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Research Article

Risk from *Staphylococcus aureus* in informally marketed raw cow milk

Desislava Bangieva[1]✉, Valentin Rusev[1]

[1]*Department of Food Hygiene and Control, Veterinary Legislation and Management. Faculty of Veterinary Medicine. Trakia University. Stara Zagora. Bulgaria*

Abstract

This study aimed to investigate the presence of *Staphylococcus spp*. and *Staphylococcus aureus* in raw cow milk samples taken from peddlers of five regions in Bulgaria. The results showed that all 44 samples tested were positive for *Staphylococcus spp*. All isolates were tested for coagulase production and subjected to PCR analysis. PCR amplification of *16S rRNA* and *nuc* genes found the presence of *Staphylococcus aureus* in 23 (52.3%) of a total of 44 raw milk samples. The number of *Staphylococcus spp.* ranged from $3x10^2$ to $1.08x10^6$ cfu.ml^{-1}, and that of *Staphylococcus aureus* from $1.5x10^2$ to $3.19x10^5$ cfu.ml^{-1}. It is concluded that control over the hygiene of handling and processing raw milk is essential for its safety.

Keywords: *Staphylococcus spp*., coagulase-positive staphylococci, PCR, foodborne pathogen, food safety

Abbreviations:

CPS - coagulase-positive Staphylococci
CNS - coagulase-negative Staphylococci
GMP - good manufacturing practices
PCR - Polymerase Chain Reaction
rRNA - Ribosomal RNA

✉*Corresponding author: Dr. Desislava Bangieva, PhD Student, Department of Food Hygiene and Control, Veterinary Legislation and Management, Faculty of Veterinary Medicine, Trakia University, Student Campus, 6000 Stara Zagora, Bulgaria, tel.: ++359 42 699 543, mobile: ++359 878 117 254; E-mail: desislava_bangieva@abv.bg*

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Introduction

Staphylococci are spherical Gram-positive, immobile, aerobic or facultative anaerobic bacteria which do not form spores. Depending on their ability to coagulate rabbit plasma, they are divided into two groups: coagulase-positive (CPS) and coagulase-negative (CNS) staphylococci. Members of the genus Staphylococcus may be present in the nasal cavities, throat, hair and skin of healthy people and are abundant in wounds, pustules and abscesses (Bergdoll 1989). *Staph. aureus* is the most important pathogen of the genus Staphylococcus. According to Wertheim et al. (2005) approximately 20% of the adult population carry *Staph.aureus* in their nose permanently, another 30% intermittently, whereas 50% are non-carriers. The pathogen causes various diseases in humans and animals, ranging from mild skin infection to severe diseases such as pneumonia and septicemia (Lowy 1998). On the other hand, *Staph. aureus* is capable of producing a wide range of thermostable enterotoxins causing staphylococcal food poisoning. Risky are food products with pathogen levels above 10^5 cfu.g^{-1} or cfu.ml^{-1} (Jablonski and Bohach 1997). Symptoms of staphylococcal food poisoning usually occur rapidly (after 1-6 hours), and most often include nausea, vomiting, diarrhea and abdominal pain (Balaban and Rasooly 2000). Intoxication with *Staph. aureus* ranks third in food poisoning worldwide (Zhang et al. 1998; Asao et al. 2003). Due to its composition, milk is an excellent nutrient medium for the growth of staphylococci because of its high nutritional value, neutral pH and high water activity and therefore it is often implicated in staphylococcal foodborne intoxication (De Buyser et al. 2001). According to Katsuda et al. (2005), *Staph. aureus* is the most widespread and economically significant pathogen causing intermammary infections in dairy cows. Coagulase-positive staphylococci can enter milk by direct excretion from udder with clinical and subclinical staphylococcal mastitis or by environmental contamination during the handling and processing of raw milk (Jørgensen et al. 2005).

A number of studies (Donkor et al. 2007; Kouamé-Sina et al. 2012; Makita et al. 2012) have reported the risk of *Staph. aureus* in informally marketed raw milk in different countries. Research literature indicate that *Staph. aureus* is one of the most common causes of foodborne illness in the world. The practice of illegally selling raw milk is maintained in our country. Therefore, the study aimed to investigate the risk of *Staphylococcus spp*. and identify *Staph. aureus* in informally marketed raw cow milk.

Materials and Methods

Samples collection. From November 2016 to July 2017, a total of 44 raw milk samples were collected from five regions located in Southern Bulgaria (Sofia, Blagoevgrad, Stara Zagora, Haskovo and Smolyan). Samples were kept in an ice box and transported directly to the Laboratory of Animal Food Safety and Control, Trakia University, Stara Zagora.

Isolation and identification of *Staph. aureus.* Isolation of *Staph. aureus* was performed according to ISO 6888-1:1999. The presumptive colonies were sub-cultured onto Baird-Parker Agar (base) (Merck, Germany) to get pure culture. These isolates were submitted to the following tests: Gram staining (HiMedia, India), catalase reaction with 3% hydrogen peroxide and clotting of rabbit plasma (BB - NCIPD Ltd., Sofia, Bulgaria). PCR amplifications were performed with a pair of primers specific for *16S rRNA* gene of *Staphylococcus spp*. and *nuc* gene of *Staph. aureus.*

Isolation of bacterial DNA. The genomic DNA was extracted by boiling method. An overnight culture of *Staph. aureus* was taken in 500 µl of distilled and deionised water, mixed well and boiled for 15 min. After boiling the tubes were centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant containing DNA was used as template DNA in the PCR mixture.

Identification of *Staph. aureus* by PCR. PCR reactions were performed using *16S rRNA* and *nuc* genes. All the primer pairs used in this study are shown in Table 1. Each reaction mixture contained 1 µl bacterial DNA, 1x Reaction Buffer (Thermo Scientific, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM of each of the two primers (Eurofins

Table 1. Primers used in this study for detection of *16S rRNA* and *nuc* genes.

Gene	Primer	Oligonucleotide sequence (5'-3')	Product (bp)	Reference
16S rRNA	16s rRNA forw. 16s rRNA rev.	GTA GGT GGC AAG CGT TAT CC CGC ACA TCA GCG TCA G	228	Monday and Bohach (1999)
nuc	nuc forw. nuc rev.	GCG ATT GAT GGT GAT ACG GTT AGC CAA GCC TTG ACG AAC TAA AGC	279	Brakstad et al. (1992)

Genomics, Germany) and 1 U/rxn Taq DNA Polymerase (VWR International, Belgium). The final volume was adjusted to 20 µl by adding nuclease free water. Amplification was proceeded with thermocycler (QB-96, Quanta Biotech) with an initial denaturation at 94°C for 3 min, followed by 35 cycles of amplification (denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 1.30 min), ending with a final extension at 72°C for 7 min. The amplified PCR products were separated by electrophoresis in 2,5% agarose gel at 100 V for 1 hour, stained with peqGREEN (VWR International, Belgium) and finally visualized and documented under UV Transilluminator (ImageQuant 150, GE Healthcare) (Fig. 1).

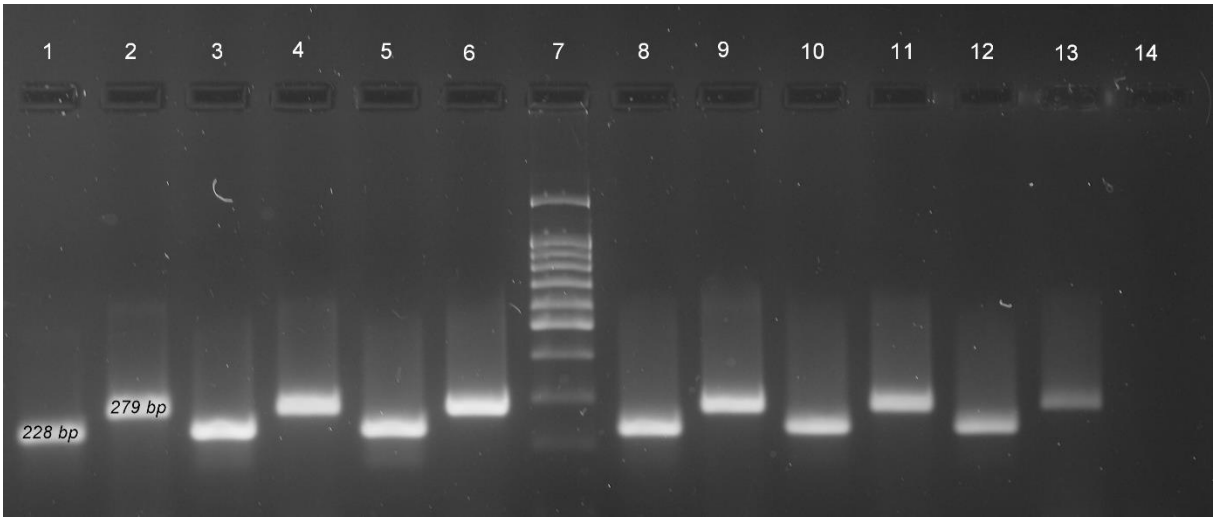

Figure 1. PCR products indicating the presence of *Staph. aureus* in informally marketed raw cow milk.

Lanes and samples: 1, 3, 5, 8, 10, 12 - PCR products of *16S rRNA* gene (228 bp); 2, 4, 6, 9, 11, 13 - PCR products of *nuc* gene (279 bp); 7 - DNA molecular size marker (100-bp ladder); 14 - negative control.

Results and Discussion

Table 2 shows that all 44 (100%) of the samples tested were positive for *Staphylococcus spp*. Contamination levels of *Staphylococcus spp*. in samples ranged from $3x10^2$ to $1.08x10^6$ cfu.ml^{-1}, with an average value of $5.49x10^4$ cfu.ml^{-1}. Coagulase-positive staphylococci (CPS) were found in 29 (65.9%) and coagulase-negative (CNS) in 15 (34.1%) of all samples analyzed. PCR amplification of *16S rRNA* and *nuc* genes identified *Staph. aureus* in 23 (52.3%) of all raw cow milk samples. Counts of the pathogen ranging from $1.5x10^2$ to $3.19x10^5$ cfu.ml^{-1} with an average value of $3.14x10^4$ cfu.ml^{-1} were detected in milk samples. Milk is considered to be a category of food at high risk of potential microbial contamination caused by insufficient animal health control, inadequate training of food business operators for milk hygiene and weaknesses in refrigeration chain during processing and storage.

Table 2. Distribution of *Staph. aureus* in raw cow milk.

No. of samples analyzed (n)	***Staphylococcus spp.***			**No. of CPS samples n (%)**	***Staph. aureus* *16S rRNA/nuc***		
	No. of positive samples n (%)	Contamination levels cfu.ml^{-1}	Average cfu.ml^{-1}		No. of positive samples n (%)	Contamination levels cfu.ml^{-1}	Average cfu.ml^{-1}
44	44 (100%)	$3x10^2$ – $1.08x10^6$	$5.49x10^4$	29 (65.9%)	23 (52.3%)	$1.5x10^2$ – $3.19x10^5$	$3.14x10^4$

Our results show that all (n=44; 100%) samples tested were positive for *Staphylococcus spp*. Coagulase-positive staphylococci (CPS) (65.9%) were more often isolated than coagulase-negative (CNS) (34.1%) in raw cow milk. *Staph. aureus* was found in 52.3% of all samples analyzed. Similar results for *Staph. aureus* prevalence showed the studies of Pourhassan et al. (2011), Fadaei (2014) and Kalmus et al. (2015) of 52%, 41.66% and 57.1%, respectively. The high prevalence confirms the widespread of the pathogen in environment and the high probability of contamination of food products. Most literature indicates that *Staph. aureus* appears in milk from cows suffering from intermammary infections. It is suggested (Roberson et al. 1998) that persistent colonization of the teat skin occurs, which is a significant predisposing factor for milk contamination with the pathogenic microorganism. The prevalence of 52.3% can be attributed to poor sanitation practices, lack of cooling system and storage at room temperature leading to intense *Staph. aureus* proliferation. Food safety hazards such as foodborne pathogens are best controlled through proper implementation of good manufacturing practices (GMP). Compared to our results for *Staph. aureus* prevalence (52.3%), some authors reported higher values (Gucukoglu et al. 2012 - 75%; Kamal et al. 2013 - 94%; Santos et al. 2014 - 71.2%; Rola et al. 2015 - 62%; Doss and Vijayasanthi 2016 - 64.70% and Shamila-Syuhada et al. 2016 - 100%), and others lower ones (Jackson et al. 2012 – 20.10%; Meshref 2013 – 23.7%; Thaker et al. – 6%; Jahan et al. 2014 – 25.53%; Ngasala et al. 2015 - 33% and Taherikalani et al. 2015 – 17.7%)

Reported CNS prevalence of 34.1% is due to poor hygiene during milking, processing, transport, storage and marketing of milk. As opportunistic microorganisms CNS are a part of the normal skin flora of humans and animals, and some species are also free-living in the environment (Normanno et al. 2005). Therefore, they are a common cause of clinical and subclinical infections and for contamination of milk and dairy products. The results of our study show that the contamination levels of raw cow milk with *Staph. aureus* range from $1.5x10^2$ to $3.19x10^5$ cfu.ml^{-1}, with an average of $3.14x10^4$ cfu.ml^{-1}. They are similar to the findings of Pyz-Łukasik et al. (2015) (from $1.6x10^3$ to $5.1x10^4$ cfu.ml^{-1}) and Rola et al. (2015) (from $1.0x10^0$ to $1.0x10^5$ cfu.ml^{-1}). Lower than our values are reported by Hill et al. (2012) in New Zealand, Jackson et al. (2012) in the United States, Meshref (2013) in Egypt, Fadaei (2014) in Iran, El-Leboudy et al. (2015) in Egypt and Shamila-Syuhada et al. (2016) in Malaysia. Data from Gucukoglu et al. (2012) and Kamal et al. (2013) show *Staph. aureus* levels in raw cow milk exceeding the risk limit of 10^5 cfu.ml^{-1}, where the amount of enterotoxin formed may cause disease. Although pasteurization kills the pathogen cells the thermostable staphylococcal enterotoxins retain their biological activity. Considering that the average level of contamination is $3.14x10^4$ cfu.ml^{-1}, the milk consumed in these regions is a serious problem for the public health. Studies show that *Staph. aureus* prevalence varies with different countries. Application of good hygiene practices during milking, storage, collection and transport to the point of sale or processing will reduce the spread of *Staph. aureus*. Strict adherence to good manufacturing and hygiene practices throughout the food chain is essential to prevent staphylococcal food intoxication.

Conclusions

Our findings indicate a high incidence of *Staph. aureus* (52.3%) in informally marketed raw cow milk. Incorrect cooling process during transport and selling of such milk is a problem that allows the growth of the pathogen and the production of thermostable enterotoxins. Consumption of contaminated milk is an important factor in the occurrence of food intoxication in humans, because of which keeping strict hygiene and sanitary standards throughout the food chain is important. Therefore, the competent authorities should adopt measures to prevent and control possible informal sale of milk. To determine the presence of enterotoxigenic strains of *Staph. aureus* and their ability to produce enterotoxins in informally marketed raw cow milk further research is needed.

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