The role of staphylococci in subclinical mastitis of cows and lytic phage isolation against Staphylococcus aureus

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Abstract

Aim: This study was conducted to determine the role of Staphylococcus in the formation of subclinical mastitis in cows and to isolate the phage against isolated Staphylococcus aureus strains.

Materials and Methods: In this study, 400 milk cows were screened by California Mastitis Test (CMT) for subclinical mastitis and 235 udders of 96 cows, which were determined to be positive, were evaluated for Staphylococcus. Milk samples were evaluated using conventional and molecular methods. In addition, phage isolation studies were performed against S. aureus strains causing mastitis.

Results: At the result of cultural examination, of 235 milk samples that were found as positive for mastitis by CMT, a total of 117 (49.7%) Staphylococcus spp. were isolated as a distribution of 74 (63.24%) coagulase-positive staphylococci and 43 (36.75%) coagulase-negative staphylococci. Of these isolates, 76 (64.95%) were characterized as S. aureus both conventional and molecular techniques. Lytic bacteriophages against two S. aureus strains which were isolated from mastitic milk samples were obtained from wastewater samples.

Conclusion: The results of this study show that a significant portion of subclinical mastitis was formed by staphylococci. In addition, phage isolation against S. aureus strains isolated can be considered as one of the steps to be applied in the prophylaxis and treatment of such infections.

Keywords: bacteriophage, dairy cow, Staphylococcus aureus, subclinical mastitis.

Introduction

Mastitis is an inflammation of mammary gland and characterized by physical, chemical, and bacteriological changes in milk and pathologic changes in the glandular tissue. In this, particular subclinical mastitis is one of the most common forms of disease of highly productive dairy animals [1]. Mastitis has a great economic importance in dairy industry due to the causes as decreasing in milk yield and quality [2]. It has also a serious zoonotic potential due to the distribution of bacteria and toxins through the milk [3].

Mastitis caused by a series of pathogen is classified as contagious and environmental epidemiologically. In dairy cows, more than 140 microorganisms have been found as the cause of mastitis [3,4]. Contagious agents use infected udder as a reservoir, spread from cow to cow during milking, and can transform to chronic subclinical infections with the appearance of clinical cases. Staphylococcus aureus, Streptococcus agalactiae, Mycoplasma spp., and Corynebacterium bovis are placed among the contagious pathogens [5].

The most important microorganism causing mastitis is Staphylococcus spp., and particularly, S. aureus is responsible for about one-third of cases of clinical and subclinical mastitis [4,6]. Staphylococci are cocci which facultative anaerobe, Gram-positive, catalase-positive in the family Micrococccaceae. Staphylococci are a part of normal bacteria flora in mammals and birds. In veterinary medicine, S. aureus causes mastitis in animal species such as cattle, sheep, goats, and horses; dermatitis in sheep and goats; and botryomycosis in pigs and horses [7].

The reasons for the failure of treatment in S. aureus-associated mastitis include the coinfection of several breast lobes, the length of treatment period, and infection occurrence due to S. aureus producing beta-lactamase. Furthermore, S. aureus forms an abscess surrounded by thick fibrous capsules in the mammary gland. This can prevent the adequate accumulation of antibiotic on target site, and thus, bacterial destroying is interrupted [8,9].

Microorganisms can gain resistance to the antibiotics used in mastitis treatment [10]. Penicillin resistance of S. aureus in mastitis cases was reported as 50% in the USA, 71.4% in Ireland, and 67.3% in...
Isolation and identification

For isolation of Staphylococcus spp., 100 μl of milk samples were homogenized by vortexing and then streaked on to Blood Agar (Oxoid CM0271) enriched with 7% sheep blood and incubated at 37°C for 24-48 h in aerobic condition. After incubation, cultures were evaluated in terms of colony morphology, pigment production, and hemolysis, and Gram-staining features were examined, as well. Suspicious colonies were identified by subjecting to the classical biochemical tests (i.e., catalase, oxidase, urease, and nitrate reduction tests) [15].

Molecular diagnosis

DNA extraction from Staphylococcus spp. isolated from milk samples was performed by the phenol-chloroform method [16]. Molecular typing of isolates was carried out according to the polymerase chain reaction (PCR) method developed by Riffon et al. [17]. S. aureus reference strain (ATCC 6538) was used as a positive control and ddH₂O was used as a negative control. In this study, it was used Sau 327 (‘-GGACGACATTAGACGAATCA-3’) and Sau 1645 (‘-CGGGCACATTTTTTATC-3’) primers for the 1318 base pairs (bp) length amplification of 235 rRNA gene specific for S. aureus. Specific bands were photographed after 1.5% agarose gel electrophoresis of the amplified products.

Phage isolation

Mastitic milk and farm wastewater samples were used as material, and field S. aureus isolates were used as host for lytic phage isolation against S. aureus. The method was performed according to the methodology developed by Oliveira et al. [18]. Briefly, 10 ml 10× Brain Heart Infusion (BHI) broth and 100 μl bacterial culture obtained by adding of 3 ml of BHI broth onto the 24 h fresh slant agar culture of S. aureus were added into the bottles containing 90 ml of mastitic milk or farm wastewater samples and the bottles were incubated at 37°C for 24 h in shaker incubator. After the incubation, suspensions were centrifuged and the supernatants were filtered through a 0.22 μm filter. To determine the phage-host sensitivity, bacterial eluate was prepared by adding 3 ml BHI broth onto the fresh S. aureus slant culture and vortexed vigorously. 100 μl of eluate was added onto 3 ml of soft agar and plated on BHI agar plates. 15 μl of suspected phage suspension was dropped on the agar plates containing host bacteria, and the plates were incubated aerobically at 37°C for 24 h. Bacteriophage activity was evaluated by the presence of plaque formation on BHI agar plates.

Purification of phages

To purify the phage, the double-layer agar method was used [19]. For this purpose, serial dilution of phage was prepared till to 10⁻⁷. Then, 1 ml sample was taken from each dilution and mixed with 100 μl fresh target bacterial culture, and 3 ml of soft agar was added thereto. The mixture was spreaded on BHI agar plates and incubated at 37°C for 24 h. Different size phage plaques on agar plates were observed and picked up and transferred separately onto the 1 ml of BHI broth by sterile pipet tips and vortexed thoroughly by adding 1 ml BHI broth. The double-layer agar method was repeated for at least 5 times until a uniform plaque was formed.

Results

Isolation and identification of S. aureus

As the results of cultural examination, of 235 milk samples that were found mastitic by CMT, a total of 117 (49.79%) Staphylococcus spp. were isolated as a distribution of 74 (63.24%) coagulase-positive staphylococci (CPS) and 43 (36.75%) coagulase-negative...
staphylococci (CNS). Of these isolates, 76 (64.95%) were characterized as S. aureus both conventional and molecular techniques (Figure-1). Among the S. aureus isolates, 7 were found having coagulase-negative activity. Apart from these, the 5 isolates identified as CNS by conventional tests were found not to be S. aureus by PCR, as well.

**Phage isolation and purification**

While the phage isolation from mastitic milk samples was not achieved, using the wastewater samples, phage was isolated against to two S. aureus strains within 35 strains selected randomly among S. aureus strains isolated from mastitic milk samples. These phages were found having lytic activity on S. aureus field isolates and then purified and concentrated for future uses (Figure-2).

**Discussion**

Bovine mastitis is an economically important disease of the dairy industry worldwide. Although various pathogens are listed, *Staphylococcus* spp., in particular *S. aureus*, is considered as one of the most important agents of clinical and subclinical mastitis. The cases caused by the antibiotic-resistant variants (methicillin-resistant *S. aureus* [MRSA] or vancomycin-resistant *S. aureus*) of *S. aureus* are more important due to the tendency to replicate chronically [20]. In a study conducted in Sweden, 31% S. aureus and 27% CNS were isolated at the result of cultural examination of 583 milk samples from subclinical mastitic cow in 226 different farms [21]. In a different study in Ireland, samples taken from infected breast lobes of 285 cattle from 15 different farms were evaluated and 61 (21%) S. aureus and 26 (9%) CNS were reported [22]. Tel et al. [23] isolated 84 (32.5%) S. aureus and 71 (27.5%) CNS from 258 milk samples in cattle in a study in Şanlıurfa region. In a study conducted by Sevinti and Şahin [24] in Kars Province, 23 (34.3%) S. aureus and 19 (28.3%) CNS were isolated from 79 cow milk samples from mastitic breast lobes. In the present study, 117 (49.7%) *Staphylococcus* spp. were isolated from 235 cow milk samples with subclinical mastitis. Of 117 isolates, 74 (63.24%) and 43 (36.75%) were determined as CPS and CNS, respectively. By species-specific PCR, 76 (64.95%) of these isolates were identified as *S. aureus*. Among the *S. aureus* isolates, 7 were found having coagulase-negative activity. Apart from these, the 5 isolates identified as CNS by conventional tests were found not to be *S. aureus* at the result of species-specific PCR, as well. In this study, *S. aureus* is the bacterium which was isolated at the highest rate among the bacterial agents causing mastitis, as well. This seems a good harmony with the other studies’ isolation results.

Bacteriophages have a significant potential as an antibacterial agent. Bacteriophage can be used in animal husbandry as an additive therapy besides of infection management of antibiotic-resistant *Staphylococcus* spp. Slanetz and Jawetz [25] showed the presence of lytic staphylococcal bacteriophages from mastitic cow milks but suggested that these phages were not freely in milk, always linked to some particles. Gill et al. [26] have identified those proteins which are carriers of phages in whey. Researchers showed that these proteins inhibit phage binding to the cell surfaces and thus prevent the phage adsorption and cell lysis. While specific bacteriophages are isolated against *S. aureus*, make thought that their lytic activities and *in vitro* conditions can be used to control of many pathological events. In a study, it was showed that phage efficiency is insufficient against subclinical mastitis caused by *S. aureus* [27]. In another study, Gill et al. [26] used 18 breast lobes with mastitis caused by *S. aureus* of 13 cows. Ten ml bacteriophage with a concentration of $1.25\times10^{10}$ pfu/ml were given into infected breast lobes for over 5 days, and no pathogen presence was determined in 3 of 18 breast lobes. This ratio was not statistically significant, and thus, it was suggested that phages may be inactive in the mammary gland. Recently, Kwiatek et al. [10] have isolated lytic bacteriophages against *S. aureus* from the milk through standard enrichment using a mixed culture of randomly selected *S. aureus* ATCC 43300 and ATCC 25923 and *S. aureus* MRSA strains.
O’Flaherty et al. [28] gave three phage cocktails at 10^8 pfu/ml concentration into the cow’s milk and showed that it could not provide detectably increase in the number of somatic cell counts indicating an immune response for a high number of phages. In an in vivo study conducted by Wills et al. [29], it was showed that the numbers of phages in treated animals were higher than initially given, suggested that phage proliferation was successfully performed in animal tissues. Capparelli et al. [30] tested a phage against S. aureus in an experimental study in mice and showed that 97% of the infected animals achieved success as a result of the study over 10 days. In the current study, bacteriophage isolation from mastitic milks against S. aureus strains isolated from mastitic cattle milks could not be achieved, but it was successfully carried out from waste farm waters. Phages were isolated and purified using double-layer agar method and showed lytic activity in vitro conditions against S. aureus strains isolated from mastitic cattle milks. These purified phages need to be titrated and some experimental studies to determine their effect on mastitis are required, and quite frankly, it is not considered in this research plan, but it is thought that this could be a separate study topic.

Conclusion
The results of this study reveal that a significant (49.7%) proportion of subclinical mastitis of the cows was formed by staphylococci and that S. aureus was the predominant (64.95%) species. In addition, lytic phage isolation from farm wastewater samples specific to S. aureus can be considered as a preliminary step to investigate its usefulness in the prophylaxis and treatment of such infections.

Authors’ Contributions
AGS carried out the study, SO and MŞ planned, designed, and supervised the experiment. EÇ, ÖÇ, and DA carried out the sampling and species identification. All authors read and approved the manuscript.

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Competing Interests
The authors declare that they have no competing interests.

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