



Detection of *mecA* gene of methicillin resistant *Staphylococcus aureus* by PCR assay from raw milk

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Received: 10 July 2015; Accepted: 23 September 2015

ABSTRACT

The occurrence of *Staphylococcus aureus* and MRSA in foods of animal origin, pose a serious threat to the well-being of humans due to innumerable clinical implications. There is a potential risk of transmission of *S. aureus* and methicillin-resistant *Staphylococcus aureus* transmission to humans through raw milk if consumed without maintaining adequate hygienic standards. This study was conducted to assess the prevalence of *S. aureus* and MRSA from raw milk samples *vis-à-vis* their phenotypic and genotypic characterization for antimicrobial resistance pattern and presence of *mecA* gene in raw milk samples of cattle, buffalo and goat in the Jammu city of Jammu and Kashmir. Samples (60) were subjected to California mastitis test to check for their mastitic status. The organisms were cultured and identified on the basis of their cultural, morphological, staining and various biochemical characteristics. The amplification of the *mecA* gene generated a product with a band size of 533bp upon agarose gel electrophoresis. The *S. aureus* prevalence was 60, 52 and 60% in raw milk of cattle, buffalo and goat, respectively. Out of 34 *S. aureus* isolates, 44.1% were MRSA positive.

Key words: Antimicrobial resistance, MRSA, PCR, Prevalence, *Staphylococcus aureus*

Mastitis, a highly prevalent disease in dairy cattle, is one of the most important threats affecting the world's dairy industry (Wallenberg *et al.* 2002). *Staphylococcus aureus* (*S. aureus*) usually causes a chronic, subclinical mastitis in dairy cattle worldwide (Coelho *et al.* 2011). It is generally accepted that the primary source of *S. aureus* in a dairy herd is the udder of infected animals (Blood *et al.* 1989). Cumbersome preventive and control measures have to be taken on farms with *S. aureus* mastitis problems, and the treatment often shows poor success (Radostits *et al.* 2000, Sutra *et al.* 1990).

The main sources of this intra-mammary infection in cattle are infected animals in the herd, workers, equipment and the utensils, used for milking (De Vlieghe *et al.* 2012). The infected udders of animals which are often chronic carriers/reservoirs shed the bacterium in milk intermittently or continuously (Blood *et al.* 1989). Health risk to consumers can be associated with milk, due to the presence of zoonotic pathogens and antimicrobial drug residues in

milk used in the treatment of primary diseases (Bradely 2002). The occurrence of *S. aureus* and MRSA in foods of animal origin poses a serious threat to the well-being of humans due to innumerable difficult to treat clinical implications. In this regard, this study was carried out to generate data about the presence of *Staphylococcus aureus* and MRSA in raw milk in Jammu.

MATERIALS AND METHODS

Mastitic milk samples (60) were collected aseptically from veterinary clinics and dairy farms in pre-sterilized test tubes and transported in ice within 2 h of collection to the laboratory for further processing and analysis. Each sample was enriched in peptone water and incubated at 37°C for 24 h. Each inoculum was cultured on Baird Parker agar (selective medium for *Staphylococcus*), mannitol salt agar and incubated at 37°C for 24 h. All positive samples were subjected to coagulase test and biochemical characterization of the presumptive *S. aureus* isolates for confirmation of *Staphylococcus aureus* (Monica 1991, Bennett and Lancette 2001). Thus, confirmed *S. aureus* isolates were inoculated and cultured on Oxacillin resistance screening agar (ORSA) for detection of methicillin resistant *Staphylococcus aureus* (Pereira *et al.* 2009) and the presumptive blue colour colonies of MRSA on ORSA were further inoculated in MRSA alert test kit for MRSA confirmation.

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Molecular characterization and detection of *mecA* gene specific for MRSA

Preparation of DNA template by boiling and snap chilling method: A loopful of colonies from ORSA plate, were suspended in 400 µl of nuclease free water and suspension was prepared by gently vortexing the tubes. The suspension was boiled in a water bath for 10 min followed by snap chilling on ice for 10 min and centrifugation at 14,000 rpm for 5 min. The supernatant was transferred to autoclaved nuclease free tubes and was used as template for PCR reaction.

Details of the primers used: Detection of *mecA* gene by the polymerase chain reaction is considered as the “Gold standard” in the diagnosis of MRSA. The primers used in this regard were as per Pereira *et al.* (2009). The forward and reverse oligonucleotide primers sequence used was 5' AAAATC GAT GGT AAA GGT TGGC 3' and 5' AGT TCT GCA GTA CCG GAT TTGC 3' with a product size of 533bp.

The reaction mixture used in PCR for the detection of *mecA* gene for methicillin resistance of *S. aureus* was prepared from the stock solution of the various reagents. Titration of MgCl₂ (25mM) was carried out at 1.5, 2, 2.5 and 3mM to obtain the clear bands of 533bp on agarose gel electrophoresis. 0.5µl of 25µM forward and reverse primers each was used to make a final reaction mixture of 25µl. Reaction mixture included 10× PCR buffer (vol. 5µl and a final concentration of 1×), MgCl₂ (25µM) vol. 3l and final concentration of 3µM, dNTP mix (10µM each) 0.5 µl and final concentration of 0.2µM, forward and reverse primers (25µM each, with a volume of 0.5 µl with a final concentration of 0.5µM, 1 ml of template DNA, 0.5 µl of 1.5 U Taq DNA polymerase (3U/µl) and finally 14 µl of NFW. Amplification parameters as described for *mecA* PCR (Murakami *et al.* 1991) were used. The amplification cycle consisted of denaturation at 94°C for 5 min, followed by 40 cycles, each consisting of initial denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 2 min followed by final extension at 72°C for 5 min. The confirmation of PCR product was done by electrophoresis of amplified products in 1.0% agarose gel in 0.5× Tris-borate-EDTA buffer (TBE) in horizontal electrophoresis unit.

RESULTS AND DISCUSSION

Our findings revealed highest prevalence of *S. aureus* in cattle and goat milk owing to 60% each. Higher positivity was observed in the milk samples of cattle milk (60%) and goat milk (60%), than that of buffalo milk (52%) as shown in the Table 1.

Table 1. Prevalence of *S. aureus* and MRSA in raw milk samples

Species	No. (n)	<i>S. aureus</i> +ve (%)	MRSA +ve (%)
Cattle	25	15 (60%)	8 (32%)
Buffalo	25	13(52%)	6 (24%)
Goat	10	6 (60%)	1 (10%)

Table 2. Biochemical characteristics of *S. aureus* and MRSA isolates from milk

Biochemical tests	<i>S. aureus</i> (n =34)	MRSA (n=15)
Gram's staining	+	+
Motility	-	-
Coagulase	+	+
Oxidase	-	-
Catalase	+	+
Mannitol fermentation	+	+
Indole production	+	+
Methyl red test	+	+
VP-test	+	+
Citrate utilization	+	+
Urease production	+	+
Haemolysis on blood agar	+	+
MRSA alert test kit	±	+

S. aureus showed typical pale yellow, friable, large opaque colonies on mannitol salt agar and phenol red pH indicator detected the acidic metabolic product of mannitol fermentation by *S. aureus*. On Baird Parker agar media, which is a selective medium for *S. aureus*, jet black colonies surrounded by a light halo were considered as presumptive *Staphylococcus* (Table 2).

On microscopic examination all the *S. aureus* isolates were found to be Gram positive, non-spore forming, non-motile cocci giving a clustered bunch of grape like irregular appearance.

Molecular characterization of MRSA isolates: The amplification of the *mecA* gene generated a product of approximate molecular band size of 533bp on agarose gel electrophoresis (Fig. 1), using the primers (Pereira *et al.* 2009).

It established an agreement between the phenotypic and molecular techniques used for *S. aureus* and MRSA detection.

Kumar and Prasad (2010) reported 18.52% *Staphylococcus* ascribing to contaminated environment and unhygienic handling or preparation in milk and milk products in Pantnagar. However, findings of study corroborate with the findings of Farzana *et al.* (2004) who observed 40% milk samples contaminated with *Staphylococcus aureus* in Multan city in Pakistan. Our findings are nearly in agreement with the Abera *et al.* (2010)

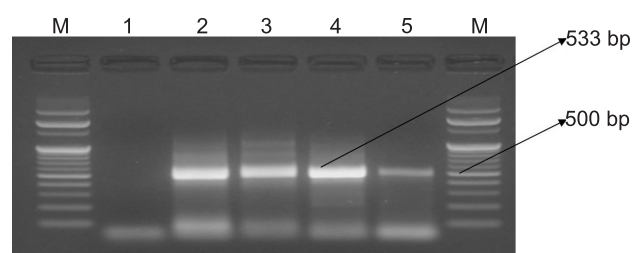


Fig. 1. Agarose gel showing amplification of the 533bp *mecA* gene of MRSA on polymerase chain reaction. Lane M: 100bp molecular weight marker; lane 1, negative; lane 2, 3, 4 and 5, amplified product of 533bp of *mecA* gene.

who found 59 (42.1%) isolates of *S. aureus* from 140 milk samples on bacteriological examination in Adama town, Ethiopia. The findings also showed near agreement with other research findings carried out in Ethiopia on the prevalence of *S. aureus* in raw milk, which was reported to be 52.8% around Sebeta, 53.35% in South Wollo, 53.5% in Kallu province, 61.11% in South Wollo, 63% and 68.1% in Addis Ababa by Hundera *et al.* (2005), Haile (1995), Tolossa (1987), Tolla (1996) and Zerihun (1996), respectively. The high prevalence of *S. aureus* was attributed to the wide distribution of the organism inside mammary glands and on the skin of teats and udders (Jones *et al.* 1998). *S. aureus* is reported to survive in udder and establishes chronic and subclinical infections, from where it is shed into the milk, serving as a source of infection for healthy cows during the milking process (Radostitis *et al.* 1994).

Our findings were also in agreement with studies of Adlan *et al.* (1980), Varrst and Envoldsen (1997), Kerro and Tareke (2003), Hundera *et al.* (2005) and Mekonnen *et al.* (2005), as they reported *S. aureus* to be the predominant isolate in raw milk from clinical and subclinical mastitis cases. Boerlin *et al.* (2003) reported 58.45% positive for *Staphylococcus aureus* in Switzerland. Mubarak *et al.* (2010) reported 65% of raw milk samples positive for *Staphylococcus* spp. among the 80 raw milk samples in Coimbatore district in Tamil Nadu ascribing it to unhygienic practices being followed by the handlers of milk. Our study, however, revealed higher prevalence of *S. aureus* in milk compared to 21.4% reported by Singh *et al.* (2011) in Paonta Sahib in Himachal Pradesh. The findings of this study showed comparatively much lower prevalence of *S. aureus* in raw milk samples than reported by Haran *et al.* (2012) who studied the herd prevalence of *S. aureus*, including MRSA, from bulk tank milk (BTM) from Minnesota farms. Prevalence of methicillin-susceptible *S. aureus* (MSSA) was 84%, while for MRSA, the herd prevalence was 4%. They isolated a total of 93 MSSA (Methicillin Sensitive *Staphylococcus aureus*) and 2 MRSA isolates from 150 BTM samples. However, the MRSA reported in our study is much higher than reported by Haran *et al.* (2012). Our findings were also higher than as reported by Kumar *et al.* (2011) who studied the distribution of MRSA and antibiotic resistance in 107 strains of *S. aureus* from regions around U.P. They isolated *S. aureus* from milk samples of 195 infected udders from Sahiwal cattle. A high prevalence of MRSA (13.1%) was observed in the tested isolates whereas our finding showed a prevalence of 44.1% MRSA in raw milk samples. Addis *et al.* (2011) investigated the prevalence of *Staphylococcus* spp., distribution and characterization in raw bovine milk samples in DebreZeit, Ethiopia. The identification results showed 33 and 46% prevalence of *Staphylococcus* spp. in raw bucket milk and tank milk, respectively, with an overall prevalence of 39.5% (79/200) which is again not in agreement with our findings. The study nearly corroborates with the findings of Mir (2012) wherein out of 200 samples of milk and milk products, 32% (64) tested positive for *S. aureus* in Srinagar

city, Jammu and Kashmir. MRSA prevalence found in our study was 44.1% (15/34), however some countries have reported far lower rates of prevalence of MRSA in milk, being 1.4% in Switzerland, 1.5% in Japan, 2.4% in Korea and 5.1 to 16.7% in southwest Germany. Kumar *et al.* (2011) reported 13% of *S. aureus* isolates (14 of 107 samples) from cattle in Haryana being MRSA, which is much less than the findings of our study. Presently, antimicrobial therapy is one of the primary control measures in reducing the *Staphylococcal* morbidity and mortality. However, the indiscriminate use of antimicrobials has led to the development of resistance against many such antimicrobials. The resistance of *S. aureus* can be attributed to the presence of *mecA* gene that leads to synthesis of an altered penicillin binding protein PBP2a. The drug sensitivity of *S. aureus*, studied against 14 commonly used antibiotics, revealed high percentage of resistance with penicillin G (76.47%), ampicillin (67.64%), streptomycin (55.88%), and ceftriaxone (29.41%). The *Staphylococcus aureus* isolates were most sensitive to amikacin (64.7%), enrofloxacin (61.76%), cephalothin (58.82%) followed by gentamicin (55.88%). Intermediate resistance was shown by gatifloxacin (52.94%) followed by ceftriaxone and cefuroxime both 44.11%.

The higher *S. aureus* and MRSA prevalence in our study may be attributed to varying unhygienic conditions such as improper cleaning of utensils, dirty udders, animals with sub-clinical mastitis, milk handling techniques and improper/lack of refrigeration and storage that are known to increase the proportion of *Staphylococcal* spp. in raw milk and milk products. There is a potential risk of *S. aureus* and MRSA transmission to humans through raw milk if such milk is consumed without maintaining adequate hygienic standards. MRSA transmission can occur to animals from infected farms, dairy and clinical environments. The major reason of high prevalence of MRSA in our study may be environmental contamination and indiscriminate use of antimicrobial drug therapy to treat animals in the Jammu division of Jammu and Kashmir state. Future studies are required to elucidate the transmission potential and magnitude of the problem.

ACKNOWLEDGEMENT

The authors are thankful to the Dean, F.V.Sc & A.H, SKUAST, Jammu and Head of the Department, Veterinary Public Health and Epidemiology for the necessary facilities which were required for the investigation. Help from the farm workers who facilitated the sample collection is also acknowledged.

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