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Full Length Research Paper

Features of *Staphylococcus lugdunensis* isolated in western region of Turkey

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***Staphylococcus lugdunensis* is a member of the coagulase-negative staphylococci (CNS) which has clinical and laboratory characteristics that resemble those of *Staphylococcus aureus*. The purpose of this study was to investigate the frequencies, microbiological characteristics and antibiotic susceptibilities of *S. lugdunensis* isolates. Forty-one (41) *S. lugdunensis* isolates, which were collected between 2006 and 2009 in our laboratory, were retrospectively included. Twenty-one (21) re-cultured isolates were investigated about several conventional biochemical tests and antibiotic susceptibility tests. The species identification was confirmed by 16S rRNA gene sequencing. Most of *S. lugdunensis* isolates (71%) were isolated from skin-soft tissue samples. Nine 9(43%) out of 21 isolates were clumping factor positive. All of the isolates were tube coagulase-negative, pyrrolidonyl arylamidase (PYR) and DNase positive. The ornithine decarboxylase (ODC) test, beta-hemolysis and synergistic hemolysis were positive in 19 (90%) isolates. Slime production was found in 5 (24%) isolates. Most isolates were susceptible to the antibiotics tested, although 14% were β-lactamase positive and could be identified by the disk diffusion method for penicillin G. Methicillin resistance and vancomycin tolerance did not detected. *S. lugdunensis*, is an unusual pathogen which may be can be misidentified with *S. aureus* by some laboratory characteristics, but which in many instances may be treated with narrow-spectrum antibiotics.**

Key words: *Staphylococcus lugdunensis*, infection, characteristics, identification, susceptibility.

INTRODUCTION

Staphylococcus lugdunensis, a coagulase-negative staphylococcus (CNS), has clinical characteristics that resemble those of the coagulase-positive *Staphylococcus aureus*, since it often causes aggressive and rapidly progressive infections (Hellbacher et al., 2006; Tan et al., 2008). There are several case reports of serious infective endocarditis (Tan et al., 2008; Ebright et al., 2004; Patel et al., 2000; Sotutu et al., 2002; Zinkernagel et al., 2008), septic shock and bacteremia (Ebright et al., 2004; Castro and Dowdy, 1999; Pareja et al., 1998), meningitis secondary to ventriculo-peritoneal shunt infections (Elliott et al., 2001; Kaabia et al., 2002), osteomyelitis (Greig and Wood, 2003; Murdoch et al., 1996), prosthetic joint infections (Weightman et al., 2000), skin and post-surgical wound infections (Hellbacher et al., 2006; Herchline and Ayers, 1991), breast abscesses (Hellbacher et al., 2006; Asnis et al., 2003) and peritonitis associated with continuous ambulatory peritoneal dialysis (Schnitzler et al., 1998) due to *S. lugdunensis.*

S. lugdunensis can be misidentified as *S. aureus* in the routine microbiological laboratory, since the DNase test can be weakly positive, the agglutination test for clumping factor can be positive, and the colony morphology often resembles that of *S. aureus*. Compared with other CNS, *S. lugdunensis* is generally more sensitive to antibiotics used against staphylococci. It is often possible to use penicillin for treatment of infections caused by these organisms (Hellbacher et al., 2006).

There is a well description of the microbiological

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characteristics and clinical significance of *S. lugdunensis* (Frank et al., 2008). In light of the foregoing, we decided to investigate the laboratory characteristics and clinical significance of this bacteria isolated in our laboratory. The aim of this study was to investigate the clinical spectrum, frequency, microbiological characteristics and antibiotic susceptibility patterns of *S. lugdunensis* isolates collected in our hospital.

MATERIALS AND METHODS

A total of 41 *S. lugdunensis* isolates, which were collected between January, 2006 and June, 2009 in Microbiology Laboratory of Kocaeli University were retrospectively included in present study. VITEK 2 (bioMérieux, France) automated system was used for identification and susceptibility testing. In total, 21 stored isolates were re-cultured. Several conventional biochemical tests including the detection of tube coagulase (with human plasma), slide coagulase (clumping factor), DNase (Salubris, Turkey), synergistic hemolysis, ornithine decarboxylase (ODC) (Salubris, Turkey), pyrrolidonyl arylamidase (PYR) (Oxoid, United Kingdom) and slime production were performed (Bannerman and Paecock, 2007).

The species identification of all *S. lugdunensis* isolates was confirmed by 16S rRNA gene sequencing. A 25 µL polymerase chain reaction (PCR) mixture consisting of 1X PCR buffer, 0.2 mM of each deoxyribonucleotide triphosphates (dNTPs), 0.5 µM of each primer, 1.25 mM $MgCl_2$, 1.5 units of Taq polymerase, 2 µL of genomic DNA was prepared by boiling a touch of bacteria in water for 10 min followed by a short centrifugation. An initial 5 min denaturation at 94°C was followed by 35 cycles of 30 s denaturetion at 94°C, 1 min annealing at 58°C and 1 min 30 s elongation at 72°C. PCR were ended with 10 min final elongation at 72°C (Becker et al., 2004). PCR products (1400 bp) were analyzed by agarose gel electrophoresis, cleaned with a PCR purification kit (Qiagen, USA) and sequenced (Iontek Inc., İstanbul, Turkey). Sequencing was performed with dye terminator cycle sequencing with the ABI Prism BigDye Terminator kit (Applied Biosystems, Foster City, Calif.) used to obtain the sequences. The assay was carried out according to the standard protocol. Data was collected on an ABI 377 automated fluorescence sequencer. The sense and antisense primers used were 5'-TGGCTCAGATTGAACGCTGGCGGC and 5'-TACCTTGTTACGACTTCACCCCA respectively (Lee et al., 2008).

Disk-diffusion tests (Oxoid, England) for penicillin G (10 µg) and cefoxitin (30 µg) were performed and interpreted according to Clinical Laboratory and Standards Institute (CLSI) (CLSI, 2008). Tests for β-lactamase production were performed with nitrocefin disks (Oxoid, England). Minimal inhibitor concentration (MIC) determinations of penicillin G, oxacillin and vancomycin were performed by Etest (AB Biodisk, Solna, Sweden). Microbroth dilution test was also performed for detection of vancomycin MIC and minimal bactericidal concentration (MBC). Vancomycin tolerance is typically defined as an MBC/MIC ratio of ≥32 (Moody and Knapp, 2004). MBC testing was repeated five times. *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 were used as control strains.

RESULTS

A total of 41 *S. lugdunensis* were isolated between January, 2006 and June, 2009, which were 2% of all the CNS isolates. 30 (73%) of the *S. lugdunensis* isolates were from outpatients and 11 (27%) of them were from inpatients. 29 (71%) of *S. lugdunensis* were isolated from wounds-abscesses, 4 (10%) from blood, 4 (10%) from urine, 3 (7%) from peritoneal dialysis catheter and 1 (2%) were isolated from cerebrospinal fluid.

Microbiological characteristics of 21 re-cultured isolates

Hemolysis

A total of 19 isolates (90%) demonstrated hemolysis on blood agar containing sheep erythrocytes within 24 h, additionally, synergistic hemolytic activity was observed in these 19 isolates. Two (2) isolates were nonhemolytic after 4 days of incubation.

Coagulase

Nine (9) (43%) of 21 *S. lugdunensis* isolates were slide coagulase (clumping factor) positive (which 4 were weakly, and 5 were strongly positive). All of the isolates were tube coagulase-negative.

Biochemical profile

All isolates were DNase positive, PYR positive and except for 2 of them, the others were positive for ODC.

Biofilm formation

Slime production was found in 5 (24%) of the 21 *S. lugdunensis* isolates, one of which was isolated from peritonal dialysis catheter and the others were from skin-soft tissue samples.

Species identification by 16S rRNA gene sequencing

All isolates were verified as *S. lugdunensis* by 16S rRNA gene sequencing. Raw sequences obtained were manually edited and assembled in Vector NTI (Invitrogen, USA) to obtain 1400 bp nucleotide sequence. The sequences were then subjected to Basic Local Alignment Search Tool (BLAST) by using blastn search algorithm in NR database. There was 100% sequence coverage with 99 to 100% identity and the sequence retrieved from the NR database belonged to *S. lugdunensis* N920143 (Accession number: FR870271.1).

Antimicrobial susceptibilities

Twenty-eight (28) of 41 *S. lugdunensis* isolates (68%) were susceptible to all of the 22 antimicrobial agents tested by VITEK 2. The antibiotic susceptibilities of 41

Table 1. Antimicrobial resistance patterns of *S. lugdunensis* isolates.

Antimicrobial resistance pattern	Number (n=41)
ERY,CLI,CIP,GM,TOB,FOF,FA,MUP,MXF,RIF,TMP,TET,VAN,TEC,LZD,Q-D susceptible	28
Only CLI resistant	1
Only FOF resistant	3
Only RIF resistant	1
Only TET resistant	2
ERY and CLI resistant	4
ERY, CLI, FOF, TET, FA resistant	1
ERY, CLI, FOF, TET, FA, CIP resistant	1

ERY, Erythromycin; CLI, clindamycin; CIP, ciprofloxacin; GM, gentamycin; TOB, tobramycin; FOF, fosfomycin; FA, fusidic acid; MUP, mupirocin; MXF, moxifloxacin; RIF, rifampycin; TMP, trimethoprim/sulfamethoxazole; TET, tetracycline; VAN, vancomycin; TEC, teicoplanin; LZD, linezolide; Q-D, quinupristin/dalfopristin.

Table 2. Antimicrobial susceptibility test results of β-lactamase positive and negative isolates.

Antimicrobial susceptibility test	β-lactamase positive isolates (n=3)	β-lactamase negative isolates (n=18)
Penicilin disk diffusion	10 - 14 mm	40 - 54 mm
Penicilin MIC	2 - >32 µg/ml	0.016 - 0.125 µg/ml
Cefoxitin disk diffusion	30 mm	23 - 34 mm
Oxacillin MIC	0.38 - 0.75 µg/ml	0.023 - 0.125 µg/ml

clinical isolates are shown in Table 1. All *S. lugdunensis* isolates were methicillin susceptible by VITEK 2, except for 2 isolates which were methicillin susceptible as well in further analysis.

Among the 21 re-cultured isolates, 3 isolates (14%) were β-lactamase positive which were also resistant to penicillin.

Penicillin zone diamaters and MIC values were between 10 to 14 mm and 2 to 32 µg/ml in β-lactamase positive isolates while 40 to 54 mm and 0.016 to 0.125 µg/ml were in β-lactamase negative isolates. Cefoxitin zone diamaters were between 23 to 34 mm in all strains. Oxacillin MIC values were between 0.38 to 0.75 µg/ml in β-lactamase positive isolates and 0.023 to 0.125 µg/ml in β-lactamase negative isolates (Table 2).

All isolates were susceptible to vancomycin (MIC range was 0.25 to 2 µg/ml). In all isolates, vancomycin MBC values were within two doubling dilutions of the MIC values and MBC/MIC ratios were between 2 to 4 suggesting no vancomycin tolerance.

DISCUSSION

S. lugdunensis is the most virulent CNS, with percentile of 91% among all isolates associated with clinically significant infections (Tan et al., 2006).

Skin and soft tissue infections are the most common infections caused by *S. lugdunensis* (Herchline and Ayers, 1991; Frank et al., 2008). Skin infections mainly occur in the perineal or inguinal region (Hellbacher et al., 2006; Frank et al., 2008; Tan et al., 2006; Van der Mee-Marquet et al., 2003). It has been suggested that the breast area is an additional site of *S. lugdunensis* contamination and infection (Hellbacher et al., 2006; Van der Mee-Marquet et al., 2003). It can also cause invasive diseases, especially fulminant valve involvement endocarditis with an aggressive evolution compared to other CNS and even *S. aureus*. In contrast, *S. lugdunensis* bacteraemia without endocarditis does not always cause a serious, aggressive illness. In the nosocomial setting, endocarditis is far less frequent, and *S. lugdunensis* bacteremia is usually associated with a catheter or other foreign material (Hellbacher et al., 2006; Ebright et al., 2004; Zinkernagel et al., 2008; Frank et al., 2008). In present study, most of *S. lugdunensis* isolates (71%) were isolated from skin and soft tissue samples. Unfortunately, we could not detect the anatomic area of all samples because of the retrospective nature of the study. But still, two *S. lugdunensis* isolates were from breast abscesses. We could not detect sourse origin of endocarditis and all four blood isolates had nosocomial origin.

S. lugdunensis, a CNS, is as virulant and destructive as *S. aureus*. For this reason, if *S. lugdunensis* is suspected during clinical course of infection, especially in sterile sites, the prompt identification to the species level is life saving (Frank et al., 2008). *S. lugdunensis* can be discriminated from other CNS by a negative tube coagulase test, a positive PYR reaction, and positive

ODC activity. *S. lugdunensis*, is PYR positive as *Staphylococcus haemolyticus*, *Staphylococcus schleiferi*, and *Staphylococcus intermedius*. ODC is an important determinant of *S. lugdunensis* identification, which ≥ 90% of isolates are positive. However, a small number of *Staphylococcus epidermidis* isolates are also ODC positive (Frank et al., 2008; Bannerman and Paecock, 2007). Two (2) isolates of *S. lugdunensis* were ODC negative in our study which were also nonhemolytic and verified as *S. lugdunensis* by 16S rRNA gene sequencing.

S. lugdunensis can be misidentified as *S. aureus* in the routine microbiological laboratory, since the DNase test can be weakly positive, test for clumping factor can be positive, and the colony morphology often resembles that of *S. aureus* (Hellbacher et al., 2006). Human plasma is preferred for detection of clumping factor in *S. lugdunensis* and *Staphylococcus schleiferi* (Bannerman and Paecock, 2007). However, testing for clumping factor in *S. lugdunensis* identification is not a reliable method (Frank et al., 2008; Mateo et al., 2005). In this study, 9 (43%) out of 21 *S. lugdunensis* isolates were slide coagulase-positive while all of the isolates tested were tube coagulase-negative. All of the isolates which we tested were also DNase positive**.**

S. lugdunensis showed delayed moderate to wide zone of hemolysis within 48 to 72 h in different studies (Tan et al., 2008; Zinkernagel et al., 2008; Frank et al., 2008; 2007; Bannerman and Paecock, 2007; Freney et al., 1988; Hebert, 1990). Additionally, a synergistic hemolytic activity can be observed in 73 to 95% of *S. lugdunensis* strains (Frank et al., 2007; Hebert, 1990; Vandenesch et al., 1998). In the present study, complete hemolysis was observed in 19 isolates (90%) of *S. lugdunensis* and synergistic hemolytic activity was also observed in the same isolates.

Biofilm (slime) formation by CNS is found in commensal isolates as well as in nosocomial isolates. Infections caused by slime producing CNS, are difficult to treat, because of the high levels of the antimicrobial resistance (Frank et al., 2008). Frank et al. (2007) investigated the ability of 10 antistaphylococcal agents at concentrations traditionally tested in MIC assays to significantly reduce the number of bacteria recovered from biofilms of 15 *S. lugdunensis* isolates. They were found that biofilms were resistant to high concentrations of most of the drugs (Sotutu et al., 2002). Hebert (1990) reported that 4 (11%) of 38 clinical isolates of *S. lugdunensis* were positive for slime production. In another study, slime positivity was reported in 4 (22%) of 18 *S. lugdunensis* isolates (Koksal et al., 2006). In our study, 5 (24%) of 21 isolates were slime positive. They were isolated from skin-soft tissue infections except for one which was from peritoneal dialysis catheter.

S. lugdunensis, unlike most CNS, generally has been characterized as being susceptible *in vitro* to most antibiotics (Tan et al, 2008; Frank et al., 2008). Frank et al. (2007) reported that all clinical *S. lugdunensis* isolates were susceptible to 10 antistaphylococcal antimicrobial agents (cefazolin, daptomycin, linezolid, moxifloxacin, nafcillin, rifampin, quinupristn/dalfopristin, tetracycline, trimethoprim-sulfamethoxazole and vancomycin) with the exception of only one isolate which was found resistant to trimethoprim-sulfamethoxazole (Frank et al., 2007). In another study, 15 bloodstream isolates were exhibited susceptibility to many common antimicrobials, including penicillin (Ebright et al. 2004). In our study, 68% of *S. lugdunensis* isolates were susceptible to all 22 antimicrobial agents tested. Whereas two of the strains exhibited oxacillin resistance (MIC range 0.5 to 2 mg/L) by the Vitek 2 system; none of the strains was resistant to oxacillin and cefoxitin in MIC and disc testing, respectively. Because isolates exhibiting oxacillin MICs of 0.5 to 2 mg/L usually lack the *mecA* gene, oxacillin resistance detected by commercial systems is not indicative of the presence of the *mecA* gene (Mateo et al., 2005; Hussain et al., 2000). Methicillin resistance is rare among *S. lugdunensis,* with only three descriptions of *mecA* in among *S. lugdunensis* isolates in English literature (Tan et al., 2008; Becker et al., 2006; Tee et al., 2003). In the past, description methods of methicillin resistance and oxacillin breakpoints were under debate. In 2005, CLSI raised the oxacillin resistance breakpoints for *S. lugdunensis* to ≥4 µg/ml as in *S. aureus*. Consequently, falsely classification of *S. lugdunensis* isolates with MICs of 0.5 to 2 µg/ml but lacking the *mecA* gene as being oxacillin resistant was prevented (Tee et al., 2003). Currently, *S. lugdunensis* strains which have oxacillin MICs of ≤ 2 µg/ml are considered susceptible, whereas those that have oxacillin MICs of ≥4 µ/ml are considered as resistant. The CLSI has also recommended the use of a cefoxitin disk (30 µg) instead of an oxacillin disc (1 µg) for the detection of methicillin resistance in *S. lugdunensis* (CLSI, 2008). Current CLSI breakpoints for oxacillin MIC and cefoxitin disc testing clearly differentiate *mecA*-positive strains of *S. lugdunensis.* The latex detection of *pbp2* also is a viable alternative (Tan et al., 2008).

It has been suggested that penicillin resistance is rare in *S. lugdunensis* due to the lack of β-lactamase and the mecA gene (Mateo et al., 2005). Several studies reported β-lactamase positivity and penicillin resistance rates of 12 to 40% (Castro and Dowdy, 1999; Pareja et al., 1998; Asnis et al., 2003; Frank et al., 2008; Bannerman and Paecock, 2007; Becker et al., 2004; Moody and Knapp, 2004; Mateo et al., 2005; Koksal et al., 2006; May et al., 1998). In our study, 3 (14%) of 21 isolates were penicillin resistant due to β-lactamase production.

There was good agreement between the disk-diffusion results for penicillin G and β-lactamase positivity/ negativity. All β-lactamase-positive isolates had a zone diameter ≤ 14 mm, while those that were β-lactamase-negative had zone diameters ≥ 40 mm. Similarly, penicillin MIC values were between 2 to 32 µg/ml in

β-lactamase positive isolates and 0.016 to 0.125 µg/ml in β-lactamase negative isolates. Measurement of zone diameters in disk-diffusion tests appeared to be a reliable method for detecting β-lactamase production. Hellbacher et al. (2006) reported that all β-lactamase- positive isolates had a zone diameter ≤ 22 mm, while those that were β-lactamase-negative had zone diameters ≥ 38 mm.

In the present study, oxacillin MIC values were between 0.38 to 0.75 µg/ml in β-lactamase positive isolates and 0.023 to 0.125 µg/ml in β-lactamase negative isolates, although all were in suscepibility ranges. The higher MIC values were probably caused by β-lactamase production, comparable to the phenomenon of *S. aureus* isolates designated as borderline oxacillin-resistant *S. aureus* (BORSA) (Hellbacher et al., 2006).

Vancomycin has bactericidal activity against staphylococci. But it should be reserved for situations in which other antimicrobials are not effective (Frank et al., 2008). Current *S. lugdunensis* isolates usually remain susceptible to methicillin and other antistaphylococcal antibiotics. Therefore, the use of glycopeptides for *S. lugdunensis* infections is usually limited to the initial days of empirical treatment when a possibly methicillin-resistant *Staphylococcus* infection has to be considered and to patients with a beta-lactam allergy (Bourgeois et al., 2007). Vancomycin tolerance is a phenomenon in which bacteria with susceptible MICs are refractory to killing in MBC assays. It has been studied in *S. aureus* endocarditis isolates, and it has been suggested that tolerance should be considered when treating endocarditis (Frank et al., 2007). The high rate of vancomycin tolerance among *S. lugdunensis* isolates has also been reported as well (Frank et al., 2008; Frank et al., 2007; Bourgeois et al., 2007). Tolerance to vancomycin in 93% of *S. lugdunensis* isolates has been reported (Frank et al., 2007). Bourgeois et al. (2007) were found tolerance to vancomycin in 6 of 13 *S. lugdunensis* isolates by time-kill curve methodology. An unexpected outcome in the present study was the lack of vancomycin tolerance in *S. lugdunensis* isolates. It was reported that time-kill curves have the crucial advantage of providing dynamic data and are the most reliable approach to detect tolerance (Bourgeois et al., 2007). May et al. (1998) reported that, because of the poor reproducibility of MBC determinations, MBC/MIC ratios should be used with caution for the detection of vancomycin tolerance. However, a low MBC/MIC ratio can be used to exclude tolerance. We repeated MBC testing five times to avoid the errors.

Conclusion

S. lugdunensis, is an unusual pathogen which may be misidentified with *S. aureus* by the clinical course of infected patients as well as *in vitro* certain laboratory characteristics, but which in many instances may be treated with inexpensive and narrow-spectrum antibiotics such as penicilin G (Ebright et al., 2004). The species identification is very important especially in invasive infections such as endocarditis. Although, *S. lugdunensis* can be differentiated from other CNS by a positive PYR reaction, and positive ODC activity as reported by several studies, it must be remembered that it may be ODC negative as in our study. *S. lugdunensis* isolates are generally susceptible to most of the antibiotics and disk diffusion test with penicillin G is a reliable indicator of β-lactamase production. Although the high rate of vancomycin tolerance among *S. lugdunensis* isolates has been reported, we did not detect any vancomycin tolerance. Further studies about vancomycin tolerance should also be performed especially in respect of endocarditis.

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