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Determination of some virulence factors in staphylococci isolated from milk and meat products

Bestimmung von Virulenzfaktoren aus Staphylokokken-Isolaten von Milch- und Fleischprodukten

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Summary

The aim of this work is to characterize *Staphylococcus* spp. isolated from meat and milk products in Ankara, Turkey. A total of 232 isolates were isolated from raw calf meat (minced), chicken drumsticks, raw milk, ice cream, and white cheese samples. The isolates were identified as *S. aureus* (10.3 %), *S. intermedius* (7.8 %), *S. hyicus* (1.7 %), *S. cohnii* (23.2 %), *S. hominis* (18.1 %), *S. xylosus* (8.1 %), *S. simulans* (7.8 %), *S. capitis* (6 %), *S. epidermidis* (5.6 %), *S. haemolyticus* (4.3 %), *S. auricularis* (3.9 %), *S. warneri* (2.1 %) and *S. saprophyticus* (0.9 %). A high proportion of *Staphylococcus* isolates had virulence factors such as slime formation (42.7 %), biofilm formation (35.3 %), DNase activity (32.8 %) and hemolytic activity (31 %). Our findings indicate that meat, milk and their products represent potential hazardous sources of virulent *Staphylococcus* species.

Keywords: Staphylococcus, virulence factors

Zusammenfassung

Ziel der vorliegenden Arbeit war es, *Staphylococcus* spp. von Fleisch- und Milchprodukten aus Ankara (Türkei) zu isolieren und zu charakterisieren. Insgesamt 232 Isolate wurden aus rohem Kalbfleisch (fein zerkleinert), Hähnchenkeulen, Rohmilch, Speiseeis und weißem Käse isoliert. Die Isolate wurden als *S. aureus* (10,3 %), *S. intermedius* (7,8 %), *S. hyicus* (1,7 %), *S. cohnii* (23,2 %), *S. hominis* (18,1 %), *S. xylosus* (8,1 %), *S. simulans* (7,8 %), *S. capitis* (6 %), *S. epidermidis* (5,6 %), *S. haemolyticus* (4,3 %), *S. auricularis* (3,9 %), *S. warneri* (2,1 %) und *S. saprophyticus* (0,9 %) identifiziert. Ein hoher Anteil der *Staphylococcus*-Isolate zeigte Virulenzfaktoren wie Schleimbildung (42,7 %), die Bildung von Biofilmen (35,3 %), DNase-Aktivität (32,8 %) und hämolytische Aktivität (31 %). Unsere Ergebnisse zeigen, dass Fleisch, Milch und daraus hergestellte Produkte eine potenzielle Gefahrenquelle von virulenten *Staphylococcus*-Arten darstellen.

Schlüsselwörter: Staphylokokken, Virulenzfaktoren

Introduction

Staphylococci are ubiquitous bacteria reported as part of the normal microbiota of air, soil, water, humans and animals, but are also isolated from a wide range of foodstuffs such as meat, cheese and milk (Irlinger, 2008). *Staphylococcus aureus* is a common pathogen associated with serious community and hospital acquired diseases and has for long been considered as a major problem of public health. Some strains of this organism can cause food-poisoning by production of enterotoxins when growing in foods (Pereira et al., 2009).

Coagulase-negative staphylococci (CNS) are often present in food related samples and especially in fermented products (Irlinger, 2008). Although CNS are generally considered as having a positive role in the production of some fermented food (Irlinger, 2008), the identification of certain risk factors in some CNS strains, as well as the existence of nosocomial and urinary tract infections related to *Staphylococcus epidermidis* and *S. saprophyticus,* have raised questions about the presumption of safety of the species belonging to this group (Zell et al., 2008).

S. aureus has the ability to produce several exoenzymes that contribute to virulence (Vasudevan et al., 2003). The virulence factors include surface proteins that promote colonization of host tissues, invasions that promote bacterial spread in tissues (leukocidin, hyaluronidase), surface factors that inhibit phagocytic engulfment (capsule), biochemical properties that enhance their survival in phagocytes (catalase production), and membrane-damaging toxins that lyse eukaryotic cell membranes (hemolysins, leukotoxin). Similar activities have been observed in different species of CNS (Vasudevan et al., 2003; Zell et al., 2008).

The detection of virulence factors is useful in determining the significance of isolates. The clinical significance of staphylococci has been well established, but their potential as a public health problem in food needs more attention.

The purpose of the present study was to investigate the production of DNase, slime, biofilm and hemolysins of staphylococci isolated from meat, milk, and their product.

Materials and Methods

Sample collection and preparation

A total of 232 staphylococci isolates isolated from 100 raw calf meat (minced), chicken drumsticks, raw milk, ice cream, and white cheese samples. The samples were purchased from various supermarkets, dairy plants and pastry shops, in Ankara, Turkey, between June 2009 and August 2010. White cheese and meat samples were collected in sterile polyethylene packs and milk samples were collected in disposable plastic bottles, transported on ice to the laboratory, and analyzed within 2 h. Ice cream samples were collected in sterile jars and transported to the laboratory in a deep freezer and stored at –18 °C. The samples were kept at 4 °C for 10 min before microbiological analyses.

Isolation and identification of isolates

Food samples of 25 g or 25 ml were diluted with 225 ml of 1 % buffered peptone water (BPW; Oxoid, Basingstoke, Hampshire, UK) and homogenized in a stomacher (Lab. Lemco 400, Seward, Worthington, UK) for about 10 min. From each prepared sample, 0.1 ml was streaked on to 5 % sheep blood agar and incubated aerobically at 37 °C for 48 h. After incubation, suspect colonies were examined by Gram staining. The colonies with morphologies compatible with *Staphylococcus* spp. were transferred to tryptic soy broth (TSB; Oxoid, Basingstoke, Hampshire, UK) and tryptic soy agar (TSA; Oxoid, Basingstoke, Hampshire, UK). After growth, *Staphylococcus* spp. were identified on the basis of colony characteristics, Gram staining, pigment production, hemolysis and the following biochemical reactions: catalase activity, coagulase test (rabbit plasma), oxidase test, O/F test with glucose, resistance to bacitracin (0.04 U), mannitol fermentation on Chapman Agar, urease, nitrate reduction, novobiocin resistance, phosphatase, deoxyribonuclease (DNase) test and carbohydrate (xylose, sucrose, trehalose, maltose, fructose, lactose, mannose) fermentation tests (Murray et al., 2003). The API ID Staph (Bio Merieux SA, Marcy-I'Etoile, France) was used to determine the species more precisely. The isolates were kept frozen at –20 °C in skim milk containing 15 % (v/v) glycerol, until assayed. The *S. aureus* ATCC 25923 was used as a control strain.

Determination of some virulence factors

a. Coagulase Test

Coagulase activity was determined by the method described by Quinn et al., (1994). This test was performed as a Tube Coagulase (TC) test. Several colonies of each organism were mixed with 0.5 ml of citrated rabbit plasma in a sterile test tube. The tube was incubated at 37 °C and examined after 4 and 24 h. Clot formation at either reading was recorded as positive.

b. Determination of slime formation by the Congo Red Agar method (CRA)

Production of slime from all isolates was studied by cultivation of the isolates on Congo Red Agar (CRA). CRA plates (sucrose 50 g (Sigma, St. Louis, MO), brain heart infusion broth 37 g (Oxoid, Basingstoke, Hampshire, UK), agar 10 g, congo red 0.8 g (Sigma, St. Louis, MO), distilled water 1000 ml) were incubated at 37 °C for 24 h. After incubation, bright black colonies were established as slime positive (Gundogan et al., 2006).

c. Determination of quantitative biofilm formation by the microplate method (MP)

Biofilm-forming ability was measured by determination of adhesion to polystyrene microtiter plates according to the protocol of Christensen et al., (1985). Briefly, isolates were inoculated in TSB (tryptic soya broth, Oxoid) and incubated for 18 h at 37 °C. After a 1:40 dilution in TSB supplemented with 0.25 % glucose, 200 µl of each dilution were distributed in flat-bottom 96-well polystyrene plates (Oxyvital, Hong Kong, China). The plates were incubated for 18 h at 37 °C, washed 3x with phosphate buffer saline (PBS), pH 7.0, air-dried for 1 h at 60 °C and stained with 0.25 % crystal violet for 1 min. After washing, optical density (OD) of each well content was measured at 570 nm using an automated Multiskan reader (GIO. De Vitae, Rome, Italy). We defined the cut-off OD (OD_c) for the microtiter-plate test as three standard deviations above the mean OD of the negative control. The adherence ability of the tested strains was classified into four categories based on the OD: "$OD \leq OD_c$: non–adherent, $OD_c < OD \leq 2xOD_c$: weakly adherent, $2xOD_c < OD \leq 4xOD_c$: moderately adherent, $4xOD_c < OD$: strongly adherent". All tests were carried out three times and the results were averaged.

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d. DNase test

DNase Agar (Oxoid, Basingstoke, Hampshire, UK) was used to determine DNase activity. *Staphylococcus* isolates were incubated on DNase agar at 35 °C for 18 h. After overnight incubation, 1 N HCI was poured on the plates and colonies with clear color were considered DNase positive (Gundogan et al., 2006).

e. Hemolysin production

Hemolysin activity was determined on Blood Agar Base (BAB; Oxoid, Basingstoke, Hampshire, UK) containing 5 % defibrinised sheep blood. After incubation for 48 h at 37 °C, the presence or absence of zones of clearing around the colonies was interpreted as β-hemolysis (positive hemolytic activity or γ-hemolysis (negative haemolytic activity), respectively. When observed, greenish zones around the colonies were interpreted as α-hemolysis and taken as negative for the assessment of hemolytic activity (Barbosa et al., 2010).

TABLE 1: *Origin of isolates and species distribution of the staphylococci.*

Species	Ice cream	Raw milk	White cheese	Calf meat	Chiken meat	Total	%
S. aureus	–	9	9	6	–	24	10.3
S. intermedius	–	8	3	3	4	18	7.8
S. hyicus	–	4	–	–	–	4	1.7
S. cohnii	30	4	12	2	6	54	23.3
S. hominis	11	11	7	6	7	42	18.1
S. xylosus	–	–	–	19	–	19	8.2
S. simulans	1	5	2	–	10	18	7.8
S. capitis	2	1	–	8	3	14	6.0
S. epidermidis	4	4	–	2	3	13	5.6
S. hemolyticus	2	3	5	–	–	10	4.3
S. auricularis	–	3	4	–	2	9	3.9
S. warneri	4	–	1	–	–	5	2.1
S. saprophyticus	2	–	–	–	–	2	0.9
Total	56	52	43	41	40	232	100

Statistical analysis

The Chi-square (χ^2) tests were used to determine statistically significant differences in the prevalence of CPS and CNS in food samples. Significant differences between CPS, CNS and virulence factors were also determined. P values of less than 0.05 were considered significant.

Results

Isolation and identification of isolates

Origin of isolates and species distribution of the staphylococci are given in Table 1.

Out of the total 232 staphylococci isolates, 46 (19.8 %) isolates were identified as coagulase positive staphylococci (CPS), whereas 186 (80.2 %) isolates were coagulase negative staphylococci (CNS) by the tube coagulase test.

The 46 CPS isolates were identified as *S. aureus* (10.3 %), *S. intermedius* (7.8 %) and *S. hyicus* (1.7 %). The 186 CNS isolates were identified as *S. cohnii* (23.3 %), *S. hominis* (18.1 %), *S. xylosus* (8.2 %), *S. simulans* (7.8 %), *S. capitis* (6 %), *S. epidermidis* (5.6 %), *S. haemolyticus* (4.3 %), *S. auricularis* (3.9 %), *S. warneri* (2.1 %) and *S. saprophyticus* (0.9 %). Present study showed that the prevalence of CNS species in food samples is significantly higher than those CPS species ($\rho < 0.05$).

Virulence factors

The production rate of DNase, slime, biofilm and hemolysin of *Staphylococcus* species is shown in Table 2. The frequency of positive DNase tests, slime and biofilm formation and hemolysis for CPS strains were 60.9 %, 52.2 %, 32.6 %, 47.8 %, and for CNS strains were 25.8 %, 40.3 %, 36 %, 26.9 %, respectively.

The statistical analyses revealed that the prevalence of DNase activity and β-hemolysin production in CPS was significantly higher than those CNS ($\rho < 0.05$). On the other hand, the prevalence of slime and biofilm production was not significantly different ($\rho > 0.05$).

Much higher numbers of *S. aureus* (87.5 %), *S. hyicus* (75 %) and *S. warneri* (60 %) strains had DNase activity. DNase activity was also found in *S. xylosus* (42.1 %), *S. haemolyticus* (30 %), *S. hominis* (26.1 %), *S. cohnii* (26 %), *S. intermedius* (22.2 %), *S. auricularis* (22.2 %), *S. simulans* (16.7 %), *S. epidermidis* (15.4 %) and *S. capitis* (14.3 %) strains, but none of the *S. saprophyticus* strains has DNase activity.

β-hemolytic activity was mostly found in *S. aureus* (75 %) followed by *S. cohnii* (59.3 %). β-hemolytic activity

TABLE 2: *The production of DNase, slime, biofilm and hemolysins among Staphylococcus species.*

Species		DNase		Slime formation		Biofilm formation		β-Hemolysis	
		n	(%)	n	(%)	n	(%)	n	(%)
S. aureus	(24)	21	(87.5)	17	(70.8)	11	(52.3)	18	(75)
S. intermedius	(18)	4	(22.2)	4	(22.2)	3	(16.7)	4	(22.2)
S. hyicus	(4)	3	(75)	3	(75)	1	(25)	–	–
S. cohnii	(54)	14	(26)	26	(48.1)	29	(53.7)	32	(59.3)
S. hominis	(42)	11	(26.1)	20	(47.6)	18	(42.9)	7	(16.7)
S. xylosus	(19)	8	(42.1)	6	(31.6)	4	(21)	–	–
S. simulans	(18)	3	(16.7)	6	(33.3)	6	(33.3)	–	–
S. capitis	(14)	2	(14.3)	3	(21.4)	4	(28.6)	4	(28.6)
S. epidermidis	(13)	2	(15.4)	4	(30.8)	4	(30.8)	5	(38.5)
S. hemolyticus	(10)	3	(30)	4	(40)	2	(20)	2	(20)
S. auricularis	(9)	2	(22.2)	1	(11.1)	–	–	–	–
S. warneri	(5)	3	(60)	5	(100)	–	–	–	–
S. saprophyticus	(2)	–			–	–	–	–	–
Total	(232)	76	(32.8)	99	(42.7)	82	(35.3)	72	(31)

of *S. epidermidis* (38.5 %), *S. capitis* (28.6 %), *S. intermedius* (22.2 %), *S. haemolyticus* (20 %) and *S. hominis* (16.7 %) were found in much lower incidence. None of the *S. hyicus, S. xylosus, S. simulans, S. auricularis, S. warneri* and *S. saprophyticus* strains had hemolytic activity.

Slime was produced by all of the *S. warneri* strains and 70.8 % of the *S. aureus* and 75 % of the *S. hyicus* strains produced slime. The slime production was also detected in 48.1 % *S. cohnii,* 47.6 % *S. hominis,* 40 % *S. haemolyticus,* 33.3 % *S. simulans,* 31.6 % *S. ylosus,* 30.8 % *S. epidermidis, S. intermedius* (22.2 %), *S. capitis* (21.4 %) and *S. auricularis* (11.1 %) strains, but none of the *S. saprophyticus* strains.

In this study, biofilm producers were divided into three groups, namely strong, moderate, and weak biofilm producers. Of the 82 isolates, 35 (42.7 %) were assessed as weak, 32 (39.0 %) as moderate, 15 (18.3 %) as strong biofilm producers (data not shown). *S. cohnii* was the highest biofilm-producing strain (52.3 %), followed by *S. aureus* (52.3 %), *S. hominis* (42.9 %), *S. simulans* (33.3 %), *S. epidermidis* (30.8 %), *S. capitis* (28.6 %), *S. hyicus* (25 %), *S. xylosus* (21 %), *S. haemolyticus* (20 %) and *S. intermedius* (16.7 %). None of the *S. auricularis, S. warneri* and *S. saprophyticus* strains had biofilm formation.

Discussion

Our results show that most of the *Staphylococcus* species have the ability to produce exoenzymes which are known to contribute to virulence (Vasudevan et al., 2003).

DNase activity is important to distinguish between pathogenic staphylococci and nonpathogenic resident flora. DNase is as important as coagulase for pathogenesis (Pfaller and Herwaldt, 1988). In the study of Devriese and Oeding (1975), it was found that there was a strong association of DNase and coagulase with *S. aureus.* Both of these tests were positive 96 % of all cases. In this study, the frequency of positive DNase test was 60.9 % for CPS and 87.5 % for *S. aureus.* Similarly, Citak et al. (2003) and Gundogan et al. (2006) found that 93.6 % and 94.5 %, respectively, of *S. aureus* isolated from food samples had DNase activity. We found that 25.8 % of the CNS isolates were DNase producers. However, Citak et al., (2003) reported that only 15 (10.2 %) of 147 CNS isolated from raw milk had DNase activity. Several studies have also found DNase producers among animal and human clinical CNS isolates (Turkyilmaz and Kaya, 2006; Cunha et al., 2006). This enzyme is considered a virulence factor because of its ability to break down DNA.

Hemolysin plays an important role in staphylococcal virulence, as it may increase the possibility of the occurence of the infection (Ike et al., 1987). Hemolysins of pathogenic microorganisms have been shown to have potent toxic effect on lymphocytes, macrophages, neutrophils, epithelial cells, fibroblasts and other cell lines (Ali-Vehmas et al., 2001). The results concerning hemolysins by staphylococcal food isolates were not found in the literature. In the present study, it was determined that 47.8 % of CPS and 26.9 % of CNS isolates have beta hemolysis. These values were much lower than the 100 % hemolysin incidence of *S. aureus* and CNS isolated from clinical and subclinical mastitis cows reported by Ebrahimi et al., (2009). Similar to our results, Turkyilmaz and Kaya (2006) reported that 58.9 % of CPS and 28.9 % of CNS isolates isolated from animal clinical isolates had hemolysis characteristics. The incidence of hemolysin production among food *S. aureus* isolates in our study (75.0 %) seems to be much higher than that reported for clinical strains by Ali-Vehmas et al., (2001) (24 %). However, our results suggest that staphylococcal isolates isolated from foods had β-hemolysis and that this trait is not exclusive to clinical isolates.

Slime production may reflect the microorganisms capacity to adhere to specific host tissues and thereby to produce invasive microcolonies (Schlegelova et al., 2008). In our study, the rate of CRA and MP methods positiveness was for CPS 52.1 % and 32.6 % and for CNS 40.3 % and 36.0 %, respectively. Ciftci et al., (2009) found that only 22 of 59 (37.2 %) of *S. aureus* isolated from mastitic milk samples were slime producing. On the other hand, Vasudevan et al., (2003) found that 32 (91.4 %) of 35 *S. aureus* isolated from bovine mastitis were slime positive. Other investigators suggest that CNS (42.2 %) isolates produce a slime more often than *S. aureus* (5.1 %) (Citak et al., 2003). In this study, 39.0 % and 18.3 % of staphylococcus isolates were classified as moderate and strong biofilm producers, respectively. Marino et al., (2010) reported that only 19.9 % of the *S. aureus, S. epidermidis* and *S. pasteuri* strains had moderate to strong biofilm formation on the polystyrene microtitre plates. The results of our study do not confirm these findings. However, some of the previous studies have shown that the nutrient content of the growth medium influences slime/biofilm development (Vasudevan et al., 2003; Zell et al., 2008). In food processing environments, biofilm formation by staphylococci, which could carry virulence factors and even resistances to several antibiotics, is a matter of concern.

Human nares and fingers are the main sources of *S. aureus.* Considering that CNS inhabit at the human skin and mucous membranes, it is obvious that these microorganisms can contaminate food if not handled properly (Udo et al., 1999). Altay et al., (2003) reported that 44 CPS (30 *S. aureus,* 9 *S. delphini,* 3 *S. intermedius,* 1 *S. schleiferi,* 1 *S. hyicus*) and 74 CNS (16 *S. simulans,* 11 *S. hyicus,* 8 *S. saprophyticus,* 6 *S. epidermidis,* 4 *S. arlettae,* 4 *S. lentus,* 4 *S. gallinarum,* 3 *S. chromogenes,* 3. *S. warneri,* 3 *S. haemolyticus,* 2 *S. caprae,* 2 *S. auricularis,* 2 *S. xylosus,* 2 *S. cohnii*) isolates isolated from poultry. In this study, similar strains were identified. On the other hand, the incidence of *S. aureus* (10.3 %) in the present study was much lower than the rates of 52.2 % reported by Schlegelova et al., (2008), 60 % by Da Silva et al., (2004), 53.3 %–61.1 % by Gundogan et al., (2005, 2006), 47.2 % by Citak and Duman (2011) as well as 33.4 % by Guven et al., (2010), but higher than 6 % reported by Can and Celik (2012) and 6.7 % by Cepoglu et al., (2010).

Nevertheless, the results of the present study revealed that the majority of the staphylococcal species isolated from foods had virulence factors and might have an important role in the pathogenesis of infections. In combination with proper hygiene and production methods may prevent further proliferation of these bacteria in our foods.

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