THE PREVALENCE OF RESISTANCE TO ANTIMICROBIAL AGENTS OF *STAPHYLOCOCCUS SPECIES* ISOLATES FROM MILK PRODUCTS IN MASHHAD, IRAN

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ABSTRACT

Staphylococci are widely distributed in the nature. Although most of *Staphylococcus* spp. are saprophytes, but some are pathogenic to both human and animal species. Staphylococci, especially *S. aureus* are the leading cause of nosocomial infections. These organisms frequently reveal a high natural, intrinsic resistance to antimicrobials. In this study, the prevalence of coagulase positive and coagulase negative staphylococcal contamination of milk products and antibiotic resistance patterns of isolated *Staphylococcus* spp. in Mashhad-Iran were investigated. For this purpose, 300 samples from different dairy products were purchased directly from the market. A total of 54 *Staphylococcus* spp. were isolated of which 18 strains (33.3%) were coagulase positive, and 36 strains (66.7%) were coagulase negative. The antimicrobial susceptibilities of these isolates were determined against 10 antibacterial agents using the disk diffusion method. Thirteen different antibiotic resistance patterns were observed. The highest level of antibiotic resistance was observed against penicillin (92.6%) and cephalexin, (66.6%). None of the isolates was resistant to norfloxacin and gentamycin. In conclusion, regarding high levels of staphylococcal contamination in different dairy products and remarkable levels of resistance against some antibacterial agents, these products may play a substantial role in prevalence of antibacterial resistance and related hygienic problems.

KEYWORDS: Coagulase-positive; Coagulase-negative Staphylococci; antibiotic susceptibility; milk

1. INTRODUCTION

Staphylococci are important causes of mastitis [1]. The efficacy of the antibiotic used is paramount for sucessful elimination of infection. When causing mastitis, *S. aureus* can also be found in milk, where high levels of contamination can be rapidly achieved if conditions are favorable. During growth in milk, enterotoxigenic *S. aureus* strains are able to produce thermostable enterotoxins. When ingested, they can cause nausea, vomiting and diarrhea [2].

The quality of milk is determined by aspects of composition and hygiene. Due to its complex biochemical composition and high water activity, milk serves as an excellent culture medium for the growth and multiplication of many kinds of microorganisms. The contamination of milk and milk products is largely due to human factor and unhygienic conditions. Staphylococci are ubiquitous, living in dust, environmental surfaces, and on humans and animals worldwide. Staphylococcal food poisoning results from the ingestion of *Staphylococcus* enterotoxin which has been produced in contaminated food [3,4]. Milk products may contain *Staphylococcus* spp., although most of them are saprophytes, however some are pathogenic to both human being and animals. These bacteria may become resistant to different antibiotics. Development of resistance in zoonotic bacteria constitute a public health risk, primarily via the increased risk of treatment failures.

Gram-positive cocci still predominate as a cause of nosocomial and community-acquired infections. These organisms frequently reveal a high natural, intrinsic resistance to antimicrobials. Additionally, these bacteria are able to acquire resistance to frequently used drugs rapidly through selective pressure of the environment and via the genetic evolution of bacteria. The wide application of

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antimicrobials in medical and veterinary practice, usage of antibiotics in agriculture and common usage of antiseptics and disinfectants result in selective pressure. The use of antibiotics directly selects resistant variants to different antibiotics or disinfectants [5].

Almost as soon as penicillin was introduced, penicillin resistant strains of *S. aureus* began to appear. This resistance was due to the production of inducible, narrow spectrum β-lactamase, efficient at hydrolysing penicillins. The number of penicillinase producing staphylococcal strains continues to increase, and they now predominate in hospitals (about 80%), but in some community acquired infections of many strains remain sensitive. Because of the ineffectiveness of penicillin, β-lactamase resistant penicillins such as methicillin, isoxazolyl penicillins and cephalosporins were introduced.

Use of antimicrobial agents will select for resistance genes in nonpathogenic bacteria, which may later transfer the acquired resistance to different pathogenic bacterial species. If antibiotic use is the major selective pressure encouraging the development of antibiotic resistance, then reducing antibiotic use should result in decreased antibiotic resistance. Hence, in this research, antibiotic resistant strains of *Staphylococcus* spp., isolated from five different milk products, were identified.

2. MATERIALS AND METHODS

2.1 Sample collection.

A total of 300 samples of five different dairy products consisting of 80, 70, 70, 50 and 30 samples of traditional cheese, traditional icecream, cream, pasteurized milk and pasteurized icecream respectively, were taken from the market in Mashhad, Iran according to the standard methods [6]. Briefly all the samples were collected under aseptic conditions in sterilized containers, and immediately transported at 4 °C to the Food Microbiology Laboratory, Ferdowsi University of Mashhad to immediate assays.

2.2 Isolation and identification procedure.

After preparation of samples according to standard methods [7], the presence of *Staphylococcus* spp. was determined by culturing 0.01 ml of each sample on Baird-Parker (Difco) and blood agar plates and incubated overnight at 37°C. The microbial strains were streaked on blood agar to obtain a pure culture and then presumptively identified on the basis of morphology, haemolysis pattern and Gram staining. Gram positive cocci were tested for catalase and coagulase production [7]. All isolates were kept frozen in TSB (Triticase Soy Broth) with glycerol for antibiotic susceptibility testing.

2.3 Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using the Kirby-Bauer disk diffusion method [8] on Mueller-Hinton agar (Oxoid) with 10 different disks (Oxoid) containing 10 IU penicillin, 30 μg chloramphenicol, 23.75 μg sulfamethoxazole/1.25 μg trimethoprim, 30 μg tetracycline, 10 μg gentamycin, 30 μg cephalexin, 5 μg novobiocin, 30 μg vancomycin, 15 μg erythromycin or 10 μg norfloxacin, each.

Initially, all isolates were inoculated in Tripticase Soy Broth (TSB, Difco) and incubated for 6 hours at 37°C until reached a turbidity of 0.5 McFarland standard. The culture suspensions were swabbed onto 150 mm petri dishes containing Mueller-Hinton agar and then antibiotic disks were applied. Plates were incubated at 35°C for 24 hours. Zones of growth inhibition were evaluated after overnight incubation according to the standard values [8]. ATCC strain 25923 was used for quality control.

3. RESULTS AND DISCUSSION

A total of 54 strains (18%) of *Staphylococcus* spp. were isolated from 300 samples of five different dairy products.Thirty three percent of all *Staphylococcus* isolates were identified as coagulase positive, and 66.7% as coagulase negative strains (Table 1). A total of 100% of the isolated strains showed resistance to least one or more of the antibacterial agents used. Prevalence of antibiotic resistance in coagulase positive and coagulase negative staphylococci strains isolated from milk products is shown in Table 2. As represented in Table 2, the highest level of antibiotic resistance was observed against penicillin (92.6%). Lower levels of resistance were subsequently observed with cephalexin (66.6%),

novobiocin (44.4%), tetracycline (31.4%), erythromycin (18.5%), sulfamethoxazole/trimethoprim (14.8%), vancomycin (5.5%), and chloramphenicol (3.7%) (Table 2). All isolates were found susceptible to gentamycin and norfloxacin. Different antibiotic resistance patterns in isolates are shown in Table 3. Accordingly, 13 different patterns of resistance were observed.

Milk and milk products may contain *Staphylococcus* spp. Although most of them are saprophytes, but some of them are pathogenic to human and animal species [3]. An individual who carries the bacteria and works with food is likely to be a main source of food contamination. People who eat this food will develop food poisoning especially if the food has not been kept under the correct environmental conditions [4,9].

In this study, a total of 18 strains (33.3%) were coagulase positive staphylococci. Coagulase is a pathogenic factor of staphylococci. Therefore, the presence of these strains in milk products is very important. A total of 36 strains (66.7%) were coagulase negative. Due to the possibility of toxin production in these strains, all products must be controlled microbiologically. As a result of unhygienic conditions from producers and distributors, contamination rate is high. A close relationship exists between the rate of resistance development and the quantities of antibiotics used [10]. Worldwide, large quantities of antimicrobials are used for growth promotion and prophylactic purposes and more moderate amounts for therapy [10]. Development of resistance in zoonotic bacteria constitutes a public health risk, through the increased risk of treatment failures.

In the present study, resistance to penicillin (92.6%) was the most common, which is in agreement with previous studies [11-13]. The occurrence of resistance to penicillin among *Staphylococcus* spp. isolated from milk products in this study was higher than previous reports [14,15]. The high prevalence of resistance to penicillin, could be related to extensive use of this antibiotic and formation of bacteria with β-lactamase enzyme.

A total of 31.4% of the resistant isolates from milk products were resistant to tetracycline, which is in agreement with previous observations [13,15]. Tetracycline has been one of the most commonly used antibiotics for the treatment of animal infections. Therefore, the very frequent occurrence of resistance among almost all of the bacterial species is probably a consequence of this phenomenon. In the present study, 44% of *Staphylococcus* isolates were resistant to novobiocin. It genetically related to resistance of some staphylococci. Resistance to novobiocin is a diagnostic marker of *S. saprophyticus*.

Our study revealed that, resistance of isolated strains were relatively low to sulfamethoxazole/trimethoprim (14.8%) and erythromycin (18.5%). Also in this study, 5.5% of isolates were resistant to vancomycin and 3.7% to chloramphenicol, which is in agreement with previous observations [13,16,17].

A total of 66.6% of isolated strains were resistant to cephalexin, which is in accordance with previous studies [13], perhaps due to low effectiveness against cell wall of the bacteria, and high rate of this antibiotic used. In aggreement with previous observations for isolates from mastitic milk [12,17], the results reported in this study indicated that 100% of *Staphylococcus* spp. isolates from milk products were susceptible to gentamycin and norfloxacin.

The results of the present study revealed 13 antibiotic resistance profiles. Resistance to penicillin was the most prevalent. All of the isolates were resistant to one or more of the antimicrobial agents, with none being resistant to norfloxacin and gentamycin.

Table 2. Prevalence of antibiotic resistance between CoPS and CoNS isolates.

Staphylococcus isolates		The number of isolates with resistant to different antibiotics									
		CN[1]	Te[2]	P[3]	G[4]	NB[5]	SxT[6]	V[7]	NOR[8]	E[9]	Ch[10]
CoPS[a] (18 strains)	RI[c]	15	6	18	0	3	3	1	0	6	1
	%	83.3	33.3	100	0	16.7	16.6	5.5	0	33.3	5.5
CoNS[b] (36 strains)	RI	21	11	32	0	21	5	2	0	4	1
	%	58.3	30.5	88.9	0	58.3	13.8	5.5	0	11.1	2.8
Total (54 strains)	RI	36	17	50	0	24	8	3	0	10	2
	%	66.6	31.4	92.6	0	44.4	14.8	5.5	0	18.5	3.7

[a]CoPS=Coagulase positive staphylococci, [b]CoNS=Coagulase negative staphylococci,
[c]RI= Resistant Isolate, [1]CN=Cephalexin, [2]TE=Tetracycline, [3]P=Penicillin,
[4]G=Gentamycin, [5]NB=Novobiocin, [6]SXT=Sulfamethoxazole/Trimethoprim,
[7]V=Vancomycin,
[8]NOR=Norfloxacin, [9]E=Erythromycin, [10]Ch=Chloramphenicol.

Table 3. Antibiotic resistance profiles between coagulase positive and coagulase negative staphylococci isolated from milk products.

4, 5 and 6 Resistant Determinant	1, 2 and 3 Resistant Determinant
P, CN, TE, NB, SXT, V	P, TE, NB
P, CN, SXT,CH,E	P, TE, CN
P, CN, NB, TE, V	TE, P
P, CN, NB, SXT, E	P, CN
P, CN, TE, SXT	P, NB
P, CN, NB, E	P
P, NB, CN	

4. CONCLUSIONS

In conclusion, because *S. aureus* is one of the most important pathogens responsible for food intoxication, the presence of multi-resistant strains in the community, particularly in countries where antibiotic availability and use is not well regulated, is a principle problems in the public health. Inappropriate use of antibiotics is suspected to be a major contributing factor in the relatively high level of resistance to antimicrobial agents observed in this study. Therefore, milk and milk products can act as a good source of antibiotic resistant *Staphylococcus species* posing a threat to consumers and public health. Thus, control of antimicrobial resistance in dairy herds should consider overall management practices rather than just antimicrobial usage. Additional studies are needed to identify the individual management practices that can influence antimicrobial resistance levels in dairy herds.

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