermomicrobium roseum* has been reported by Sharma and Vijay (1996) Navarro et al. (2011), Sharma and Satish (2012) and Gea et al. (2012), Bavistin increased the number of fruit bodies and yield. Results of present study are in agreement with that which revealed that Carbendazim advanced spawn run by 6-7 days, pinhead initiation by 5 days resulting in 67.80 to 75.84% increase in yield. Other chemicals, Thiophanate–methyl Dithane Z-78 and Dithane M- 45 also significantly increase the mushroom yield, shortened spawn run period and pinhead initiation as compared to the control.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Screening for exopolysaccharide-producing strains of thermophilic lactic acid bacteria isolated from Algerian raw camel milk

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Exopolysaccharides synthesized by lactic acid bacteria play a major role in the manufacturing of fermented dairy products as thickening agents. Exploration of the biodiversity of wild lactic acid bacteria from natural environments is currently the most suitable approach to search for the desired exopolysaccharide-phenotype. A total of 82 thermophilic lactic acid bacteria strains were isolated from Algerian raw camel milk. The isolation of strains was carried on modified Chalmers agar medium, under semi anaerobic conditions at 42°C. Bacterial isolates were phenotypically characterized and grouped into four genera: *Lactobacillus* (31.7%), *Enterococcus* (30.5%), *Streptococcus* (24.4%) and *Pediococcus* (13.4%). Based on the mucous type of the colonies, thirty EPS-positive strains were selected to be screened for their ability to produce exopolysaccharides. The production of polymers was carried out on Man, Rogosa and Sharpe (MRS) broth, supplemented with lactose and glucose as carbon sources. Yields quantification of soluble exopolysaccharides using a colorimetric method, showed that the selected strains produce yields ranging between 160 and 740 mg/l for *Lactobacillus* strains, between 126 and 319 mg/l for *Streptococcus* strains, between 70 and 242 mg/l for *Enterococcus* strains and between 132 and 134 mg/l for *Pediococcus* strains. This suggests that some strains have potential to be used as new culture starters for this and possibility other dairy products.

Key words: Camel milk, thermophilic lactic acid bacteria, exopolysaccharides.

INTRODUCTION

Microbial polysaccharides have been investigated in detail during the last few decades. Today, there is an increasing demand in food industries for live microbes producing polysaccharides (Patel et al., 2010). Bacterial polysaccharides can be divided into intracellular polymers, structural polymers and extracellular polymers or exopolysaccharides (EPS) (Kumar et al., 2007). The bacterial EPS vary greatly in their composition and hence in their chemical and physical properties (Sutherland, 1999). Many lactic acid bacteria (LAB) are able to produce EPS. The dairy LAB used in the manufacture of fermented milks such as *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactococcus lactis* subsp. *cremoris* were extensively studied in the last years (Cerning, 1995). EPS synthesized by LAB play a major role in the manufacturing of fermented

dairy products (Duboc and Mollet, 2001; Jolly et al., 2002). These molecules are economically important because they can impart functional effects on foods and confer beneficial health effects (Welman and Maddox, 2003). When suspended or dissolved in aqueous solution, EPS provide thickening and gelling properties (Marshall and Rawson, 1999; Laws and Marshall, 2001). The polymer-producing ability is an extremely unstable property; it seems to be linked to the presence of plasmids of varying size in mesophilic lactic acid bacteria, whereas most of the EPS-producing strains of thermophilic lactic acid bacteria (TLAB) do not harbor plasmids (Cerning, 1995). Some EPS confer on LAB a ropy character that can be detected in cultures that form long strands when extended with an inoculation loop. When EPS are produced *in situ* during milk fermentation, they can act as natural bio-thickeners, giving the product a suitable consistency, improving viscosity. The increasing demand by consumers of novel dairy products requires a better understanding of the effect of EPS on existing products and at the same time, the search for new EPS-producing strains with desirable properties. Therefore, exploration of the biodiversity of wild LAB strains from natural environments is currently the most suitable approach to search for the desired EPS-phenotype (Ruas-Madiedo and de los Reyes- Gavilán, 2005). The screening of ropy strains and the isolation and quantification of EPS have led to the application of a large variety of techniques (Goh et al., 2005; Ruas-Madiedo and de los Reyes-Gavilán, 2005). The amounts of EPS produced by the dairy strains vary considerably (Ludbrook et al., 1997; Laws et al., 2001; Badel et al., 2011). A wide range of bacteria are known to produce EPS. Several LAB produce exopolysaccharides that are secreted into the growth media (Cerning et al. 1986, 1988). Most bacteria produce EPS under all conditions, but the quantities and the composition of EPS are strain dependent and affected by the nutritional and environmental conditions (Garcia-Garibay and Marshall, 1991). Up to now, camel milk was not deeply investigated for the characterization of thermophilic bacteria. The purpose of this investigation was to obtain the efficient TLAB strains isolated from raw camel milk which produce high amount of EPS. In the current study, eighty two (82) strains of TLAB isolated from Algerian raw camel milk were taxonomically characterized using the phenotypic methods. On the other hand, these strains were screened according to their ability to produce EPS on solid and liquid media, and a colorimetric method was used for quantifying EPS yields.

MATERIAL AND METHODS

Sampling and isolation of TLAB strains

Twenty eight (28) samples of raw camel milk were obtained from two locations in the south of Algeria. Samples were collected in sterile bottles and immediately transported to the laboratory in ambient temperature, the pH of each sample was measured and the microbiological analysis was performed on the arrival. The strains isolation was carried out after milk acidification to retrieve a large diversity of TLAB (Khedid et al., 2009). 10 ml of each sample were mixed with 90 ml of sterile yeast water (10% w/v, Oxoid) and serial decimal dilutions were carried out. Isolation of TLAB was performed by the standard pour-plate method, using modified Chalmers-agar medium (Vanos and Cox, 1986). Plates were incubated semi anaerobically for 48 or 72 h at 42°C. The LAB colonies were picked and purified on MRS-agar plates (De Man et al., 1960), and strains were kept frozen at -20°C in MRS broth supplemented with 25% glycerol.

Preliminary identification of TLAB isolates

TLAB strains were identified according to many recommended methods (Sharpe, 1979; Samelis et al., 1994; Harrigan, 1998; Badis et al., 2004; Khedid et al., 2009). All isolates were initially Gram stained and examined for cell morphology and motility, then were examined using different kinds of tests; growth at different temperatures (10, 15, 30 and 45°C) and at different pH (4.2 and 9.6), as well as salt tolerance (6.5 and 18% of NaCl) in MRS broth (Oxoid), catalase reaction, gas production from glucose, ammonia from arginine hydrolysis, acetoin production (Voges-Proskauer test), utilization of citrate and heat resistance at 60.5°C for 30 min. Tests were repeated two times to avoid confusing results in the identification.

Screening test for mucoidy and ropiness

Screening test was carried on customized MRS-agar medium (Degeest and De Vuyst, 1999; Degeest et al., 2001, 2002). TLAB Strains were plated and incubated under semi anaerobic conditions at 42°C for 48 h. At the end of incubation, mucoidy of colonies was determined by visual appearance, and ropiness was determined by touching them with a sterile inoculation loop (Ricciardi et al., 1997; Welman et al., 2003; Ruas-Madiedo and de los Reyes-Gavilán, 2005), and confirmed by ethanol precipitation method. Colonies which have mucoid and ropy phenotype were picked up and purified by following the streaking method, then preserved at 4°C on MRS agar slants (Vijayendra et al., 2008) and selected for the next step.

Exopolysaccharides production

Customized MRS broth was used for fermentations. It contained (in grams/liter): lactose (75), glucose (25), peptone (30), yeast extract (12), Lab Lemco (8), K_2HPO_4 (2), sodium acetate (5), tri-ammonium citrate (2), $MgSO_4$-$7H_2O$ (0.2), $MnSO_4$-H_2O (0.038) and Tween

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Abbreviations: EPS, Exopolysaccharides; **LAB,** lactic acid bacteria; **TLAB,** thermophilic lactic acid bacteria; **OD,** optical density; **BSA,** bovine serum albumin.

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Table 1. Phenotypic characters of TLAB strains isolated from raw camel milk.

Character	*Lactobacillus*	*Enterococcus*	*Streptococcus*	*Pediococcus*
Motility	-	-	-	-
Gram stain reaction	+	+	+	+
Cell morphology	R	C	C	Ct
Presence of spore	-	-	-	-
Catalase activity	-	-	-	-
Growth at: pH 4.2	+	v	+	+
Growth at: pH 9.6	-	+	-	-
Growth in: 2% NaCl	+	+	+	+
Growth in: 6.5% NaCl	-	+	-	-
Growth in: 18% NaCl	-	-	-	-
Growth at temperature:10°C	-	+	-	-
Growth at temperature:15°C	-	+	-	-
Growth at temperature:30°C	-	+	-	-
Growth at temperature:45°C	+	+	+	+
Survive at 60.5°C for 30min.	v	v	+	-
Glucose fermentation	+	+	+	+
Production of acetoin	-	v	-	-
CO_2 from glucose	-	-	-	-
NH_3 from arginine	-	+	-	+
Utilization of citrate	-	-	-	-
Strains number	26	25	20	11

R: Rod, C : Cocci, Ct: Cocci/tetrads, +: More than 90% of strains showed a positive result, - : More than 90% of strains showed a negative result, v: Between 10 and 90% of strains showed a positive or negative result.

80 (1 ml/L). The unfermented medium was ultra-filtered under 10 000 Da., using a tangential filtration system, in order to eliminate polysaccharides from yeast extract which would have interfered with the purification and determination of EPS composition (Ricciardi et al., 2002; Shene et al., 2008). Sterilization was performed by microfiltration under 0.22 µm using a steritop (Millipore). The selected mucoid strains were stored at -20°C in MRS broth (Oxoid), containing 25% (v/v) glycerol.

Bacterial strains and culture conditions

The bacterial inoculants were also prepared in 10 ml of customized MRS inoculated with 100 µl of freshly prepared cultures. After incubation at 40°C for 24 h, they were adjusted to OD_{600} = 1 and transferred into 500 ml Erlenmeyer flasks containing 90 ml of fermented medium. Fermentations were performed at 40°C for 24 h. Agitation was maintained at 100 rpm to provide adequate dispersion. Growth was monitored by measuring the final optical density (OD) at 600 nm, and bacterial biomass can be determined from a standard curve of absorbance. Acidification was estimated with the measurement of final pH of cultures (Gancel and Novel, 1994; Vaningelgem et al., 2004).

Isolation and quantification of exopolysaccharides

Exopolysaccharides were purified from the various culture strains using conventional method of Ruas-Madiedo and de los Reyes-Gavilán (2005), with some modifications. Grown cultures were heated in boiling water for 15 min to inactivate enzymes, and then cooled down to room temperature, centrifuged (20 min, 10 000 g) to remove cells and coagulated proteins, and the supernatant was collected. EPS were precipitated from the supernatant with three volumes of cold ethanol (96%) followed by an overnight incubation at -20°C. After centrifugation (20 min, 10 000 g, 4°C), the precipitates were re-suspended in hot ultrapure (Milli-Q) water and dialyzed (molecular weight cut-off: 10000 Da.) for 2 days against ultrapure (Milli-Q) water (changed twice each day). EPS solution was then frozen at -80°C and lyophilized. The EPS powder was determined by measuring the dry weight of the precipitate, and stored for further analysis. Total sugar content was measured according to the phenol-sulfuric acid method of Dubois et al. (1956) using glucose as standard. Proteins content was determined according to Bradford (1976) method using bovine serum albumin (BSA) as standard. Experiments of EPS production, isolation and quantification were repeated three times for each studied strain. In order to estimate the precision of the mean of a variable, the standard error of the means was calculated by using of the EXCEL program.

RESULTS

Isolation and preliminary identification of TLAB strains

After the preliminary characterization, a total of eighty two (82) Gram positive, catalase negative, no spore forming and homo-fermentative isolates, obtained from modified Chalmers medium (incubated at 42 °C for 2 or 3 days), were investigated for their phenotypic characters on the MRS medium (Table 1). The isolates were preliminary

subdivided into four (4) groups. Twenty six (26) rod shaped strains, homo-fermentative and Gram positive, catalase negative, which grew at 45°C but not at 15°C, absence of gas production from glucose, were considered as *Lactobacillus*. Twenty five (25) cocci shaped strains, homo-fermentative and Gram positive, catalase negative, which grew at 10 and 45°C, grew in the presence of 6.5% of NaCl and at pH 9.6 were considered as *Enterococcus*. Twenty (20) cocci shaped strains, in pairs or in chain cells, homo-fermentative and Gram positive, catalase negative, which grew at 45°C but not at 10°C, and resist heating at 60.5°C for 30 min, were considered as *Streptococcus*. Eleven (11) cocci shaped strains, in pairs or in tetrads, homo-fermentative and Gram positive, catalase negative, which grew at 45°C but not at 10 °C, were considered as *Pediococcus*.

Screening for EPS-producing phenotype

A set of 82 TLAB strains were screened for mucoidy and ropiness on customized MRS-agar medium, examined by the traditional pick test and confirmed by ethanol precipitation method. The results revealed the presence of thirty (30) mucoid and ropy strains in our culture collection, twelve (12) *Lactobacillus* of twenty six (26) tested strains, nine (9) *Streptococcus* of twenty (20) tested strains, seven (7) *Enterococcus* of twenty five (25) tested strains and two (2) *Pediococcus* of eleven (11) tested strains.

EPS production, isolation and determination

The investigation in the second step of screening for EPS production by TLAB isolated from raw camel milk, showed that 96.66% of selected strains produced EPS with more than 100 mg/l; *Lactobacillus* EPS yield ranged between 160-740 mg/l, *Streptococcus* EPS amount ranged between 126-319 mg/l, *Enterococcus* EPS yield ranged between 70-242 mg/l and *Pediococcus* EPS yield ranged between 132 and 134 mg/l. *Lactobacillus* strain (*L*115) had the highest EPS yield, while the *Enterococcus* strain (*E*28) had the lowest EPS yield (Figure 1a).

The total sugar content in EPS was in the range of 19.49 to 77.37% for *Enterococcus* and 50.57 to 58.69% for *Pediococcus* strains. Whereas, proteins accounted for lower than 4.86% for all studied strains (Figure 1b). For the cultures conditions and parameters, the final OD ranged between 2.85-4.55 for *Streptococcus* strains, 2.66-8.25 for *Lactobacillus* strains, 2.74 - 4.17 for *Enterococcus* strains and 3.44 - 3.61 for the *Pediococcus* strains. The final pH was estimated at a range of 4.3- 4.5 for *Streptococcus* strains, 3.7-5.4 for *Lactobacillus* strains, 4.1- 4.3 for *Enterococcus* strains and 4.2 for *Pediococcus* strains (Figure 1c).

DISCUSSION

All bacterial strains isolated from the raw camel milk samples fit the classification of lactic acid bacteria. TLAB were present in fermented raw camel milk, because of their ability to produce high levels of lactic acid as well as being able to survive under high acidic conditions. It was noted that except *Enterococcus* isolates, all TLAB strains isolated from the fermented raw camel milk were unable to grow at temperature of 30°C. The high level of TLAB in raw milk can be favored by low pH conditions (Badis et al., 2004). In this study, it was noted that the biodiversity of 82 thermophilic lactic acid bacteria isolated from fermented camel milk is limited to the four genera: *Lactobacillus* (31.7%), *Enterococcus* (30.5%), *Streptococcus* (24.4%) and *Pediococcus* (13.4%). These findings can be compared to those obtained in raw dromedary milk of Morocco (Benkerroum et al., 2003) which showed 99 isolated strains of LAB belonging to five genera: *Enterococcus* (58.6%), *Pediococcus* (28.3%), *Streptococcus* (4%), *Lactococcus* (8.1%) and *Leuconostoc* (1%). And also, our results can be compared with data obtained by Khedid et al. (2009), who have isolated 120 LAB strains from raw camel milk, grouped into six genera; they were clearly dominated by the genus *Lactobacillus* (37.5%), followed by the genus *Lactococcus* (25.8%) and *Leuconostoc* (11.7%), *Enterococcus* (10.8%), *Streptococcus* (9.2%) and *Pediococcus* (5%). In the same topic, Kacem and Karam (2006) isolated 216 LAB in camel milk from arid regions of Algeria, which were identified in four genera: *Lactobacillus* with (46.9%), followed by the genus *Lactococcus* (22%), *Enterococcus* (19.3%) and *Leuconostoc* (11.5%). Abdelgadir et al. (2008) isolated 180 LAB in the Sudanese fermented camel milk, they were clustered by rep-PCR into three genera: *Streptococcus*, *Enterococcus* and *Lactobacillus*.

The first stage of screening for EPS-producing phenoltype revealed that 36.6% of the studied thermophilic lactic acid bacteria strains show a mucous aspect of colonies. This phenotypic character can be related to the production of EPS on solid media (Gomez, 2006). Therefore, the presence of a translucent or creamy material involving a mucous colony is an indicator of EPS production potential. The production of polymers was confirmed by mixing each colony in absolute ethanol. Precipitate formation indicates the presence of EPS. The discriminatory value of the methods to test mucoidy and ropiness of bacterial colonies, were relatively low. Different EPS screening methods have been reported for LAB. The visual inspection of bacterial colonies on agar plates is most probably the easiest method, but it is insensitive. This method is unable to detect LAB strains that produce low amounts of EPS (Smitinont et al., 1999).

In the second stage of screening of various TLAB strains on the MRS broth, data showed that all the 30 selected mucous strains from 82 TLAB examined isolates, were able to produce exopolysaccharides. The amount of EPS production differs between genera and

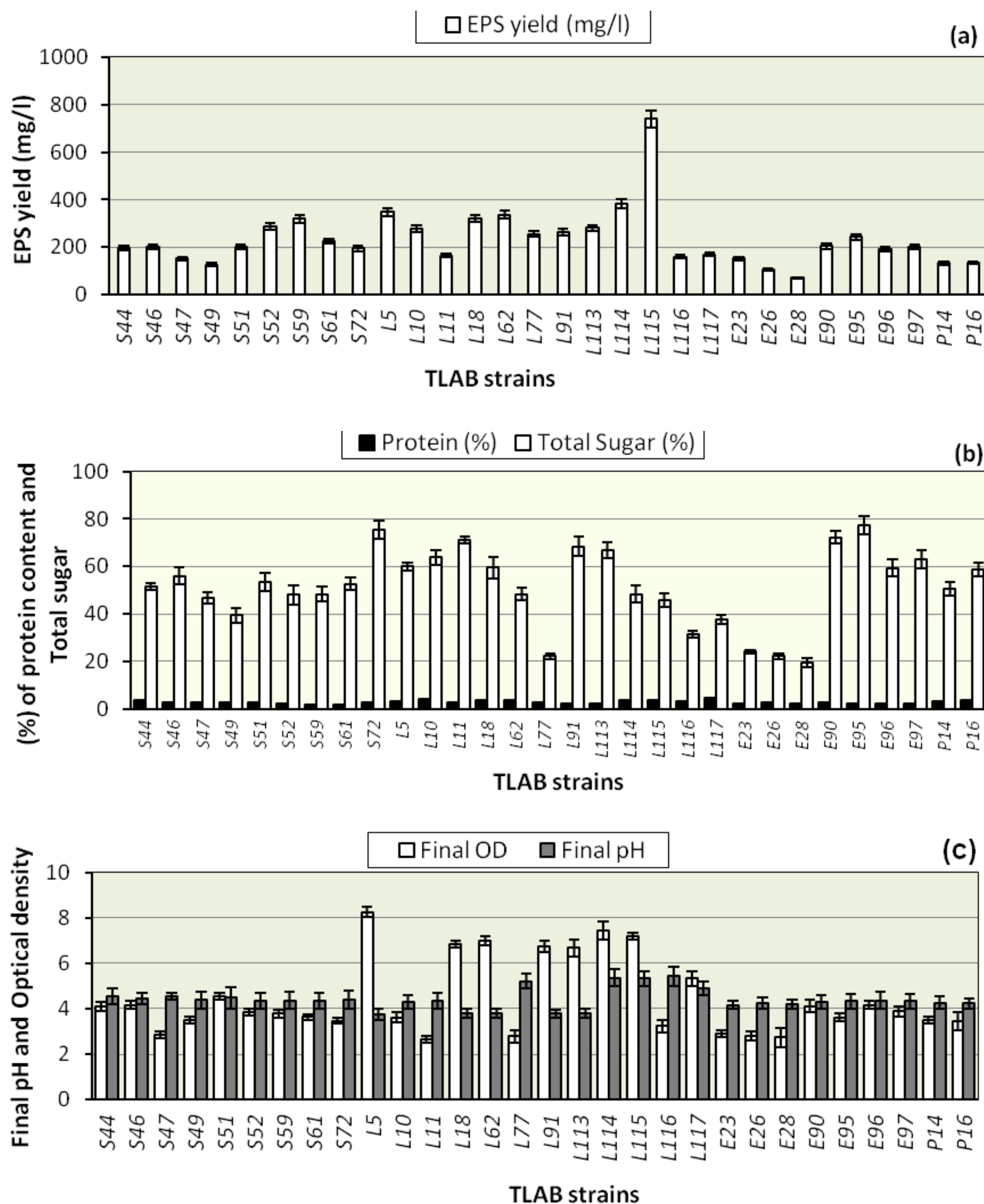

Figure 1. Screening of the TLAB strains for EPS production and partial characterization of produced exopolysaccharides, (a): EPS yields (mg/l) of TLAB screened on customized MRS broth, (b): Total sugar and proteins content of TLAB exopolysaccharides, (c): Final optical density (OD) and final pH of TLAB cultures. *L*: *Lactobacillus* strains, *S*: *Streptococcus* strains, *E*: *Enterococcus* strains, *P*: *Pediococcus* strains. Bars on the histogram represent the standard error of means.

varies within a genus. These findings approve the results on EPS from lactic acid bacteria reported by Van den Berg et al. (1993) in which 30 strains out of 607 tested showed the ability to produce exopolysaccharides. *Lactobacillus* strains produce the highest yields of exopolysaccharides, range between 160 and 740 mg/l. Our results can be compared positively with those reported by Laws and Marshall (2001), who obtained EPS yields of 175 mg/l produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* LY03 and less than 5 mg/l by *L.*

delbrueckii subsp. *bulgaricus* LY58. Similarly, Frengova et al. (2000) reported that the EPS contents were recorded between 58 and 540 mg/l for various strains of *Lactobacillus bulgaricus*. Other data reported by Xu et al. (2010) shows EPS yields ranged between 67 and 238 mg/l for *Lactobacillus paracasei* HCT. *Streptococcus* strains produce EPS yields between 126 and 319 mg/l. These data can be compared to those obtained by Laws and Marshall (2001), with EPS yields of 100 mg/l produced by *Streptococcus thermophilus* SY102 and less than 15 mg/l produced by *S. thermophilus* SY60. Similarly, Frengova et al. (2000) reported that the polysaccharide yields were recorded between 40 and 270 mg/l for various strains of *S. thermophilus*. In synthesis, we can conclude that our values of EPS yield did not differ significantly to those obtained by other authors. However, there is no report recorded for EPS production by both following genera: *Enterococcus* and *Pediococcus*. Hence, our results revealed that *Enterococcus* strains produce EPS yields ranging between 70 and 242 mg/l, and also, both *Pediococcus* strains (P14, P16) which produce EPS amounts of 132 and 134 mg/l, respectively. The isolated EPS powders had a total sugar content ranging between 22 and 71% for *Lactobacillus* strains, between 39 and 75% for *Streptococcus* strains, between 19 and 77% for *Enterococcus* strains and between 50 and 58% for both *Pediococcus* strains. Hence, protein content was negligible and ranged between 1.6 and 4.8% for various TLAB strains. These findings can be partially compared with those reported by Shene et al. (2008) for *Streptococcus* strains, having a total of sugar ranging between 20 and 60%, and protein content ranging between 0.3 and 3.6%. For the growth conditions tested, it was noted that the final pH of all cultures was decreased and ranged between 3.75 and 5.43, while the final optical density values ranged between 2.66 and 8.25. The EPS-yields of TLAB strains have shown moderate correlation with the bacterial growth, but, they were low in correlation with values of total sugar fraction. We suggest that this correlation is not necessary because the bacterial EPS may contain a non-carbohydrate moiety. These findings shows that fermented camel milk can be a potential source of thermophilic lactic acid bacteria that produce exopolysaccharides.

Conclusion

Our results demonstrate the diversity of TLAB in Algerian raw camel milk. This dairy product contains several genera of LAB, which were preliminary identified, and have a potential for EPS-producing activity with high yields. These strains can be used as starter culture with predictable characteristics and contribute to the development of fermented milk with stable consistent quality. As perspectives, three approaches are required: Firstly, genotypic characterization of isolates to determine the number of distinct strains among the described isolates of TLAB in our collection, PCR and DNA sequencing will be undertaken. The results will then be compared with other data obtained for other strains of TLAB. Secondly, optimization of our thermophilic lactic acid bacteria based on their technological properties and their use as starters (alone or in association) for dairy products. Finally, EPS-producing strains can be also examined for their ability to form biofilms, then, exopolysaccharides can be characterized, and applied according to the physicochemical characteristics.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Properties of *Enterococcus faecalis,* a new probiotic bacterium isolated from the intestine of snakehead fish (*Channa striatus* Bloch)

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The present study aimed to isolate and characterize the lactic acid bacteria (LAB) from the intestine of snakehead (*Channa striatus*) fingerling to be used as a new probiotic in aquaculture. The total colony count of bacteria in the fish intestine was 2.1 × 10^6 cfu/g. Five LAB were isolated from the intestine of twenty fish and one of these isolates, LAB-4 was identified as *Enterococcus faecalis* by conventional and molecular techniques. Probiotic properties showed that this LAB could grow from pH 3 to 8, but the best growth was observed at pH 7. *E. faecalis* grew at 0.15 and 0.3% bile salt concentrations, from 15to 45°C and at 4% NaCl in de Man Rogosa and Sharp (MRS) broth. This bacterium showed *in vitro* inhibitory activity against three fish pathogens viz., *Aeromonas hydrophila, Pseudomonas aeruginosa* and *Shewanella putrefaciens*. Antibiotic sensitivity tests indicated that *E. faecalis* was resistant instantly to: streptomycin, gentamycin and kanamycin, intermediate to tetracycline, and sensitive to chloramphenicol, amoxicillin and ampicillin antibiotics. Moreover, significantly (*P*<0.05) improved survival of fish was observed when fed with *E. faecalis*-fortified diet in an *in vivo* challenge test using *A. hydrophila*. Based on the results, it can be concluded that *E. faecalis* is a promising probiotic for snakehead fish against pathogenic infestation.

Key words: Isolation, characterization, probiotic, *Enterococcus faecalis*, snakehead fish.

INTRODUCTION

Since the use of antibiotics has negative effects on animals and environment, several alternative strategies such as probiotic bacteria have been suggested (Lauzon et al., 2008; Pan et al., 2008). The use of lactic acid bacteria (LAB) as main probiotics can control potential pathogens in aquaculture (Ringø and Gatesoup, 1998;

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Aly et al., 2008; Kim and Austin, 2008). Some LABs are normal microbiota in the gastrointestinal (GI) tract of healthy aquaculture animals that can be used as probiotic (Kim and Austin, 2008). Probiotics can prevent the growth of harmful bacteria by colonization in the gut and produce organic acids and antimicrobial compounds (Ruiz-Moyano et al., 2008; Das et al., 2010). In addition, probiotic bacteria appears to have a wide variety of benefits such as lactose digestion, resistance to enteric pathogens, anti-colon cancer effect, small bowel bacteria overgrowth, allergy, immune system modulation and reduction in serum cholesterol to the host (Cebeci and Gurakan, 2003; Salminen et al., 2004). Some properties such as acid and bile salt tolerance, antibacterial activity against pathogens and antibiotic susceptibility are important tools to be investigated, when selecting potential probiotic bacteria (Cebeci and Gurakan, 2003; Balcázar et al., 2008; Pan et al., 2008). Furthermore, challenge tests have been suggested as a golden standard to be included when evaluating probiotics (Aly et al., 2008) and the resistance to enteric pathogens (Cebeci and Gurakan, 2003).

Snakehead (*Channa striatus*) is a popular food fish in Southeast Asian countries (Jais et al., 2002; Rahman et al., 2012, 2013; Muntaziana et al., 2013).

As there is less information accessible on the bacterial community in the gastrointestinal tract of fish (Navarrete et al., 2009; Zhou et al., 2009; Wu et al., 2012) and no information available on bacteria in the intestine of snakehead, the first aim of the present study was to isolate and identify LAB from the intestine of snakehead fingerlings. The second aim was to evaluate the characteristics of an isolated LAB. As *Aeromonas hydrophila*, a common freshwater fish pathogen is causing high mortality in different life stages of fish (Aly et al., 2008; Rengpipat et al., 2008); the third aim of the present study was to show if dietary supplementation of a LAB had any effect in a challenge study using *A. hydrophila*.

MATERIALS AND METHODS

Sampling

A total of 60 healthy snakehead fingerling fish (*Channa striatus*) with the average weights of 5.0-6.0 g were collected over three times from a fish farm in Seri Kembangan, Selangor, Malaysia (3.0333° N, 101.7167°E), and transferred to the Aquatic Animal Health Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM). The fishes were maintained in a fiberglass tank (1000 l) at UPM and after 14 days of collection, twenty fish were randomly selected, anesthetized with tricaine methanesulphonate (100 mg/l) (Sigma, Chemical Co. St. Louis, MO, USA), disinfected with alcohol (70%), and dissected under antiseptic conditions. The intestines were removed and homogenized in a sterile saline solution (0.85% v/w), as described elsewhere (Rengpipat et al., 2008).

Isolation of LAB from the fish intestine

Homogenized intestine samples were serial diluted (up to 10^{-4}) and 0.1 ml of each dilution was spread onto triplicate tryptic soy agar (TSA) (Sigma, USA) plates and incubated at 30°C for 48 h to count the colony forming units (cfu) of bacteria. The homogenized stomach samples were also immersed in de Man Rogosa and Sharp (MRS) broth (Sigma, USA) and incubated at 30°C for 24 h. After incubation, 0.1 ml of the cultured broth was spread onto MRS agar containing bromo-cresol purple (0.17 g/l, Sigma, USA) (Rengpipat et al., 2008). The plates were incubated at 30°C for 48 h under anaerobic conditions (anaerobic jar, Oxoid, USA). Colonies of yellow appearance were transferred to MRS agar and sub-cultured three times to obtain pure colonies (Nguyen et al., 2007; Kopermsub and Yunchalard, 2010).

Antagonistic effect test for the selection of LAB

Primary antibacterial activity of the isolates was studied by disc diffusion technique using cell-free cultured broth to select one isolate with the highest inhibitory activity against *A. hydrophila*, a procedure previously suggested by Aly et al. (2008). *A. hydrophila* was cultured in Tryptic Soy Broth (TSB, Sigma, USA), incubated at 30°C for 24 h and then streaked on TSA plates. Bacterial cells of the cultured MRS broths of five LAB isolates were precipitated at 4°C and 8586 g for 5 min (Eppendorf, 5810R, Germany). Sterile discs were immersed in the supernatants, air dried, and placed on TSA plates. The plates were incubated at 30°C for 24-48 h to observe inhibition zones (Lauzon et al., 2008).

Identification of selected LAB by conventional and molecular techniques

The carbohydrate fermentation pattern of the most promising LAB isolate from the antagonistic test was determined using an API kit (50 CH, API 50 CHL medium, bioMérieux, France) to identify the selected LAB (Aly et al., 2008). Further identification of the LAB isolate was carried out using 16S rRNA gene sequencing as described by Pond et al. (2006). Briefly, the genomic DNA of the isolates was extracted using a DNA extraction kit (Genomic DNA Mini kit, Genaid, bioMérieux, France). Polymerase chain reaction (PCR) was used to amplify the 16S rRNA of the extracted DNA using the primers pAF 5' AGA GTT TGA TCC TGG CTC AG 3' as forward and phR 5' AAG GAG GTG ATC CAG CCG CA 3' as reverse primers. The purified products were sequenced by NHK Sequencing Service Laboratory in South Korea (NHK Bioscience Solutions SDN BHD) using the specific primers (pAF and phR). In 16S rRNA gene sequencing, approximately 1500 bp was analyzed by BioEdit software and then compared with BLAST data from GenBank in the National Center for Biotechnology Information.

Probiotic properties

pH tolerance

Acid tolerance of the selected bacterium at different pH levels was investigated. MRS broths with different pH levels; 2, 3, 4, 5, 6, 7 and 8 were prepared using 1% HCl (Sigma, Chemical Co. St. Louis, MO, USA) and 1 N NaOH (Sigma, USA), and distributed into 25 ml bottles. The broth media and the control bottles were autoclaved at 121°C for 15 min and soon after cooling, they were inoculated with an overnight culture (30 µl) of the selected strain in the MRS broth followed by incubating at 30°C. Optical density at 600 nm (OD_{600}) was measured by a spectrophotometer (Shimadzu, UV-1601, Japan) after 2, 4 and 8 h of incubation. The viability of the isolate was also controlled by duplicate inoculation on MRS agar plates as described elsewhere (Balcázar et al., 2008; Kim and Austin, 2008).

Bile salt tolerance

Bile salt tolerance was tested in MRS broth with 0, 0.15 and 0.3% (w/v) Oxgall bile salt (Sigma, USA). Duplicate bottles (25 ml medium) of MRS broth containing different concentrations of filtered bile salt were inoculated by 30 µl of the cultured strain and incubated at 30°C. Growth rate was assessed by measuring OD_{600} after 0, 2, 4 and 8 h post-incubation (Balcàzar et al., 2008; Kim and Austin, 2008).

Growth at different NaCl concentrations

Growth rate of the LAB strain at different sodium cloride concentrations was determined in MRS broth by adding 0, 1, 2, 3 and 4% NaCl (Sigma, USA). The duplicate bottles (25 ml medium) containing different levels of NaCl were inoculated with 30 µl cultured bacterium and incubated at 30°C. OD_{600} was measured after 0, 2, 4, 8, 16 and 24 h of incubation as described by Kim and Austin (2008).

Growth at different temperature levels

Growth of the selected LAB strain was evaluated at nine different temperatures, viz., 10, 15, 20, 25, 30, 35, 40, 45 and 50°C. 30 µl of an overnight MRS broth culture was transferred to duplicate MRS broth bottles and incubated at 30°C. OD_{600} was measured after 0, 4, 8, 16 and 24 h of incubation according to Balcázar et al. (2008).

Antibacterial activity against three fish pathogens

Three freshwater fish pathogens; *Aeromonas hydrophila, Pseudomonas aeruginosa* (obtained from the pure stock kept at Aquatic Animal Health Unit, Faculty of Veterinary Medicine, UPM, Malaysia) and *Shewanella putrefaciens* (ATCC-49138, Lot: 4987125) were used to test the antibacterial potential of the LAB; *in vitro* growth inhibition of the target bacteria. This was tested using disc diffusion and well diffusion techniques previously described by Balcázar et al. (2008). The pathogenic bacteria were cultured in TSB and incubated at 30°C for 24 h. Subsequently, 30 µl of the culture with 10^3 cfu/ml cells were spread onto duplicate TSA plates. The selected LAB strain was cultured in MRS broth at 30°C for 18 h. The cells were harvested by centrifugation at 7155 g and 4°C (Eppendorf, 5810R, Germany) for 5 min and the supernatant was used for antibacterial activity by the disc and well diffusion methods.

Antibiotic sensitivity test

Antibiotic sensitivity of the selected strain were tested against eight common antibiotics [gentamycin (GM, 10 µg), streptomycin (S, 10 µg), amoxicillin (AMX, 25 µg), tetracycline (TE, 30 µg), chloramphenicol (C, 30 µg), ampicillin (AM, 10 µg), erythromycin (E, 15 µg) and kanamycin (K, 30 µg)] using disc diffusion technique (Akinjogunla et al., 2010). Fifty µl of the 24 h broth cultured strain were spread on MRS agar and subsequently, antibiotic Bio-discs (bioMérieux, France) were placed on duplicate plates using the Oxoid Disc Dispenser System (USA). The plates were incubated at 30°C for 24-48 h to measure the inhibition zones (Kim and Austin, 2008). The interpretations of zone sizes were expressed based on the standard table of the Kirby-Bauer Test (Bauer et al., 1966).

Experimental design in challenge test

In total, 120 snakehead fingerlings with an average weight of 6.5 ± 0.3 g were randomly distributed into 12 aquaria each (45 × 30 × 30 cm) containing 10 fish. The experiment was set up with a completely randomized design in treatments, each of which was triplicated. Four treatments were used: (T_c) LAB was not included in the diet and the fish were not injected with the pathogen, (T_1) LAB was supplemented to the diet at 10^7 cfu/g and the fish were not injected with the pathogen, (T_2) LAB was included in the diet; similar level as for T_1 and the fish were injected (10^7 cfu/ml) with the pathogen and (T_3) LAB was included in the diet; similar level for T_1 and the fish were not injected with the pathogen. To prepare experimental diets, the LAB was cultured in MRS broth (Sigma, USA) and incubated at 30°C for 18 h. The LAB was harvested using refrigerated centrifuge (Eppendorf 5810R, Germany) at 4°C and 1207 g for 30 min. The bacterial pellet was washed twice with sterile saline solution and adjusted at 10^7 cfu/ml based on optical density and total plate count of the LAB during 24 h. The prepared suspension was mixed with commercial feed by adding 200–300 ml distilled water per kg diet; dried at room temperature (25°C); stored in sterile plastic bags and placed in refrigerator at 4°C. The LAB-fortified diet preparation was repeated every two weeks during the five-week feeding trial. Commercial dry feed (MAY FISH FEED LTD SDN BHD) was served as a basis of the experimental diet. Proximate composition of the diet including dry matter (DM), crude protein (CP), crude fiber (CF), lipid and ash were analyzed according to AOAC (2000) and were 92.56, 33.81, 3.12, 7.73 and 3.7%, respectively. The experimental fish were acclimatized for two weeks prior to use for the experiment. All fish were fed twice (10 am and 4 pm) daily at the rate of 20 g/kg of estimated biomass for five weeks.

Intraperitoneal injection

Intraperitoneal injection was used to introduce *A. hydrophila*. Briefly, *A. hydrophila* was cultured in TSB and incubated at 30°C for 18 h. The cultured broth was centrifuged (Eppendorf, 5810 R, Germany) at 1006 g and 4°C for 30 min and pellet bacteria were washed two times with sterile saline solution. Then, the concentration of *A. hydrophila* was adjusted to 10^7 cfu/ml by total plate count (TPC) and optical density. After two weeks of feeding with or without LAB-fortified diet, a 0.1 ml aliquot of *A. hydrophila* was injected to T1 (fish fed LAB in the diet) and T3 (no addition of LAB in the diet) in the morning before feeding. As in the control, 0.1 ml sterile water was injected to T2 (with LAB and no pathogen) and Tc (no LAB and no pathogen) to make uniform condition for injection stress (Aly et al., 2008; Abdel-Tawwab and Ahmad, 2009). The challenge experiment was terminated two weeks after injection.

Investigation of infected fish in challenge test

During the challenge test, dead fish were dissected. The anatomy of internal organs; intestine, liver, kidney, spleen and abdomen were investigated to study the symptoms of infection. In addition, infected organs were cultured (by swab) on TSA plates and incubited at 30°C for 24 h and sub cultured two times. Three to five pure colonies from infected organs of each treatment were Gram stained and API-20E kit (bioMérieux, France) tested. This was conducted to confirm *A. hydrophila* infection according to Rengpipat et al. (2008).

Statistical analysis

The statistical analysis was conducted to compare the quantitative results in probiotic properties and *in vivo* challenge test by the

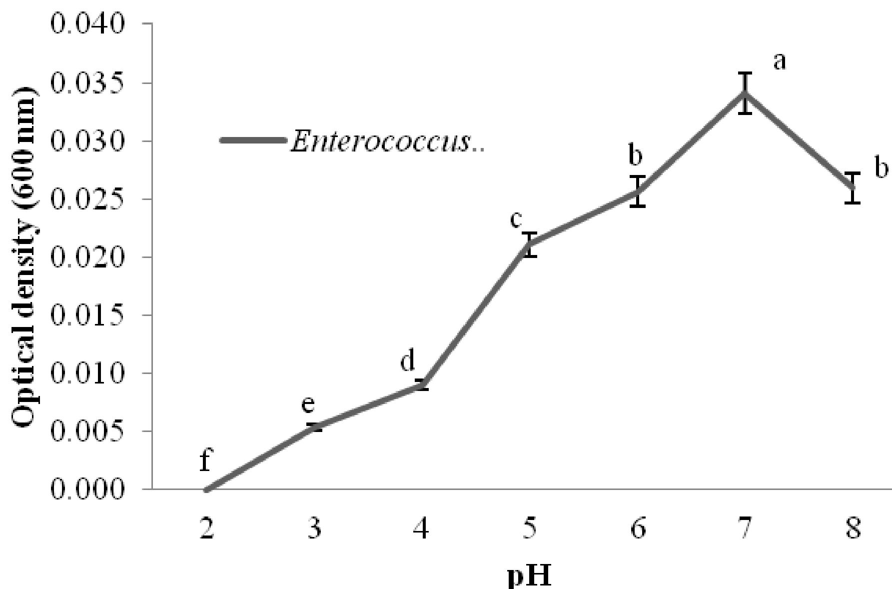

Figure 1. pH tolerance of *E. faecalis* after 2 h of incubation at different pH. Vertical bars indicate ± SE. Means with the same letter are not significantly different ($P > 0.05$).

analysis of variance (ANOVA) using the SAS program (Version 8.2). Duncan's multiple range test was performed to determine the differences among the treatment means (α = 0.05) (SAS, 2001).

RESULTS

Isolation, selection and identification of LAB

Plate counts of bacteria indicated that LAB were a minor part of the microbiota in the stomach of snakehead as they accounted for only 12.2% of the total bacterial count of 2.1×10^6 cfu/g in the intestine. Five yellow colonies of LAB coded as LAB-1 to LAB-5 were isolated from the intestine of the snakehead fingerlings. The isolates were Gram-positive, catalase- and oxidase- negative and were short rod or cocco- bacilli shaped. The antibacterial test, LAB-4 showed a significantly higher ($P< 0.05$) inhibition zone against *A. hydrophila* than the other LAB. Based on this criterion, strain LAB-4 was selected for further identification and probiotic characterization. 16S rRNA gene sequence analysis of LAB-4 showed that the bacterium was closely related to *E. faecalis* (100% similarity) with accession no. HM579789.

pH tolerance

pH tolerance of *E. faecalis* showed that the growth rate of this strain significantly ($P < 0.05$) changed when grown at different pH; 2 to 8 (Figure 1). There was no growth and viability at pH 2 after 2 h incubation, but the strain grew well at pH 7.

Bile salt tolerance

Three bile salt concentrations (0, 0.15 and 0.3%) were studied to find out the tolerance of *E. faecalis* after 2, 4 and 8 h of incubation. This bacterium not only showed viability but also exhibited proliferation in all three concentrations for all incubation times (Figure 2). As bile salt concentration increased, the growth rate of *E. faecalis* significantly ($P < 0.5$) decreased after 2 h of incubation. A similar trend was also observed after 4 and 8 h post-incubation.

Growth in different NaCl concentrations and temperature levels

E. faecalis showed good viability and growth rates in 0 to 4% NaCl after 4, 8, 16 and 24 h incubation. However, the growth decreased with increasing NaCl concentrations. Moreover, the growth rate of *E. faecalis* was significantly ($P < 0.05$) increased with increasing temperature up to 30°C, but decreased at 40°C. No growth was observed at 10 and 50°C and the viability was observed to be nil at 50°C.

Antibacterial activity test

Results from the disc diffusion technique showed that *E. faecalis* significantly ($P < 0.05$) inhibited *in vitro* growth of *A. hydrophila* and *S. putrefaciens,* but had no impact on *P. aeruginosa* growth. *E. faecalis* showed higher inhibi-

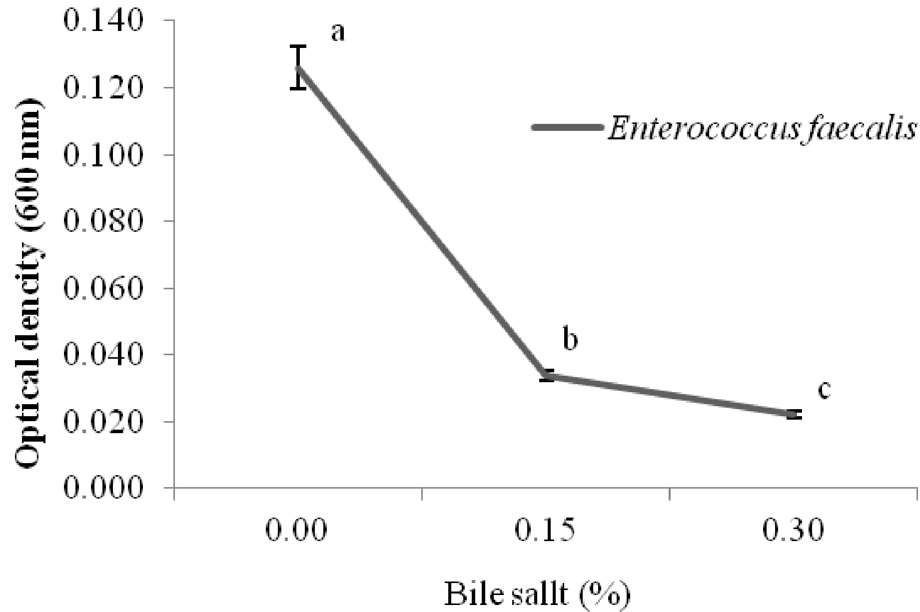

Figure 2. Bile salt tolerance of *E. faecalis* after 2 h incubation in different concentrations. Vertical bars indicate ± SE. Means with the same letter are not significantly different ($P > 0.05$).

tory activity against the three pathogens when tested by the well diffusion technique and a significant ($P < 0.05$) higher effect was noted against *A. hydrophila* as compared to *P. aeruginosa* and *S. putrefaciens* (Figure 3). The inhibition zones against three pathogenic bacteria by using the well diffusion method was significantly ($P < 0.05$) higher than the results of the disc diffusion method.

Antibiotic sensitivity test

With respect to antibiotic susceptibility profiles test, *E. faecalis* was found to be resistant (R) to tetracycline (TE), streptomycin (S), gentamycin (GM) and kanamycin (K), intermediate (I) to erythromycin (E) and sensitive (S) to chloramphenicol (C), amoxicillin (AMX) and ampicillin (AM)

Effect of *A. hydrophila* challenge test on experimental fishes

All fish in treatment group T_3 (without the supplementation of LAB to the diet and injected with *A. hydrophila*) were dead in the three replicate tanks after 48 h (Table 1). The survivability of snakehead fingerlings was 100% for the control group (Tc) and treatment group T_2 (LAB-fortified diet without injection of *A. hydrophila*). Treatment group (T_1) fed with *E. faecalis* and injected with the pathogen showed 56.6% mortality at 48 h after injection; afterwards no mortality was observed. Statistical analysis of the survival rate of fish fed fortified diet with *E. faecalis* and exposed to *A. hydrophila* (T_1) was significantly ($P < 0.05$) improved as compared to fish fed non LAB-fortified diet but exposed to *A. hydrophila* (T_3). In the latter group, 100% mortality was observed 48 h post-injection.

The anatomy of dead (infected) fish in group T_3 showed hemorrhage in kidney, spleen, eye and abdominal muscles in all fishes. In addition, swollen abdomen with yellowish liquid was observed. The results of the challenge test confirmed that *A. hydrophila* is capable of inducing mortality in snakehead fish, but the survival of fish fed with LAB supplemented diet was significantly improved.

DISCUSSION

Total colony count of bacteria in intestine

The results of the present study showed a low population level of culturable bacteria (2.1×10^6 cfu/g) in the intestine of snakehead. This level is higher than that reported in the foregut (7×10^3 to 7×10^4 cfu/g), midgut (4×10^3 cfu/g) and hindgut (4.5×10^4 to 4.5×10^5 cfu/g) of Atlantic cod (*Gadus morhua* L.) (Ringø et al., 2006). Furthermore, the current study showed that LAB was a minor part of microbiota in snakehead intestine. Ringø et al. (2006) described that the gut microbiota of fish are less diversed than in terrestrial animals.

pH and bile salt tolerance

Kim and Austin (2008) described that one of the most important criteria for characterization of probiotic bacteria

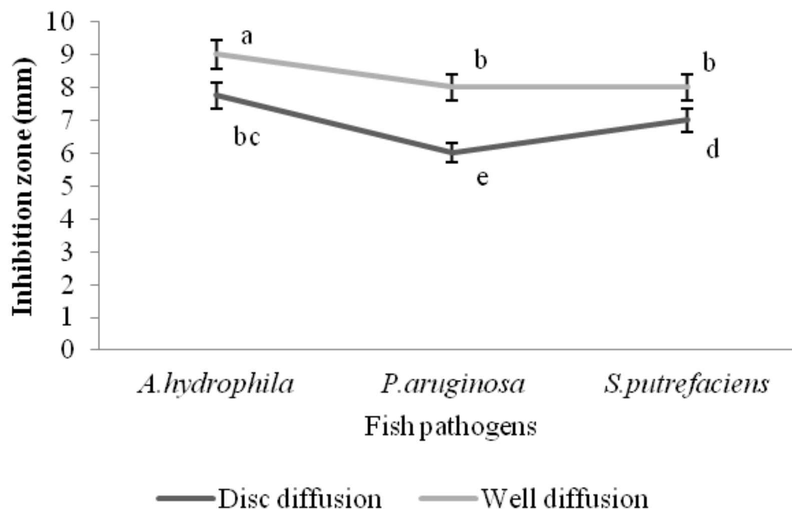

Figure 3. Comparison of disc and well diffusion techniques in antagonistic effect test with *E. faecalis* against pathogenic bacteria. Vertical bars indicate ± SE. Means with the same letter are not significantly different ($P > 0.05$).

Table 1. Effect of four experimental groups: with and without *E. faecalis* in diet, and with and without pathogen injection on fish survival (means ± SE).

Treatment	Survival (%)
Tc	100 ± 4.51^{a}
T_1	53.4 ± 3.32^{b}
T_2	100 ± 4.77^{a}
T_3	0 ± 0.01^{c}

SE: Standard error, Tc: fish fed without *E. faecalis* in the diet and without pathogen injection, T_1: fish fed with *E. faecalis* in the diet and with pathogen injection, T_2: fish fed with *E. faecalis* in the diet and without pathogen injection, T_3: fish fed without *E. faecalis* in the diet with pathogen injection. Means with the same letter in columns are not significantly different ($P > 0.05$), ($n = 30$).

is their tolerance to acidic conditions. The results of the present study displayed that *E. faecalis* was able to grow at pH from 3 to 8. This result is in agreement with Cebeci and Gurakan (2003) and Nguyen et al. (2007) who reported the viability of *L. plantarum* at pH 4 to 10 and Balcázar et al. (2008) reported the growth activity of *L. fermentum* and *L. plantarum* at pH 2.5 to 6.5.

Bile salt tolerance has been suggested as an important criterion for probiotic bacteria to grow and survive in fish intestine (Balcázar et al., 2008). The results of the present study are in agreement with Cebeci and Gurakan (2003), Nguyen et al. (2007) and Balcàzar et al. (2008), who reported the tolerance of *Lactobacillus* species to different bile salt concentrations. Probiotics that tolerate at low pH and bile salt levels are able to pass through the stomach and then colonize and grow in the intestine as well as survive there in stress conditions (Cebeci and Gurakan, 2003).

Growth in different NaCl concentrations and temperatures

E. faecalis in the present study showed high potential proliferation in an environment up to 4% NaCl. Nguyen et al. (2007) reported that *L. plantarum* PH04 could grow at 6% NaCl and at temperatures between 25 and 45°C. Kim and Austin (2008) reported that two probiotic carnobacteria strains isolated from rainbow trout intestine were able to grow in up to 15% (w/v) NaCl and at temperatures ranging from 10 to 37°C. The growth ability of *Carnobacterium* strains isolated from brown trout (*Salmo trutta*) was limited in 8% NaCl but they grew at temperatures between 4 and 45°C (Gonzalez et al., 2000). Similar results were also reported by Samelis et al. (1994) and Thapa et al. (2006). The results of this study showed that *E. faecalis* could grow within a wide range of temperature (15 - 45°C).

Antibacterial and antibiotic susceptibility tests

The selected strain, *E. faecalis* showed *in vitro* growth inhibition against the three tested fish pathogens, especially *A. hydrophila* and these results are in accordance with Rengpipat et al. (2008), who reported inhibition activity against *A. hydrophila* using cell-free cultured broths of five LAB. Kim and Austin (2008) demonstrated antibacterial ability of *Carnobacterium* strains (isolated from rain-

bow trout intestine) against *A. hydrophila* and *A. salmonicida*. Antibiotic susceptibility test can indicate resistance or sensitivity to specific antibiotics. LAB showing resistance to specific antibiotics indicates that these bacteria can be included in the diet at the same time if antibiotic treatment is required. Antibiotic resistance is an advantageous capacity as the intestinal microbiota can quickly recover after antibiotic treatment (Cebeci and Gurakan, 2003; Kim and Austin, 2008).

Challenge test

The challenge test indicates that snakehead fish was infected readily by *A. hydrophila* but the survival was improved when they were fed with dietary *E. faecalis*. All infected fish showed hemorrhage in internal organs with swollen abdomen. Similar observations were also reported by Rengpipat et al. (2008) and Aly et al. (2008) in their studies with sea bass (*Lates calcarifer*) and Nile tilapia (*Oreochromis niloticus*), respectively. According to Abdel-Tawwab and Ahmad (2009), the number of *A. hydrophila* cells were declined after an artificial challenge in fish with Spirulina (*Arthrospira platensis*) and that bacterial numbers were lower in the liver and kidney of fish treated with probiotic than the control. Therefore, *E. faecalis* can be used as a high potential probiotic to inhibit *A. hydrophila* activity in snakehead fish culture.

Conclusion

The present study revealed that *E. faecalis* has potential probiotic properties. In addition, it suggests that *E. faecalis* is a safe alternative to antibiotics to inhibit *A. hydrophila* activity in snakehead fish culture.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Bacteriological assessment of the quality of *Brassica oleracea* var. *capitata* grown in the Accra Metropolis, Ghana

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Bacterial and other microbial contamination of fresh vegetables from the farm or garden to the market and to the final consumer remain a problem worldwide. This study was designed to evaluate the various possible bacterial species responsible for the contamination of *Brassica oleracea* var. *capitata* (cabbage) in the Korle-Bu vegetable garden and Agbogbloshie market in the Accra Metropolis, Ghana. Sixty (60) cabbage samples were collected and investigated bacteriologically using standard Food and Agriculture Organization (FAO) of the United Nations (UN) total aerobic plate count methods.Cabbage samples from the vegetable garden were found to be more contaminated than the market with a total mean colony count of 2.43×10^6 CFU/g and 1.53×10^6 CFU/g respectively. *Staphylococcus aureus* was the most predominant bacteria isolated with a high percentage occurrence of 51% followed by *Escherichia coli* (28%), *Bacillus* sp. (12%), *Streptococcus* sp. (5%), and *Pseudomonas aeruginosa* (4%). From the study, bacterial contamination of cabbage grown at the Korle-Bu vegetable garden and the Agbogbloshie market were all above the recommended standard levels especially *E. coli* which should be less than 10 bacteria per gram. Therefore it is recommended that these vegetables be thoroughly washed with safe water or saline solutions before processing and consumption especially where they are not going to be heated or cooked before consumption.

Key words: Vegetables, Cabbage, *Staphylococcus, Escherichia*.

INTRODUCTION

Bacteria and other microbial contamination of fresh vegetables from the farm or garden where they are grown to the market where they are displayed, sold, and finally to the consumer still remain a major problem throughout the world especially in the developing countries including Ghana. Cabbage known scientifically as *Brassica oleracea* var. *capitata* was first identified by Linn. The plant is one of the major vegetables grown in most parts of Ghana and beyond. There are three main varieties namely, white headed cabbage, red headed cabbage,

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and savoy headed cabbage around the world. Leaves can be eaten fresh in salads, as green cooked vegetables or fermented. In Ghana and other parts of the world, the leaves of the white headed cabbage is one of the components that are used for preparing ready to eat (RTE) foods like salad and also for the preparation of vegetable stew in the various houses. The vegetable has a high nutritive value capable of supplying the human body with essential vitamins, proteins, carbohydrates, vitamins, and also can signal genes to increase the production of enzymes involved in detoxification (Leja et al., 2006; Mariga et al., 2012).

The city of Accra is the national capital of the Republic of Ghana and is predominantly an industrial community. Sanitation in Accra is a major problem due to overpopulation and also several industrial waste produced by most of the industries. As a result, most of the river bodies as well as the lagoons have been turned into dumping sites. Therefore it is difficult for the vegetable farmers in the Accra Metropolis to get a suitable water to water their farm produce and end up using the dirty or contaminated water from gutters for watering the vegetables. It is possible that several microorganisms that are harmful to the human health in the dirty water may find their way into the vegetables and infect humans after eating such vegetables. Agbogbloshie market is located at the central part of Accra and is not far from the Korle lagoon which has been turned into a dumping site.

The mode of harvesting, transportation, and storage methods used for the vegetables can also contribute a lot in the introduction of those microorganisms into the vegetables. For instance, there are reports that cross-contamination can occur by the use of dirty harvesting equipments, unhygienic handling during sorting, packaging, transport, improper storage, and display (Beuchat, 2006). The presence of cut surfaces provides an increased surface area for contamination growth and allows microbial infiltration of the tissue. Exposing vegetables to various types of cutting has been shown to result in a six to seven-fold increase in microbial numbers (O'Brien et al., 2001).

The health hazards associated with the eating of fresh vegetables like cabbage is underestimated due to the several nutritional benefits obtained from that vegetable. Gastrointestinal infections, for example, are the most common diseases caused by enteric bacteria. Although a lot of studies have been done on bacteriological analysis of fresh vegetables in several countries, bacterial contamination of food remains a risk factor for gastrointestinal infections in Ghana and with the recent outbreak of cholera in Ghana (Anonymous, 2012) there is the need to conduct this study to find out the level of bacterial contamination of cabbage which is a major component of food in Ghana. Therefore this study was designed to find out the various possible bacterial species responsible for the contamination of cabbage in the Korle-Bu vegetable garden and the Agbogbloshie market of the Accra Metropolis, Ghana.

MATERIALS AND METHODS

Sample collection

Cabbage samples for the study were collected from the Korle-Bu vegetable garden and the Agbogbloshie market, all in the Accra Metropolis of Ghana. Only cabbage samples grown at the Korle-Bu vegetable garden and cabbage samples from retailers at the Agbogbloshie market who buy their cabbage from the Korle-Bu vegetable garden were analysed. A total of 60 cabbage samples were collected in sterile plastic bags from the Korle-Bu vegetable garden (30 samples) and the Agbogbloshie market (30 samples). In the Agbogbloshie market, the samples were collected from four different retailers in the market who confirmed that they buy their cabbages from the Korle-Bu vegetable garden. The samples were collected in a period of two weeks. Each cabbage sample was given a specific code number which corresponds to number for each media plate used for the analysis. Each day's collected samples were sealed in sterile plastic bags and transported to the laboratory immediately for the bacteriological analysis.

Bacteriological analysis

Total aerobic plate count

The total aerobic plate counts of bacteria from the cabbage samples were evaluated using a modification of the Food and Agriculture Organization (FAO) of the United Nations (UN) standard food and nutrition methods by Andrews (1992). First, the leaves of the cabbage samples were selected with the aid of a sterile forceps and washed with sterile distilled water. Then, 10 g of the leaves of each sample were weighed and rinsed for 8 min in a 250 ml beaker containing 90 ml of sterile distilled water to obtain 10^{-1}. Ten-fold serial doubling dilutions of the samples through to 10^{-5} were made as follows: four additional sterile test tubes were appropriately labelled and serially arranged on the test tube rack for each sample. Sterile distilled water (9 ml) was introduced into each test tube with the aid of micropipette with sterile tips. Using separate sterile pipette tips, 1 ml of the rinsed test sample was introduced into the first test tube (10^{-2}) and mixed thoroughly. After mixing, 1 ml of the contents of 10^{-2} test tube was pipetted and introduced into the second test tube (10^{-3}) and mixed thoroughly. The same procedure was repeated for the rest of the tubes. Then, 0.1 ml of each dilution were pipetted using a micropipette with sterile tips and dropped on the surface of a pre-labelled plate count agar (PCA: Oxoid Limited, Basingstoke, UK) and Difco MacConkey agar (DMA: Becton, Dickinson and Company, Sparks, MD 21152, USA) plates in accordance with the labelling on the cabbage samples. A sterile glass spreader was used to spread the sample dilutions uniformly over the surface of the agar plates. The plates were then incubated at 37°C for 24 - 48 h.

After overnight and subsequent incubations, the plates were examined for evidence of bacteria growth and the number of colonies counted. The isolated bacterial index on each agar plate was expressed as CFU/g by multiplying the number of colonies with the dilution factor. Counting was done with the aid of a hand lens.

Identification of isolates

The identification and characterization of the isolated bacterial species in the present study were done using colonial morphology,

Table 1. Mean bacteria colony count values (×10^6 CFU/g) of isolated bacteria in cabbage from the vegetable garden and the market.

Bacterial specie	Garden	Market	Total mean colony count
Staphylococcus aureus	1.19	0.81	2.0
Escherichia coli	0.67	0.42	1.09
Bacillus sp.	0.31	0.17	0.48
Streptococcus sp.	0.13	0.09	0.22
Pseudomonas aeruginosa	0.13	0.04	0.17
Total	2.43	1.53	3.96

Gram staining reactions, catalase, indole, oxidase, motility, citrate utilization, methyl red (MR), VogesProskauer (VP), triple iron sugar (TSI), and coagulase tests according to the Food and Agriculture Organization (FAO) of the United Nations (UN) standard food and nutrition methods by Andrews (1992) and cross referenced with Bergey's manual of determinative bacteriology (Holt et al., 1994).

Statistical analysis

Results obtained from the experiments were entered into a database and analysed statistically using Statistical Package for *Social Sciences (SPSS)* version 20 statistical software for windows and a summary was presented using the descriptive statistics such as means and percentages. Factor analysis was performed on samples from the vegetable garden and those from the market to establish their level of correlation or variability in terms of mean colony counts. Also the student's t-test was used to find out significant difference between the parameters studied. P-values >0.05 were taken as statistically insignificant difference.

RESULTS

Mean bacterial counts of cabbage samples from the vegetable garden and the market

Vegetables more especially cabbage are essential part of people's diet all round the world. Sometimes these cabbage are consumed raw and often without heat treatment or thorough washing and as such have been known to serve as vehicle for the transmission of pathogenic microorganisms associated with human diseases. In the present investigations, the highest mean bacterial colony count were observed for the samples from the Korle-Bu vegetable garden (2.43 x 10^6 CFU/g) representing 61.4% of all the isolated bacteria in the study.

The mean colony count values of isolated bacteria ranged between 0.13 to 1.19×10^6 CFU/g for the samples from the vegetable garden and 0.04 to 0.81×10^6 CFU/g for the samples from the market (Table 1). *Staphylococcus aureus* was the predominant bacteria with mean colony count values of 1.19×10^6 and 0.81×10^6 CFU/g from the vegetable garden and the market, res-pectively (Table 1). Other bacteria including *Escherichia coli*, *Bacillus* sp., *Streptococcus* sp., and *Pseudomonas aeruginosa* were also isolated. For example, with *E. coli*, a mean colony count values of 0.67 ×10^6 and 0.42 ×10^6 CFU/g were isolated from the cabbage samples from the vegetable garden and the market whiles for *Bacillus* sp. a mean colony counts values of 0.31 ×10^6 and 0.17 ×10^6 CFU/g were also isolated from the two study sites, respectively. However, for *Streptococcus* sp., a mean colony count values of 0.13×10^6 and 0.09 ×10^6 CFU/g from the vegetable garden and the market, respectively were isolated in the study. A mean colony count values of 0.13 ×10^6 and 0.04 × 10^6 CFU/g were also isolated for *P. aeruginosa* as presented in Table 1.

Bacterial index in cabbage samples

Five groups of bacteria including *S. aureus*, *E. coli*, *Bacillus* sp., *Streptococcus* sp., and *P. aeruginosa* were isolated and identified from the cabbage samples from the Korle-Bu vegetable garden and the Agbogbloshie market of the Accra Metropolis. Factor analysis performed to verify the significance of the differences in counts of bacteria was statistically significant (p<0.005). *S. aureus* was the most predominant bacteria isolated with a total mean colony count value of 2.00×10^6 CFU/g of all the bacterial contaminants isolated from all the cabbage samples representing 51% of all the bacteria isolated and identified in this investigation (Table 1 and Figure 1). The second most predominant bacteria isolated in this study was *E. coli* with a total mean colony count value of 1.09×10^6 CFU/g representing 28% of all the bacterial isolates identified. *Bacillus* sp. had a total mean colony count value of 0.48×10^6 CFU/g representing 12% of all the bacterial contaminants isolated and identified. *Streptococcus* sp. and *P. aeruginosa* were the least isolated bacterial contaminants with a total mean colony count values of 0.22×10^6 and 0.17×10^6 CFU/g representing 5 and 4%, respectively of all the bacterial contaminants isolated (Table 1 and Figure 1).

DISCUSSION

Bacterial contamination of cabbage in the Accra Metropolis investigated showed total mean bacterial colony count values of 2.43×10^6 and 1.53×10^6 CFU/g in the vegetable garden and the market, respectively. The results of the present study is similar to a previous research conducted by Frank-Peterside and Waribor (2006) which reported that bacteria load on leafy vegetables increase

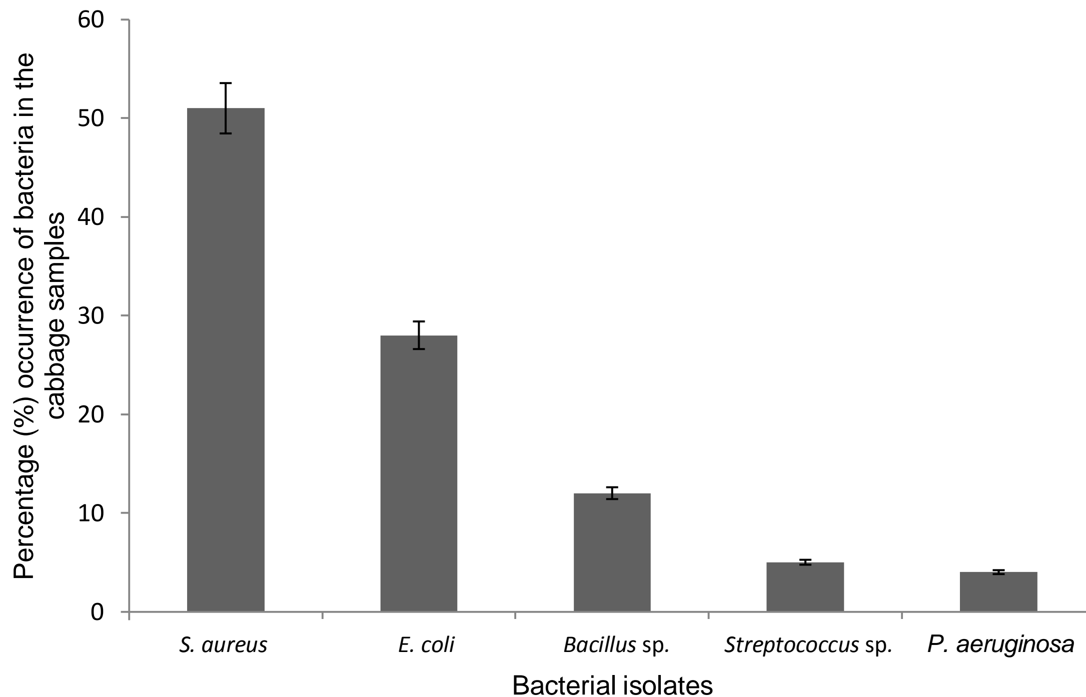

Figure 1. Percentage distribution of contaminants isolated from the cabbage samples.

with time during storage. However, the decrease in the bacterial contamination in the samples from the market may be attributed to the storage conditions of the vegetable. Some of the market women when orally interviewed confirmed that, they occasionally sprinkle salt water on the cabbage to prevent them from quick spoilage and also to help kill microorganisms that may be present in the cabbage during harvesting as previously proposed by Abdullahi and Abdulkareem (2010). This act by the market women may have resulted in the low bacterial colony count values in the cabbage samples from the market.

Among the isolated bacterial pathogens, *S. aureus, E. coli*, *Bacillus* sp., *Streptococcus* sp. and *P. aeruginosa* were the predominant bacterial species found to be associated with the cabbage in the vegetable garden and also during the storage and selling processes in the Agbogbloshie market in the Accra Metropolis. This finding indicates gross contamination from the vegetable garden and the market until it finally reaches the consumer. The high prevalence of *S. aureus* on the cabbage samples may be due to pre and post-harvest handling; for it is known that, *S. aureus* is an opportunistic pathogen found living in the nasopharynx and skin of up to 50% of normal people (Enright, 2003; Guignard et al., 2005). Therefore the high frequency of the bacteria found in this study may be attributed to the bacteria being present as a normal flora of humans and can contaminate the vegetables as a result of poor hygiene of farmers and sellers.

E. coli was found in 28% of all the samples from the vegetable garden and the market analysed (Figure 1). The presence of *E. coli* on the cabbage samples from all the sampling sites with a high amounts on those from the vegetable garden may be as a result of faecal contamination because the bacteria is present in sewage, faeces, soil, water, and commonly come in contact with vegetables as result of the water used during the growing processes of the vegetables. During the investigation, the water used by the farmers for watering the cabbage was examined macroscopically on each sampling day and they were found out to be just wastewater from drainages around the vegetable gardens as previously reported by other research workers (Drechsel et al., 2006; Ackerson and Awuah, 2010). Also work by Solomon et al. (2003) reported that repeated spraying of crops with contaminated irrigation water increases the chances of crop contamination and this may also account for the high bacterial contamination of the cabbage samples investigated.

Although *Bacillus* sp. was isolated from the cabbage samples from the vegetable garden and the market, respectively in this study (13 and 11%, p-value > 0.05) other research workers did not isolate the bacteria in their investigations (Ibrahim and Jude-Ojei, 2009; Taura and Habibu, 2009). However, Abdullahi and Abdulkareem (2010) working on RTE vegetables in Sabon-Gari, Zaria, Nigeria also observed the presence of *Bacillus* sp. The isolation of *Bacillus* sp. may be due to environmental factors and the ability of the bacteria to form spores (Gupta et al., 2013; Merghni et al., 2014).

Percentage occurrence of *Streptococcus* sp. from the cabbage samples from the vegetable garden and the market in this study was recorded (5 and 6%, p-value > 0.05) as presented in Figure 1 and Table 2. However, the

Table 2. Percentage occurrence (%) of isolated bacteria in cabbage from each site sampled.

Bacterial isolate	Garden	Market	P-value
Staphylococcus aureus	49	53	0.756*
Escherichia coli	28	27	0.930*
Bacillus sp.	13	11	0.811*
Streptococcus sp.	5	6	0.861*
Pseudomonas aeruginosa	5	3	0.692*
Total (%)	100	100	

*p-values were considered insignificant (> 0.05)

isolation of *P. aeruginosa* (5 and 3%) from the cabbage samples from the vegetable garden and the market, respectively (p-value > 0.05) may come from the environment. In a similar related work done by Itohan et al. (2011) who also isolated the bacteria in cabbage and the vegetables they analysed. *P. aeruginosa* is widely distributed in nature and is commonly present in moist environments. It can also colonize normal humans, in whom it is a saprophyte. It only causes disease in humans with low immune defences system (Stover et al., 2000). Therefore cross-contamination of the cabbage samples by *P. aeruginosa* can occur during storage, preparation, dirty harvesting equipment, unhygienic handling, and improper storage (Codex Alimentarius Commission, 2007).

Conclusion

This study have shown that all the cabbage samples investigated have high bacterial contamination and their persistence and proliferation is a reflection of the use of unsafe or contaminated water in watering these vegetables. It is therefore recommended that these vegetables be thoroughly washed with safe water or saline solutions before processing and consumption especially where they are not going to be heated or cooked before consumption.

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Declaration of interest: None

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Full Length Research Paper

Application of bacterial biomass as a potential heavy metal bio-removal agent

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Water has been the most important element for saving life; the major global health problem has been water pollution that may be due to the wastewater discharge into the water bodies. Several techniques have been used for water treatment that is, physical, chemical and biological methods. Recently, the third method was the most effective one for the wastewater treatment. In this work twenty bacterial isolates were isolated from River Nile, Egypt to study their capability to remove some heavy metals from its solution. Agar plates amended with different concentrations of some heavy metals were used for screening the bacterial capability for removing the tested heavy metals. According to the identification procedures based on the BIOLOG system the bacterial isolate MSNIOF11 showed a similarity of 97% to *Bacillus subtilis* var. *globigii*, so it was given the name as *Bacillus subtilis* var. *globigii* MSNIOF11. The heavy metal removal process was pH and temperature dependent, where the maximum growth and heavy metal removal was recorded at 30°C with neutral pH (7.0). In the first 24 h there was an increase of the metal removal and there was no significant change after 30 h.

Key words: Bioremoval, heavy metals, *Bacillus subtilis*, biomass, biolog, MicroPlats.

INTRODUCTION

Water pollution is an acute problem in the River Nile. In the rise of the increasing urbanization and industrialize-tion, the pollution potential of the River is gaining momen-tum day by day. Dumping wastewater and toxic wastes into the main channel of the River has caused serve pollution in the River to the extent that its water is posing a threat to the survival of aquatic flora and fauna (Dalman et al., 2006).

Water quality has been decreased during this century due to discharge of wastewater into water channels as well as environmental pollutants. This is considered as one of the major global health problems, and cross adap-tation of microbial population to structurally related che-micals may play an important role in the practical deve-lopment and application of bioremediation techniques (Liu and Jones, 1995; Monachese et al., 2012). Pollution of the natural environment by heavy metals is a world-wide problem because these metals are indestructible

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and most of them have toxic effects on living organisms when they exceed a certain concentration (MacFarlane and Burchett, 2000; Ekeanyanwu et al., 2010; Raphael et al., 2011; Aikpokpodion et al., 2012; Ahmed et al., 2013). Heavy metals are of high ecological significance since they are not removed from water as a result of self purification, but accumulate and enter the food chain which inevitably affects the human health resulting in extremely disrupted biological processes (Loska and Wiechula, 2003; Igwe and Abia, 2006; Akpor and Muchie, 2010; Young et al., 2012).

The bio-removal/ processes could offer the possibility to destroy or render various contaminants using natural biological activity. As such, it uses relatively low-cost, low technology techniques, generally have a high public acceptance and can often be carried out on site using a microbial source which has received much attention recently due to the awareness of environmental problems (Saithong and Poonsuk, 2002; Rani et al., 2010; Abioye, 2011). Microorganisms such as bacteria (Daboor and Sabae, 2007; Nanda et al., 2011; Lin and Harichund, 2011; Samarth et al., 2012), algae (El-Sherif et al., 2008; Tamilselvan et al., 2011; Mane and Bhosle, 2012; Kumar and Oommen, 2012), fungi (Selvam et al., 2002; Joshi et al., 2011; Simonescu and Ferdes, 2012; Hemambika et al., 2011) and yeast (Abdul Rehman et al., 2008; Machado et al., 2009, 2010) persist a variety of mechanisms exist for the removal of heavy metals from aqueous solution. Regarding the same process Zhou et al. (2007) used *Bacillus* cells as a factor to remove the chromium ions from the aqueous solution. Jarosławiecka and Piotrowska-Seget (2014) reported that the bacterial extracellular polysaccharides are involved with the lead adsorption.

The main objective of the present study was to isolate and screen heavy metal tolerance of bacterial isolates and evaluate their competence to remove heavy metals from its solution and detect the suitable conditions for the maximum activities under laboratory conditions.

MATERIALS AND METHODS

Sampling and strain isolation

Water samples were collected from five stations at Demitta Branch of RiverNile. These stations are highly polluted with high concentrations of heavy metals due to discharge of industrial effluent. Twenty bacterial isolates (12 as Gram positive and eight as Gram negative) were isolated after seeding the samples on Glu-cose Mineral Salt (GMS) agar plates, (Daboor and Sabae, 2007).

Heavy metal

Heavy metals solutions of zinc sulfate ($ZnSO_4$), lead chloride ($PbCl_2$) and cadmium chloride ($CdCl_2$) were prepared with a final concentration 100 mg/L and kept sterilized for further use.

Screening for bacterial isolates resistant to metal ions

The bacterial isolates were separately streaked on GMS having 100 mg/L of each of the metal solution. After two days of incubation at 30°C the plates were checked for bacterial growth (Daboor and Sabae, 2007).

Heavy metal removal efficiency detection

The bacterial isolates that showed a positive growth on the agar plates (having heavy metals solutions) were sub cultured again onto modified T-medium (Duxbury, 1981) amended with mixture of heavy metal ions Cd^{2+}, Pb^{2+} and Zn^{2+} (50 mg/L of each one). The plates were swabbed and the growth was measured by the periodic determination of culture density absorbance at wave length of 600 nm using a spectrophotometer (Ultrospec 1100-pro, Amersham Pharmacia Biotech) based on McFarland's scale (Sutton, 2011). The most resistant isolates were inoculated (0.2 g) into 250 mL conical flask containing 50 mL T- medium which has metal ions with 50 mg/L final concentration of each metal ion and incubated in shake condition (50 rpm) for 24 h at 30°C.

Identification and characterization of the metal resistant isolates

Bacterial colonies from the plates showed highly intensiveness growth were picked and streaked on Tryptic Soy Agar (TSA) medium, after overnight incubation at 30°C pure colonies were stained with Gram stain, hence figure out which Biolog Micro Plate could be used.

According to the manufacturer's directions of MicroLog System (Biolog, Hayward, CA, USA) pure bacterial isolates were transferred into Biolog Universal Growth (BUG) medium (Biolog, Hayward, CA, USA) and incubated overnight at 30°C. Bacterial growth were collected and suspended in 0.15 M NaCl using cotton swabs. (Tanase et al., 2011).

Biolog MicroPlates preparation and identification

The isolate was tested to utilize 95 different carbon sources in Gram-positive (GP) MicroPlate (Biolog, Hayward, CA, USA) as recommended by the manufacturer's manual. A plastic disposable loop was used to collect colonies carefully so that there would be minimum carryover of nutrients from the agar when the growth was suspended in 0.15 M NaCl. The turbidimeter (which measured the turbidity at wave length at 590 nm) was blanked with a tube of uninoculated saline. The suspension was then adjusted to fall within the low-limit and high-limit GP MicroPlate turbidity standards supplied by manufacturer. The inoculum, which was always used within 10 min. of preparation, was poured into a disposable plastic reservoir just prior to use. MicroPlates (GP) were inoculated with an eight-channel multi-pipette, with 150 µl of the inoculum being dispensed per well; plates were then generally incubated at 30°C. The carbon source utilization patterns were read with a MicroPlate reader and analyzed for the differentiation of bacterial strains by a cluster analysis program using Biolog database and software (Biolog, Hayward, Calif), with the MicroLog GP data base colour formation in the individual cells of the microtitre plates was measured at 590 nm (Miller and Rhoden, 1991; Holmes et al., 1994).

Biolog system provides identifications if the similarity index of the genus or species was 0.750 or greater after four hours incubation. When a lower similarity value is obtained, the user is prompted to

continue the incubation for 24 h. In this study, all MicroPlates were read at both four hours and confirmed after 24 h even when identification was reported at four hours, a similarity index of less than 0.50 results in an instrument report of not identified (NI). Similarity indices of 0.50 result in a computer report of identification to either the genus or the species level (Miller and Rhoden, 1991; Holmes et al., 1994).

Effect of temperatures on bacterial cells and heavy metals removal

The effect of temperature degree was investigated by using the isolate *B. subtilis var. globigii* MSNIOF11. Heavy metal removal was conducted in 250 mL conical flasks containing 50 mL of GMS broth having heavy metals with final concentration 150 mg/L (50 mg/L of each metal). The flasks (three replicates) were inoculated with 0.2 g bacterial cells and incubated for 48 h at 20, 25, 30, 35 and 40°C. Samples were taken and centrifuged at 10.000 rpm for half hour. The supernatant was analyzed to state the heavy metal remaining in the solution (Daboor and Sabae, 2007) and heavy metal removal was calculated based on its initial concentration according to the equation of Kuycak and Volesky (1988).

$$Q = (C_I - C_F)*V/V_1$$

Q: metal removal; C_I: initial metal concentration; C_F: final metal concentration; V: volume of reaction and V_1: total volume

Effect of pH on bacterial cells and heavy metals removal

The effect of different pH values (5.0, 6.0, 7.0, 8.0 and 9.0) was investigated. Adjusting the pH of the medium using 0.1 N HCl and 0.1 N NaOH, and incubating for 24 h at 30°C, remaining heavy metals in the solution were calculated as described previously based on the equation of Kuycak and Volesky (1988).

Effect of incubation time on bacterial cells and heavy metals removal

To study the effect of different incubation periods on both bacterial growth and heavy metals removal, the pH was adjusted to pH 7.0. After several intervals of time 12, 18, 24, 30 and 36 h of incubation at 30°C, heavy metals residue in the solution were calculated following the method reported by Kuycak and Volesky (1988).

Statistical analysis

The arithmetic means of the three replicates estimations were tabulated and the least significant difference (L.S.D.) at 0.05% confidence limit was calculated according to Pielou (1966).

RESULTS

Biolog MicroLog identification

Only one of the twenty bacterial isolates showed a very good growth in the presence of heavy metals high concentration (data not shown). This isolate was rood shaped positive to Gram staining. After four hours and 24 h the color change within each well in the MicroPlate was red by the automated reader. The tested strain showed 97% similarity with *Bacillus subtilis* var. *globigii* and the data also revealed a very low similarity (2.8%) with *B. pumilus.* The relationship between *Bacillus* strains by carbon sources utilization pattern was shown in Figure 1. The analyzed data by Biolog software illustrated the arrangement and distances of the *Bacillus* species, clarified that both B3 (the selected strain) and B2 have the same unit of taxonomic distances, hence B3 is *Bacillus subtilis* var. *globigii*, for differentiation between the selected strain and others it given the name *Bacillus subtilis* var. *globigii* MSNIOF11.

Effect of different temperatures

Effect of incubation temperatures on the growth of *B.. subtilis* var. *globigii* MSNIOF11 and heavy metal removal were represented in Figure 2. The temperature effect represented significant differences (P>0.05) between the percentage of metal ion removal, where maximum activity of the heavy metal removal was recorded at 30°C with the values of 72.30, 68.30 and 70.00% of Cd, Pb and Zn ions.

Effect of different hydrogen ion concentrations

pH values play an important role on heavy metal removal by *B. subtilis* var. *globigii* MSNIOF11,as shown in Figure 3. Hydrogen ion concentrations - pH values- affected both the growth and heavy metal removal by the bacterial isolate. At pH levels of 5.0 and 9.0 no obvious growth was seen and metal removal was indemonstrable, nevertheless, the maximum growth and the maximum metal removal were obtained at pH of 7.0, the logarithmic numbers of viable cells /ml were 7.70, 8.39 and 9.20 for Cd^{2+}, Pb^{3+} and Zn^{2+} at 30°C, respectively and at the same time metal removal percentage was in the order of Cd^{2+}>Pb^{2+}> Fe^{2+} with 69.30, 68.70 and 67.00%,, respec-tively. It was also clear that, there was a significant drop in both growth and metal uptake when the pH was shifted towards both acidic and alkaline media (pH of 6.0 and 8.0).

The percentages of metal uptake were 36.30, 56.70 and 48.70% at pH 6.0, while it was 53.30, 43.30 and 46.6% at pH 8.00 for Cd^{+2}, Pb^{+2} and Zn^{+2}, respectively. The maximum values for Cd, Pb and Zn ions were recorded at pH 7.0, where the removal percentage was in the order Cd^{+2}> Zn^{+2}> Pb^{+2} .

Regarding to the effect of the incubation periods the data represented in Figure 4 showed that after 12 h incubation the microbial growth gradually increased. The metal removal was increased by time until 24 h at which

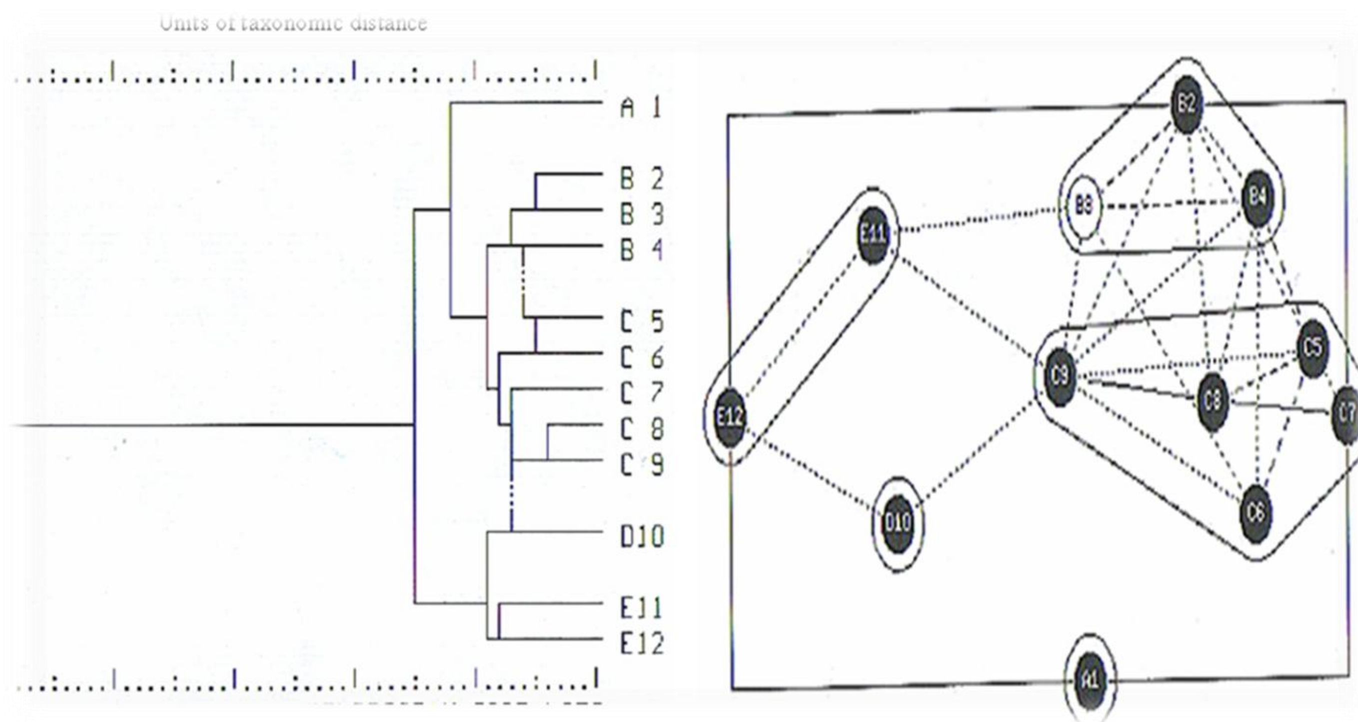

Figure 1. Dendrograms distance generated by Biolog MicroLog identification system; A1: *Bacillus maroccanus,* B2: *B. subtilis* var. *globigii,* B3*: Bacillus* sp.$_{\mathbf{MSNIOF11}}$*, B4*: *B. pumilus,* C5: *B. subtilis,* C6: *B. amyloliquefaciens,* C7: *B. alcalophilusss halodurans,* C8: *Bacillus coagulans***,** C9: *B. licheniformis,* D10: *B. circulans,* E11: *B. azotoformans*and E12: *B. coagulans.*

the highest values of Cd^{2+}, Pb^{2+} and Zn^{2+} removal were detected, but there was no significant effect with time increasing up to 30 h.

DISCUSSION

The results here illustrated the ability of some bacterial isolates to resist the heavy metals toxicity. Only one bacterial isolate showed resistant to the toxicity of all the tested heavy metals, this may be due to biochemical and structural properties, physiological and/or genetic adaptation of microorganisms and environmental modification of metal specification control the surviving of microorganisms in solutions having toxic metals **(**Cooksey, 1993; Blackwell et al., 1995). Hence this bacterial isolate was selected for the heavy metal bioremoval study and identification. It is not possible to isolate and culture microorganisms from their natural habitat and stay behind identification. This will lead to shortage of microbial community composition and function information (Wagner et al., 1993).

In this study, different carbon source profiles were generated by inoculating Biolog GP microtitre plates. The colour development in each well of the GP Biolog microtitre plates reflected the ability of the bacterial community to utilize that specific carbon source. The data indicated that metabolic diversity (substrate utilization), as determined with the Biolog system, could be used to learn more about functional diversity (number of different substrates utilized) and evenness (distribution of species abundance within the community) in natural habitats (Miller and Rhoden, 1991, Heerden et al., 2002; De Paolis and Lippi, 2008).

Some of the *B. subtilis* could survive in the presence of Cd^{+2}, Pb^{+2} and Zn^{+2} at concentration 100 mg/L, while others cannot. Bioaccumulation of heavy metals such as copper, zinc, cadmium, and nickel were reported by several *Bacillus* species **(**Mayer and Beveridge, 1989, Samarth et al., 2012; Odokuma and Akponah, 2012). Heavy metal bioremoval includes the formation of stable complexes between heavy metals and nuclides of microbial biomass and these complexes are generally the result of electrostatic interactions between the metal ligands and negatively charged cellular biopolymers which were produced in both Gram negative and Gram positive bacteria (Ledin and Pedersen, 1996).

The metal bioremoval capacity of living cells from aqueous solutions was influenced by environmental growth conditions, such as temperature, pH value, biomass concentration, and incubation time. It was clear from the present data that, the temperature degree had an important effect on the bioremoval percentage of heavy metal by *B. subtilis* var. *globigii* MSNIOF11 where

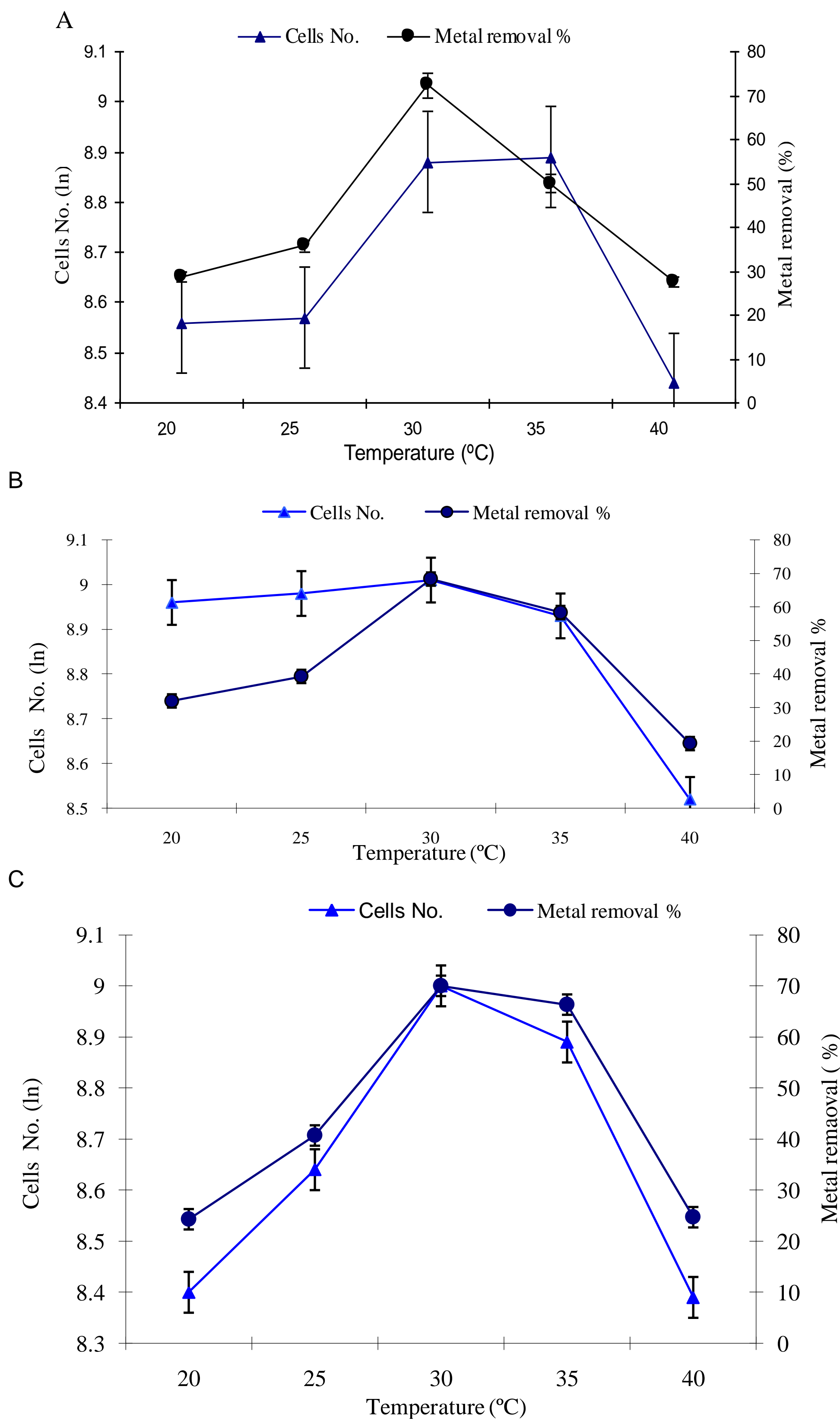

Figure 2. Heavy metal removal and bacterial cell number affected by various temperatures, **(A)** Cadmium, **(B)** Lead and **(C)** Zinc ions.

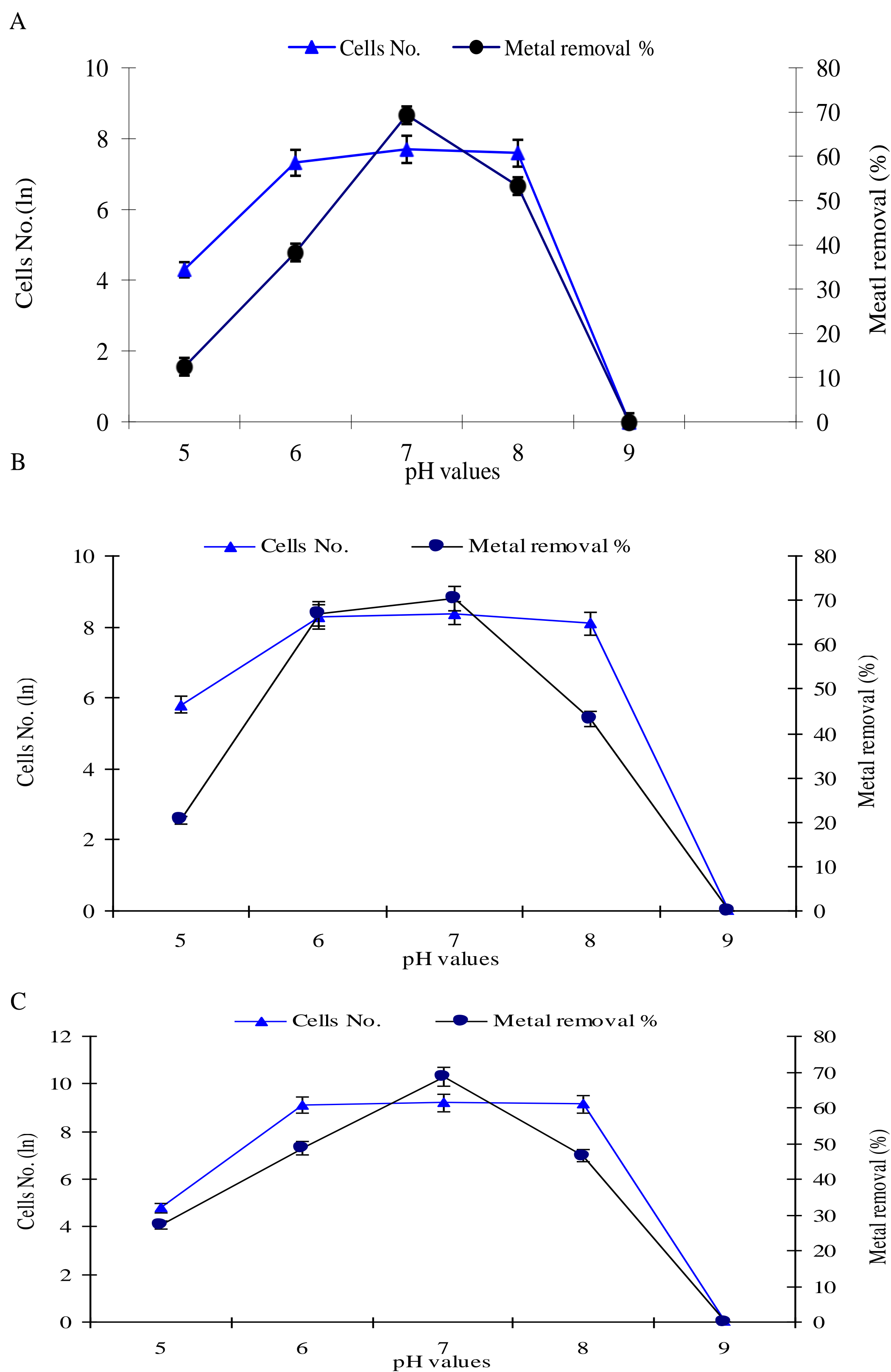

Figure 3. Heavy metal removal and bacterial cell number affected by various pH values, (A) Cadmium, (B) Lead and (C) Zinc ions.

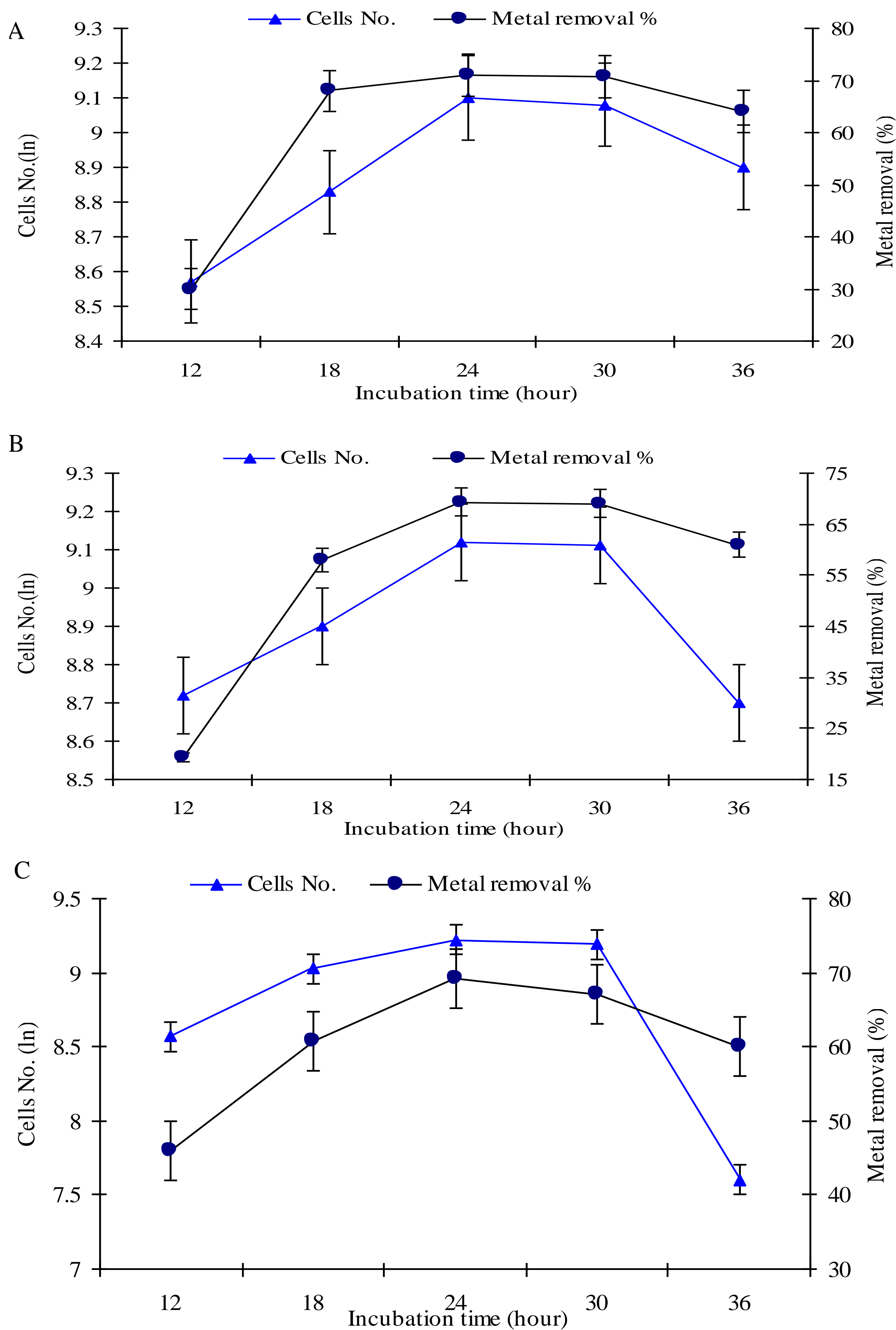

Figure 4. Heavy metal removal and bacterial cell number after various incubation periods, **(A)** Cadmium, **(B)** Lead and **(C)** Zinc.

the uptake of the tested metals increased gradually by increasing temperature from 20 to 30°C and decreased at the higher temperature 35 and 40°C. The maximum metals (Cd^{+2}, Pb^{+2} and Zn^{+2}) bioremoval were recorded at 30°C, which may depend on cell metabolism that are most likely to be inhibited by low temperature, meanwhile the higher temperature also affect the integrity of the cell membrane, these present results are in line with those reported by Brady and Duncan (1994 a,b).

The metal binding to bacterial cell was influenced by many factors including pH, buffer type, ionic strength and incubation time. The present results showed the maximum removal percentage of Cd^{2+}, Pb^{2+} and Zn^{2+} ions by *B. subtilis* var. *globigii* MSNIOF11 at pH7.0. The change of pH values from five to seven may result in an increase in the bacterial cell wall negative charge which favored electrochemical attraction and adsorption of metal (Gourdon et al., 1990). The results here were in accordance with those of Lo et al. (2001), they reported that, the biosorption increased by increasing pH from two to seven, and as the pH value was increased the solubility of the metal decreased which enhanced metal sorption (Tsezos and Volesky, 1982**)**. On the other hand at low pH most of nitrogen containing groups at the bacterial cell wall would be neutral and so preclude the metal sorption (Elliot et al., 1986; Korenevskii et al., 1999) and the high concentrate of hydrogen ion compete with the cations of sorption sites (Mclean and Beveridge, 1990). The results also revealed that, both growth and metal bioremoval were not observed at pH five. In this respect Mera et al. (1992) suggested a delicate competition between H^+ and metal ions for binding into the cells in the presence of competitive cations (Cd^{2+}, Pb^{2+} and Zn^{2+}) would alter energy states of these cells. Also, metal removal was completely inhibited at pH nine that may be due to the formation of insoluble oxides, hydroxides and carbonates at pH above neutrality which reduced the free metal ions (Brady and Duncan, 1994 a, b).

The present results showed that the metal removal also varied with incubation time. When cell suspension of *B. subtilis* var. *globigii* MSNIOF11was exposed to heavy metal in their solutions, the utmost removal was recorded after 24 h the increase of incubation time had a little effect on the metal removal, the stage of the life cycle and cultural conditions affected metal accumulation, the structural features of the cell wall as affected by cell age provide a mechanism to immobilization metals and prevent their entry into the cell (Remacle, 1990; Delgado et al., 1996).

In conclusion the selected bacterial isolate *B. subtilis* var. *globigii* MSNIOF11 had the capability of accumulation of the test metals. The bioremoval process of the tested heavy metals from its solutions was pH and temperature dependant. *B. subtilis* var. *globigii* MSNIOF11 recorded maximum growth and heavy metal removal at 30°C with neutral pH (7.0) during the first 24 h. By passing time that is, after 30 h there was no significant change. For that reason the major directions in the bioremediation technology research contains, studying the microbial communities from contaminated sites with special emphasis on those strains that play major functional roles in pollutant removal. In this respect, characterization of the catabolic potential, as well as accurate taxonomical identification of such types of bacteria is very important. In this study, we identified phenotypic and metabolic traits.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Rapid detection of virulence associated genes in Streptococcal isolates from bovine mastitis

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In the present study, 15 *S. agalactiae* out of 56 streptococcal isolates recovered from 98 milk samples collected from clinical cases, one organized farm and two unorganized sectors in and around Bangalore. All the streptococcal isolates were confirmed at genus level using genus specific primers targeting *tuf* gene of *Streptococcus*. Species level identification for *S. agalactiae*, *S. dysgalactiae* and *S. uberis* was done using 16S rRNA. Primers were designed for targeting *cfb* gene of *S. agalactiae*, *mig* gene of *S. dysgalactiae*, whereas for targeting *sip*, *hyl* gene of *S. agalactiae* and *skc*, *pau*A gene of *S. uberis* either published or designed earlier were used to screen for virulence genes of streptococcal isolates and reference strains. Desired amplicons for the virulence genes were obtained. All the *S. agalactiae* isolates were also screened for CAMP factor phenotypically by employing CAMP test which was demonstrable in fourteen isolates but *cfb* gene encoding for CAMP factor was detectable by PCR in all the isolates. The study ultimately helps us to understand the virulence characteristics and mechanisms behind emergence of new strains or shifts in mastitis epidemiology in response to control measures, including antibiotic treatment and vaccination.

Key words: Streptococci, virulence factors, bovine mastitis, CAMP factor.

INTRODUCTION

Bovine mastitis is one of the most problematic diseases and continues to have major economic impact on the dairy industry throughout the world. Several bacterial genera and species capable of causing mastitis are widespread in the environment of dairy cows. *Streptococcus* species are one of the most important causative agents of mastitis. Usually the mastitis caused by Streptococci is of the subclinical type, so early detection of such mastitis cases is of paramount importance. Among *Streptococcus* species, *S. agalactiae*, *S. dysgalactiae* and *S. uberis* are the predominant group of organisms isolated from mastitis next to *Staphylococcus* species. In spite of its high prevalence of Streptococci in both clinical and subclinical bovine mastitis, little is known about factors that contribute

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to the virulence of *Streptococcus* species. The ability of an invading pathogen to initiate growth *in vivo* and stably infect a host requires acquisition of virulence factors capable of neutralizing the mechanisms of the host's defense. These factors include structural components, toxins and enzymes that serve to overcome the otherwise effective nonspecific defensive measures of the host (Brubaker, 1985). These factors can exert a direct effect on stromal cells while others can thwart one or more host defense mechanisms to allow for survival and persistence of the pathogen in the invaded tissue (Woolcock, 1988). Several cell-associated and extracellular factors of *Streptococcus* species have been identified during last decade and Streptococci can interact with several plasma and extracellular host derived protein such as immunoglobulin G, fibrinogen, victronectin, collagen, plasminogen and α_2 – macroglobulin. These interactions are mediated by bacterial virulence factors such as pore forming protein, surface expressed Mig protein, hyluronidase and fibrinolysin which are involved in promoting dissemination of organism into the host. Yet, the relative importance of these factors in the transmission and pathogenesis of mastitis caused by Streptococci has not been understood (Calvinho et al., 1998).

The identification and characterization of virulence factors of Streptococci causing bovine mastitis will enhance our understanding of the pathogenesis of intra-mammary infection. In addition, the antibiogram of Streptococci needs to be studied which would indicate the pattern of resistance to various antibacterials contributing to their virulence properties. These may in turn contribute to the development of methods to minimize the production losses due to mastitis. Further, the study of evolution of strain-specific transmission and virulence characteristics including antibiotic resistance in Streptococci isolated from bovine mastitis may help us to understand mechanisms behind emergence of new strains or shifts in mastitis epidemiology in response to control measures, including antibiotic treatment and vaccination.

MATERIALS AND METHODS

Isolation and biochemical characterization

A total of 72 subclinical milk samples based on Electrical Conductivity (EC) using Oriental Instruments, Japan and somatic cell count (SCC) using ChemoMetec, Denmark were subjected for bacteriological examination. About 0.1 ml of milk sample having SCC more than 5,00,000 cells/ml and EC more than 6.5 mS/cm were inoculated in *Streptococcus* selection broth, with 10% CO_2 tension for 6 h to obtain sufficient growth of the organisms. Then the growth from *Streptococcus* selection broth was streaked on to blood agar plates (M/s. Hi-Media, Mumbai), incubated at 37°C for 48 h under 10% CO_2 tension to obtain pure culture. These pure cultures were again streaked onto secondary blood agar plates and then onto BHI agar (M/s. Hi-Media, Mumbai) for further identification procedures. Pure cultures thus obtained were subjected for the primary test like catalase test. Further, all the streptococcal isolates which are negative for catalase test were subjected for biochemical tests such as Voges Proskauer test, esculin hydrolysis, hippurate hydrolysis, sugar fermentation and PYR test according to the method described by Collee et al., 1996.

Phenotypic characterization

Determination of CAMP reaction

For this purpose, the test culture was streaked horizontally on a blood agar plate and a known β-hemolytic *S. aureus* was streaked vertically 3 to 5 mm above the test culture streaking. A positive reaction was recorded after incubation for 18 to 24 h at 37°C which results in half moon shaped zone of complete hemolysis in the zone of incomplete staphylococcal- β- hemolysis.

Determination of streptokinase activity

For this, about 0.5 ml of *S. uberis* (the reference strain AD2 and AD6) supernatant, a known producer of streptokinase enzyme was suspended with equal volume of 1:5 diluted rabbit plasma along with known coagulase producer such as *S. aureus* supernatant, incubated at 37°C and results were recorded at hourly intervals for 6 h. Absence of coagulation indicated positive reaction. Paralleling, supernatant of reference strain was added in equal quantity to the coagulated plasma produced by a known Coagulase producer such as *S. aureus.* Fibrinolysis as indicated by the dissolution of the clot was recorded as positive reaction.

Bacterial strains

Reference Streptococci namely, *S. agalactiae* (AD1) Genbank accession no. HM355961, *S. dysgalactiae* (AD3), HC359248 and *S. uberis* (AD2) HC355971 and (AD6) HC355972 procured from Project Directorate on Animal Disease Monitoring and Surveillance (PD_AADMAS), Bangalore, and *E. coli* Genbank accession no. JF926686, *S. aureus* Genbank accession no. JN247783.1 maintained in the Department of Veterinary Microbiology, Veterinary College, Bangalore were used.

Preparation of bacterial DNA

Bacterial DNA was purified using the "QIAamp DNA Mini and Blood mini kit" as per the manufacturer's instructions.

Designing of virulent gene primers

The Genus specific (*tuf* gene) and species specific (16S rRNA gene) primers for *S. agalactiae*, *S. dysgalactiae* and *S. uberis*, primer targeting surface immunogenic protein (*sip*) and *Plasminogen* activator (*pauA*) gene for *S. agalactiae* and *S. uberis* respectively were designed at the Department of Veterinary Microbiology under NAIP scheme and were used for screening of *Streptococcus* isolates. Further, CAMP factor (*cfb*), surface-expressed mig protein (*mig*) gene based primers were designed using "Lasergene DNA STAR" software for *S. agalactiae* and *S. dysgalactiae*, respectively; streptokinase (*skc*) gene based primers for *S. uberis* designed at Molecular Virology Laboratory, IVRI, Bangalore under NAIP scheme; hyluronidase (*hyl*) gene based published primers (Sukhnanand et al., 2005) for *S. agalactiae* were used for molecular studies and the working concentration of the primers for PCR was 20 pmol/µL. The primer sequences and the lengths of the amplified products are detailed in Table 1.

PCR amplification

The PCR reaction mixture contained 2.5 µL of 10X PCR *Taq* Buffer A, 1 µL (20 pmol) of each *Saga sip* F & R/ *Saga* CAMP F & R/ *Saga*

Table 1. Nucleotide sequences and product length of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* virulence gene specific primers.

Name of the primer	Sequence 5'─ 3'	Product length (bp)	Reference
VM *Saga sip*- F VM *Saga sip*- R	ACTATTGACATCGACAATGGCAGC GTTACTGTCAGTGTTGTCTCAGGA	266	Nithinprabhu et al. (2010)
VM *Saga* CAMP- F VM *Saga* CAMP- R	CAAAGATAATGTTCAGGGAACAGATTATG CTTTTGTTCTAATGCCTTTACATCGTT	320	-
VM *Saga hyl* -F VM *Saga hyl*-R	CATACC TTAACAAAGATATATAACAA AGATTTTTTAGAGAATGAGAAGTTTTTT	950	Sukhnanand et al. (2005)
VM *Sdys mig* F VM *Sdys mig* R	CGTTTTTAGTTTCGGGAGCA TGCCTTCAATTGAGTCTGCTG	188	-
VM *Sub pauA*-F VM *Sub pauA*-R	TGCTACTCAACCATCAAAGGTTGC TAGCAGTCTCAGTAGGATGAGTGA	439	Nithinprabhu et al. (2010)
VM *Sub skc*-F VM *Sub skc*-R	TCCGGATTTTGGGTCCTTAGCCA AGTCGACTTTGCGCCTGATGCAC	475	-

hyl F & R/ *Sdys mig* F &R/ *Sub pau*A F & R and *Sub skc* F & R primers and 1 µL (100 µM) of each dNTPs, 3 µL (150 ng) of streptococcal DNA and filtered quartz water was added to make a final volume of 25 µL. The amplification reactions were carried out in 0.2 ml micro centrifuge tubes using a programmable thermal cycler (Master Cycler pro, M/s Ependorff, Germany). The amplification was programmed for 30 cycles with temperature cycles of denaturation at 94°C for 30 s annealing at 52, 55, 55, 52, 54 and 52°C, respectively for 30 s and extension at 72°C for 30 s. An additional cycle with an extension step of 10 min was included to complete the synthesis of unfinished products. After the completion of the reaction, PCR products were electrophoresed on a 1.8% agarose gel and the images were captured (Gel Doc XR, M/s, BioRad., U.S.A).

RESULTS

A total of 147 bacterial isolates were recovered from 86 milk samples including 14 clinical and 72 subclinical milk samples. Of these, majority of the isolates recovered were *S. aureus* (45), CoNS (23), Streptococci (56) followed by *E. coli* (23). For identifying *Streptococcus* isolates, *tuf* gene based primer was used at genus level with an amplicons size of 110 bp (Figure 3) and 16S rRNA based primer was used at species level with an amplicons size of 329 bp (Figure 4), 549 bp (Figure 5) and 854 bp (Figure 6), to identify *S. agalactiae*, *S. dysgalactiae* and *S. uberis*, respectively. Out of 56 *Streptococcus* isolates, only fifteen isolates (Genbank accession no. JN998527.1), and reference *S. agalactiae* (AD1) yielded 329 bp amplicon of 16S rRNA gene confirming them as *S. agalactiae*, none of the isolates yielded 549 bp amplicon which would confirm them as *S. dysgalactiae* and none of the isolates yielded 854 bp amplicon confirming it to be *S. uberis.* However, the reference culture yielded the desirable amplicons. All the fifteen isolates and the reference strain (AD1) yielded specific amplicon of 266 (Figure 7), 320 (Figure 8) and 950 bp (Figure 9) confirming the presence of *sip*, *cfb* (Genbank accession no. JN657311.1, JN657312.1, JN378717.1) and *hyl* gene (Genbank accession no. JN247792.1, JN247784.1, JN247791.1, JN256018.1 and JN120257.1), respectively. The screening of *S. dysgalactiae* reference strain (AD3) revealed the presence of *mig* gene which yielded specific amplicon of 188 bp (Figure 10). Reference strains of *S. uberis* (AD2 and AD6) were also screened for the virulence gene *pau*A and *skc* by earlier designed primers. The screening of reference strains revealed the presence of *pau*A and *skc* gene in both the reference strain AD2 and AD6 yielded specific amplicons of 439 (Figure 11) and 475 bp (Figure 12), respectively.

Majority of the isolates in the present study could not be speciated based on biochemical tests; they were neither *S. uberis* nor *S. dysgalactiae*. Sequence specific primer for identification of these isolates was designed at the Lead centre, PD_ADMAS Bangalore and used for PCR amplification. The amplified products were sequenced and NCBI BLAST results indicated that these isolates belong to *S. bovis-equinus* complex.

The reference cultures were used as the positive controls whereas; *S. aureus* and *E. coli* were used as negative controls. The PCR amplified products were then sequenced and the primer specificity was confirmed by sequence BLAST analysis. Furthermore, these sequences were aligned by Clustal V method using MegAlign program of the same software with sequences available in NCBI and phylogenetic analysis revealed the genetic

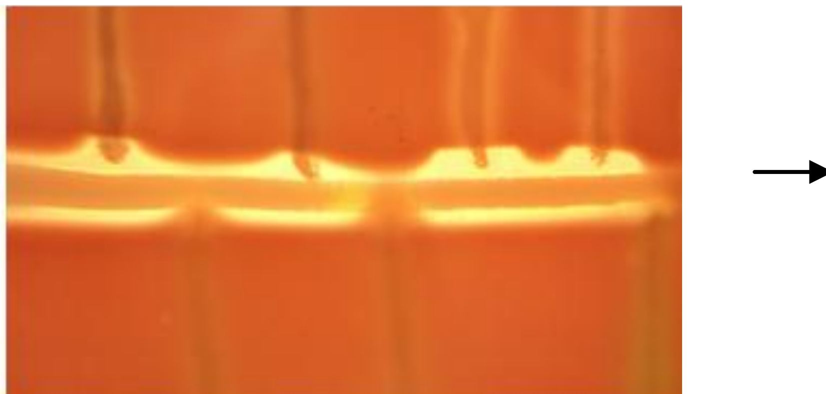

Figure 1. CAMP test showing half-moon shaped zone of complete hemolysis on a blood agar by *S. agalactiae* along with *S. aureus*.

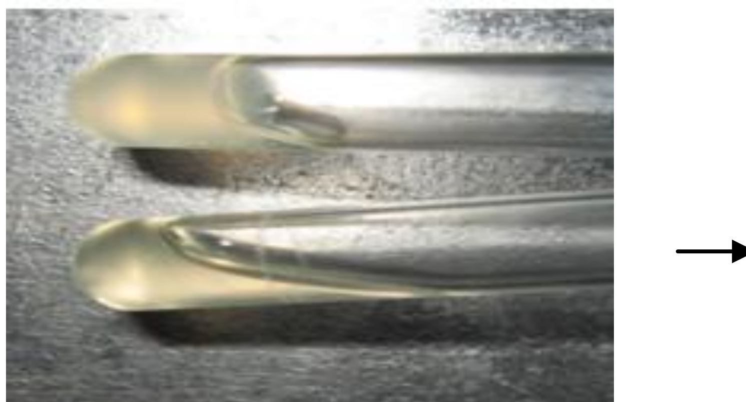

Figure 2. Streptokinase assay showing absence of coagulation or fibrinolysis as indicated by the dissolution of the clot produced by known coagulase producer *S. aureus.*

diversity among the isolates.

Of the fifteen *S. agalactiae* isolates tested, only fourteen isolates were phenotypically positive for CAMP factor as detected by CAMP test (Figure 1); whereas, all the isolates were positive genotypically as detected by PCR by targeting *cfb* (CAMP factor) gene.

DISCUSSION

In India, improvisation in quality and quantity of milk produced is a prerequisite for export of milk and milk products. However, it is threatened by mastitis which continues to be a cause of significant economic loss to the dairy industry not only in India, but also internationally. In the present study, the highest prevalence of SCM at 82% was observed in organized sector, which comprised of crossbred animals. The observations made in this study, despite thorough biochemical characterization of streptococcal isolates, could not lead us to precise identification of these isolates up to the species level due to variability in their biochemical profiles; hence, the findings emphasize the need for development of molecular methods for precise identification of streptococci as this is one of the most useful tools applied to the revision of the bacterial classification system (Facklam, 2002). Rapid nucleic acid amplification and detection technologies are quickly displacing the traditional assays based on pathogen phenotype rather than genotype. The development of the PCR based methods provides a promising tool for the rapid identification of bacteria. The *tuf* gene provided a better discrimination over the 16S rRNA at the streptococcal genus level, which is particularly useful for the identification of very closely related species. Thus, this peculiarity of the streptococcal *tuf* gene was used in the present study. Interestingly, nine isolates were obtained from fifteen clinical cases (60%) which signify their role in clinical mastitis. Streptococcal isolates detected in the present study were either from the clinical or subclinical cases which indicated their potential to cause the

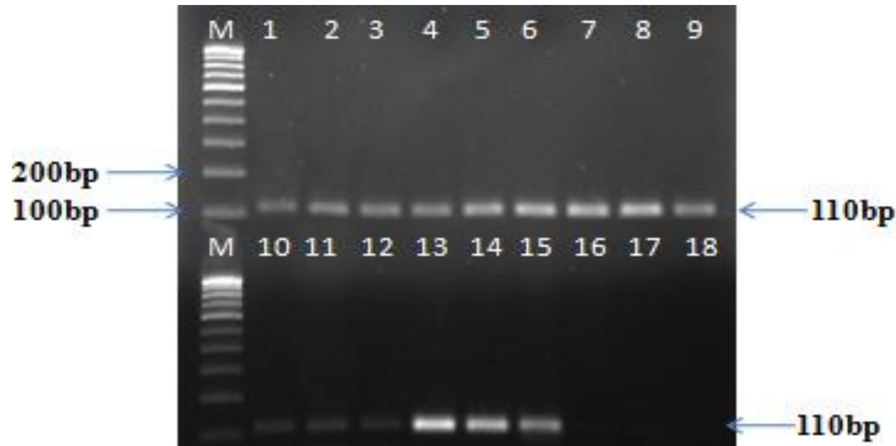

Figure 3. PCR amplification of 110 bp *tuf* gene of *Streptococci* isolated from bovine mastitis cases, Lane M: 100 bp DNA ladder, Lanes 1-12: *Streptococci* isolate No. 41 to 52, Lanes 13-15: Positive control (*S. agalactiae* AD1, *S. dysgalactiae* AD3, *S. uberis* AD6 respectively), Lanes 16-18: Negative controls (*S. aureus*, *E. coli*, NTC respectively).

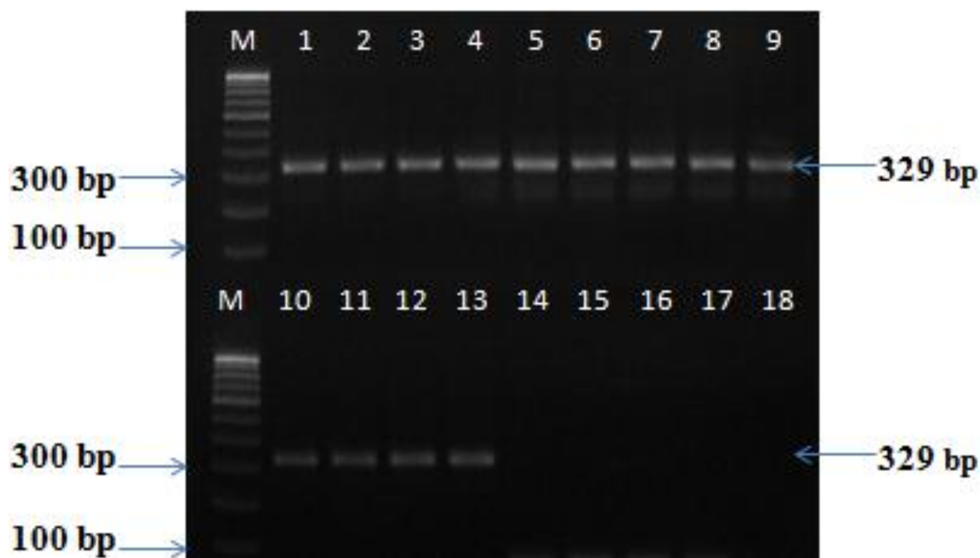

Figure 4. PCR amplification of 329 bp 16S rRNA gene of *S. agalactiae* isolated from bovine mastitis cases, Lane M: 100 bp DNA ladder, Lane 1: Positive control *S. agalactiae* AD1, Lanes 2-13: *S. agalactiae* isolate No. 55, 56, 57, 61, 86-92, 95, Lanes 14-18: Negative controls (*S. dysgalactiae* AD3, *S. uberis* AD6, *S. aureus*, *E. coli*, NTC respectively).

infection. *S. agalactiae* was the major species among streptococci reported even in previous studies (Mallikarjunaswamy and Murthy, 1997; Ross et al., 2001; Balakrishnan et al., 2004).

Sip (surface immunogenic protein) is an antigenic protein localized on the surface of *S. agalactiae* which is capable of raising an antibody response. It is also known that *sip* is highly conserved at the gene level. The *sip* gene based primers amplified all fifteen isolates of *S. agalactiae* and a reference *S. agalactiae* (AD1) precisely without any ambiguity. Cell surface protein like pore forming protein encoded by CAMP factor/*cfb* gene, was found to produce a classical CAMP phenomenon with the typical half moon forming hemolytic zones on cattle or sheep blood agar plates by the influence of β-lysin of *S. aureus* and exosubstances of non-hemolytic streptococci (Christie et al., 1944). CAMP factor genes are described to be fairly widespread among streptococci, at least in serogroups A, B, C, G, M, P, R and U (Gase et al., 1999). The results of the present study are in accordance with

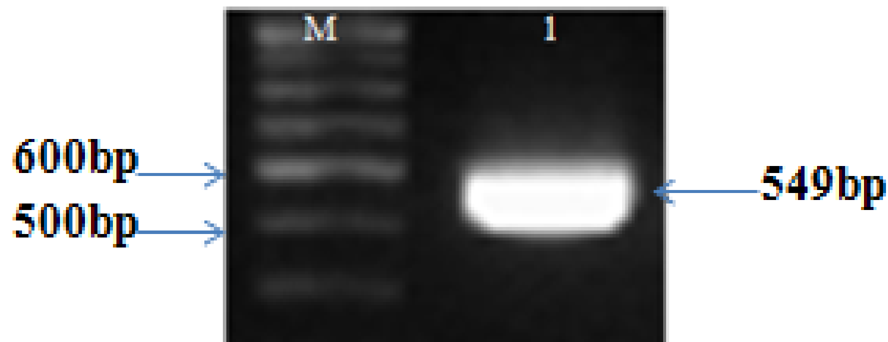

Figure 5. PCR amplification of 549 bp 16S rRNA gene of *S. dysgalactiae*, Lane M: 100 bp DNA ladder, Lane 1: Positive control *S. dysgalactiae* (AD3).

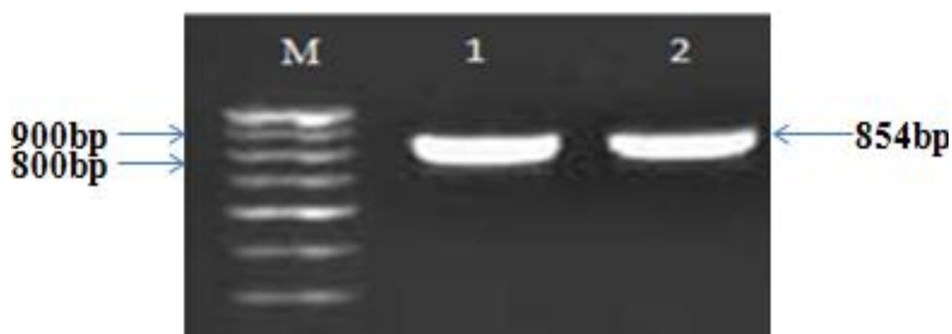

Figure 6. PCR amplification of 854 bp 16S rRNA gene of *S. uberis*, Lane M: 100 bp DNA ladder, Lane 1: Positive control *S. uberis* (AD2), Lane 2: Positive control *S. uberis* (AD6).

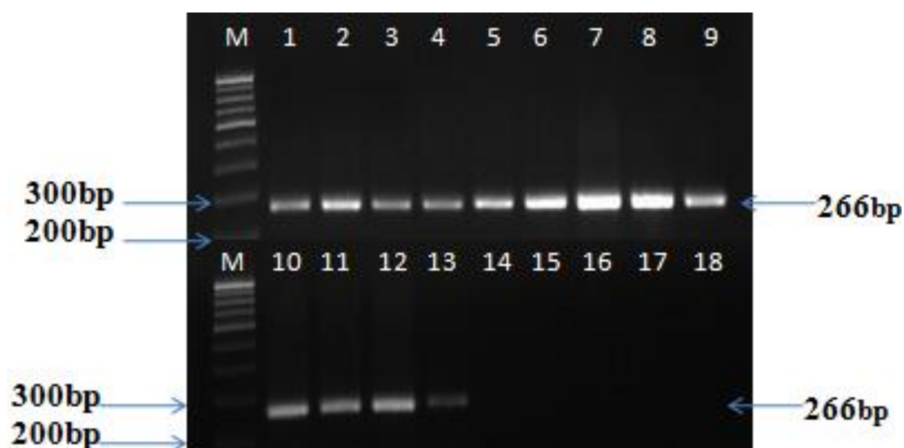

Figure 7. PCR amplification of 266 bp *sip* gene of *S. agalactiae* isolated from bovine mastitis cases, Lane M: 100 bp DNA ladder, Lane 1: Positive control (*S. agalactiae* AD1), Lanes 2-13: *S. agalactiae* isolate No. 55, 56, 57, 61, 86-92, 95, Lanes 14-18: Negative controls (*S. dysgalactiae* AD3, *S. uberis*, *S. aureus*, *E. coli*, NTC respectively).

the earlier reports confirming wide prevalence of CAMP factor possessing *S. agalactiae.* Phenotypic property was not demonstrable in one isolate out of 15, which may be due to lack of expression of the gene. This could be due to the absence of complete open reading frame (ORF). Although, it is reported in earlier studies that the CAMP

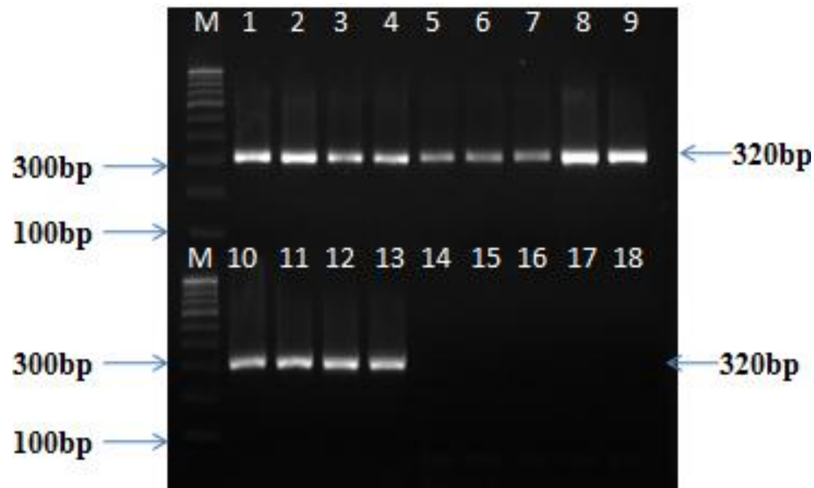

Figure 8. PCR amplification of 320 bp *cfb* gene of *S. agalactiae* isolated from bovine mastitis cases, Lane M: 100 bp DNA ladder, Lane 1: Positive control (*S. agalactiae* AD1), Lanes 2-13: *S. agalactiae* isolates No. 55, 56, 57, 61, 86-92, 95, Lanes 14-18: Negative controls (*S. dysgalactiae* AD3, *S. uberis* AD6, *S. aureus*, *E. coli*, NTC respectively).

Figure 9. PCR amplification of 950 bp *hyl* gene of *S. agalactiae* isolated from bovine mastitis cases, Lane M: 100 bp DNA ladder, Lane 1: Positive control (*S. agalactiae* AD1), Lanes 2-13: *S. agalactiae* isolates No. 55, 56, 57, 61, 86-92, 95, Lanes 14-18: Negative controls (*S. dysgalactiae* AD3, *S. uberis* AD6, *S. aureus*, *E. coli*, NTC respectively).

factor is not essential for systemic virulence of GBS (Hensler et al., 2007), its phenotypic detection for presumptive identification of GBS in clinical laboratory is of immense diagnostic value. *S. agalactiae hyl*B encodes hyaluronate lyase (hyaluronidase), a putative virulence factor facilitate the spreading of bacteria in host tissues (Akhtar and Bhakuni, 2004). The hyaluronidase activity in *S. agalactiae* is associated with host specificity (Lin et al., 1994). The secreted and putative virulence gene (*hyl*B) was used as a target for DNA sequencing-based sub-typing and often provided a higher discriminatory power and might provide insight into the evolution of virulence-related characteristics (Cai et al., 2002). The results of the present study are in accordance with the earlier reports (Cai et al., 2002; Correa et al., 2010).

The study confirmed wide prevalence of three important virulence genes in *S. agalactiae* isolates obtained from both organized as well as unorganized sectors, including both subclinical and clinical cases of mastitis from different geographical locations. The findings of the study emphasize the role of virulent gene possessing *S. agalactiae* in causing clinical as well as subclinical cases of bovine mastitis. In continuation, it is necessary to scan *S. agalactiae* for other virulence genes and their possible

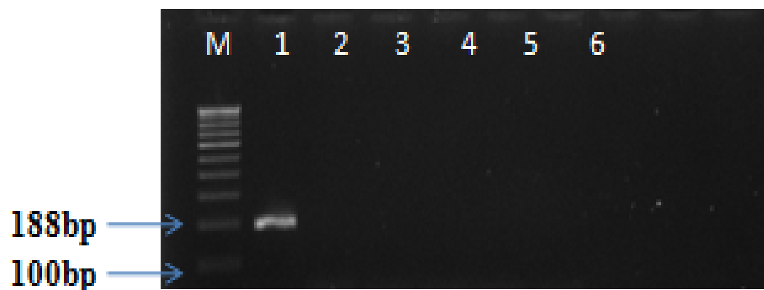

Figure 10. PCR amplification of 188 bp *mig* gene of *S. dysgalactiae*, Lane M: 100 bp DNA ladder, Lane 1: *S. dysgalactiae* (AD3), Lanes 2-6: Negative controls (*S. agalactiae* AD1, *S. uberis* AD6, *S. aureus*, *E. coli*, NTC respectively).

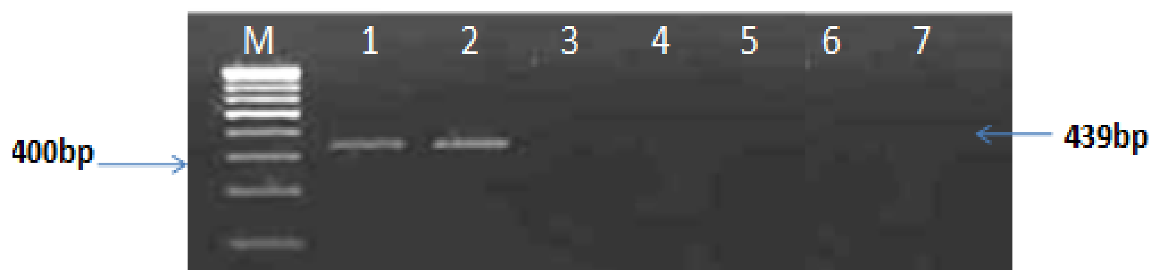

Figure 11. PCR amplification of 439 bp *pau*A gene of *S. uberis*, Lane M: 100 bp DNA ladder, Lanes 1 and 2: *S. uberis* AD2 and AD6, Lanes 3-7: Negative controls (*S. agalactiae* AD1, *S. dysgalactiae* AD3, *S. aureus*, *E. coli*, NTC respectively).

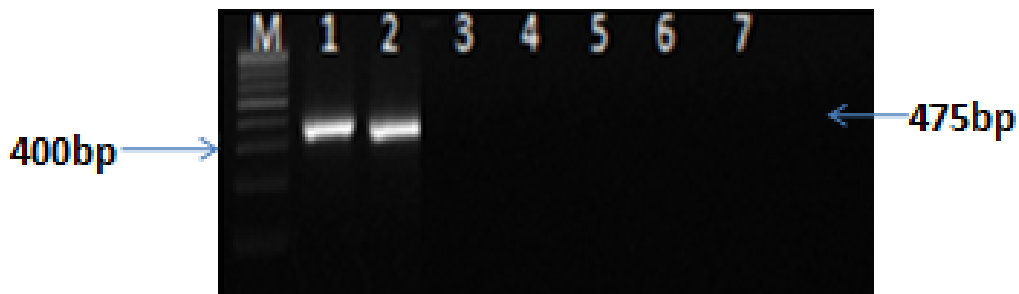

Figure 12. PCR amplification of 475 bp *skc* gene of *S. uberis*, Lane M: 100 bp DNA ladder, Lanes 1 and 2: *S. uberis* AD2 and AD6, Lanes 3-7: Negative controls (*S. agalactiae* AD1, *S. dysgalactiae* AD3, *S. aureus*, *E. coli*, NTC respectively).

role in causing mastitis. The Mig protein is involved in resisting phagocytosis by bovine neutrophils (PMNs) in the presence of bovine serum (Song et al., 2001). Thus, the Mig protein, an M-like protein, is considered as a potential virulence factor of *S. dysgalactiae*. This protein could act as the sensory component of a multiple component system, whereby, binding of IgG and or IgA to Mig could trigger a conformational change on this protein, resulting in the activation of secondary proteins with histidine-kinase activities that result in the modulation of gene expression of factors involved in virulence. The DNA sequence encoding the α_2-M receptor portion of the *mig* gene was different from other *Streptococcus* and which was highly specific to *S. dysgalactiae* (Jonsson et al., 1994). All of them possessed DNA fragments that hybridized to the IgG probe suggesting that the IgG-binding sequence of *mig* is highly conserved in these strains. Surprisingly, none of the streptococcal isolates obtained in our study were identified as *S. dysgalactiae* by PCR in contrast to the biochemical assays. Further, *mig* gene pri-

mer based PCR revalidated the earlier identification process by using 16S rRNA gene based PCR and it is a good tool to ascertain virulence properties of *S. dysgalactiae* with reference to Mig protein.

Streptokinase, a bacterial plasminogen activator is produced by a variety of pathogenic *Streptococcus* species and is needed for degradation of extracellular matrix proteins and subsequent colonization. Notably, streptokinases isolated from different strains of streptococci possess an intrinsic species specificity for their target plasminogen molecules that parallels the host range of the microorganisms (Mccoy et al., 1991). It has also been reported that the amino acid sequence of streptokinase gene (*skc*) of *S. uberis* was highly conserved within the species (Johnsen et al., 1999). However, *skc* gene based PCR standardized in this study is a useful assay for identification of virulent *S. uberis.* An effort was made to standardize the procedure for streptokinase assay using two reference strains of *S. uberis* (AD2 & AD6). This procedure could be used even for the clinical isolates to study their potential to produce the enzyme streptokinase. The ability of the streptokinase enzyme to cause fibrinolysis or prevent the formation of coagulation in rabbit plasma produced by a known coagulase producer such as *S. aureus* was tested. The reference strain of *S. uberis* used for the streptokinase assay was able to cause fibrinolysis as well as prevent the formation of coagulation. Streptokinase which activates bovine plasminogen might be an essential virulence factor of *S. uberis*, allowing its rapid growth in the bovine mammary gland (Leigh and Field, 1993). Hence, the phenotypic detection of streptokinase enzyme produced by streptococci could serve as an indicator of pathogenicity of the isolates under study (Figure 2).

The *pau*A is a putative virulence factor of *S. uberis* and encodes the plasminogen activator which converts plasminogen in blood plasma and tissues in cattle to plasmin (Leigh, 1999, 2000). However, *pau*A gene specific PCR provides useful supplementary data to differentiate *S. uberis* from closely related species. It was also shown that *pau*A gene based PCR (Zadoks et al., 2005) could be used for rapid species identification, since *pau*A is *S. uberis* species-specific and absent in other *Streptococcus* species or other bacteria commonly associated with bovine mastitis (Ward and Leigh, 2004). However, none of the streptococcal isolates obtained in our study were identified as *S. uberis* by PCR in contrast to the biochemical assays. To summarize, phylogenetic and sequence pair distance analysis revealed high genetic variation among the streptococci isolates with respect to the virulence genes as observed in the present study. This provides a virulence gene based tool to study the molecular epidemiology of streptococcal mastitis in bovines which would in turn help us to understand mechanisms behind emergence of new strains or shifts in mastitis epidemiology in response to control measures, including antibiotic treatment and vaccination.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Effect of carbon and nitrogen sources on exopolysacharide production by rhizobial isolates from root nodules of *Vigna trilobata*

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Twenty five (25) rhizobial strains were isolated from root nodules of *Vigna trilobata* cultivars grew in soils collected from all districts of Andhra Pradesh, India. Five out of 25 rhizobial strains which produced copious amount of Exopolysaccharides (EPS) on Yeast Extract Mannitol Agar (YMA) medium with congo red were identified by sequencing of their 16S rDNAs. The amount of EPS produced by these five strains increased during the first 72 h of incubation but it declined afterwards. The amount of EPS produced correlated positively with increase in mannitol concentration from 1 to 3% (m/v). However, there was a decrease in EPS production when mannitol concentration was equal or higher than 4%. All the five strains studied preferred mannitol and sodium nitrate as the best carbon and nitrogen sources for EPS production. *Sinorhizobium kostiense* MRR104 produced maximum levels of EPS 892 mg/100 ml when mannitol was used as carbon source, while *S. xinjiangense* MRR110 produced maximum levels of EPS 377 mg/100 ml when sodium nitrate was used as nitrogen source. Variation among the rhizobial strains in utilization of carbon and nitrogen sources for EPS production was clearly evident from this study. All the strains analysed in the present study can be exploited for production of commercial biopolymer (gum), due to their non pathogenic nature and their ability to produce copious amounts of EPS when compared to strains studied in earlier reports.

Key words: Rhizobial strains, *V. trilobata,* Exopolysaccharides (EPS), carbon and nitrogen sources.

INTRODUCTION

Rhizobium spp. are known to synthesize a variety of cell surface polysaccharides, including exopolysaccharides (EPS), lipopolysaccharides (LPS), and cyclic glucans (CG). In addition, *Sinorhizobium* spp. produces K-antigen polysaccharides (KPS). All these four polysaccharides are important for bacterial performance in both free life and symbiosis (Margaret et al., 2011). Rhizobial exopolysaccharides (EPS) play an important role in plant root invasion during nodule formation and promotes growth of the plants by chelating various metal ions. EPS helps in creation of near anaerobic conditions in the microenvironment surrounding the rhizobial cell surface,

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before infecting the root hair, to protect the nitrogenase enzyme (Gupta et al., 1982) and enhances nodulation (Olivares et al., 1984). EPS protects the producing organism against desiccation (Sayyed et al., 2011) toxic compounds, and osmotic stress, helps in the formation of biofilms (Kucuk and Kivanc, 2009) and serves as energy source to be catabolised during nutrient deficiency. Apart from its role in symbiosis and plant growth, the EPS synthesized in culture have importance in many industries including food, oil and pharmacy. Microbial EPS have been commercialized as possible future industrial commodities for food as thickening agents and in agriculture for the encapsulation of somatic embryos, which offer a greater feasibility for precise delivery of plant growth regulators, fungicides and pesticides (Mathur and Mathur, 2001). EPS has been proved to be a potential biopolymer used as emulsifier in the degradation of hydrocarbons, stabilizers, binders, coagulants and separating agents in a variety of industries (Staudt et al., 2011). Bacterial EPS exhibit antioxidant activity (Mahendran et al., 2013). Recently rhizobial EPS were patented as best bioproduct for skin treatment (US Patent 20,130, 302, 261- 2013). EPS produced by bacteria are commercially exploited so far, however, much of the present day research is concentrating on the industrial application of rhizobial EPS. Rhizobial biopolymers are preferred in the food industry by virtue of their non pathogenic nature and copious production (Bomfeti et al., 2011). Rhizobial EPS are species or strain specific hetero polysaccharides consisting of repeating units of sugars (Mandal et al., 2007) the majority of which are hexoses and uronic acids as well as non carbohydrate substituents such as acetate, pyruvate, hydroxyl butyrate and succinate (Aman et al., 1981; Cunningham and Munns, 1984). However, the most common types of EPS produced by Rhizobia are EPS1 and EPS2. EPS1 type are high molecular weight succinoglycans and EPS II are low molecular weight galactoglucans (Oliveira et al., 2012). Several factors influence the production of EPS by rhizobial strains, such as carbon and nitrogen sources as well as incubation period (Duta et al., 2004, 2006).

Vigna trilobata commonly called Pillipesara, was mainly cultivated as short term pasture and green manure crop in India, Pakistan, Indonesia and Sudan. Though nodulation in *Vigna trilobata* was first reported in Japan by Asia and in India by Raju in 1936 (Allen and Allen, 1981) the comprehensive studies on rhizobial symbiont characterization and other related studies are meagre. The present investigation was aimed to study the diversity of rhizobial strains producing exopolysaccharide. Although much work was published on EPS production, characterization and factors effecting EPS production by rhizobial strains from different host legumes, our study is the first of its kind on rhizobial strains isolated from nodules of *V. trilobata.* In this paper, for the first time, rhizobial species nodulating V. *trilobata* have been identified by 16S rDNA sequencing.

MATERIALS AND METHODS

Isolation of rhizobia

Soil samples were collected from agricultural fields under the cultivation of *V. trilobata* from all the 25 districts of Andhra Pradesh. Certified seeds of *V. trilobata* were purchased from the National Seed Corporation (NSC) Guntur. Plants were grown in earthen pots filled with these district soils and were maintained properly in the Botanical garden of Acharya Nagarjuna University. After 90 days of germination, healthy root nodules from gently uprooted plants, surface sterilized with 0.1% mercuric chloride and 70% alcohol and washed thoroughly by sterile distilled water were used for isolation (Vincent, 1970). Rhizobial strains were isolated from root nodules of *V. trilobata* plants, using selective medium Yeast Extract Mannitol Agar (YMA) with congo red and pure cultures were maintained after sub culturing on the same medium. Pure cultures of all the 25 isolates were authenticated as rhizobia by performing the appropriate biochemical tests (Somasegaran and Hoben, 1994) and nodulation ability on homologous hosts by plant infection tests (Vincent, 1970). Out of the 25, the five strains which produced higher amounts of EPS were further identified up to the species level through 16S rDNA sequencing (Macrogen, South Korea) and the sequences were deposited in the gene bank. The strain names with allotted accession numbers, used in this study are *Rhizobium sp.*MRR103-JX576499 (isolated from Guntur district soil); *Sinorhizobium kostiense* MRR104 - KC428653 (isolated from Chittor district soil); *Sinorhizobium xinjiangense*MRR110 -KC415691 (isola-ted from Kadapa district soil); *Rhizobium sp.*MRR 123 - KC503884 (isolated from Nellore district soil); *Ensifer sp.*MRR125 - KC503885 (isolated from Mahaboobnagar district soil).

Exopolysaccharide (EPS) production

For production of EPS, all the five strains were inoculated into Erlenmeyer flasks (250 ml) containing 100 ml of YMB supplemented with 1% Mannitol (m/v). The flasks were incubated at room temperature on an orbital shaker at 200 rpm for 72 h. After incubation, the broth was centrifuged at 3000 x *g* and the culture supernatant was mixed with 2 volumes of chilled acetone. The crude polysaccharide precipitated was collected by centrifugation at 3000 x *g* for 30 min. The EPS was washed with distilled water and acetone alternately, transferred into a filter paper and weighed after overnight drying at 105°C (Damery and Alexander, 1969). The strain that produced maximum amount of EPS, *S. kostiense* MRR104 was used in the studies on optimization of the conditions for maximum EPS production, like the incubation period and the concentration of mannitol (carbon source in the YMA medium).

Effect of mannitol concentration

To optimize the concentration of Mannitol for maximum production of EPS production, the test cultures were inoculated with five different concentrations of Mannitol (1, 2, 3, 4 and 5 %) including the concentration prescribed in the original YMB medium (1%). All the inoculated flasks were incubated for 72 h at room temperature on an orbital shaker at 200 rpm and the amount of EPS produced was estimated by the method described by Damery and Alexander (1969).

Effect of incubation period

For the study of EPS production at different incubation periods, the test culture was inoculated into YMB medium (1% mannitol) and incubated at different periods from 12 to 84 h with 12 h intervals. The

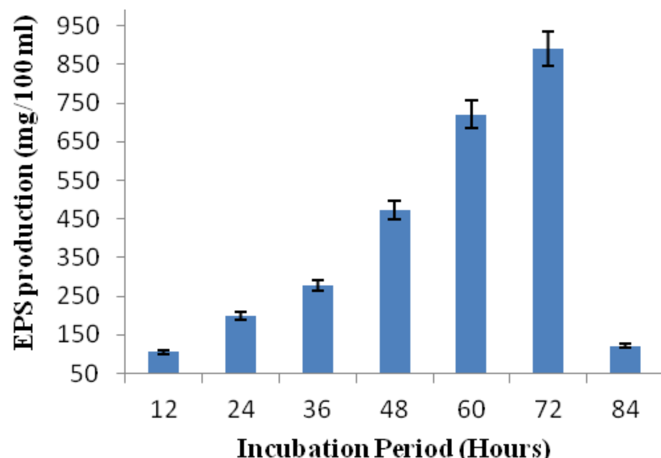

Figure 1. Effect of incubation period on EPS (mg/100 ml) **production by** *Sinorhizobium kostiense* MRR104.

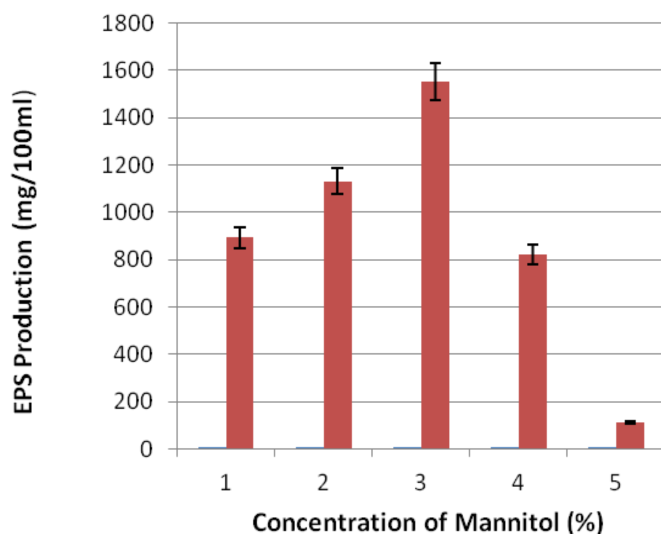

Figure 2. Effect of Mannitol concentration on EPS (mg/100 ml) production by *Sinorhizobium kostiense* MRR104 after 72 hof incubation.

The inoculated flasks were incubated at room temperature on an orbital shaker at 200 rpm and the amount of EPS produced was estimated by the method described by Damery and Alexander (1969).

Effect of Carbon and Nitrogen sources on EPS production

To study the effect of carbon source on production of EPS by all the five strains of rhizobacteria, ten carbon sources including six monosaccharides – arabinose, galactose, glucose, fructose, raffanose and xylose; three disaccharides – sucrose, maltose, lactose and one sugar alcohol – mannitol, were used in by replacing, in same concentration (1%), the mannitol in the original YMA medium. Control was maintained without any carbon source. All the five strains viz., MRR 103, MRR 104, MRR 110, MRR 123, MRR 125 which produced maximum EPS were used in this study. Rhizobial cultures were inoculated separately into 100 ml of YMB medium containing different carbon sources and the flasks (250 ml) were incubated on a orbital shaker at 200 rpm for 72 h. After incubation, the amount of EPS produced was estimated by the method described by Damery and Alexander (1969).

To study the effect of nitrogen source on the production of EPS, five different nitrogen sources - ammonium sulphate, glycine, sodium nitrate, potassium nitrate, and L-asparagine were selected. All the nitrogen sources were added to the medium by replacing the 0.1% yeast extract of the original YMA medium composition. Rhizobial strains were inoculated separately into the flasks (250ml) containing 100ml of YMB supplemented with different nitrogen sources. All the inoculated flasks were incubated for 72 h on an orbital shaker at 200 rpm. After incubation the amount of EPS produced was estimated by the method described by Damery and Alexander (1969).

Three replicates were used for each treatment. Statistical analyses of the data were performed using SPSS software (version 20). Correlation coefficient and ANOVA were calculated for the data wherever necessary. Duncan's test was used for multiple range analyses to determine the significant difference between groups of data. The results were considered to be significant at $P<0.05$.

RESULTS AND DISCUSSION

In the present study, all the 25 rhizobial strains isolated from *Vigna triolobata* produced EPS by utilizing the available carbon source in the YMB medium. Five out of 25 strains, producing copious amounts of EPS, were selected for further analyses. Fernandes et al. (2011) reported that six out of 38 isolates from *Cajanus cajan* produced more than 200 mg EPS/L. However, our strains produced much higher amounts of EPS (>600 mg/100ml) than those isolated from *C. cajan.* In *S. kostiense* MRR104, EPS production was minimum at 12 h of incubation and the maximum was recorded at 72 h of incubation (Figure 1) and decreased at 84 h. Hence, a period of 72 h of incubation was considered as optimum for maximum EPS production by the isolates of *V. trilobata*. In our experiments, it was statistically proved that incubation period positively correlated with growth (r=0.59) and EPS production (r= 0.47) during the first 72 h. This is not the case for other rhizobial strains studied previously. Kucuk and Kivanc (2009) reported a maximum EPS production after an incubation period of 8 days for *Rhizobium ciceri*, while 6 days was reported by Sayyed et al. (2011) for *Rhizobium* sp. Mannitol as good carbon source supported the rhizobial strains in growth and production of EPS. In the present study, the EPS production increased gradually when mannitol concentration was increased up to 3% and showed a decline from 4% onwards (Figure 2). *S. kostiense* MRR104 produced maximum amount of EPS 1550 mg/100 ml at 3% of mannitol and a minimum (112 mg/100 ml) at 5% mannitol. This decrease in EPS may be due to the mobilization of EPS by the organism itself probably under the influence of EPS hydrolase (Basu and Ghosh, 1999). The influence of mannitol concentration and period of incubation varies among different rhizobial strains. Thus, Mukherjee

Table 1. EPS (mg/100ml) produced by rhizobial strains in YMB supplemented with different carbon sources.

Carbon sources (1.0%)	*Rhizobium* sp. MRR 103	*Sinorhizobium kostiense* MRR 104	*Sinorhizobium xinjiangense* MRR 110	*Rhizobium* sp. MRR 123	*Ensifer* sp. MRR 125
Control	5	14	21	10	21
Mannitol	723	892	692	816	719
Arabinose	364	243	266	305	166
Galactose	307	249	266	171	196
Sucrose	228	285	184	360	122
Maltose	286	397	208	377	378
Lactose	239	319	218	266	226
Glucose	454	650	262	720	412
Fructose	302	311	281	228	293
Raffinose	388	237	270	242	116
Xylose	114	137	149	106	185

et al., (2011) reported that EPS production by *Rhizobium* sp. isolated from *Crotalaria saltiana* was maximum at 2% (m/v) of mannitol, whereas Datta and Basu (1999a) described that EPS production by *Rhizobium* sp. isolated from *C. cajan* reached its maximum at 4% (m/v) of mannitol with an incubation period of only 65 h.

All the five rhizobial strains utilized the 10 different carbon sources and produced significantly high amount of EPS than when a control medium without carbon source was employed (Table 1). Though all the carbon sources supported EPS production, maximum production was observed when sugar alcohol - mannitol was used as carbon source indicating that mannitol supported maximum EPS production by all the strains of *V. trilobata.* This is in agreement with previous reports by Ghosh et al. (2005a) in *Rhizobium* sp. from *Dalbergia lanceolaria,* Kucuk and Kivanc (2009) in *R. ciceri*Rc5 and Mandal et al. (2007) in *Rhizobium* sp. from *Vigna mungo*. However, it has been also described that other rhizobial species show maximum EPS production using different carbon sources. Glucose was preferred as carbon source for maximum EPS production by rhizobial sp. from *Crotalaria saltiana* (Mukherjee et al., 2011) and rhizobial sp. from *Melilotus alba* (Datta and Basu,1999b) while xylose was preferred by isolates from *C. cajan* (Fernandes et al., 2011) and galactose by *Rhizobium* sp. SS5 from *Sesbania sesban* (Sridevi and Mallaiah, 2007). Ghosh et al. (2011) reported that sucrose (1.5%) induced the maxi-mum EPS production though maximum growth was observed with mannitol by *Rhizobium* sp. from *Phaseolus mungo.* Among the monosaccharides tested, after manni-tol, glucose was preferred by most of the strains in the present study while Nirmala et al. (2011) reported that sucrose was preferred next to mannitol by *Rhizobium* sp. from *V. mungo*. Among the disaccharides used, maltose was preferred next to glucose by MRR 104, MRR 123 and MRR 125 strains. From the present study, it is evident that rhizobial strains isolated from *V. trilobata* preferred sugar alcohol, mannitol for maximum EPS production and gave second preference to monosac-charide and less to the disaccharide carbon sources. Among the five strains studied, *S. kostiense* MRR104 produced maximum EPS of 892 mg/100 ml followed by *Rhizobium* sp. MRR 123 with 816 mg/100 ml.

Variation in EPS production by *Aschenomenon aspera* isolates when different carbon sources used was previously reported by Ghosh et al. (2005b). Similarly, significant variations in the amount of EPS produced among the different carbon sources, among the strains and also within the strains were recorded in the present study with the strains of *V. trilobata*.

Statistically there were significant differences between the carbon sources used and rhizobial strains in the production of EPS (p = <0.05). The Duncan test reveals that the highest EPS production occurred for *S. kostiense* MRR104 when mannitol was used as carbon source.

Effect of different nitrogen sources was studied by replacing 0.1% Yeast extract of the original YMA medium with five different nitrogen sources. All the strains studied efficiently utilized those different nitrogen sources and produced high amount of EPS over the control (Table 2). This clearly shows the significant role played by the nitrogen source on EPS production. Sodium nitrate was preferred for maximum production of EPS followed by potassium nitrate by all the strains studied. Similar results were reported previously by Kucuk and Kivanc (2009) in *R. ciceri,* Sridevi and Mallaiah, (2007) in *Rhizobium* sp. SS5 from *Sesbania sesban*. In contrast, potassium nitrate was preferred by *Rhizobium* sp. from *V. mungo* (Nirmala et al., 2011), rhizobial sp. from *Melilotus alba* (Datta and Basu, 1999b) and *Rhizobium* sp. from *Dalbergia lanceolaria* (Ghosh et al., 2005a).

The maximum EPS production of 377 mg/100 ml was recorded with strain *S. xinjiangense* MRR110 followed by 325 mg/100 ml by *Ensifer* sp. MRR125. Significant differences (P = <0.05) between the nitrogen sources on EPS production were recorded in the present study. The Duncan's test reveals that strain *S. xinjiangense* MRR110

Table 2. EPS (mg/100 ml) produced by rhizobial strains in YMB supplemented with different nitrogen sources.

Nitrogen sources (0.1%)	*Rhizobium sp.* MRR103	*Sinorhizobium kostiense* MRR 104	*Sinorhizobium xinjiangense* MRR110	*Rhizobium sp* MRR123	*Ensifer sp.* MRR125
Control	0.005	0.041	0.008	0.006	0.022
Ammonium Sulphate	133	207	165	141	130
Glycine	72	120	92	118	80
Sodium Nitrate	197	284	377	272	325
Potassium Nitrate	167	214	202	203	102
L-Asparagine	80	121	176	111	111

produced maximun EPS when sodium nitrate was used as nitrogen source. Glycine and L- asparagine were less preferred by the strains in the present study. In contrast, *Rhizobium* sp. from *Phaseolus mungo* preferred glycine (Ghosh et al., 2011) and *Rhizobium* sp. from *Vigna mungo* preferred L-asparagine (Mandal et al., 2007) for maximum production of EPS. In the present study, *S. xinjiangense* MRR110 proved to be a more efficient EPS producer than those described in earlier reports of *Sinorhizobium* TR1 from *Trigonella foenum-graecum* (Tank and Saraf, 2003) which produced only 20 μgml^{-1}. Thus, the fact that rhizobial strains exhibit high variations in utilization of carbon and nitrogen sources for the production of EPS was proved among the strains isolated from *V. trilobata* cultivars by the present study. This variation can be attributed to the strain adaptability to the different environmental conditions that prevailed in the soil at geographically different areas from which they were isolated. From the present investigation, it is evident that the rhizobial strains from *V. trilobata* with high EPS production definitely could be added to the list of very few rhizobial strains –*Mesorhizobium pluriforuium* BR3804 with 1.44 g/l and *Rhizobium tropici* CIAT899 with 3.0 g/l EPS production, which were identified as most suitable for commercial production of gum (Bomfeti et al., 2011).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Short Communication

Modified and simple method for isolation of genomic DNA from fungal culture

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A modified and easy method for isolation of intact, good quality genomic DNA from fungal culture is reported. The method was originally designed for the isolation of DNA from plant sample by Kim et al. (1997) which was tried in the present case for the fungal mycelium with slight modification and found worth applying. The main objective of the present work was to provide a simple method of genomic DNA isolation from fungal culture without any contamination.

Key words: DNA isolation, fungus, methodology.

INTRODUCTION

Fungi are large group of eukaryotic microorganisms such as yeasts and molds. They encompass enormous diversity of taxa with varied life cycles, ecology and morphology. Very little information is available on their true biodiversity, that is, 5% of them are formally classified on the basis of morphology and physiology of estimated 1.5 to 5 million species. Advances in molecular genetics have opened the way for DNA analysis to be incorporated into taxonomy. Polymerase chain reaction (PCR) is the common technology employed to characterize the microbial communities (Madigan et al., 2000). The foremost priority for molecular study is an availability of efficient method for the extraction of good quantity and purity of DNA. Different methods are available for the isolation of genomic DNA from fungi but they are time consuming, their quality and quantity is not satisfactory. The major constraint while extracting DNA from fungal culture is breaking the rigid chitin cell walls, as it is resistant to classical DNA isolation method (Fredricks et al., 2005). In addition, the nucleases and high polysaccharide contents create problems for the isolation (Zhang et al., 1996; Muller et al., 1998).

Now a days, researchers are using kits for the extraction of genomic DNA as traditional methods available are inefficient in terms of yield and they are time consuming but the kits are costly and it restrict its use to smaller number of samples per day (Fernandez et al., 2008; Gursel et al., 2009; Dieguez et al., 2009)

The aim of the present study was to extract good quality and quantity of the DNA from any fungal culture. The present method is simple and efficient to extract good quality and quantity of DNA from fungal culture which has been modified from the plant genomic DNA extraction method for the application of molecular biology.

MATERIALS AND METHODS

A pure culture of *Fusarium oxysporum* f. sp. *lycopersici* was obtained from Indian Type Culture Collection (ITCC), New Delhi (ITCC, F-1322) and the isolate RBS-1 was collected from the

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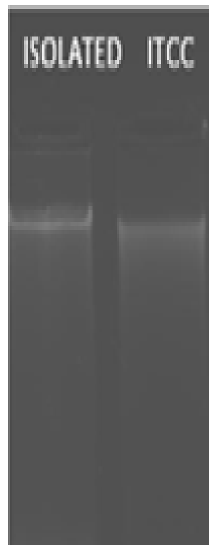

Figure 1. Genomic DNA extracted from two fungal strains (F-1322, ITCC and RBS-1, isolated).

Fusarium wilt infected field, Anand, Gujarat, India. The isolates were multiplied at 28°C on potato sucrose agar (PSA) at pH 6.0-6.5 (200 g/l potato, 20 g/l sucrose and 20 g/l Agar). After inoculation the cultures were incubated at 28°C for 3 to 4 days until a uniform fluffy mycelial growth was obtained. The mycelial met was collected with the help of filtration, grinded to fine powder in mortar and pestle with liquid nitrogen and stored at 0°C until its downstream processing.

1000 mg of mycelia powder were weighed and thoroughly homogenized with 5 ml of extraction buffer (250 mM NaCl, 25 mM EDTA (pH 8.0), 0.5% SDS, 200 mM Tris HCl) and 50 µl of β-mercapto-ethanol in mortar and pestle. The homogenate was transferred to the tube and incubated at 65°C for 1 h. The volume of the homogenate was measured and polyvinylpyrollidone was added (6% of final volume). To this half, the volume of 7.5 M ammonium acetate was added and incubated in ice for 30 min. The mixture was centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was collected after centrifugation and equal volume of absolute ethanol was added with incubation at -20°C for 30 min subsequently. The homogenate was centrifuged at 10,000 rpm for 10 mins at 4°C. The supernatant was discarded this time and the pellet was allowed to air dry. The pellet was resuspended in TE buffer (10 mM Tris HCl: 1mM EDTA, pH-8), to this 20 µL/ml of RNase was applied and kept at 37°C for 30 min for the removal of RNA contamination. After incubation, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) mixture was added and the homogenate was centrifuged at 10,000 rpm for 10 min. The supernatant was collected, to this equal volume of chloroform: isoamyl alcohol (24:1) was added and the mixture was centrifuged at 10,000 rpm for 10 min at 4°C to remove the traces of phenol. Aqueous phase was obtained, mixed with equal volume of chilled ethanol and incubated at -20°C for 60 min. Again it was centrifuged at 10,000 rpm for 10 min at 4°C. The pellet obtained was washed twice with 70% ethanol and it was finally air dried and redissolved in TE buffer. The concentration and purity of DNA was determined by measuring the A260/280 ratio with UV spectrophotometer. (A260/280 of DNA = 1.8 indicates high purity of DNA). The DNA samples were also separated in 0.8% agarose and visualized after staining with ethidium bromide to ascertain their integrity.

Isolated DNA was then subjected to PCR for the amplification reaction in turn its race identification using primer set SP 13(FP 5’GTCAGTCCATTGGCTCTCTC3’, RP 5’TCCTTGACACCATCACAGAG3’) and SP 23(FP 5’CCTCTTGTCTTTGTCTCACGA3’, RP 5’GCAACAGGTCGTGGGGAAAA3’) based on reported sequences (Di Pietro and Roncero, 1998; Posda et al., 2000). The PCR reaction mixtures (12.5 µl) contained 2 µl of fungal genomic DNA (ca. 50 ng), 1X PCR buffer, 2.5 mM each dNTP, 1 U Taq DNA polymerase and 10 pmoles of each primer. Cycle composed of initial denaturation at 94°C for 2 min followed by 29 cycles of denaturation at 94°C for 1 min, annealing for 1 min at an appropriate temperature and elongation at 72°C for 1 min. Final extension was carried out at 72°C for 2 min. Ten microlitres of PCR reaction product was electrophoresed in a 1.5% Agarose gel, which was then stained with Ethidium bromide for identification of the amplicon.

RESULTS AND DISCUSSION

A good quality DNA from *F. oxysporum* f. sp. *lycopersici* (F-1322 and the isolate (RBS-1) was extracted following Kim et al. (1997) method with slight modification of incubation of homogenate after extracting it in mortar and pestle at 60°C instead of room temperature and use of phenol reagent with chloroform : isoamyl (25:24:1) mixture. Figure 1 shows the intact and pure (without the RNA contamination) DNA. The method is basically designed to isolate the genomic DNA from plant samples but the author tried it for the fungal culture and it showed a good result.

Amer et al. (2011) proposed non liquid nitrogen based method for the extraction of DNA from filamentous fungi. The mycelia met was homogenized in extraction buffer after its collection from Petri plate, the present method also checked for the simplicity of non-liquid nitrogen based but it was observed that with use of liquid nitrogen, the yield of DNA obtained will be higher than the other; this is due to better homogenization of mycelial powder crushed first in liquid nitrogen.

Mahuku et al. (2004) developed a rapid method for the extraction of DNA from plant pathogenic fungi devoid of application of hazardous chemicals phenol and chloroform but the present method employ it because without its use, the protein content will not be removed and it will create a problem with amplification using PCR techniques. The methods must be optimized but not at the cost of quality and quantity of the DNA.

Isolated genomic DNA was further checked for the amplification reaction by deducing the race of the fungal culture employing PCR method. Two primers pairs labeled SP13 and SP23 specific to race classification of *F. oxysporum* f. sp. *lycopersici* were used to detect the race of the fungal culture used in the study. Figure 2 shows amplification pattern obtained from both strains. A 445 bp band could be obtained with SP13 where as SP 23 resulted in no amplicon indicating that the fungus belongs to race 1; in turn it proves that the DNA is up to the mark for the amplification reaction.

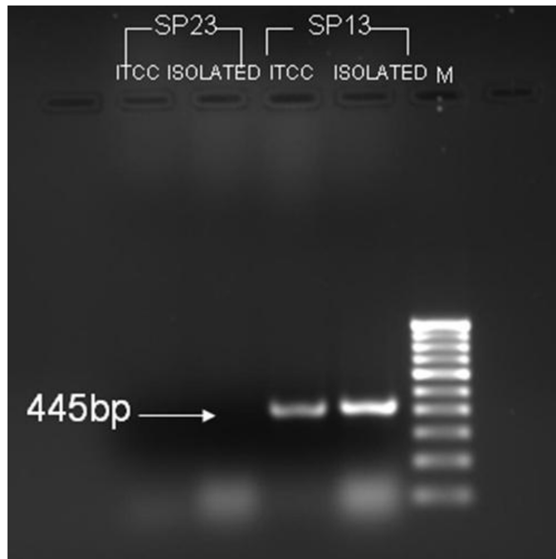

Figure 2. Identification of *F. oxysporum*f. sp. *lycopersici* (FOL) race by polymerase chain reaction (PCR) with primer sets SP13 and SP23. ITCC: Indian Type Culture Collection (New delhi) strain F-1322, M: 100 bp marker.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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