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Full Length Research Paper

Detection of biofilm formation of a collection of fifty strains of *Staphylococcus aureus* isolated in Algeria at the University Hospital of Tlemcen

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The burden of disease caused by *Staphylococcus aureus* continues to grow; this organism has the ability to form biofilm and it is also a frequent cause of medical device and implant-related infections. The objective of this study was to evaluate the biofilm-forming ability of a collection of clinical isolates of *S. aureus*. In a total of 240 *Staphylococcus* spp. isolated from catheters, retrieved at five services (neonatology, internal medicine, pneumology, pediatric and neurology), only 50 (20.83%) strains were identified by conventional microbiological methods as *S. aureus* species; these strains were screened by microtiter plate assay for detection of biofilm formation. Of the 50 clinical isolates, 16 (32%) were non adherent, 20(40%) weakly, 10 (20%) moderately and 4(8%) strongly adherent. The quantitative method of microtiter plate can be involved as a simple, rapid, inexpensive and reproducible assay to assess biofilm formation which is further an important feature of pathogenecity of *S. aureus* in the clinical setting.

Key words: Microbial biofilm, *Staphylococcus aureus*, catheter, microtiter plate assay.

INTRODUCTION

Staphylococci are most often associated with chronic infections of implanted medical devices (Dunne, 2002; Raad, 2000). Such infections are predominately caused by *Staphylococcus aureus* and *Staphylococcus epidermidis*. The first one is known as an ubiquitous bacteria. It also has an inherent ability to form biofilms on biotic and abiotic surfaces (McCann et al., 2008; Begun et al., 2007). The biofilms protect the cells not only from host immune response but also from antimicrobial agents (Donlan et al., 2002). Indeed, biofilm formation is a major concern in nosocomial infections because it protects microorganisms from opsonophagocytosis and antibiotics, leading to chronic infection and sepsis (Martí et al., 2010). These qualities have converged to make *S. aureus* a significant burden on our current health care system (Hobby et al., 2012). One of the patient populations most vulnerable to *Staphylococcus aureus* infection are those with implanted medical devices such as central venous catheters, cardiac valves and pace-makers, artificial joints and various orthopedic devices (Hobby et al., 2012). Therefore, once biofilm-associated *S. aureus* infections occur, they are difficult to be treated by conventional procedures (Trampuz and Widmer, 2006).

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In fact, the biofilm formation involves the production of a polysaccharide intracellular adhesion (PIA) (Ziebuhr et al., 2001; Mack et al., 1996) which is the formal name of slime. This polysaccharide depends on the expression of the intercellular adhesion (*icaADBC)* operon, which encodes three membrane proteins (IcaA, IcaD and IcaC) with enzymatic activity and one extracellular protein (IcaB) (Djordjevic et al., 2002; Christensen et al., 1985). The *icaADBC* gene locus has also been detected in *S. aureus* and a range of other coagulase-negative staphylococci (Allignet et al., 2001; Cramton et al., 1999; Knobloch et al., 2002; McKenney et al., 1999). In addition, several surface proteins have been involved in the biofilm formation process, including biofilm associated protein (BAP) (Cucarella et al., 2001), *S. aureus* surface protein G (SasG) (Montanaro et al., 2011; Corrigan et al., 2007), Fibronectin-binding proteins (FnBPs) (Vergara-Irigaray et al., 2009; O'Neill et al., 2008) or Staphylococcal protein A (Spa). It is now suggested that protein-mediated biofilm formation under in vivo conditions is also an important virulence factor (Merino et al., 2009).

It is estimated that approximately 65% of all bacterial infections in humans are caused by biofilms (Costerton and Stewart, 2000) and Christensen et al. (1982) showed that 63% of the pathogenic strains produced slime, and only 37% of the nonpathogenic strains produced slime (Costerton et al., 1995). In the laboratory, Christensen et al. (1982) demonstrated that only one slime-producing cell per 16 000 non-slime-producing cells results in a culture that produces a gross amount of slime. Furthermore, there is increasing recognition that biofilm growth gives rise to a significant population of bacteria with a diverse set of phenotypes, often termed "variants" (Yarwood et al., 2007). This phenomenon has been explained by the ''insurance hypothesis,'' which posits that the presence of diverse subpopulations increases the range of conditions in which the community as a whole can thrive (McCann, 2000; Yachi and Loreau, 1999).

A biomaterial can be defined as any substance, natural or synthetic, used in the treatment of a patient that at some stage, interfaces with tissue (Wollin et al., 1998). Although, any medical device easily inserted and removed (catheters, contact lenses, endotracheal and nasogastric tubes) or long-term implants (cardiac valves, hip joints and intraocular lenses) represents potentially a favorable support to microbial biofilms formation. Whereas, it is now well documented that biofilms are notoriously difficult to eradicate (Diani et al., 2014) and are often resistant to systemic antibiotic therapy and removal of infected device becomes necessary (Lewis, 2001; Souli and Giamarellou, 1998). Anyway, the skin surrounding the catheter insertion site has been implicated as the most common source of central venous catheters (CVC) colonization (Raad et al., 1993).

In order to study bacterial biofilms, a large variety of experimental direct (including microscopy techniques) and indirect observation methods have been developed. The microtiter plate procedure is an indirect method for estimation of bacteria *in situ* and can be modified for various biofilm formation assays (An and Friedman, 2000). This method has been investigated using many different organisms and stains (Hobby et al., 2012; Ramage et al., 2001; Stepanovic et al., 2000; Christensen et al., 1985; Deighton and Balkau, 1990; Miyake et al., 1992) in which the optical density (OD) of the stained bacterial film is measured with an automatic spectrophotometer.

In this study, we screened our original collection of 50 clinical isolates of *S. aureus* from intravenous catheter-associated infections by the polypropylene microtiter plate method for determining their ability to form biofilm. Parallelly, it is known that the genes that are crucial for biofilm formation are a subset of the genes involved in pathogenesis. This work was realized for the first time at the university hospital of Tlemcen. Our aim was to assess biofilm-forming ability of our collection, knowing that this organism is difficult to control and causes several constraints in different services of the hospital.

MATERIALS AND METHODS

Staphylococcus aureus isolates

In a total of 240 clinical isolates of *Staphylococcus* spp. isolated from catheters from four different services (neonatology, internal medicine, pneumology, pediatric and neurology service) at the university hospital of tlemcen (North-West Algeria) during a period of two years (from 2009 to 2011), 50 strains were identified as *S. aureus* on the basis of standard and conventional microbiological techniques including Gram stain, catalase and coagulase tests. The identification was completed with API Staph gallery (bioMérieux, Marcy l'Etoile, France).

Microtiter plate assays

In the present study, we screened the fifty clinical isolates of *S. aureus* for their ability to form biofilm by microtiter plate method according to the works of Christensen et al. (1985) with some modifications.

Strains from fresh agar plates were inoculated in 3 ml of brain heart infusion (BHI) with 1% glucose (Mathur et al., 2006) and incubated for 24 hours at 37°C in stationary conditions and diluted 1 in 20 with fresh medium. Individual wells of sterile, propylene, 96 well Microplate were filled with 200 µl of the diluted cultures and 200 µl aliquots of only BHI + 1% glucose were dispensed into each of eight wells of the column 12 of microtiter plate to serve as a control (to check non-specific binding and sterility of media). After incubation (24 h at 37°C), the microtiter plates content of each well was removed by tapping the bottom plates. The wells were washed four times with 200 µL of phosphate buffer saline (1 ×PBS pH 7.2) to remove planktonic bacteria. The plates were then inverted and blotted on paper towels and allowed to air dry for 15 min (Broschat et al., 2005). Adherent organisms forming-biofilms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v) (Borucki et al., 2003; Mathur et al., 2006) and allowed to incubate at room temperature for 15 min. After removing the crystal

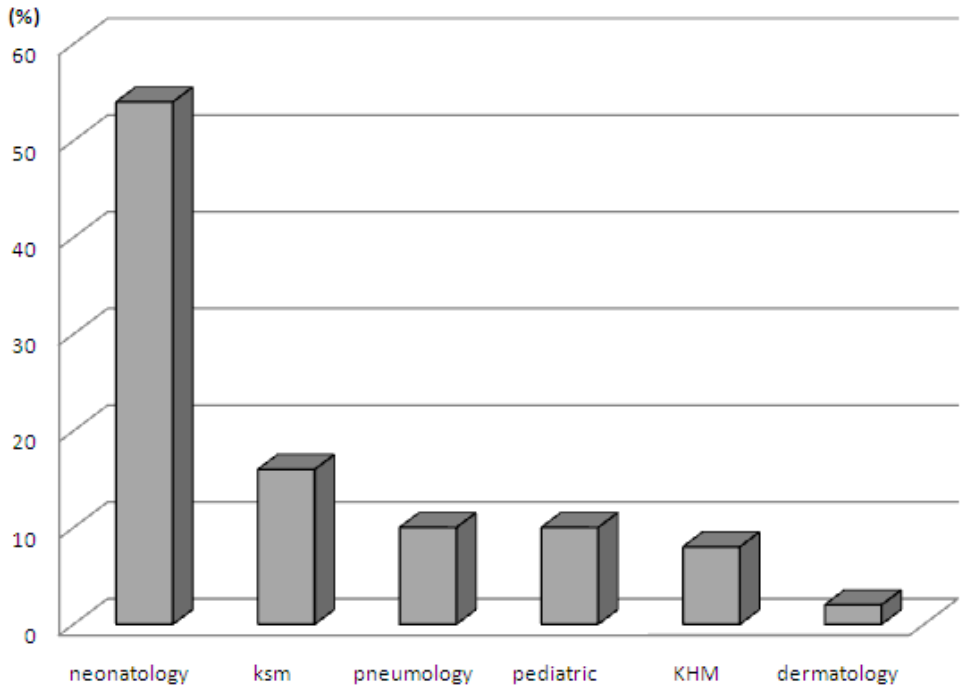

Figure 1. Distribution of the fifty studied clinical isolates of *S. aureus* according to different services of the university hospital of Tlemcen during a period of two years.

violet solution, wells were washed three times with 1 × PBS to remove unbound dye. Finally, all wells were filled by 200 µl ethanol (95%) to release the dye from the cells. Optical density (OD) of stained adherent bacteria was determined with an Absorbance Microplate Reader (model EL×800) at wavelength of 630 nm. To correct background staining, the OD values of the eight control wells were averaged and subtracted from the mean OD value obtained for each strain. The experiment was repeated three times separately for each strain and the average values were calculated with standard deviation (SD).

Classification of adherence

The mean values of OD obtained for blank tests were subtracted from the mean values of OD obtained for each test strain to correct the background staining of microtiter plate. The Absorbance Microplate Reader (model EL×800) used in this study has a dynamic range from 0 to 3.0 OD. According to the classification of Christensen et al. (1985) using the microtiter-plate, strains are divided into three categories: non-adherent, weakly adherent and strongly adherent. However, our clinical isolates were classified into four categories (Stepanovic et al., 2000): non-adherent (OD < ODc); weakly-adherent (ODc < OD < 2xODc); moderately-adherent (2xODc < OD < 4xODc); strongly-adherent (4xODc < OD); with ODc: the cut-off OD (three standard deviations above the mean OD of the blank test). The averaged OD values and standard deviations were made by Excel computer software.

RESULTS

As can be shown in Figure 1, of the fifty (20.83%) clinical strains of *S. aureus*: 27 (54%), 9 (18%), 5 (10%), 5 (10%) and 4 (8%), were respectively isolated from the following services: Neonatology, pneumology, pediatric, neurology, and internal medicine.

The results of microtiter plate assay used for assessment of biofilm-forming ability of the fifty clinical isolates of *S. aureus* are presented in Figure 2. The method applied in this study allowed us to measure biofilm formation after growth in BHI 1% glucose for 24 h at 37 °C. Spectrophotometric measurement of optical densities (OD) of adherent cells enabled us to classify our clinical isolates collection into four categories (Figure 2); non adherent (OD ≤0.2), weakly (0.2<OD≤0.4), moderately (0.4<OD≤0.8) and strongly (0.8<OD) adherent strains (Figure 3). Of the 50 clinical isolates studied, 16 (32%) were designated as non adherent, 20 (40%) as weakly 10 (20%) as moderately and 4 (8%) as strongly adherent.

DISCUSSION

The *Staphylococcus* genus acquires a huge importance in implant-related infections (Campoccia et al., 2006). Elsewhere, the number of diseases caused by *S. aureus* continues to grow. One of the reasons why *S. aureus* is such a ubiquitous pathogen is that it colonizes the anterior nasopharynx in 10 to 40% of humans and can be easily transferred to the skin (Williams, 1963). Biofilm-forming ability is one of the crucial ways that enable this microorganism to express it pathogenecity. It was found

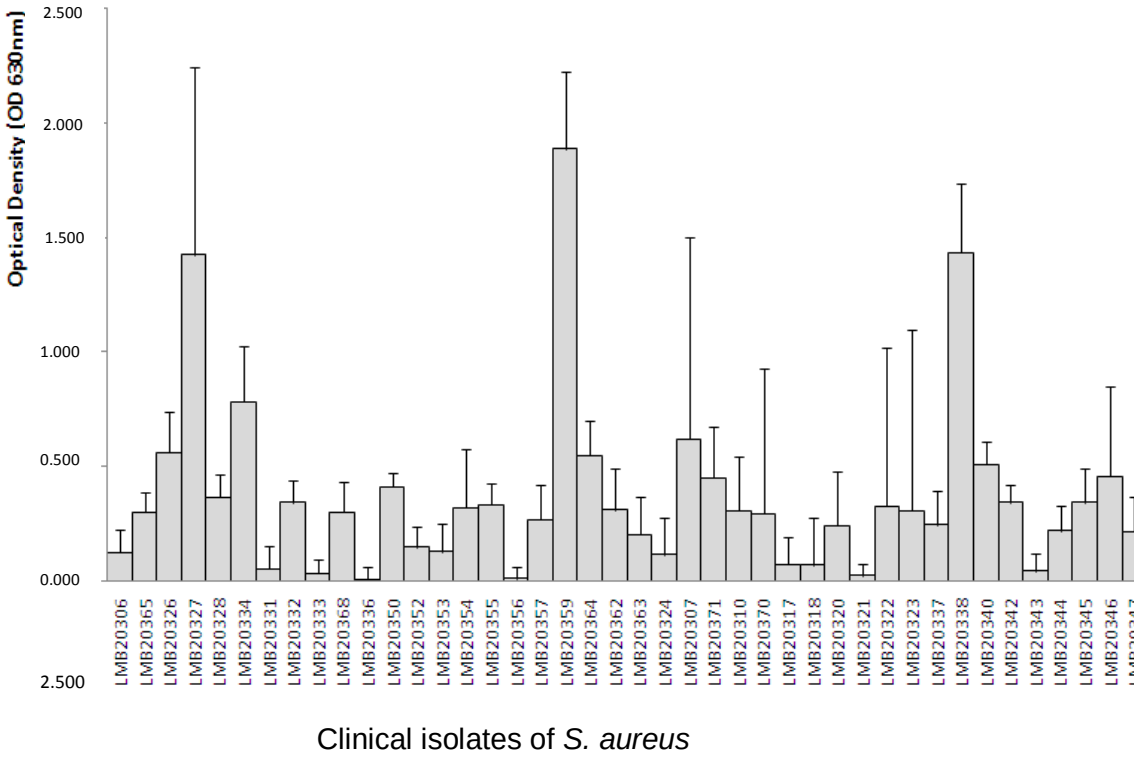

Figure 2. Biofilm-forming ability on polypropylene microtiter plate of the fifty clinical isolates of *S. aureus* following growth for 24 h at 37°C in brain heart infusion 1% glucose. Bars represent mean values of OD (measured at wavelength of 630 nm) and their standard deviations.

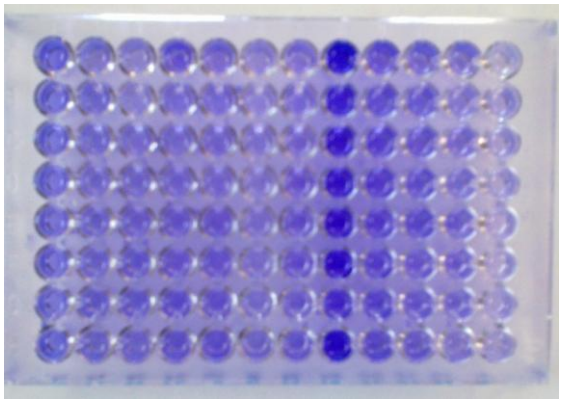

Figure 3. Screening of biofilm formation with crystal violet staining by the 96 well microtiter plate: (I) high, (II) moderate (III) weak and (IV) non adherent.

that the virulence of the organism does indeed vary with its ability to adhere to plastic tissue culture plates (Baddour et al., 1984). Furthermore, as the process of adherence is the initial event in the microbial pathogenesis of infection, failure to adhere will result in removal of the microorganism from the surface of an implanted medical device and avoidance of device-related infection (Ofek and Beachey, 1980). Moreover, biofilm formation by *S. aureus* is influenced by environmental factors like sugars (glucose and/or lactose) or proteases present in the growth medium and depends also on the genetic make-up of a particular *S. aureus* isolate (Melchior et al., 2009). Therefore, according to several researches it was supposed that assessing for biofilm formation could be a useful marker for the pathogenicity of staphylococci. Their active adhesion mechanisms are currently regarded as crucial virulence factors and frequently considered for the characterization of the clinical isolates in studies of molecular pathogenesis and epidemiology (Campoccia et al., 2006). However, some authors considered that there is a little or no correlation between biofilm formation *in vitro* and the clinical outcome of the infection (Kotilainen, 1990; Perdreau-Remington et al., 1998).

In this study, the largest number of clinical isolates of *S. aureus* was collected from neonatology services (n=27), followed by internal medicine (n=9), pneumology and pediatric services (n=5) and finally the neurology services (n=4) (Figure 1). Furthermore, investigation of the correlation between the isolation sites and biofilm-forming ability was not highlighted in this work but it would be efficient to note that among the four strains of *S. aureus* recognized as strongly adherents, two are from the neonatology services.

Various methods have been used to quantify adhesion

of microorganisms to different surfaces. Direct methods allow the *in situ* observation of microbial colonization, including microscopy techniques (laser-scanning confocal, transmission electron and scanning electron microscopy) and indirect methods such as microtiter plate assay, Tube method (TM) and Congo red agar (CRA). Among these various methods, we have used in this study a simple *in vitro* microtiter pate method to quantify the biofilm formation of 50 clinical isolates of *S .aureus*. This method has the advantage of enabling researchers to rapidly analyze adhesion of multiple bacterial strains or growth conditions within each experiment (Djordjevic et al., 2002).

It is known that the direct observation by microscopic techniques is the most important method to study adhesive cells and biofilms, but we think that the microtiter plate assay can be used alternatively as an accurate, rapid, reproducible and inexpensive primer screening method. Thus, this simple quantitative method enables us to assess simultaneously a big number of strains for their biofilm-forming ability. However, in order to complete and enhance the final results obtained in this study, it would be efficient to carry out other experiments, such as PCR for detection of *ica*ADBC genes in the isolates and comparison with the microtiter plate assay results; and animal infection test especially among the four strongly adherent stains to assess the relationship between the biofilm formation and the pathogenicity.

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Antibiotic resistance and plasmid profile of *Leuconostoc* spp. isolated from carrot

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In the present study, a total of 56 isolates were isolated from different root vegetables. Out of these, 17 isolates were identified as *Leuconostoc* spp. All the 17 isolates were checked for antibiotic sensitivity against different antibiotics. Results revealed that majority of the isolates were resistant to Penicillin G, Vancomycin, Oxacillin and Ceftazidime. Four isolates (S-9, S-13, S-37 and S-42) were resistant to methicillin. However, all the isolates were highly sensitive to Imipenum. Carbenicillin and Amoxicillin sublactam showed antibacterial sensitivity against all the isolates except S-13 and S-B_2C_2, respectively. Electrophorogram revealed that among the different 17 *Leuconostoc* isolates, S-B_2C_2 showed the presence of multiple plasmids (six) corresponding to the molecular weights of 1.5, 1.9, 2.0, 2.6, 3.2 and 10 kb, respectively. Endonuclease restriction analysis study was carried out with purified plasmid using four endonucleases (*Alu* I, *Bam* HI, *Hae* III and *Hind* III). Treatment with *Alu* I resulted in the disappearance of all the 6 plasmid bands, indicating complete digestion of the plasmids. Restriction analysis of plasmid DNA of isolate S-B_2C_2 revealed complete digestion of two plasmids (2.6 and 1.5 kb) when treated with *Hind* III. However, a new band of molecular weight equivalent to 1.7 kb did appear. Data presented in the paper indicates the multiple plasmid availability in bacteria and their diversity in response to restriction sites available on them.

Key words: Antibiotic resistance, plasmid, restriction digestion, root vegetables.

INTRODUCTION

Antibiotic resistance in bacteria which was rare before the dawn of antibiotic era has increased tremendously mainly because of over-use/misuse of antibiotics and transfer of resistance genes horizontally among bacteria (Levy, 1997). Today, antibiotic resistance among pathogens emerges shortly after the introduction of every new antimicrobial compound. Studies on the selection and dissemination of antibiotic resistance have mainly been focused on clinically relevant bacterial species. However, the recent findings that antibiotic resistance is amply present in commensal bacteria such as *Lactobacillus* (Teuber et al., 1999; Erdogrul and Erbilir, 2006), *Leuconostoc* (Rodriguez, 2009) and *Bifidobacterium* (Ammor et al., 2007; D'Aimmo et al., 2007) has also attracted the attention of food microbiologists.

Lactic acid bacteria may also be involved in horizontal transfer of antibiotic resistance as they are consumed live together with food and live in close association with diverse organisms in various ecological niches. Leuconostocs are heterofermentative lactic acid bacteria

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Table 1. Sources of selected *Leuconostoc* spp. isolates.

Source	Isolate numbers
Carrot (*Daus carota* sub sp. *sativus*)	S-9, S-13, S-41, S-CH, S-B_2C_2
Black carrot (*Daus carota* sub sp. *carota*)	S-33
Beet (*Beta vulgaris*)	S-21, S-23
Turnip (*Brassica rape* sub sp. rape)	S-28, S-37, S-38, S-42
Raddish (*Raphanus sativus*)	S-15, S-31, S-35, S-36
Cabbage (*Brassica oleracea* Linne.)	S-YCB

All vegetables were collected fresh from farmers to isolate LAB. Isolation was done by enrichment culture technique.

that occur naturally in milk, grass, herbage, grapes and many vegetables (Teuber and Geis, 1981). Several members of this group are used in dairy fermentations to produce aroma compounds (Cogan, 1985). Though common inhabitants are food and food products, much attention has not been paid on the antibiotic resistance of *Leuconostoc* spp. Antibiotic resistance to methicillin in *Leuconostoc mesenteroides* isolated from meat (Vidal and Collin-Thompson, 1987) and to vancomycin in *Leuconostoc* spp. (Hamilton-Miller and Shah, 1998; Simpson et al., 1988) have also been reported.

One of the major and common problem faced by the medical microbiologist, now a days, is the development of resistance to various antimicrobials which pose a challenge to public health. Thus understanding the routes of dissemination of antimicrobials resistant bacterial strains and resistance encoding genetic sequence is crucial to effectively control and minimize the problem. Food and food products are thus effective sources for the acquisition of drug resistant bacteria and genes involved in drug resistance resulting in the uncontrolled dissemination of resistance among the animals including human beings. Transfer of antibiotic resistance from animals to humans through food products derived from animals colonized by resistant bacteria is quite possible (Gonzalez-Zorn and Escudero, 2012). However, the role of LAB as reservoir of antibiotic resistance determinants with transmission potential to pathogenic species is now increasingly acknowledged (Marshall et al., 2009; van Reenen and Dicks, 2011).

Lactic acid bacteria are closely associated with some root vegetables such as carrot, turnip, beet and radish. These are consumed raw or are used to produce fermented products. However, LAB associated with these vegetables have been studied with respect to their role in fermentation of these vegetables. However, much attention has not been paid toward antibiotic resistance and nature of resistance in these organisms (Table1).

MATERIALS AND METHODS

Isolation of lactic acid bacteria

Lactic acid bacteria were isolated by using enrichment culture technique. The root vegetables were washed thoroughly first with tap water and then with sterile distilled water to remove the dirt, dust and micro-organism present on the surface. The vegetables were chopped in to small pieces and were put in to 500 ml Erlenmeyer flasks containing 3% brine adjusted to pH 5.0. The flasks were incubated at ~15°C. After incubation for 3-4 days, 100 µl of the brine was spread on MRS medium (de Man et al., 1993) containing bromothymol blue. LAB were identified with small colonies (2-5 mm in diameter) with entire margins, convex, smooth glistening and yellow in colour with a yellow zone around them.

Antibiotic sensitivity test

A loop full of freshly grown bacterial culture was suspended in 1 ml sterile distilled water. Aliquots of 100 µl of these bacterial suspensions (~1 x 10^6 cfu/ml) were spread on Petri plates containing MRS Agar. The plates were incubated at 30°C for 15 min and thereafter, discs of different antibiotics were placed with the help of sterilized forceps on the surface of inoculated plates. The plates were incubated at 30°C and observed for zone of inhibition after 24 h.

Plasmid isolation

Plasmids were isolated using HiPura Plasmid DNA Miniprep Purification Spin Kit procured from HiMedia Pvt. Ltd. Mumbai, India.

Agarose gel electrophoresis

The DNA isolated was electrophoresed on agarose gel (1.0%). Aliquots of 5 µl of sample along with 2 µl of 6X loading dye were loaded in wells and allowed to run at 80-100 V for 1-2 h. The bands were visualized on UV-trnsilluminator (Genei Pvt. Ltd.).

Restriction digestion of plasmid DNA

Aliquots of 8 µl of plasmid DNA sample were taken in microcentrifuge tubes and 4-5 µl of restriction enzymes (*Alu* I, *Bam* HI, *Hae* III and *Hind* III) was added to each tube. Tubes were incubated at 37°C for 3 h. Reaction was terminated by adding stop solution (0.5M EDTA). Samples were then electrophoresed on agarose gel (1.0 %) to observe the restriction pattern.

RESULTS

Isolation and confirmation of lactic acid bacteria (LAB)

On the basis of the colony characteristics 56 isolates

Table 2. Antibiotic resistance profile of *Leuconostoc* spp. isolates.

Strains	Tested Antibiotics														
	P	Ox	Va	M	I	A	Ck	Ca	Cb	Cf	AMS	B	Ak	Rf	Ce
S-9	R	R	R	R	+++	R	++	+	++	R	++	+	R	++	+
S-13	R	R	R	R	+++	R	R	R	R	R	++	+	R	++	R
S-15	+	R	R	+	++	+	++	R	+++	++	+	+++	++	++	++
S-21	+	R	R	+	++	+	++	+	++	+	++	+	+	++	++
S-23	R	R	R	+	++	+	++	R	++	+	++	+	+	++	R
S-28	+	R	R	+	++	+	++	R	+++	++	++	+	+	++	R
S-31	R	R	R	+	+++	+	++	R	+++	++	++	++	+	++	++
S-33	R	R	R	+	+++	+	++	R	++	R	++	+	+	++	++
S-35	++	+	++	++	+++	+	++	+	++	R	++	+	+	++	++
S-36	R	R	R	+	+	+	+	R	++	+	++	+	+	++	R
S-37	R	R	R	R	++	+	+	R	++	+	++	+	+	++	+
S-38	R	R	R	+	++	+	+	R	++	+	++	+	+	++	+
S-41	+	R	R	++	+++	++	+	R	+++	++	+++	+	++	++	++
S-42	R	R	R	R	+	+	+	R	++	+	++	+	++	++	R
S-CH	R	R	R	++	+++	++	++	+	+++	+	++	+	R	+	++
S-B_2C_2	R	R	+	+	+++	R	R	+	++	+	R	R	R	+++	R
S-YCB	++	R	R	+	++	++	+	R	++	+	++	+	+	++	+

1-6 mm Resistant (R); 7-15 mm - susceptible (+); 16-25 mm - intermediate susceptible (++); 26-35 mm - highly susceptible (+++). P- Penicillin (10 mcg/disc), Ox– Oxacillin (1 mcg/disc), M-Methicillin (30 mcg/disc), Va–Vancomycin (30 mcg/disc), I- Imipenum (10 mcg/disc), A- Ampicillin (2 mcg/disc), Ck- Ceftizoxime (30 mcg/disc), Cb- Carbenicillin (100 mcg/disc), Ca- Ceftazidime (30 mcg/disc), Cf- Ciprofloxacin (5 mcg/disc), AMS- Amoxicillin Sublactam (30/15 mcg/disc), B- Bacitracin (0.05 µ/disc), Ak- Amilkacin (30 mcg/disc), Rf- Rifampicin (15 mcg/disc), Ce- Cephotoxime (30 mcg/disc).

were picked, purified and characterized. Out of 56 isolates, 17 were identified as *Leuconostoc* spp. All the 17 isolates were found to be Gram positive, small rod or cocco-bacilli, non-spore forming, non-motile, catalase negative. These were also negative for indole production and produced extracellular dextran in the presence of sucrose.

All the 17 isolates were checked for antibiotic sensitivity against 16 different antibiotics (Table 2). Result of this study revealed that majority of the 17 isolates were resistant to Penicillin G, Vancomycin, Oxacillin and Ceftazidime, 4 isolates *viz.* S-9, S-13, S-37 and S-42 were resistant to Methicillin, whereas others were sensitive though slightly only. None of the isolates showed resistance against Imipenum as all the isolates were highly sensitive to this drug. Carbenicillin showed antibacterial sensitivity against all the isolates except one (S-13). All the isolates were intermediate to highly sensitive to Rifampicin. Likewise Amoxicillin Sublactam showed antibacterial sensitivity against all the isolates except one isolates, S-B_2C_2 which was found to be resistant to this antibiotic.

Plasmid DNA isolation

Results revealed that among 17 isolates, only one isolates, S-B_2C_2 showed the presence of plasmids. Electrophorogram revealed that among the different LAB isolates, S-B_2C_2 showed the presence of multiple plasmids (six) corresponding to the molecular weights of 1.5, 1.9, 2.0, 2.6, 3.2 and 10 kb, respectively (Figure 1, Lane 2). None of the rest isolates possessed any plasmid (Figure 1).

Endonuclease restriction analysis

Endonuclease restriction analysis study was carried out with purified plasmid using four endonucleases (*Alu* I, *Bam* HI, *Hae* III and *Hind* III). Treatment with *Alu* I resulted in the disappearance of all the 6 plasmid bands (Figure 2, Lane 2), indicating complete digestion of the plasmids. When the plasmid DNA of isolate S-B_2C_2 was treated with *Bam* HI, only one plasmid of molecular weight equivalent to 2.6 kb disappeared because of complete digestion. However, the remaining 5 bands remained unaffected (Figure 2, Lane 3). Digestion with *Hae* III resulted in the loss of four plasmids out of six. Two of the plasmids (2.0 Kb and 3.2 Kb) remained undigested (Figure 2, Lane 4). Restriction analysis of plasmid DNA of isolate S-B_2C_2 revealed complete digestion of two plasmids (2.6 and 1.5 kb) when treated with *Hind* III. However a new band of molecular weight equivalent to 1.7 kb did appear (Figure 2, Lane 5).

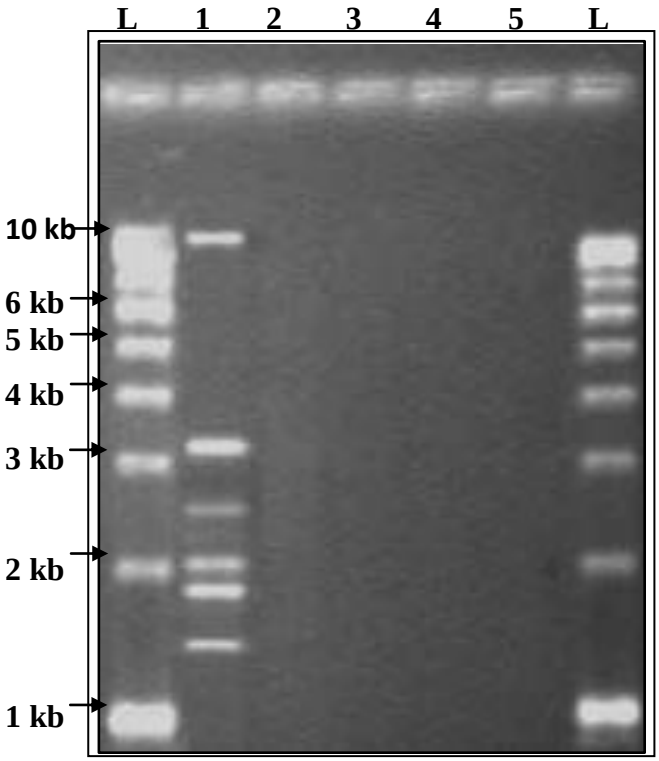

Figure 1. Plasmid profile of different LAB isolated from vegetable sources. L denotes DNA leader (1 to 10 kb), 1-5 are different LAB isolates used (1: SB_2C_2, 2: S-9, 3: S-15, 4: S-23, 5: S-38).

DISCUSSION

Lactic acid bacteria, a broad group of Gram positive, non-spore forming rods and cocci have a role as commensal on mucosal surfaces and skin and inhabit the digestive tract of many animal species including humans (Tannock et al., 1990). A large number of species of lactic acid bacteria has been detected in the digestive tract but their prevalence and distribution varied with the animal species (Vaughan et al., 2002). In general, lactic acid bacteria are the organisms which first colonize the digestive system of animals. Many lactic acid bacteria possess probiotic property and are thus widely used in probiotic preparations.

Lactic acid bacteria are common inhabitants of many vegetables and fruits and thus form a part of fermented food products prepared from these fruits and vegetables. These lactic acid bacteria from fermented products may act as reservoirs of antimicrobial resistance genes that could be transferred into pathogens either in the food web or in the gastrointestinal tract of humans and animals (Belen Florez et al., 2005). The development of antibiotic resistance in bacteria is of public concern in view of the fact that a patient could develop antibiotic resistance because of emergence of a drug resistant micro-organism in patient's body (Nagulapally, 2007). Thus, strains of micro-organisms for use in food systems as starters or probiotics need to be examined carefully for antimicrobial resistance (Teuber et al., 1999).

Since antibiotic susceptibility and resistance of lactic acid bacteria from vegetable and their products have not been studied much, the present investigation was carried out to determine the antibiotic resistance and diversity among different isolates with respect to presence of plasmids and their endonuclease restriction analysis. A total of 28 isolates of LAB were identified from root vegetables collected from 7 different locations around Dehradun town. These isolates were characterized for their morphological, cultural and biochemical characterstics and were found to belong to the category of LAB.

During biochemical characterization, all the 28 isolates were found to be negative for catalase activity, indole production and nitrate reduction. Almost all the non-lactic acid bacteria are catalase negative and do not produce indole. These tests are commonly used and described in the Burgey's Manual of Systemic Bacteriology for identification of LAB. Nitrate production is another important property of LAB. Lactic acid bacteria reduce nitrate to nitrite (Anderson, 1984). In acidic environment, nitrate may react with secondary or tertiary amines or with amides to form nitrosamines which are known for their carcinogenic effect. Some microorganism such as *Paracoccus denitrificans* has been reported to reduce

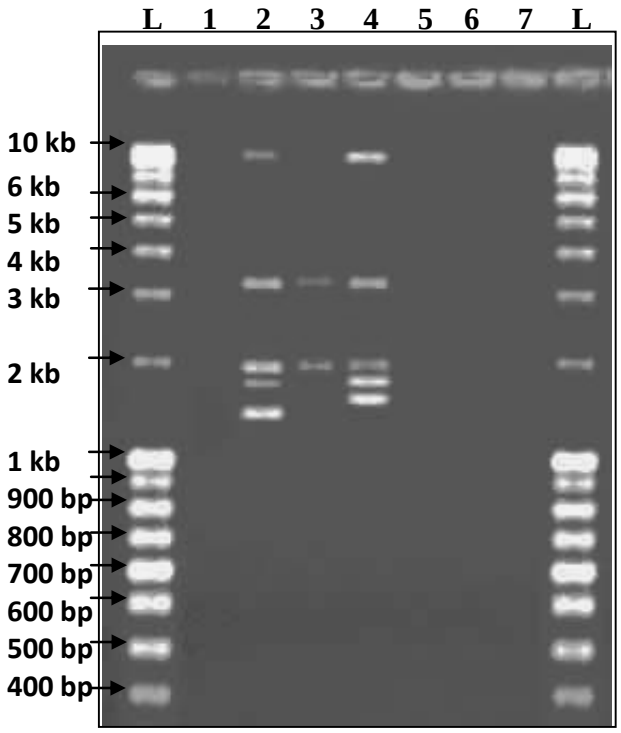

Figure 2. Plasmid restriction profile of SB_2C_2 generated by different restriction enzymes used in this study. L denotes DNA leader (100 to 10 kb), 1-5 are different LAB isolates used (1: SB_2C_2 digected with *Alu* I, 2: SB_2C_2 digected with *Bam* HI, 3: SB_2C_2 digected with *Hae* III, 4: SB_2C_2 digested with *Hind* III, 5-7: Blank).

nitrate to nitrite in commercial carrot juice (Kerner et al., 1986). Similarly, Grajek and Walkowiak-Tomczak (1997) reported that the treatment of Red beet juice with *P. denitrificans* made possible the complete removal of nitrates with limited scale changes in its flavor and color.

However, none of the isolate in our study possessed the property of nitrate reduction. Extracellular Dextran production in the presence of sucrose is the property of some of the *Leuconostoc* spp. Out of 28 isolates, 15 were positive for extracelluar dextran production in the presence of 2% sucrose on MRS medium. Thus, these 15 isolates seem to belong to the genus *Leuconostoc.*

Antibiotic resistance of all the 28 isolates was examined by disc-diffusion method and these isolates were found to be diverse in their antibiotic resistance against 16 antibiotics belonging to different groups. During this study, we observed that most of the strains of *Leuconostoc* spp. were resistant to Oxacillin, Vancomycin, Ceftazidime and Amphotericin. However, they were found to be susceptible to Imipenum, Ceftizoxime, Carbenicillin, Ciprofloxacin, Amoxicillin Sublactam, Bacitracin, Amikacin Rifampicin and Cephotoxime. Resistance to vancomycin in *Leuconostoc* spp. has been reported earlier also (Facklam et al., 1989; Orberg and Sandine, 1984). Infact this widespread resistance among the *Leuconostoc* spp. have been used by Benkerroum et al. (1993) to formulate a medium for the selective isolation of *Leuconostoc* from vegetables and dairy products using 30 µg of Vancomycin/ml as a criteria for selective isolation.

The antibiotics resistance though is present in *Leuconostoc* spp. but the isolated strains were sensitive to majority of antibiotics specially belonging to second and third generation. The development of resistance in lactobacilli including *Leuconostoc* spp. is of major concern because of possibility of horizontal transfer of resistance from these bacteria to pathogens. Increasing evidences point at a crucial role for foodborne LAB as reservoir of potentially transmissible AR genes, underlining the need for further, more detailed studies aimed at identifying possible strategies to avoid AR spread to pathogens through fermented food consumption (Devirgiliis et al., 2013).

Results revealed that among 17 isolates, only one isolate, S-B_2C_2 showed the presence of plasmids. As inferred from the electrophorogram, isolate S-B_2C_2 showed the presence of multiple plasmids (six) corresponding to the molecular weights of 1.5, 1.9, 2, 2.6, 3.2 and 10 kb, respectively. None of the rest isolates possessed any plasmid. The presence of plasmid(s) in the *Leuconostoc* spp. has been shown earlier also by several workers (Prievost et al., 1995; Biet et al., 2002). However the frequency was found to be low. Prievost et al. (1995) reported that only six strain possessed single cryptic plasmid among the 15 strains of *Leuconostoc oenos* studied.

It was recorded that isolate S-B_2C_2 showed resistance against 56% of the sixteen antibiotics used in the study. On the other hand, among the susceptible cases, only three could suppress the test organism adequately giving a zone of inhibition in between 16-35 mm. Such response of the organism against the antibiotics indicates a possible role of plasmids in such resistance behaviour. The presence of multiple plasmids may support the high resistance profile against a range of antibiotic as plasmid borne resistance is common in many microbes. It is well reported that antibiotic resistance is often plasmid borne (Svara and Rankin, 2011). Our results get support from Aslim and Beyatli (2004) who reported higher antibiotic resistance in the isolates carrying multiple plasmids. Additionally, they reported higher susceptibility in the isolates having no plasmid.

Digestion of plasmid DNA with restriction endonucleases was also carried out using 4 endonucleases, *Hind* III, *Bam* HI, *Alu* I and *Hae* III. Effect of the four endonucleases on plasmid DNA of S-B_2C_2 varied. All the six plasmids were digested when the plasmid DNA was treated with *Alu* I, where as *Bam* III could digest only one plasmid (2.6 Kb) out of six. The digestion with *Hind* III resulted in the loss of two plasmids of the molecular size of 2.6 and 1.5 kb with the appearance of new band of molecular weight equivalent to 1.7 kb.

From these studies, it appears that restriction sites on the plasmids vary from plasmid to plasmid. Whereas a large number of restriction sites were present on plasmid 4 (2.6 kb) and 6 (1.5 kb) since these plasmids are completely digested by 3 endonucleases, that is, *Hind* III, *Bam* HI, *Alu* I and *Hind* III, *Alu* I, *Hae* III respectively, plasmid 4 of the molecular size of 2.0 kb contain the least number of restriction sites since it is digested completely but by *Alu* I endonuclease only. Further investigation will reveal which of the plasmid and fragment possess the resistance gene(s) and is responsible for antibiotic resistance trait in the organism.

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