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Safety hazards in bacteriocinogenic *Staphylococcus* strains isolated from goat and sheep milk

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ABSTRACT

In this study, 28 bacteriocinogenic *Staphylococcus* strains isolated from goat and sheep milk were subjected to the PCR detection of enterotoxin genes (*sea-see*), enterotoxin-like toxin Q gene (*selq*), toxic shock syndrome toxin gene (*tst1*), and antibiotic resistance genes. They were also evaluated for phenotypic resistance against 10 antibiotics and hemolytic activity. The tyramine and histamine production was investigated using the agar plate assay and capillary zone electrophoretic analysis (CZE). Twenty-five isolates harbored at least one enterotoxin gene. The gene *sec* was the most frequent (89%). The gene *tst1* was found in 84% of *sec*-positive isolates. The occurrence of antibiotic resistance genes was in the order of *blaZ/tetK* (100%), *mecA/ermB* (86%), *ermC* (50%), and *tetM* (18%). The genes *ermA*, *aac(6′)Ie-aph(2″)Ia*, *vanA*, and *vanB* were absent in all the isolates. Nineteen isolates were phenotypically susceptible to all the antibiotics. The only isolate with phenotypic resistance to penicillin G and oxacillin was *S. epidermidis* 4S93 which had a different *Sma*I-PFGE profile from those of the other *S. epidermidis* strains. All the *S. haemolyticus* and *S. pseudintermedius* isolates were not susceptible to trimethoprim. Twenty-five isolates showed complete or partial hemolytic activity. None of the isolates was able to decarboxylate tyrosine, while CZE analysis revealed histamine formation activity in *S. haemolyticus* 4S12. The occurrence of safety risks in the isolates reinforces the need for regular monitoring of food-producing animals to mitigate the risks of multidrug resistant and zoonotic pathogens. Moreover, none of the isolates fulfilled the safety criteria to be used as starter cultures or biopreservatives.

1. Introduction

Staphylococci are widespread in the environment, found mainly on the skin, skin glands, and mucous membranes of warm-blooded animals and humans. As a consequence, they have been frequently isolated from foods of animal origin including raw milk, cheese, and fermented meat [1,2]. Besides direct contact with livestock, these products are, therefore, potential sources for transmission of staphylococci to humans [3]. It is important from the outset that most *Staphylococcus* species are considered as opportunistic pathogens causing a variety of diseases in both humans and animals ranging from relatively benign food poisoning to life-threatening toxic shock syndrome [4]. The pathogenicity of this genus is conferred by virulence genes encoding extracellular proteins (enterotoxins, toxic shock syndrome toxin-1 [TSST-1], nucleases, hemolysins, etc.), antibiotic resistance, and biogenic amine production [4,5].

The antimicrobial peptides referred to as “bacteriocins” are the other metabolites produced by some *Staphylococcus* strains. These agents have attracted considerable attention in recent years due to their potential application in medicine and food preservation; furthermore, the bacteriocin-producing strains have the potential to be used as starter cultures if they meet certain requirements such as not posing a safety risk to the consumers. However, it is noteworthy to mention that bacteriocins are, first of all, bacterial weapons against competitive microorganisms and can, therefore, increase the virulence potential of the producing strains [6]. From both points of view, safety assessment of bacteriocin-producing strains of food origin is of great significance. The most important concern is the presence of transmissible antibiotic resistance markers which could in turn contribute to resistance dissemination [2,7]. The present study, therefore, aimed to assess the pathogenic properties of 28 bacteriocinogenic *Staphylococcus* isolates using both phenotypic and molecular methods. The isolates were investigated in terms of: (1) the presence of virulence factors, (2) the potential to form biogenic amines, and (3) their phenotypic and

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genotypic antibiotic resistance profiles.

2. Materials and methods

2.1. Bacterial strains

In the previous research, 243 staphylococci were isolated from 110 raw milk samples of goat and sheep herds (n = 50) located in Fars province, Iran. The samples were derived from tribal (regions 1, 2, and 3) or rural (region 4) areas. The tribal herds grazed either on annual pastures or on perennial dwarf shrub lands far from human activities. In the rural area, animals grazed on farms or pastures contaminated with manure, untreated sewage, or wastes from residential houses. The isolates were screened for antimicrobial activity using an agar spot test method. In total, 28 isolates exhibited antagonistic activity against the indicator strain, *Micrococcus luteus* ATCC 4698, which is highly sensitive to the bacteriocins [8]. The susceptibility of antimicrobial agents to proteolytic enzymes confirmed their proteinaceous nature, and they could therefore be referred to as bacteriocin-like inhibitory substances. Furthermore, all bacteriocin-producing strains exhibited immunity to their own antimicrobial compounds; this feature differentiates bacteriocins from peptide antibiotics [9]. These 28 isolates were then identified to the species level by sequencing 16S rRNA and/or RNA polymerase B (*rpoB*) genes. They belonged to the species *S. chromogenes* (n = 11), *S. epidermidis* (n = 7), *S. haemolyticus* (n = 6), *S. pseudintermedius* (n = 2), *S. aureus* (n = 1), and *S. agnetis* (n = 1). Moreover, the genetic diversity within each species was determined by Pulse-Field Gel Electrophoresis (PFGE) genotyping [unpublished data]. Stock cultures of the isolates were stored at −80 °C in brain-heart infusion (BHI) broth supplemented with 30% (v/v) glycerol. Prior to each experiment, the isolates were reactivated in BHI broth at 37 °C for 24–48 h.

2.2. DNA extraction

Total DNA was extracted from a 24-h BHI broth culture. In brief, the cells were harvested by centrifugation (10000 × *g* for 5 min), washed by distilled water, and then resuspended in 600 μL of TE buffer (10 m*M* Tris, 1 m*M* EDTA, pH 8.0). The pellet was finally subjected to DNA isolation as described by Fontana et al. [10]. The total DNA recovered was quantified using a NanoDrop Lite Spectrophotometer (Thermo Scientific, Wiltham, USA).

2.3. Superantigenic toxin gene profiles

The isolates were examined for the presence of several staphylococcal virulence genes including classical enterotoxin genes *sea*, *seb*, *sec*, *sed*, *see* Ref. [11], enterotoxin-like toxin Q gene (*selq*), and TSST-1 gene (*tst1*). Primer pairs, PCR conditions, and amplicon sizes are listed in Table 1. For *selq*, or *tst1* detection, the monoplex PCR assay was conducted in a reaction volume of 25 μL containing 12.5 μL of Taq DNA Polymerase 2.0 × Master Mix RED (1.5 m*M* MgCl2; Ampliqon, Copenhagen, Denmark), 0.4 μM of each primer, and 50–100 ng DNA template. The remaining genes were co-amplified by multiplex PCR according to Omoe et al. [12]. *S. aureus* DSM 19040 (*sec*, *see*, and *tst1*), and *S. aureus* DSM 19041 (*sea*, *seb*, and *sed*) were used as positive control strains.

2.4. Hemolytic activity

Hemolytic activity was tested on 5% sheep blood agar after 48 h of incubation at 37 °C. The hemolysis activity was then categorized as β-hemolysis (clear halos around colonies), α-hemolysis (greenish halos around the colonies), and γ-hemolysis (absence of hemolysis) [13].

2.5. Biogenic amine (tyramine and histamine) production

The ability to produce biogenic amines was evaluated in the decarboxylase agar medium containing tyrosine or histidine as precursors [14]. *Enterococcus faecalis* E37 was used as a positive control of tyramine production.

Histamine production was further assessed by capillary zone electrophoretic analysis (CZE). In brief, a cell pellet from an overnight culture of each isolate was washed with phosphate buffer (0.1 *M*; pH 6.0), and inoculated into tryptic soy broth (TSB) supplemented with 0.5 m*M* histidine. Following 24 h incubation at 37 °C, the supernatant was collected (10,000 × *g* for 5 min at 4 °C), and then filtered through a 0.45 μm filter. A similar process was performed for histidine-supplemented medium without bacterial culture as a negative control. *S. epidermidis* TYH1 was used as positive control strain.

The sample preparation for CZE analysis was according to the method described by Numanoğlu et al. [15] with some modifications. In detail, 1 mL of the prepared supernatant was transferred into a centrifuge tube containing 2 mL of 0.1 *M* HCl. Following vortex mixing for 2 min, the mixture was centrifuged at 10000 × *g* for 5 min. The supernatant was then filtered through a Whatman No.1 filter paper. The procedure was repeated on the centrifugation sediment. The supernatants were combined, made up to 4 mL by the addition of 0.1 *M* HCl, and finally filtered through a 0.45 μm filter. The modified method of Numanoğlu et al. [15] was applied to the quantification of histamine content of the mixture. Analysis was performed using a Prince autosampler model 1-LIFT (Prince Technologies, Emmen, The Netherlands). The sample injections were made hydrodynamically at 50 mbar for 3 s.

Table 1
The primers used for detection of staphylococcal virulence genes [12].

PCR set	Target gene	Primer sequence (5′→3′)	PCR product (bp)
Multiplex I[a]	*sea*	CCTTTGGAAACGGTTAAAACG TCTGAACCTTCCCATCAAAAAC	127
Multiplex I	*seb*	TCGCATCAAACTGACAAACG GCAGGTACTCTATAAGTGCCTGC	477
Multiplex I	*sec*	CTCAAGAACTAGACATAAAAGCTAGG TCAAAATCGGATTAACATTATCC	271
Multiplex I	*sed*	CTAGTTTGGTAATATCTCCTTTAAACG TTAATGCTATATCTTATAGGGTAAACATC	319
Multiplex I	*see*	CAGTACCTATAGATAAAGTTAAAACAAGC TAACTTACCGTGGACCCTTC	178
Monoplex II[b]	*selq*	AATCTCTGGGTCAATGGTAAGC TTGTATTCGTTTTGTAGGTATTTTCG	122
Monoplex III[b]	*tst1*	AAGCCCTTTGTTGCTTGCG ATCGAACTTTGGCCCATACTTT	447

[a] Initial denaturation of 15 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 90 s at 59 °C, and 90 s at 72 °C, and a final elongation of 10 min at 72 °C.
[b] Initial denaturation of 15 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 90 s at 57 °C, and 90 s at 72 °C, and a final elongation of 10 min at 72 °C.