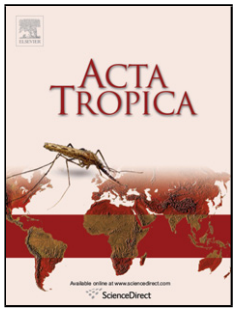

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Antibiotic susceptibilities and prevalence of Methicillin resistant *Staphylococcus aureus* (MRSA) isolated from bovine milk in Pakistan

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Running title: Prevalence and *in-vitro* therapy response of MRSA

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Highlights

- *Staphylococcus aureus* (*S. aureus*) influences are well-known to cause subclinical, clinical, and chronic mastitis.
- The genetic capabilities of methicillin-resistant *S. aureus* (MRSA) strains enhance the pathogenesis of *S. aureus* in mastitis and eludes the immune response of the host
- Bovine milk samples from district Faisalabad presented 34% of MRSA prevalence, with 30% from cattle and 38% from buffalo.
- The Ciprofloxcin, Moxifloxacin, Linezolid, and Trimethoprim plus Sulphamethoxazole drugs showed the highest efficacy to combat this pathogen in the study area

Abstract:

The study was designed to investigate bovine milk for prevalence of an emerging zoonotic pathogen Methicillin resistant *Staphylococcus aureus* (MRSA), and *in-vitro* therapeutic response of various antibiotics against MRSA. Nine hundred (900) milk samples were collected (half from cattle and half from buffalo) from private and public farms located in various tehsils of district Faisalabad, using the convenient sampling method. Milk samples were put to biochemical identification of *Staphylococcus aureus* and later oxacilline disk sensitivity testing for confirmation of MRSA. The MRSA isolates were confirmed by PCR targeting *mecA* gene in *Staphylococcus aureus*. The study found 34% prevalence of MRSA in overall bovine milk from district Faisalabad with 30 % and 38% prevalence in cattle and buffalo, respectively. Tehsil Samundari presented comparatively higher MRSA prevalence followed by tehsil

Jaranwala and tehsil Faisalabad. However, there was non-significant difference of MRSA prevalence between cattle and buffalo, and among different tehsils. All assumed risk factors except specie were significantly associated with mastitis spread. The *in-vitro* drug trial against MRSA from buffalo milk presented 100% efficacy of Ciprofloxcin, Moxifloxacin, Linezolid, and Trimethoprim plus Sulphamethoxazole combination, followed by Gentamicin and Levofloxacin presenting 90%, and Amikacin becoming 80% efficacious against MRSA from buffalo milk. The MRSA isolates of cattle milk presented similar pattern with some variations of higher susceptibility against Oxytetracycline, and Fusidic acid. The conclusion of the study states uniform prevalence of MRSA in cattle and buffalo milk in study area having assumed risk factors positively associated with disease spread, while Ciprofloxcin, Moxifloxacin, Linezolid, and Trimethoprim plus Sulphamethoxazole drugs showed the highest efficacy to combat this pathogen.

Key words: Methicillin resistant *Staphylococcus aureus* (MRSA), cattle, buffalo, milk, *in-vitro* therapeutic efficacy, risk factors

1. Introduction

Staphylococcus aureus (*S. aureus*) infections are difficult to control and are well-known to cause subclinical, clinical, and chronic mastitis, while treatment approaches frequently compromised (Barkema et al., 2009) This tolerance is also attributed to various genetic capabilities of these microorganisms, the important among them is methicillin-resistant *S. aureus* (MRSA) strains, which enhance the pathogenesis of *S. aureus* in mastitis and eludes the immune response of the host (Brady et al., 2011; Kenar et al., 2012). Methicillin-resistant *S. aureus* (MRSA) has been reported as transmissible zoonotic and humanotic importance with bidirectional diffusion (AVMA, 2014). The zoonotic spread of MRSA from animals to humans involves direct contact, handling of animal products, and contact with contaminated environment (Nunang and Young, 2007). Infectious diseases have been a serious threat for livestock (Elhaig et al., 2016; Qayyum et al., 2016; Yilmaz et al., 2016). The prevalence of MRSA in bovines has been reported 0.4% in Hungary (Juhász-Kaszanyitzky et al., 2007) and 47.6% in China (Pu et al., 2014). Poor farm management and indiscriminate use of antibiotic may augment MRSA emergence in bovine milk (Joshi et al., 2014). The initial studies on MRSA reported hospital-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA) infections. Studies have found another group, that was named livestock associated MRSA (LA-MRSA). LA-MRSA colonization in bovine may prove to be occupational risk to veterinary professional and to the people in close contact with animals (Paterson et al., 2012). LA-MRSA infections in humans are almost similar to livestock, which includes severe skin and soft tissue infections. Studies have reported 13% of MRSA linked skin and soft tissue infections from LA-MRSA in people close or in occasional contact with livestock (Layer et al., 2012). The expression of methicillin resistance may complicate the antimicrobial therapy (Lowy, 2003). It is prime need to put early attention to LA-MRSA prevalence before it goes to circle of animal to human and from humans to animal ultimately

creating various potential threat strains to infect humans and animal health (Kadlec et al., 2009). The studies on surveillance of MRSA and identification of novel antibiotic efficacy may limit MRSA threat to animal and human health (Paterson et al., 2012). The studies on MRSA prevalence are scarce in Pakistan that demands due attention to be given. The current study was aimed to investigate prevalence of MRSA along-with its *in-vitro* drug therapy.

2. Material and Methods

2.1 Aim of study:

The present study was conducted to investigate the prevalence of MRSA from bovine milk, and *in-vitro* therapeutic response to various antibiotics.

2.2 Study design:

District Faisalabad is 3rd largest city in Pakistan located 184m above sea level altitude between 31°20'-31°33' N and 73°13'-72°55' (Cheema et al., 2006). The majority of the population of district Faisalabad is involved with agriculture in rural areas.

Three tehsils of district Faisalabad (Faisalabad, Jaranwala, and Samundari) were selected as representative of whole district. The herds were categorized as small (40-90 animals), medium (91-200), and large (>200 animals) to collect total 900 milk samples (n=450 cattle, n=450 buffalo) in such a way that each tehsil was sampled with 300 milk samples (n=150 cattle, n=150 buffalo). The dairy animals were provided with concentrate in addition to silage at large farms or fresh fodder at small farms but did not have grazing practice. The animals at small farms were observed with hand milking while medium and larger farms were on machine milking practice with occasional hand milking practices too. Ten farms from each tehsil were examined for 30 milk samples (n=15 cattle, n= 15 buffalo) using convenient sampling method (Thrushfield, 2007) depending upon the willingness and accessibility to the farm and samples shipment to the laboratory.

2.3 Screening of the field samples:

Subclinical mastitis was estimated on farm by Surf Field Mastitis Test (SFMT) proposed by (Muhammad et al., 1995). The collected milk samples were transported maintaining cold chain to Laboratory of Microbiology, University of Veterinary and Animal Sciences Lahore for further processing. A questionnaire was filled to estimate assumed risk factor association with subclinical mastitis. However, all of collected milk samples (n=900) were processed for microbiological examination in order to get whole picture of methicillin resistant *S. aureus* prevalence in bovine milk.

2.4 Confirmation of *S. aureus* and *in-vitro* antibiotic sensitivity:

Each milk sample (0.5 ml) was spread out primarily on blood agar (CM0271, Thermo Scientific Microbiology Sdn Bhd) and incubated for 24-48 hours at 37^{o}C (Lafi et al., 1998). Biochemical tests were performed as per guidelines of Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) to confirm *S. aureus* isolates. The fresh growth of *S. aureus* was adjusted at 0.5 McFarland that was swabbed on Muller Hinton agar. The oxacillin disks (1 µg) were aseptically applied on Muller Hinton agar (B3374, Oxoid, Basingstoke, UK) using disk dispenser (Oxoid™). The plates were incubated for 24 hours at 37^{o}C, and zones of inhibition were measured by Vernier callipers in millimetres (Bauer et al., 1966). The zones of inhibition were compared with standard zones of susceptibility, provided by Clinical Laboratory Standard Institute (CLSI, 2016).

2.5 Molecular confirmation of *mecA* gene:

Confirmed *S. aureus,* resistant samples against oxacillin, were further processed to *mecA* gene confirmation by PCR. DNA was extracted using bacterial DNA extraction kit (GF-BA-050, GF-1 Bacterial DNA extraction kit, Vivantis Technologies Sdn. Bhd, Malaysia). Quantification of DNA was done using Nano-drop technique (NanoDrop^{2000}-Thermoscientific^{TM}). Extracted DNA was subjected to PCR reaction using primers, P1:5'-

TGGCATTCGTGTCACAATCG-3' and P2: 5'- CTGGAACTTGTTGAGCAGAG-3' and the conditions were applied as described by (Galdiero et al., 2003) at product size of 310bp. Amplified product was run on 2% agarose gel with ethidium bromide staining at the rate of 0.5 mg/ml. The stained gel was photographed under UV light (Figure 1). The PCR confirmed methicillin resistant *S. aureus* from cattle (n=20) and buffalo (n=20) were processed to antibiotic sensitivity against different drugs including, Oxytetracycline (30µg), Tylosin (15µg), Gentamicin (10µg), Amikacin (30µg), Ciprofloxcin (5ug), Levofloxacin (5µg), Moxifloxacin (5µg), Trimethoprim + Sulphamethoxazole (30µg), Linezolid (25µg), Cefoxitin (30µg), Fusidic acid (10µg), and Vancomycin (30µg) from various calsses of antibiotics using disk diffusion method (Bauer et al., 1996). The zones of inhibitions were measured and compared with their comparative latest standarad zones given by the Clinical and Laboratory Standards Institute (CLSI, 2015).

2.6 Statistical Analysis:

The data was exposed to statistical analysis using SPSS version 22.0 computer program. Prevalence of MRSA was calculated as per formula described Thrushfield, (2007), and comparisons were made at 5% probability.

3. Results

The study showed 55% (495/900) overall subclinical mastitis based on surf field mastitis test (SFMT). The isolates of *S. aureus* found resistant against oxacillin were confirmed by targeting *mecA* gene in *S. aureus* (Fig 1). The current study found 34% (306/900) of MRSA prevalence in bovine milk from district Faisalabad (Table 1). The comparison of bovine milk MRSA prevalence from different tehsils were found non-significant with however higher prevalence in tehsil Samundari (40%) followed by 31% of MRSA in each of tehsil Jaranwala and tehsil Faisalabad. Similarly, cattle milk MRSA was found higher in tehsil Samundari (36%) followed by tehsil Faisalabad (28%) and tehsil Jaranwala (26%). The highest of MRSA

prevalence was found in buffalo milk from tehsil Samundari followed by tehsil Jaranwala and tehsil Faisalabad with 44%, 36% and 34%, respectively. Non-significant difference was found between cattle and buffalo among different tehsils. Higher MRSA prevalence was found in buffalo milk samples (38%) compared to cattle (30%) in district Faisalabad. All assumed risk factors showed significant association with mastitis except the bovine species (Table 3). Significant association of use of beta-lactam group with the spread of mastitis was found in this study. Poor hygiene condition, tick prevalence, and lack of teat dipping in current study were directly linked with mastitis spread. The study noted unjustified use of beta-lactam group in general animal ailments before the veterinary professional have been consulted. Poor hygiene condition, tick prevalence, and lack of teat dipping in current study were directly linked to mastitis spread.

The *in-vitro* findings of current study revealed Ciprofloxcin, Moxifloxacin, Linezolid, and Trimethoprim+Sulphamethoxazole presenting 100%, Levofloxacin and Gentamicin showed 90%, Tylosine, Fusidic acid, and Oxytetracyline gave rise to 60%, 50%, and 40 % efficacies against MRSA obtained from buffalo (Table 2). The *in-vitro* therapeutic trial against MRSA isolates of cattle milk presented similar pattern with few exceptions, in that efficacy of Oxytetracycline, and Fusidic acid was higher than that buffalo milk MRSA. However, efficacies of Gentamicin, Tylosine, Amikacin, and Vancomycin increased from 50% to 90%, 40% to 60%, 40% to 80%, and 40% to 70% against MRSA isolated from buffalo milk compared to MRSA obtained from cattle milk. Varying degree of sensitivity of antibiotic against MRSA in milk of different species was observed during the study

4. Discussion

A study in China (Pu et al., 2014) reported 47% MRSA prevalence in bovine which is higher to that of current study i.e. 34%. However, lower prevalence of MRSA has been found in Germany 16.7% (Spohr et al., 2011), 13.1% in India (Kumar et al., 2011), 6.3% in Korea

(Lim et al., 2013), 4% in USA (Haran et al., 2012), 1.8% in Wisconsin (Makovec et al., 2003), and 0.6% in Michigan (Erskine et al., 2002). The prevalence of MRSA is correlated with farm population, farming systems, disinfectants used, and animal trading (Alt et al., 2011; Graveland et al., 2011; Cuny et al., 2012). There are some discrepancies regarding estimation of MRSA prevalence. Lower prevalence of MRSA could be because of poor expression of *mecA* gene or overproduction of beta-lactamase (Turutoglu et al., 2009) that creates diagnostic discrepancies. On the other hand culture media's parameters like pH and culture osmolality can change phenotypic expression. The limitations of conventionally used microbiological techniques may produce false positive or false negative results (Kumar et al., 2010). The divergent of *mecA* gene cannot be diagnosed with the primers that are currently used in various studies (García-Alvarez et al., 2011; Haran et al., 2012). Moreover greater variation in MRSA strains can be reason of diagnostic limitation (Haran et al., 2012).

All assumed risk factors in the study showed significant ($p<0.05$) association with mastitis except the animal species. The farms of study area mostly have practiced to keep cattle and buffaloes together that may be the reason for non-significant difference of disease among species. Moreover, farm workers involved in milking process have same exposure to cattle and buffalo so spread of pathogen have equal chances of infection between species. In this study poor hygiene condition, tick prevalence, and lack of teat dipping were found directly linked to mastitis spread. Similar findings were reported in a study on *S. aureus* from Pakistan (Aqib et al., 2017). Direct contact with contaminated milk and with environment also helps the spread of MRSA. The situation aggravates in the presence of farm mismanagement and inexcusable use of drugs in general ailments and mammary gland diseases (Nunang and Young, 2007; Juhász-Kaszanyitzky et al., 2007). Unhygienic hands of personals while milking are potential source of spread of contagious mastitis pathogen during milking practices. Tick infestation serves as source of bacterial transmission animal to animal with special perspective to *S. aurues*

like contagious pathogen (Seifu and Tafesse, 2010). Higher prevalence of *S. aureus* positively associated with higher milking frequency was in line with previous study (Aqib et al., 2017a). Unhygienic milking process which was common observed during this study, might be contributed to higher prevalence in animals that had gone through higher frequency of milking.

The findings of current study revealed Ciprofloxcin, Moxifloxacin, Linezolid, and Trimethoprim+Sulphamethoxazole presenting 100%, Levofloxacin and Gentamicin showed 90%, Tylosine, Fusidic acid, and Oxytetracyline gave rise to 60%, 50%, and 40 % efficacies against MRSA obtained from buffalo. The findings of current study regarding Linezolid were also in line with a study on epidemiology and molecular characterization of methicillin-resistant *S. aureus* (Nemeghaire et al., 2014) in which they noted all of isolates sensitive to Linezolid, while 84% sensitivity was noted against Ciprofloxacin which is congruent with findings of current study. The sensitivity of MRSA against Trimethoprim and Ciprofloxacin was in agreement with findings of Aqib et al. (2017b), but efficacy of Amikacin against MRSA was higher in current study. On the other hand, no MRSA isolate from buffalo and cattle milk were found sensitive to Cefoxitin which is in line with a study on MRSA (Nemeghaire et al., 2014) where 100% resistance was noticed.

In conclusion, the methicillin resistant *S. aureus* was found prevalent in cattle and buffalo from all the tehsils of district Faisalabad, Pakistan. The spread of MRSA was found uniform without any significant difference between bovine species or among different tehsils of studied areas. *In-vitro* therapeutic efficacy indicated Ciprofloxacin, Moxifloxacin, Linezolid, Trimethoprim + Sulphamethoxazole, levofloxacin equally effective against MRSA isolates of cattle and buffalo. However, other antibiotics gave promosing results against MRSA from bovine milk. The study found effectitve treatment options against prevalent MRSA from bovine milk.

Conflict of Interest Statement

The authors declared that they have no conflict of interest.

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REFERENCES

Alt, K., Fetsch, A., Schroeter, A., Guerra, B., Hammerl, J.A., Hertwig, S., Senkov, N., Geinets, A., Mueller-Graf, C., Braeunig, J., Kaesbohrer, A., 2011. Factors associated with the occurrence of MRSA CC398 in herds of fattening pigs in Germany. BMC Vet. Res. 7, 69.

American Veterinary Medical Association (AVMA): MRSA and animals FAQ. 2014. Available online at https://www.avma.org/KB/Resources/FAQs/Pages/MRSA-HHP-FAQs.aspx.

Aqib, A.I., Ijaz, M., Durrani, A.Z., Anjum, A.A., Hussain, R., Sana, S., Farooqi, S.H., 2017a Identification of coagulase gene in Staphylococcus aureus isolates recovered from subclinical mastitis in camels, Pakistan. Pak. Vet. J. 37 (2): 160-164.

Aqib, A.I., Ijaz, M., Hussain, R., Durrani, A.Z., Anjum, A.A., Sana, S., Farooqi, S.H., Hussain, K., Ahmad, S.S., 2017b. Prevalence and antibiogram of Staphylococcus aureus, a camel mastitogen from Pakistan. Pak. J. Zool. 49, 861-867.

Barkema, H.W., Green, M.J., Bradley, A.J., Zadoks, R.N., 2009. Invited review: The role of contagious disease in udder health. J. Dairy Sci. 92, 4717-29.

Bauer, A.W., Kirby, W.M., Sheriss, J.C., Turc, M., 1996. Antibiotic susceptibility testing by standardized single method. Am. J. Clin. Pathol. 45, 493-496.

Bauer, A.W., Kirby, W.M., Sherris, J.C., Turck, M., 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45, 493-496.

Brady, R.A., Graeme, A.O., Leid, J.G., Prior, M.L., Costerton, J.W., Shirtliff, M.E., 2011. Resolution of Staphylococcus aureus biofilm infection using vaccination and antibiotic treatment. Infect. Immun. 79, 1797-803.

Cheema, M.A., Farooq, M.U., Ahmad, R.A., Munir, H.A., 2006. Climatic trends in Faisalabad (Pakistan) over the last 60 years (1945-2004). J. Agric. Soc. Sci. 2, 42-5.

Clinical and Laboratory Standards Institute (CLSI), 2015: Performance Standards for Antimicrobial Susceptibility Testing 26th edition: Informational Supplement. CLSI Document M100-S26. Performance Standards for Antimicrobial Disk Susceptibility Test, Approved standard 12th edition. CLSI Document M02-A12. Clinical and Laboratory Standards Institute, Wayne PA.

Clinical and Laboratory Standards Institute (CLSI), 2016. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved standard M31-A3. CLSI, Wayne, PA.

Cuny, C., Friedrich, A.W., Witte, W., 2012. Absence of livestock-associated methicillin-resistant Staphylococcus aureus clonal complex CC398 as a nasal colonizer of pigs raised in an alternative system. Appl. Environ. Microbiol. 78, 1296-7.

Elhaig, M.M., Selim, A., Mahmoud, M.M., El-Gayar, E.K., 2016. Molecular confirmation of Trypanosoma evansi and Babesia bigemina in cattle from lower Egypt. Pak. Vet. J. 36, 409–414.

Erskine, R.J., Walker, R.D., Bolin, C.A., Bartlett, P.C., White, D.G., 2002. Trends in antibacterial susceptibility of mastitis pathogens during a seven-year period. J Dairy Sci. 85, 1111-8.

Galdiero, E., Liguori, G., Isanto, M.D., Damiano, N., Sommese, L., 2003. Distribution of mecA among methicillin-resistant clinical staphylococcal strains isolated at hospitals in Naples, Italy. Eur. J. Epidemiol. 18, 139–145.

García-Álvarez, L., Holden, M.T., Lindsay, H., Webb, C.R., Brown, D.F., Curran, M.D., Walpole, E., Brooks, K., Pickard, D.J., Teale, C., Parkhill, J., 2011. Meticillin-resistant Staphylococcus aureus with a novel mecA homologue in human and bovine

populations in the UK and Denmark: a descriptive study. Lancet. Infect. Dis. 11, 595-603.

Graveland, H., Duim, B., Van Duijkeren, E., Heederik, D., Wagenaar, J.A., Livestock-associated methicillin-resistant Staphylococcus aureus in animals and humans. Int. J. Med. Microbiol. 301, 630-4.

Haran, K.P., Godden, S.M., Boxrud, D., Jawahir, S., Bender, J.B., Sreevatsan, S., 2012. Prevalence and characterization of Staphylococcus aureus, including methicillin-resistant Staphylococcus aureus, isolated from bulk tank milk from Minnesota dairy farms. J Clin. Microbiol. 50, 688-95.

Holt, J.G., Krieg, N.R., Sneathm, P.H.A., Staley, J.T., Williams, S.T., 1994. Bergey's Manual of Determinative Bacteriology. 9th Ed. Baltimore, MD: Williams and Williams.

Joshi, L.R., Tiwari, A., Devkota, S.P., Khatiwada, S., Paudyal, S., Pande, K.R., 2014. Prevalence of methicillin-resistant Staphylococcus aureus (MRSA) in dairy farms of Pokhara, Nepal. Int. J. Vet. Sci. 3, 87-90.

Juhász-Kaszanyitzky, E., Jánosi, S., Somogyi, P., Dán, Á., van der Graaf-van Bloois, L., van Dulijkeren, E., Wagenaar, J.A., 2007. MRSA transmission between cows and humans. Emerg. Infect. Diseases. 4, 630-2.

Kadlec, K., Ehricht, R., Monecke, S., Steinacker, U., Kaspar, H., Mankertz, J., Schwarz, S., 2009. Diversity of antimicrobial resistance pheno-and genotypes of methicillin-resistant Staphylococcus aureus ST398 from diseased swine. J. Antimicrob. Chemother. dkp350.

Kenar, B., Kuyucuoğlu, Y., Şeker, E., 2012. Antibiotic susceptibility of coagulase-negative staphylococci isolated from bovine subclinical mastitis in Turkey. Pak. Vet J. 32, 390-3.

Kumar, R., Yadav, B.R., Singh, R.S., 2010. Genetic determinants of antibiotic resistance in Staphylococcus aureus isolates from milk of mastitic crossbred cattle. Curr. Microbiol. 60, 379-86.

Kumar, R., Yadav, B.R., Singh, R.S., 2011. Antibiotic resistance and pathogenicity factors in Staphylococcus aureus isolated from mastitic Sahiwal cattle. J. Biosci. 36, 175-88.

Lafi, S.Q., and Hailat, N.Q., 1998. Bovine and ovine mastitis in Dhuleil valley of Jordan. Veterinarski Arhiv. 68, 51–57.

Layer, F., Cuny, C., Strommenger, B., Werner, G., Witte, W., 2012. AktuelleDaten und Trends zu. Methicillin-resistenten Staphylococcus aureus (MRSA). Bundesgesundheitsbla. 55, 1377-86.

Lim, S.K., Nam, H.M., Jang, G.C., Lee, H.S., Jung, S.C., Kim, T.S., 2013. Transmission and persistence of methicillin-resistant Staphylococcus aureus in milk, environment, and workers in dairy cattle farms. Foodborne Pathog Dis. 10, 731-6.

Lowy, F.D., 2003. Antimicrobial resistance: the example of Staphylococcus aureus. J. Clin. Invest. 111, 1265-73.

Makovec, J.A., Ruegg, D.P., 2003. Antimicrobial resistance of bacteria isolated from dairy cow milk samples submitted for bacterial culture: 8,905 samples (1994–2001). J Am Vet Med Assoc. 222, 1582-9.

Muhammad, G., Athar, M., Shakoor, A., Khan, M.Z., Rehman, F., Ahmad, M.T., 1995. Surf Field Mastitis Test: An inexpensive new tool for evaluation of wholesomeness of fresh milk. Pak. J. Food Sci. 5, 91-3.

Nemeghaire, S., Argudín, M.A., Haesebrouck, F., Butaye, P., 2014. Epidemiology and molecular characterization of methicillin-resistant Staphylococcus aureus nasal carriage isolates from bovines. BMC Vet. Res. 10, 153.

Nunang, C., and R. Young. MRSA in farm animals and meat- A new threat to human health. 2007. Soil association, USA.

Paterson, G.K., Larsen, J., Harrison, E.M., Larsen, A.R,, Morgan, F.J,, Peacock, S.J., Parkhill, J., Zadoks, R.N., Holmes, M.A., 2012. First detection of livestock-associated meticillin-resistant Staphylococcus aureus CC398 in bulk tank milk in the United Kingdom, January to July 2012. Euro surveillance: bulletin Europeen sur les maladies transmissibles, European communicable disease bulletin. 17(50).

Pu, W., Su, Y., Li, J., Li, C., Yang, Z., Deng, H., Ni, C, 2014. High incidence of oxacillin-susceptible mecA-positive Staphylococcus aureus (OS-MRSA) associated with bovine mastitis in China. PLoS One. 9, e88134.

Qayyum, A., Khan, J.A., Hussain, R., Awais, M., Ahmad, N., Khan, M.S., 2016. Investigation of milk and blood serum biochemical profile as an indicator of subclinical mastitis in Cholistani cattle. Pak. Vet. J. 36, 275–279.

Seifu, E., Tafesse, B., 2010. Prevalence and etiology of mastitis in traditionally managed camels (Camelus dromedarius) in selected pastoral areas in eastern. Ethiopia. Ethiop. Vet. J. 14, 103-113.

Spohr, M., Rau, J., Friedrich, A., Klittich, G., Fetsch, A., Guerra, B., Hammerl, J.A., Tenhagen, B.A., 2011. Methicillin-Resistant Staphylococcus aureus (MRSA) in Three Dairy Herds in Southwest Germany. Zoonoses Public Health. 8, 252-61.

Thrushfield, M., 2007. Veterinary Epidemiology, (Blackwell Science, USA). pp: 180-181

Turutoglu, H., Hasoksuz, M., Ozturk, D., Yildirim, M., Sagnak, S., 2009. Methicillin ad aminoglycoside resistancein Staphylococcus aureus isolates from bovine mastitis and sequence analysis of their mecA genes. Vet. Res. Commun., 33, 945-956.

Yilmaz, R., Cangul, I.T., Onat, K., Akkoc, A., Ozyigit, M.O., Akdesir, E., 2016. Histopathological, immunohistochemical and bacteriological characterization of Mycoplasma bovis pneumonia in cattle. Pak. Vet. J. 36, 316–321.

Figure 1: PCR gel results of MRSA at 310bp level against 100bp molecular weight marker on Ethidium bromide stained 2% Agarose Gel. Lane M indicates Molecular weight marker, Lane 1-6 shows MRSA positive samples

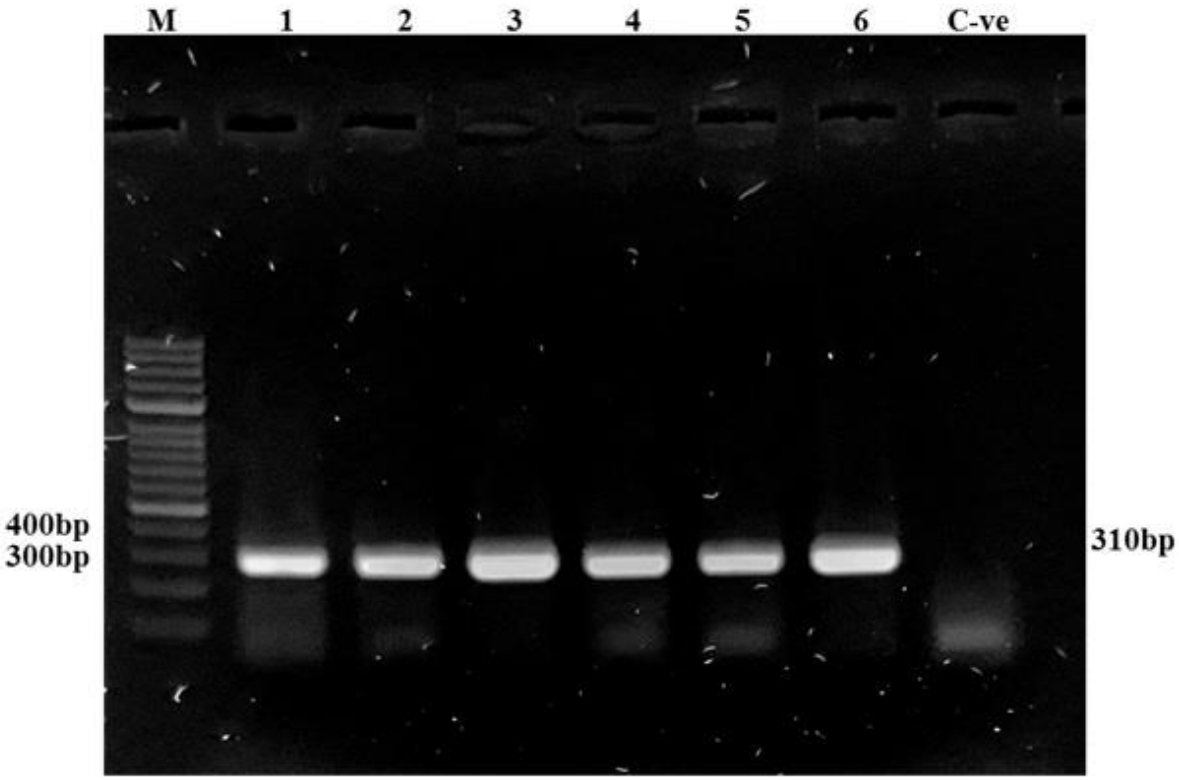

Figure1: PCR gel results for MRSA at 310bp level against 100bp molecular weight marker on ethidium bromide stained 2% agarose gel. Lane M indicates 100bp molecular weight marker, Lane 1-6 indicates MRSA positive samples and Lane C-ve indicates control negative

Table 1: Prevalence of Methicillin resistant *Staphylococcus aureus* in cattle and buffalo

**Area	*Cattle				*Buffalo				*Overall			
	Milk samples	**No. (%)**	**95% CI**	**P-value**	**Milk samples**	**No. (%)**	**95% CI**	**P-value**	**Milk samples**	**No. (%)**	**95% CI**	**P-value**
Faisalabad	150	42 (28)	20.79-35.21		150	51 (34)	26.39-41.61		300	93 (31)	25.76-36.24	
Jaranwala	150	39 (26)	18.96-33.04	0.513	150	54 (36)	28.29-43.71	0.552	300	93 (31)	25.76-36.24	0.33
Samundri	150	54 (36)	28.29-43.71		150	66 (44)	36.03-51.97		300	120 (40)	34.45-45.55	
Total	450	135 (30)	22.64-37.36	-	450	171 (38)	30.21-45.79	-	900	306 (34)	30.98-37.02	-

Values in parenthesis are percentages; P<0.05 indicate significant difference. ** *P*-values in table show difference of MRSA among different cities for cattle, buffalo, and overall basis. **P*-values of MRSA comparison between cattle and buffalo within city were, *P*=0.68 Faisalabad, *P*=0.28 Jaranwala, *P*=0.68=0.414, *P*=0.144 over all basis

Table 2: I*n-vitro* therapeutics efficacy of various antibiotics against Methicillin resistant *Staphylococcus aureus*

Antibiotic name	Potency	Buffalo			Cattle		
		R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Oxytetracycline	30µg	40	20	40	40)	20	60
Tylosine	15µg	30	10	60	30	10	40
Gentamicin	10µg	10	-	90	50	-	50
Amikacin	30µg	10	10	80	60	-	40
Ciprofloxcin	5ug	-	-	100	-	-	100
Levofloxacin	5µg	-	10	90	-	10	90
Moxifloxacin	5µg	-	-	100	-	-	100
Linezolid	25 µg	-	-	100	-	-	100
Trimethoprim + Sulphamethoxazole	30µg	-	-	100	-	-	100
Cefoxitin	30µg	100	-	-	100	-	-
Fusidic acid	10µg	50	-	50	40	-	60
Vancomycin	30µg	30	-	70	60	-	40

S=sensitive, R=resistant, I=intermediate

Table 3: Bivariate analysis of risk factors associated with mastitis in bovine

Variable	**Parameter**	**Total**	**Positive**	**Percentage (%)**	**Relative risk**	***P*-value**
Specie	Cattle	459	243	52.94	0.92	0.205
	Buffalo	441	252	57.14	1.092	
Use of teat dips	Yes	297	105	35.35	0.447	0.001
	No	603	390	64.68	1.498	
Presence of ticks	Yes	552	351	63.59	1.429	0.001
	No	348	144	41.38	0.598	
Lactation status	Dry	369	156	42.28	0.599	0.001
	Lactating	531	339	63.84	1.445	
Frequency of milking	Once/day	84	12	14.29	0.165	0.001
	Twice/day	846	456	53.90	1.154	
Milker's hands hygiene during milking	Yes	300	108	36.00	0.460	0.001
	No	600	387	64.50	1.487	
Hygienic condition during milking	Yes	315	93	29.52	0.343	0.001
	No	585	402	68.72	1.1793	
Antibiotics used in general ailment	B-lactam group	771	453	58.75	1.166	0.001
	Others	129	42	32.56	0.395	
Antibiotics used in mastitis	B-lactam group	795	468	58.87	1.171	0.001
	Others	105	27	25.71	0.283	