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Emerging discrepancies in conventional and molecular epidemiology of methicillin resistant *Staphylococcus aureus* isolated from bovine milk

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ABSTRACT

Methicillin resistant *Staphylococcus aureus* (MRSA) is an emerging public health concern from dairy milk, and its diagnosis by phenotypic methodology is experiencing higher discrepancies. The present study was planned to estimate discrepancies in phenotypic identification of MRSA and MSSA (Methicillin sensitive *Staphylococcus aureus*) in relation to *mecA*, and prevalent risk factors from various localities. *In-vitro* oxacilline antibiotic disks were used for phenotypic identification of MRSA, whereas *mecA* gene was used as MRSA marker in *Staph aureus* by PCR. Total of 900 bovine milk samples from private and public farms located in district Faisalabad using convinent sampling technique were collected. Potential risk factors for MRSA prevalence identified by non-parametric statistical tests were compared among different subdistricts. Discrepancy in MRSA was calculated as percentage of *mecA* negative strains while that of MSSA was determined as percentage of *mecA* positive strains. Molecular identification presented 17.97% (55/306) of discrepancy in MRSA in terms of negative *mecA* strains from district Faisalabad while sub-district Faisalabad, sub-district Jaranwala, and sub-district Samundary presented 13.98% (13/93), 18.28% (17/93), and 20.83% (25/120) discrepant results, respectively. On the other hand, 29.1% (55/189) of discrepancy in MSSA in terms of *mecA* positive strains from MSSA isolates was noted. MSSA results were more discrepant than that of MRSA. Hence discrepancy ratio of MSSA over MRSA was noted to be 1.53, 1.50, and 1.21 from tehsil Faislabad, Samundary, and Jaranwala. Tick infestation, lactation stage, frequency of milking, dirty milker's hands, unhygienic milking procedures, and higher use of beta lactam of antibiotics were risk factors that were prevalent in increasing order from sub-district Faisalabad > Jaranwala > Samundary. The study concluded higher prevalence of MRSA in bovine milk samples, and found remarkable discrepancies in phenotypic and genotypic identification which demand immediate attention to tackle exacerbation in resistance patterns.

1. Introduction

The infectious diseases are serious threat for animal health and productivity [14,27,28]. The emerging food-borne diseases expressing serious public health issues worldwide are entitled to get 2/3rd share from bacteria. Among bacteria, *Staph aureus* occupies major etiology of food poisoning outbreaks [18]. This pathogen is anchored to various pathogenic factors like enterotoxins, hemolysins, pathogenic enzymes, biofilm production, and drug resistant factors. *Staph aureus* brings a serious deterioration in the milk glands and appears as public health issue. The major drug resistant characteristics regarded as methicillin resistant *Staph aureus* (MRSA) has alarmingly attained rising level in milk. This strain compromises treatment therapy in animals, and in humans upon zoonosis [25]. The miserable situation appears due to lack of desired research in the identification of MRSA in milk. Moreover, the conventional techniques have been failing in accurate epidemiological inferences when compared with results of molecular techniques [11].

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The false positive results invite empirical application of a) drugs other than beta-lactams, b) treatments for decolonization of pathogen, c) patients' isolations, d) additional coercing control strategies [7]. The outbreaks of MRSA isolates having other than *mecA* genes leads to unjustified precautionary measures that develops resistance in strains, medical complications, and compromised farm economy [22]. Accurate and early identification of MRSA is necessitated for treatment and prevention from onward transfer [17]. The recent studies have reported a higher prevalence of MRSA in camel and bovine milk [2–4]. Involvement of various risk factors that may potentiate propagation of MRSA are necessarily be given due attention [4]. The rapid and accurate method for identification of MRSA will help to tackle infections and their onward spread [17]. Studies are scarce in discrepancy estimation of MRSA reporting from Pakistan The discrepancy in results of phenotypic and genotypic identification needed to be addressed. Current study was planned to investigate discrepancy in identification of MRSA and MSSA at phenotypic and genotypic level, and estimation of prevailing risk factors of MRSA in study areas.

2. Materials and methods

2.1. Study areas and sampling

Total of 900 milk samples using convenient sampling techniques [26] from public and private dairy farms of district Faisalabad were collected and processed microbiologically for the identification of *Staph aureus*. The farms located in jurisdiction of subdistrict Jaranwala, subdistrict Samundary, and subdistrict Faisalabad were approached for milk sample collection using convenient sampling technique based on willingness of farmers and ease of sample shipment to laboratory. The samples were shifted immediately to Laboratory of Medicine, Department of Clinical Medicine and Surgery, University of Veterinary and Animal Sciences, Lahore-Pakistan, where further analysis was carried out. A dichotomous questionnaire was filled with responses from dairymen regarding animal and human's end information to investigate risk factors' association with pathogenic *Staph aureus* [4]. The potential risk factors were analysed by chi-square and logistic regression [29], and later their comparison was made among all three sub-districts to check if significant variation existed.

2.2. MRSA and MSSA identification by disk diffusion method

The milk samples (0.5 mL each) were centrifuged and sediments were aseptically spread out on blood agar. Typical round colonies were put to mannitol salt agar with subsequent biochemical identification as per recommend protocols of Bergey's Manual of Systematic Bacteriology to confirm *Staph aureus* [19]. All the *Staph aureus* (fresh growth) were swabbed on Muller Hinton agar of Oxoid (B3374, Basingstoke, UK), while 1 μg disks of oxacillin were aseptically dropped with the help of disk dispenser (Oxoid™). The plates were incubated at 37 °C for 24 h s and zones (millimeters) formed around disk were measured by Vernier calipers [6]. The zones of inhibitions measured were compared with standards provided by clinical and laboratory standard institute [10]. The *Staph aureus* isolates showing zone of inhibition less than 18 mm were considered as methicillin resistant *Staph aureus* (MRSA) while those of greater than 18 mm were declared as methicillin sensitive *Staph aureus* (MSSA).

2.3. Molecular identification of mecA

All *Staph aureus* declared as MRSA or MSSA from disk diffusion method were subjected to molecular identification of *mecA* gene by PCR. Extraction of MRSA and MSSA strains was performed using bacterial DNA extraction kit (GF-1 Bacterial DNA extraction kit, Vivantis Technologies Sdn. Bhd, Malaysia, GF-BA-050). The extraction of DNA was confirmed by running extracted sample on 2% agarose gel. Further quantification was performed by Nano-drop machine (Thermoscientific™-NanoDrop2000). The extracted DNA was put to polymerase chain reaction using primers, P1:5′-TGGCATTCGTGTCACAATCG-3′ and P2: 5′- CTGGAACTTGTTGAG CAGAG-3′ with product size of 310bp [30]. The specification of PCR included DNA sample (2ul), each primer (0.5ul), Taq buffer mix (12.5ul), and sterile distilled H_2O (9.5ul) making total volume of 25ul to be used in amplification process. The reaction was completed with initial denaturation (94 °C for 4 min-single time), denaturation (92 °C for 1 minute-34 cycles), annealing (53 °C for 50 seconds-34 cycles), extension (72 °C for 1 min-34 cycles), and final extension (72 °C for 10 min-single time) using Thermal Cycler (Applied Biosystems™ 2720, Thermo Fisher Scientific, USA, catalog# 4359659). The amplified PCR products were run on ethidium bromide stained agarose gel (2%) using 80 V for 36 min. The gel was examined under UV light illuminator.

2.4. Statistical analysis

Prevalence was calculated as per formula described by [26], while risk factor analysis was done using non-parametric statistical tests. Initially, the determinants were examined by chi square, and those having association with pathogen occurrence were put to univariable and multiple logistic regression analysis for confirmation of potential risk factors [29]. The discrepancy (%) was determined as formulas given below, while discrepancy ratio between MSSA over MRSA was determined by dividing percentage discrepant results of MSSA divided by percentage discrepant results of MRSA. Percentage change in discrepant results of MSSA over MRSA was determined by dividing discrepant results of MSSA over percentage discrepant results of MRSA with subsequent multiplication with 100. The statistical inferences were made by considering 5% probability using SPSS version 22 of statistical computer programme.

$$\text{Discrepency percentage in MRSA} = \frac{\text{No. of isolates found negative for } mecA \text{ from disk diffusion MRSA}}{\text{Total MRSA identified from disk diffusion}} 100$$

$$\text{Discrepency percentage in MSSA} = \frac{\text{No. of isolates found positive for } mecA \text{ from disk diffusion MSSA}}{\text{Total MSSA identified from disk diffusion}} 100$$

3. Results

3.1. Prevalence of misdiagnosed MRSA/mecA

The study found 495 out of 900 (55%) milk samples from dairy farms located in various sub-districts of Punjab, Pakistan having been positive for subclinical mastitis. The biochemical analysis confirmed 71.52% (354/495) of mastitis milk samples harboring *Staph aureus*. The oxacillin testing using the disk diffusion method identified 86.44% (306/354) and 13.56% (48/354) of *Staph aureus* as MRSA and MSSA, respectively. On the other hands, out of 354 *Staph aureus* isolates, the study found 74.29% (263/354) and 25.71% (91/354) of *mecA* positive and *mecA* negative strains by PCR technique, respectively as shown in Fig. 3.

3.2. mecA discrepant results with MRSA

The discrepancy was found to be 17.97% for disk diffusion MRSA to appear as *mecA* negative while 25% of disk diffusion MSSA were *mecA* positive in current study (Fig. 1). The farms in various localities presented 13.98%, 18.28%, and 20.83% of disk diffusion MRSA to be negative for *mecA* gene by PCR in sub-districts Faisalabad, Jaranwala, and Sumundary, respectively, whereas 86.02%, 81.72%, and 79.17% of MRSA identified from same localities by disk diffusion test were positive for *mecA*, respectively (Table 1). The findings presented 48 MSSA