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Short communication

Virulence properties of methicillin-susceptible *Staphylococcus aureus* food isolates encoding Panton–Valentine Leukocidin gene

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ABSTRACT

In this study, three Panton–Valentine Leukocidin gene carrying methicillin-susceptible *Staphylococcus aureus* (MSSA) strains (M1-AAG42B, PY30C-b and YF1B-b) were isolated from different food samples in Kesan—Edirne, Turkey. These strains were characterized on the basis of MLST type, *spa* type, virulence factor gene contents, antibiotic susceptibilities against 21 antibiotics and biofilm formation. The genetic relatedness of the strains was determined by PFGE. In addition, the complete gene sequences of *lukS*-PV and *lukF*-PV were also investigated. All strains were found to be susceptible to tested antibiotics and they were *mecA* negative. Three strains showed the same PFGE band pattern, ST152 clonal type and t355 *spa* type. In the detection of virulence factor genes, *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *seu*, *eta*, *etb*, *set*1, *geh* and *tst* genes were not detected. All strains showed the positive results for α- and β-haemolysin genes (*hla* and *hlb*), protease encoding genes (*sspA*, *sspB* and *aur*), *lukE* and *lukD* leukocidin genes (*lukED*). The strains were found to be non-biofilm formers. By this study, the virulence properties of the strains were described and this is one of the first reports regarding PVL-positive MSSA strains from food.

1. Introduction

Staphylococcus aureus is one of the main causative agents of life-threatening infections both in humans and animals. *S. aureus* produces more than 30 different extracellular products (Rogolsky, 1979) and some exoproteins that are responsible for particular clinical manifestations, including staphylococcal enterotoxins, the toxic-shock syndrome toxin-1, the exfoliative toxins and the Panton–Valentine Leukocidin (PVL) (Dinges et al., 2000). PVL is carried on a bacteriophage and is present only in <5% *S. aureus* isolates that is responsible for skin infections such as furunculosis and severe necrotising pneumonia (Gillet et al., 2002). Studies mostly focus on methicillin-resistant *S. aureus* (MRSA), due to its clinical importance. In fact, most Community-Acquired (CA)-MRSA clones contain PVL genes and the rates were 70% to 100% (Naimi et al., 2003; Shukla et al., 2004; Naas et al., 2005). Not only MRSA strains, but also MSSA strains, which were isolated from skin and soft-tissue infections, were found to carry PVL genes (Moran et al., 2006). The Oxford *Staphylococcus* and phage type 80/81 MSSA strains were main pandemic clones, harboring PVL genes, in the 1950s and 1960s (Robinson et al., 2005; Kearns et al., 2006). Recently, in some parts of Europe, PVL-positive MSSA strains have been associated with severe skin infections (Boubaker et al., 2004; Lesens et al., 2007; Wiese-Posselt et al., 2007; Tinelli et al., 2009).

Food has been implicated as a vehicle for the transmission of MRSA strains. Kluytmans et al. (1995) identified food-initiated MRSA outbreak in hospitalized patients. In addition, an outbreak caused by CA-MRSA contaminated food and food handler has been also reported (Jones et al., 2002). In fact, although the amounts are found in very low levels, MRSA has entered the food chain and it can cause severe infections, if food (e.g. meat) is not prepared properly before consumption (Van Loo et al., 2007).

In this study, we investigated the virulence properties (the presence of virulence factor genes, antibiotic susceptibilities and biofilm formation), clonal types, complete gene sequences of *lukSF*-PV and genetic relatedness of PVL-positive MSSA strains isolated from different food samples in Turkey.

2. Materials and methods

2.1. Isolation and strain identification

Two hundred nineteen *Staphylococcus* strains were isolated from 1209 different food samples and the isolates were identified by biochemical tests (Gram staining, catalase, coagulase and DNase production on DNase Agar (Oxoid)). Following the DNA extraction (Sudagidan et al., 2008), the presence of PVL-encoding gene (Lina et al., 1999), thermonuclease gene (*nuc*) (Sudagidan and Aydin, 2009), coagulase gene (*coa*) (Hookey et al., 1998) and methicillin resistance gene (*mecA*) (Lem et al., 2001) were searched by PCR. PVL-positive strains were also tested by GenoType® MRSA DNA-STRIP® test kit (Hain-Lifescience, Germany).

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2.2. Molecular typing

PVL-positive strains were characterized by partial 16S rRNA sequencing, Multi Locus Sequence Typing (MLST) (Enright et al., 2000) and *spa* typing (Aires-de-Sousa et al., 2006). Genetic relatedness of PVL-encoding strains was determined by PFGE-RFLP. Agarose plugs were prepared as described previously (Durmaz et al., 2007). Bacterial DNA in plugs was digested with 30 U *Sma*I (Fermentas) overnight and they were run in 1% (w/v) pulsed field certified agarose (Bio-Rad) with 5–40 s pulse time, 6 V/cm, 120° angle, at 14 °C for 22 h using CHEF-Mapper PFGE system (Bio-Rad). After electrophoresis, the gel was stained with 20 μg/ml ethidium bromide and visualized with VersaDoc 4000MP image analyzer system (Bio-Rad). The band patterns were analyzed and compared with Quantity One software (Bio-Rad).

2.3. Antibiotic susceptibility testing

Susceptibility testing was performed by disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI, 2006) for the following antibiotics: vancomycin, cefoxitin, oxacillin, penicillin G, gentamicin, erythromycin, rifampicin, linezolid, fusidic acid, cefazolin, levofloxacin, chloramphenicol, co-trimoxazole, teicoplanin, clindamycin, amoxycillin/clavulanic acid, ofloxacin, kanamycin, tobramycin, imipenem and tetracycline.

2.4. Virulence factor genes

The presence of staphylococcal enterotoxin genes *sea*, *seb*, *sec*, *sed*, *see* (Johnson et al., 1991), *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sep*, *seq* (Bania et al., 2006), *sem*, *sen*, *seo* (Jarraud et al., 2002) and *seu* (Nashev et al., 2007), exfoliative toxins (*eta* and *etb*) (Johnson et al., 1991), toxic-shock syndrome toxin (*tst*) (Booth et al., 2001), α- and β-haemolysins (*hla* and *hlb*) (Salasia et al., 2004), protease encoding genes (*sspA*, *sspB* and *aur*)(Karlsson and Arvidson, 2002), lipase encoding gene (*geh*) (Saïd-Salim et al., 2003), *lukE–lukD* leukocidin gene (Jarraud et al., 2002), and enterotoxin like protein-1 (*set*1)(Salasia et al., 2004) were searched by either monoplex or multiplex PCR. The PCR products were resolved in 1.5% (w/v) agarose gel electrophoresis in 1× TAE buffer (0.04 M Tris–acetate and 0.001 M EDTA). PCR experiments were done twice for each strain. In the detection of enterotoxin genes *sea* (*S. aureus* subsp. *aureus* NCTC (National Collection of Type Cultures) 10652), *seb* (*S. aureus* subsp. *aureus* NCTC 10654), *sec* (*S. aureus* subsp. *aureus* NCTC 10655), and *sed* (*S. aureus* subsp. *aureus* NCTC 10656) strains were used as positive controls. *Seg*, *sei*, *sej*, *seh* and *seo* positive *S. aureus* strains were obtained from the Department for Veterinary Public Health, Institute for Milk Hygiene, Milk Technology and Food Science, University of Veterinary Medicine, Vienna, Austria. *Tst*, *sem*, *sen*, *seo* and *seu* positive strain *S. aureus* N315 was kindly supplied by Dr. Teruyo Ito and Prof. Keiichi Hiramatsu from Juntendo University, Japan.

2.5. Biofilm formation

Biofilm formation of the strains was tested by microplate test adapted from Stepanovic et al. (2003). Four different media (Tryptic Soy Broth (TSB), TSB containing 1% (w/v) sucrose, Brain Heart Infusion broth (BHI) and Nutrient Broth (NB)) were used. Optical densities (OD) were determined at 590 nm by microplate reader (VarioScan Flash, Thermo) and OD > 0.5 values were accepted as biofilm forming bacteria. The test was repeated twice for each media and strain. In the biofilm test, strong biofilm forming *S. epidermidis* YT-169a strain was used as a positive control (Öztürk et al., 2007).

2.6. Sequencing of lukS-PV and lukF-PV genes

The complete DNA sequence of *lukS*-PV and *lukF*-PV genes was investigated using PCR primers described by Nakagawa et al. (2005). The following conditions were used for cycle sequencing of the genes: 1 min at 96 °C, 30 cycles of 10 s at 96 °C, 5 s at 50 °C (for *lukS*-forward), 5 s at 61 °C (for *lukS*-reverse), 5 s at 48 °C (*lukF*-forward), 5 s at 58 °C (*lukF*-reverse) and 4 min at 60 °C. The DNA sequencing was carried out using Applied Biosystems 3130*xl* Genetic Analyzer.

3. Results and discussion

3.1. Identification of the strains

We screened 219 *Staphylococcus* sp. isolated from food samples collected from different regions of Turkey. Only 3 strains showed positive results for PVL-encoding gene and they were identified as *S. aureus* by both biochemical and genotypic tests (*nuc* PCR, *coa* PCR and partial 16S rRNA sequencing) (The GenBank Accession numbers: FJ895583 (*S. aureus* strain M1-AAG42B), FJ907240 (*S. aureus* strain PY30C-b) and FJ907241 (*S. aureus* strain YF1B-b)). The presence of PVL gene and identification of *S. aureus* at the species level were also confirmed by GenoType® MRSA test kit (Hain-Lifescience, Germany).

3.2. Molecular typing of the strains

The strains were characterized by the methods (MLST and *spa* typing) based on DNA sequencing. MLST results were evaluated in the website (http://www.mlst.net), developed by David Aanensen and funded by the Wellcome Trust, and it showed that all strains were ST152 clonal type. After sequencing of *S. aureus* protein A gene of the strains, *spa* types were determined by the Ridom *spa* database (www.ridom.de/spaserver/) and it was noted as *spa* type t355 (Table 1). Furthermore, PFGE analysis was applied to the strains to determine the genetic relatedness. PFGE results indicated that all strains had the same band patterns (Fig. 1). Although they were isolated from different food samples (Table 1), all of the strains showed 100% homology. When PFGE band patterns of PVL-positive MSSA strains were compared with other 15 *S. aureus* food isolates from Kesan, the strains showed different band patterns (unpublished data). Therefore, these PVL-positive MSSA strains were considered as unique strains.

The distribution of *S. aureus* strains having ST152 clonal type varies. The first ST152 clonal type was observed in a PVL-positive CA-MRSA isolate in Europe (Müller-Premru et al., 2005). In another study, ST152 was found to be the second most common (after ST8) clonal type among PVL-positive MRSA isolates in Austria (Krziwanek et al., 2007). In addition, Monecke et al. (2007) reported a PVL-positive MRSA strain, isolated from an immigrant child from Macedonia, having ST152 clonal type and t355 *spa* type. Interestingly, not only PVL-positive MRSA isolates, but also PVL-positive MSSA isolates from different countries have been reported having ST152 clonal type. More recently, Pierre-Yves Donnio has reported in MLST.net two PVL-positive MSSA strains having ST152 and t355 clonal types which were isolated from France

Table 1
The sources and clonal types of PVL-positive MSSA strains.

S. aureus strain	Date of isolation	Location	Source	MLST	*spa* type
M1-AAG42B	April 19, 2004	Kesan—Edirne, Turkey	Pasta (makarna)	ST152	t355
PY30C-b	September 18, 2007	Kesan—Edirne, Turkey	Kaşar cheese	ST152	t355
YF1B-b	August 24, 2007	Kesan—Edirne, Turkey	Thin sheet of dough (yufka)	ST152	t355

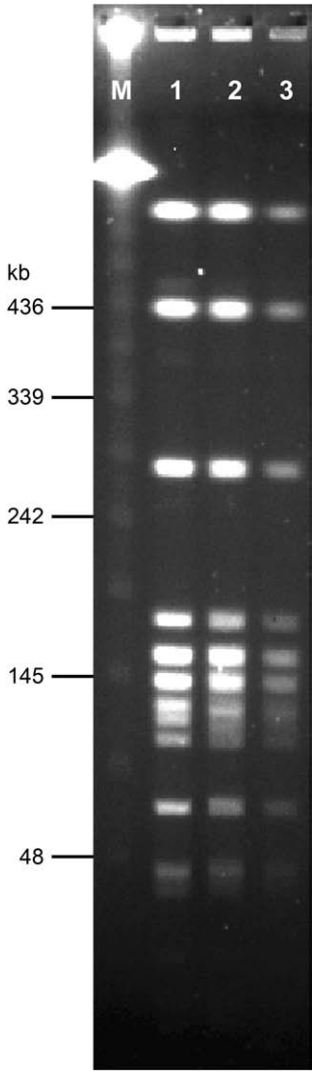

Fig. 1. PFGE analysis of PVL-positive MSSA strains. Lane M, Lambda ladder (Bio-Rad); Lane 1, *S. aureus* M1-AAG42B strain; Lane 2, *S. aureus* PY30C-b strain; Lane 3, *S. aureus* YF1B-b strain.

(unpublished data) and Algeria (Bekkhoucha et al., 2009) in clinical specimens. Moreover, Ruimy et al. (2008) reported 7 MSSA strains, which were nasal carriage isolates in Mali, showed ST152 and t355 clonal types. These results indicated that the distribution of ST152 clones is not restricted to Europe. Ruimy et al. (2008) have speculated that PVL-positive ST152 clones originated in Africa and have migrated through European countries and then acquired methicillin resistance. But, this suggestion is limited to the isolates from Mali and they do not represent the African population. The PFGE patterns may be compared to discriminate exact genetic relatedness of these strains from different countries.

3.3. Antibiotic susceptibilities of the strains

Antibiotic susceptibilities of PVL-positive strains were investigated by disc diffusion test. Each antibiotic was tested on separate Mueller–Hinton agar plates for each bacterium to determine exact zone diameter. All PVL-positive strains were determined as highly susceptible to all tested antibiotics and none of the strains were found to carry *mecA* gene. There are a limited number of studies for the examination of virulence properties and antibiotic susceptibilities of *S. aureus* food isolates in Turkey. Alisarli et al. (2003) searched the presence of *S. aureus* in 175 dairy desserts and they found that *S. aureus* was present in a total of 30 (17%) samples in Van—Turkey. Whereas, Gundogan et al. (2005) identified 80 *S. aureus* isolates in 150 meat and chicken samples in Ankara—Turkey and the overall methicillin-resistance rate for *S. aureus* was 67.5%. In another study, Gundogan et al. (2006) examined 180 samples of raw milk, pasteurised milk and ice cream from Ankara and 110 *S. aureus* were isolated. The methicillin resistance was 97.2% among *S. aureus* isolates. These data were obtained from two cities and it does not represent the whole of Turkey. Methicillin resistance in *S. aureus* food isolates is generally low. In another study, we searched methicillin resistance in 157 *S. aureus* food isolates and it was found that only one isolate was *mecA* gene positive and methicillin resistant (unpublished data).

Most of the PVL-positive CA-MRSA isolates are multidrug resistant strains and they carry the smallest SCC*mec* elements (either SCC*mec* type IV or SCC*mec* type V) (Tristan et al., 2007). In fact, the distribution of PVL-positive MRSA is worldwide. Due to intercontinental exchanges, several PVL-positive CA-MRSA clones (e.g., ST8 clone (USA300), ST1 clone (USA400), ST59 clone (USA1000), ST80 clone and ST30 clone) have spread to other continents which were previously present (Tristan et al., 2007).

3.4. Virulence factor gene contents of the strains

Interestingly, the strains did not contain important virulence factor genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *seu*, *eta*, *etb*, *set*1, *geh* and *tst* genes). However, they contained α- and β-haemolysin genes (*hla* and *hlb*), staphylococcal serine protease (V8 protease) gene (*sspA*), cysteine protease gene (*sspB*), metalloprotease (aurolysin) gene (*aur*) and *lukED* leukocidin gene. The presence of extracellular protease encoding genes might sign the infectious abilities of these strains in humans. The extracellular proteases could help the invasion of bacteria through tissues during infection process (Dubin, 2002). PVL toxins and extracellular proteases may co-operate for the progression of skin infections or necrotizing pneumonia. On the other hand, the presence of *lukE–lukD* genes in CA-MRSA isolates is common. Dufour et al. (2002) reported that approximately two-thirds of their CA-MRSA strains carry *lukED* genes that are not associated with a specific type of infection. In addition, unlike PVL genes, *lukE–lukD* leukocidin genes were found to be present in most CA-MRSA as well as HA-MRSA strains (Vandenesch et al., 2003).

3.5. Biofilm forming abilities of the strains

Biofilm formation is also accepted as a virulence factor in staphylococci. Our PVL-positive MSSA strains did not form biofilm in all tested media (TSB, TSB + 1% (w/v) sucrose, BHI and NB). We used different media to test biofilm forming ability of the strains, because the media and other environmental factors (temperature, pH, presence of CO_2 or O_2 in environment) were found to affect biofilm formation (Stepanovic et al., 2003; Fitzpatrick et al., 2005).

3.6. Gene sequences of lukS-PV and lukF-PV

The complete DNA sequencing of *lukS* and *lukF* genes revealed that there were some nucleotide changes and an amino acid change in the strains with respect to other reported PVL gene sequences (The GenBank Accession numbers: BA000033, CP000255, AB295470, AB295471, AB295472, AB303645, AB303646, and AB295473) (Takano et al., 2008). All our strains showed the same DNA sequences (The GenBank Accession numbers: FJ895584 (*S. aureus* strain M1-AAG42B), FJ895585 (*S. aureus* strain PY30C-b) and FJ895586 (*S. aureus* strain YF1B-b)). In *lukS*-PV gene at 527th nucleotide is G instead of A and this change leads to an amino acid change from CAT → histidine to CGT → arginine. In addition, there are some non-sense nucleotide differences at 663th nucleotide (T) of *lukS*-PV, at 1396th nucleotide (A) and at 1729th nucleotide (G) of *lukF*-PV. The amino acid and nucleotide differences were consisted with the previously

reported data and *lukSF* gene sequences (Takano et al., 2008). However, these nucleotide differences and amino acid changes have been identified in MRSA clones from USA and Taiwan. Furthermore, Takano et al. (2008) suggested that the amino acid changes in *lukS* region may determine host or tissue specificity of S-protein for binding. The effects of these changes on expression of PVL genes or production of potent PVL toxins have not been known. PVL destruction of immune cells and lung tissues was shown in *in vitro* and animal studies (Gillet et al., 2002). Its disease progression in humans remains to be defined (Takano et al., 2008).

In summary, this is, to our knowledge, one of the first reports related to PVL-positive MSSA strains isolated from food. Although all three *S. aureus* strains were isolated from different foods and at different dates, they showed the same results for all tests (Table 1). These results indicate that they were isolated from the same area (Kesan—Edirne) and these strains might be present locally. Furthermore, our PVL-positive MSSA strains may not have come across with any antibiotics before. Therefore, our 3 PVL-positive MSSA strains are highly susceptible to all tested antibiotics and these strains might be present in food that has not been contaminated by humans or animals. The cross-contamination among food is one of the possibilities for spreading these strains. However, other *S. aureus* strains which were isolated from the same food samples were negative for PVL genes. At this point, the sensitivity of these strains against phage infection should be elucidated. It has been suggested previously (Boyle-Vavra and Daum, 2007) to state whether PVL-positive MSSA strains are ancestors or descendants of the most prevalent PVL-positive MRSA strains is questionable. A particular attention should be applied to *S. aureus* strains from food and environmental samples in the presence of PVL genes as well as clinical MRSA and MSSA strains.

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