Indian J Dairy Sci 77(2): 117-122
https://doi.org/10.33785/IJDS.2024.v77i02.003

RESEARCH ARTICLE

Prevalence of mastitis and antibiotic resistant *E. coli* and *S. aureus* in dairy animals

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Received: 17 June 2023 / Accepted: 18 September 2023 / Published online: 23 April 2024
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Abstract: The prevalence of mastitis in milking animals and subsequent antibiotic usage is one of the major concerns in dairy sector. In this work, a baseline data was generated by collecting 675 raw milk samples from organized and individual dairy farms in Haryana. The milk samples were screened for mastitis, antibiotic residues and antimicrobial resistance (AMR) *E. coli* and *S. aureus* using rapid BD Phoenix M50 ID/ AST system and conventional methods. The study indicated 14.37% animals infected with sub-clinical mastitis and 11.25% with clinical mastitis. 79 milk samples from normal and infected animals were found contaminated with antibiotic residues with presence of enrofloxacin, streptomycin, tetracycline, sulfa drugs and multi drug residues. Out of 675 samples, 173 were infected with mastitis with involvement of *E. coli* in 18.49% and *S. aureus* in 38.72%. In *E. coli* isolates, the maximum resistance of 25% was observed against Ampicillin, Ciprofloxacin and Levofloxacin and Extended spectrum β-Lactamase (ESBL) productionwas observed in 15.65% infected samples.The maximum resistance of 32% was observed in *Staphylococcus aureus* against Ampicillin and Penicillin-G and Methicilin Resistant Staphylococcus aureus (MRSA) was found in 22.38% along with β-Lactamase resistance in 31.34% of infected samples.The main reason behind the increasing number of resistant pathogens may be due to the indiscriminate use of antibiotics by untrained veterinary professionals and lack of diagnostic facilities, hence regular screening of sub-clinical mastitis should be practiced to control the usage of antimicrobials and resistant development in dairy pathogens.

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Keywords: Antibiotic resistance; *E.coli*; ESBL; Milking animals; MRSA; Mastitis; *S. aureus*

Introduction

Mastitis is a major problem affecting all milk producing animals worldwide and is one of the main reasons for impaired milk quality (Bradley, 2002; Le Roux et al. 2003). Mastitis is the inflammation of udder; the term comes from the Greek word i.e. Masto- referring to the mammary gland and its meaning "inflammation" (Blood and Studdert., 1999). Mastitis can be sub-clinical, clinical and chronic depending on severity of inflammation. Dairy farmers face a financial burden from bovine mastitis, and preventive mitigation strategies are essential for the long-term viability of any dairy production. Controlling the infection, minimizing the risk of persistent infections, and directing antimicrobial therapy all need the identification of etiological agents (Duarte et al. 2015). It is mainly caused by bacterial pathogens which include *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, and Mycoplasma sp.* and environmental pathogens involving *E. coli* and *Klebsiella spp*. Other common pathogens include *Corynebacterium spp., coagulase-negative staphylococci* and *Pseudomonas aeruginosa* (Motwani & Kishore., 2011). Despite extensive research into controlling bovine mastitis, the occurrence of mastitis remains high, resulting in massive losses for the dairy industry (Lee et al. 2008). The pathogens responsible for producing mastitis will determine the sort of mastitis treatment that should be offered. Greater than 200,000 somatic cells per milliliter (SCC) is a sign of inflammation and subclinical mastitis (Cobirka et al. 2020). During the infection caused by the micro-organisms, the host immune response is activated to eliminate the invading microorganisms lead to inflammation and damage milk-producing tissue of the mammary gland leading to decreased in milk yield (Egyedy and Ametaj, 2022) . Milk from animals with mastitis cannot be used for human consumption because it has altered chemical composition and organoleptic proprieties (Kobayashi et al. 2013). Moreover, milk from diseased animals negatively affects the milk processing and shelf-life of final dairy products. This disease is considered to be the major cause of economic loss to dairy farmers (Tommasoni et al. 2023). Mastitis can be reduced by some aspects of dairy farming such as feeding

practices, animal husbandry, hygiene and general health care. Increasing number of infection has led to the increased use of antibiotics for treatment but their indiscriminate use by untrained veterinary professionals or quacks has resulted in increased resistance of antibiotics among the dairy animals which is a growing concern and need to be monitored carefully (Eltholth et al. 2022). The present work is focused on detection of mastitis, antibiotic residues, bacterial pathogens (*E. coli* and *S. aureus*) and their resistance pattern in raw milk samples.

Material and methods

All the experiments required for present research were run in triplicate and results were interpreted all the study carried out at National Referral Centre for Milk Quality and Safety and Dairy Microbiology Division, NDRI, Karnal.

Sample collection

A total of 675 raw milk samples were collected from different districts of Haryana i.e. Karnal, Ambala and Sonipat during the year 2018-20. Collection of milk samples was done from healthy as well as infected cows/ buffaloes. Milk sampling was carried out following aseptic procedures as described by National Mastitis Council (NMC, 2004). The time chosen for milk sample collection was before milking. All the details of the animal along with the collection date were recorded. The hands were washed properly with soap and water and the gloves were worn while sampling. Any dirt or debris present on the teat of the animal was brushed and initial few streams of milk were discarded. The teat was pre-dipped with an effective teat dip (6 part of 0.5% iodine with 1 part of glycerin) and left for few seconds. Each teat was dried properly with paper or cloth towel. The teat end was scrubbed for 15-20 seconds with cotton or cloth gauze moistened with 70% to 80% alcohol or isopropyl alcohol. The sample container was opened and immediately the sample was taken preventing the teat touching the container. The sample container was kept in ice box until delivered to lab.

Detection of Mastitis using CMT

The California Mastitis Test (CMT) is a simple indicator of the Somatic Cell Count (SCC) of milk. The procedure of CMT was followed as per the instructions given in the kit manual. 3.0 ml milk was taken in four-compartment paddle and equal amount of CMT reagent was added. After addition of reagent and sample, CMT paddle was rotated 10 times in an anti-clockwise direction and graded based on gel formation, scores as Negative (N), Trace (T), 1, 2and 3 were given.

Detection of Mastitis using Somatic cell counter

Somatic cells are purely animal body cells present in small levels in normal milk. High levels of SCCs in milk indicate poor quality milk that is caused by an intra-mammary infection. The milk analyzer (Model- Ekomilk Scan) measures the flowing time of the milk through the sample mixer capillary and determines the number of somatic cells in accordance with time. The viscosity measurement is temperature sensitive and uses Ekoprim reagent as surfactant. The somatic cells were measured as per the instruction's manual. 10mL of milk sample and 5mL of Ekoprim reagent was added in the sample bulb and the bulb rotated for few seconds after pressing the run button and displayed somatic cell count on the screen along with the time based on viscosity

Detection of antibiotics using Spore based kits

Preliminary screening of antibiotics was done using Paper strip and DPA kits developed at ICAR-NDRI, Karnal as per the test procedure given by Swathi, 2017. Test kit is working on spore germination- inhibition principle and can detect antibiotics in milk at regulatory limits set by FSSAI/ CODEX. In the presence of antibiotics, spore germination is inhibited whereas in the absence of antibiotics spores germinate leading to release of DPA/or enzyme that react with the substrate functionalized on strip resulting in color change from purple to yellow in DPA kit and colorless to blue on strip test.

Quantitative detection of antibiotic groups using AOAC approved CHARM/ ROSA

The antibiotic contaminated milk samples from normal and infected animals were tested using Rapid One step Assay (ROSA) which works on the principle of lateral flow assay. The ROSA Test uses receptors with binding to drugs. The test was performed as per instructions in operator's manual. The incubator was set at desired temperature. 300µL of milk sample was added to the strip and incubated for 8 min and results were observed on the strip and quantified using ROSA reader.

Isolation and identification of *E. coli and S.aureus*

Isolation and identification of *E. coli* was done using ISO procedure IS: 5887 Part-1:1976 (RA-2018). The milk samples were first enriched in McConkey broth and loopful was streaked on McConkey Agar and Eosin methylene blue (EMB) agar. The inoculated media was then incubated at 37°C overnight. If there was growth in McConkey broth along with fermentation of lactose, the loopful was streaked onto solid media and incubated overnight at 37°C. The suspected colonies were further identified using biochemical tests as mentioned in ISO protocol. The confirmed isolates were also tested using BD Phoenix M50. Isolation and identification of *S. aureus* was done using ISO procedure IS: 5887(Part-8/sec-1):2002 (RA-2018). The test sample was spread onto Baird Parker agar (BPA) plates and incubated for 24-48 hrs at 37°C. The suspected grey black colonies with opaque zone were identified using catalase and coagulase test. The confirmed isolates were also tested using BD Phoenix M50.

Antibiotic Susceptibility Test (AST)

The samples which were found positive for *E. coli* and *S. aureus* were further processed for their antibiotic sensitivity/ resistance using different antibiotic discs. Standard disk diffusion assay was conducted using Muller-Hinton agar and broth culture equivalent to 0.5 McFarland standards as recommended by the Clinical and Laboratory Standards Institute (2014). Antibiotic disks were chosen based on commonly used antibiotics for animal and human therapy in the study region. Antibiotics used for *E. coli* were Ceftriaxone, Ceftazidime, Cefotaxime and Cefepime, Imipenem, Meropenem, Ertapenem and Doripenem. Antibiotics used for *S. aureus* were Cefoxitin and Oxacillin. *E. coli* and *S. aureus* isolates were processed for AST using disc diffusion method for preliminary screening and further the resistance pattern was studied using confirmatory tests: double disc test for Extended spectrum β- lactamases (ESBL) detection, Modified Hodge test (MHT) and Modified carbapenamase inactivation method (mCIM)for Carbapenamase (KPC) detection and streaking on Methicillin resistant *S.aureus* (MeReSa) agar for MRSA detection. The AST pattern of positive isolates was studied using conventional method and random samples were further confirmed with BD Phoenix M50.

Statistical Analysis

Data was analyzed statistically in three replicates according to Snedecor and Cochran (1980)

Results and Discussion

A total of 675 raw milk samples from both organized and individual dairy farmers were collected from three districts of Haryana state i.e. Karnal, Ambala and Sonipat. Selection of animals in organized dairy farms and un-organized sector was random. Milk samples were collected directly into the sterile containers from cow's teat and immediately transferred to lab after proper labeling. These samples were tested for Mastitis infection, presence of antibiotic residues, bacterial pathogens i.e. *E. coli* and *S. aureus* and their phenotypic resistance profile.

Prevalence of mastitis

The study showed prevalence of 11.25% clinical mastitis and 14.37% sub-clinical mastitis (Fig.1). Among Mastitis positive samples, S.aureus was detected in 40.78% of clinical mastitis and 37.11% in clinical cases; while in case of *E.coli* 17.10% was detected in clinical mastitis cases and 19.58% in sub-clinical cases, as shown in (Fig 2).

The presence of mastitis in raw milk was 28% using CMT and 25.62% using SCC indicating strong positive correlation as also reported by Badiuzzaman et al. 2015 and Bitew et al.2010. In a similar investigation, Bhat et al. 2017 reported 11.50% and 27.81% (Maheshwari et al. 2016) respectively. The variation in sub-clinical mastitis may be attributed to the selection of animals, season, breed and sanitary conditions. Higher prevalence of sub-clinical mastitis compared to clinical mastitis in present investigation was also supported by Sori et al. 2011. Sub-clinical mastitis give invisible and silent symptoms as reported by Karimuribo et al. 2017 which is usually given less attention by farmers when it comes to treatment unlike clinical mastitis which shows visible and prominent symptoms towards which treatment and control efforts are concentrated.

Prevalence of Antibiotic Residues

The presence of antibiotic residues in milk is a serious concern keeping in view of its processing implications in terms of starter failure in fermented products and public health implications through development of antimicrobial resistance (AMR). Accordingly, surveillance study on antibiotic residues in milk was carried out. The milk samples collected from normal and infected animals were initially screened for Qualitative analysis using spore based kits (DPA/ paper strips) developed at ICAR-NDRI, Karnal. Out of 675 milk samples, normal milk used for

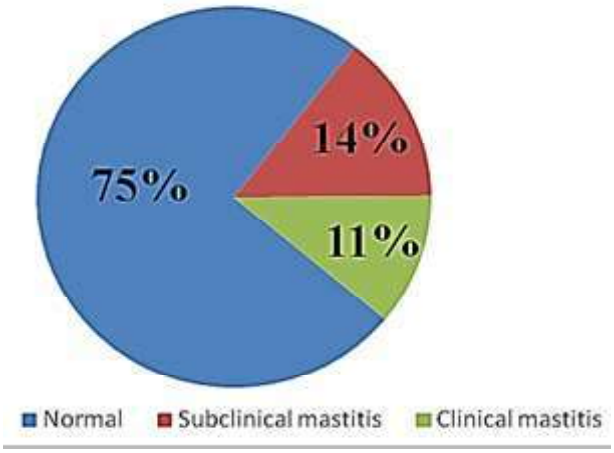

Fig. 1 Incidence of mastitis in raw milk

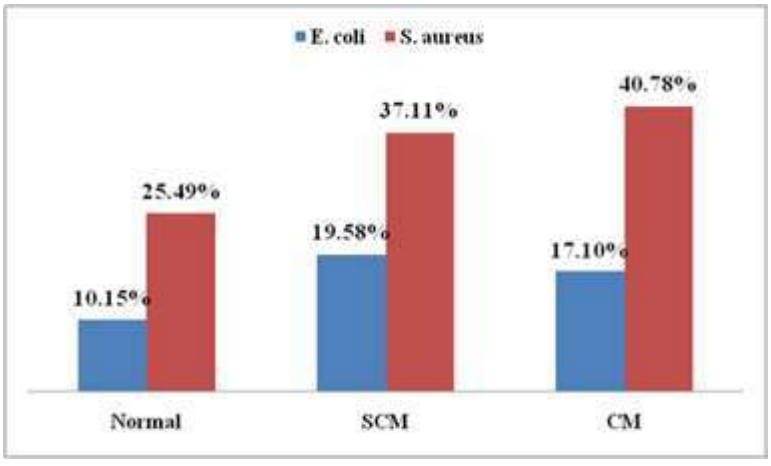

Fig. 2 Incidence of E. coli and S. aureus in raw milk

processing purpose showed presence of antibiotics in 2.96%. Milk from treated animals which is unfit for processing with sub-clinical mastitis were positive with antibiotic in 4.1 % and clinical milk samples with 4.59%. Antibiotic residues like enrofloxacin, streptomycin, tetracycline, sulfa drugs and multidrug residues were detected. In a similar study, Moudgil et al. 2019 reported 11.30% milk samples from Punjab contaminated with antibiotic residues. In recent study, FSSAI (2018) has also reported the presence of antibiotic residues in milk however, the sample size was small and needs further surveillance work to support the findings reported in the current investigation.

Isolation and Identification of *E. coli* & *S. aureus*

Out of 675 samples, 173 were infected with mastitis with involvement of *S. aureus* in 40.78% in clinical and 37.11% in sub-clinical cases. Similarly, *E. coli* was detected in 17.10 % and 19.58% respectively (Fig.2). Kumar et al. 2015 reported similar findings with incidence of 33.82%*S. aureus* and 14.91%*E. coli*. The non-infected milk samples also showed presence of *E. coli* in 10.15% and *S. aureus* in 25.49% .

Antibiotic Susceptibility Test

The AST pattern of positive isolates was studied using conventional method and random samples were further confirmed with BD Phoenix M50.

AST pattern of *E.coli* and *S.aureus* isolates

In the case of *E. coli* isolates, the highest level of resistance at 25% was observed against Ampicillin, Lowest level of resistance at 6.25% was observed against chloramphenical- and no resistance was observed against amikacin, amoxy-clav, Cefoparazone-sulbactam ,Cefoxitin, Gentamicin, Imipenem, Meropenem and Piperacillin- tazobactam(Fig. 3a). In case of *S. aureus*, the highest resistance of 32% was observed against Ampicillin and Penicillin- No resistance was observed against broad range of antibiotic (Fig. 3b).

In case of *S. aureus*, the maximum resistance of 32% was observed against Ampicillin and Penicillin-G followed by Cefazolin, Cefoxitin and Oxacillin (14%). The resistance against norfloxacin, gentamicin and tetracycline ranged between 2-8%. No resistance

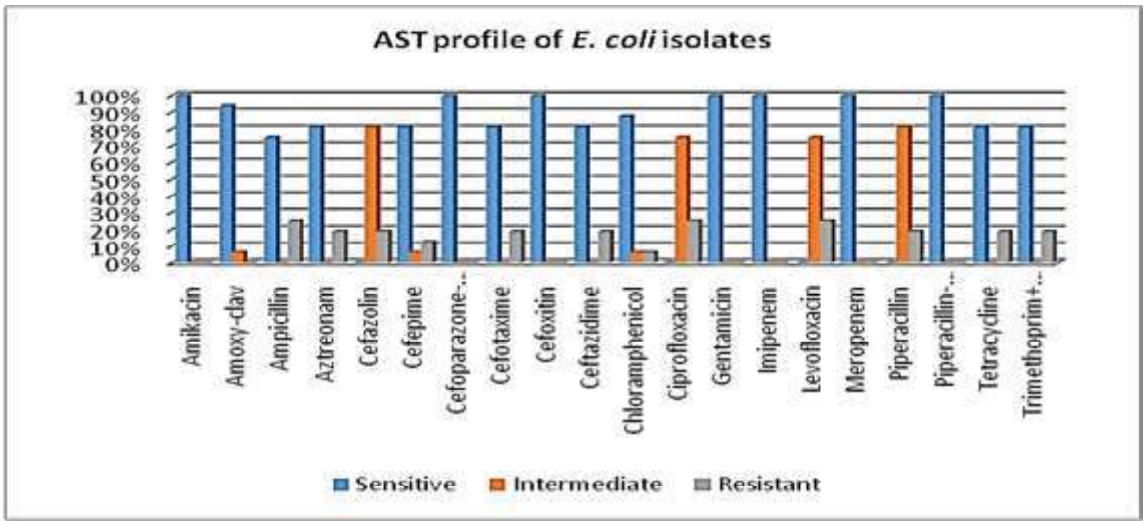

Fig. 3 (a) Graph showing AST profile of *E. coli* isolates

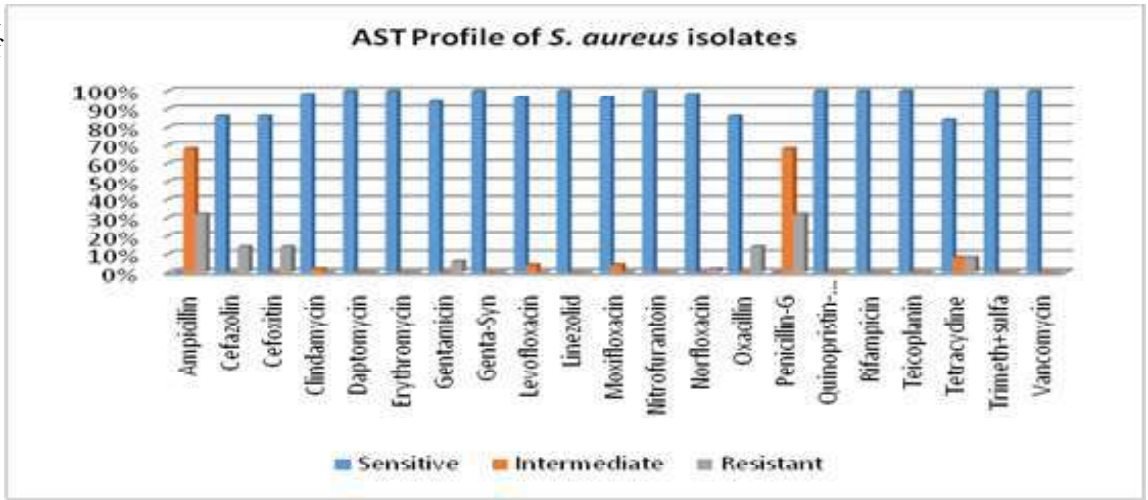

Fig. 3 (b) Graph showing AST profile of *S. aureus* isolates

was observed against Clindamycin, Daptomycin, Erythromycin, Gentamycin-Syn, Levofloxacin, Linozolid, Moxifloxacin, Nitrofurantoin, Quinopristin-dalfopristin, Rifampicin, Teicoplanin, Trimethoprim+ sulfamethaxole and Vancomycin (Fig. 3b)

The incidence of ESBL producing *E. coli was* 26.31% in sub-clinical mastitis . None of the clinical milk samples showed presence of ESBL E.coli . However , normal milk samples also showed presence of ESBL in 7.84% and carbapenase producing E.coli in 1.96 % which was considered as serious finding (Fig.4a). Our findings are in agreement with reports of Sharif et al.2017 who recorded average incidence of 20% ESBL in infected samples. Bhoomika et al. 2016 and Dewangan et al. 2017 also reported incidences of ESBL producing *E. coli* in raw milk samples as 8.22% and 7.69% in Chattisgarh.

The presence of *S. aureus* with resistance of MRSA was observed 25% in sub-clinical and 19.35% in clinical mastitis. In a similar study, Shah et al.2019 reported Methicillin resistance *Staphylococcus aureus* (MRSA) resistance in *S. aureus* with 25% in infected samples. β-lactamase resistance (BLACT) in *S. aureus* was also investigatedwith presence of 36.11% in sub-clinical and 25.80%in clinical mastitis (Fig.4b).The presence of MRSA and BLACT was also detected in normal samples wherein incidence of 9.37% and 13.28% was observed respectively. In a recent study carried out by Deepak et al. 2020 reported 9.3% presence of MRSA in bovine milk collected from healthy cattle in Chennai.

Normal milk samples showed ESBL presence at 7.84% and carbapenase producing E.coli at 1.96 % which was considered a notable finding Fig 4a. β-lactamase resistance (BLACT) in *S. aureus* was also probed at 36.11% in sub-clinical and 25.80%in clinical mastitis (Fig.4 b).

Conclusion

The prevalence of mastitis in milking animals is one of the growing concerns in dairy sector. The current investigation was carried out in infected milk samples collected from organized as well as un-organized sector keeping in view of the fact that prevailing hygienic conditions are widely different across the country. For its understanding, a baseline data was generated by collecting 675 raw milk samples from organized and individual dairy farms in Haryana. The presence of mastitis in raw milk was 28% using CMT and 25.62% using SCC indicating significant correlation. The study showed prevalence of 11.25% clinical mastitis and 14.37% sub-clinical mastitis. The variation in sub-clinical mastitis may be attributed to the selection of animals, season, breed and sanitary conditions. Sub-clinical mastitis give invisible and silent symptoms which is usually given less attention by farmers when it comes to treatment unlike clinical mastitis which shows visible and prominent symptoms towards which treatment and control efforts are concentrated. 675 milk samples were analyzed for the presence of antibiotic residues and enrofloxacin, streptomycin, tetracycline, sulfa drugs and multidrug residues were detected. Study on ID profile indicated the presence of *E. coli* in 18.49% and *S. aureus* in 38.72% infected milk samples. The AST profile revealed that *E. coli* isolates showed maximum resistance of 25% against Ampicillin, Ciprofloxacin and Levofloxacin and Extended spectrum β-Lactamase (ESBL) production in 15.65% infected samples. The maximum resistance of 32% was observed in *Staphylococcus aureus* against Ampicillin and Penicillin-G and Methicilin Resistant *Staphylococcus aureus* (MRSA) was found in 22.38% along with β-Lactamase resistance in 31.34% of infected samples. *S. aureus* remains the major pathogen in infected samples which may pose a threat to public health. The main reason behind the increasing number of resistant pathogens may be due to the indiscriminate use of antibiotics by untrained veterinary professionals and lack of diagnostic facilities; hence regular screening of sub-clinical mastitis should be practiced to

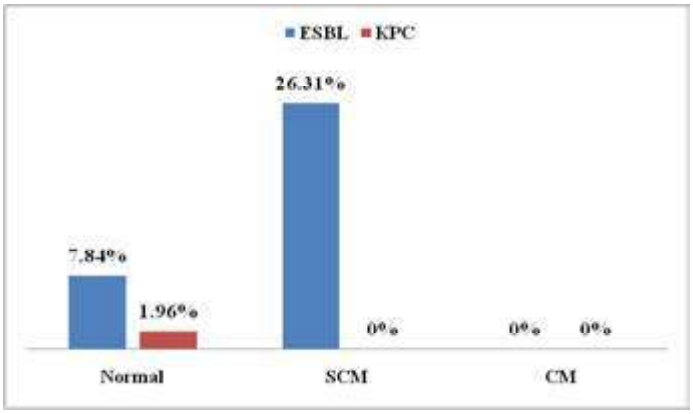

Fig. (4a): Resistance pattern of E. coli isolates at different stages of mastitis

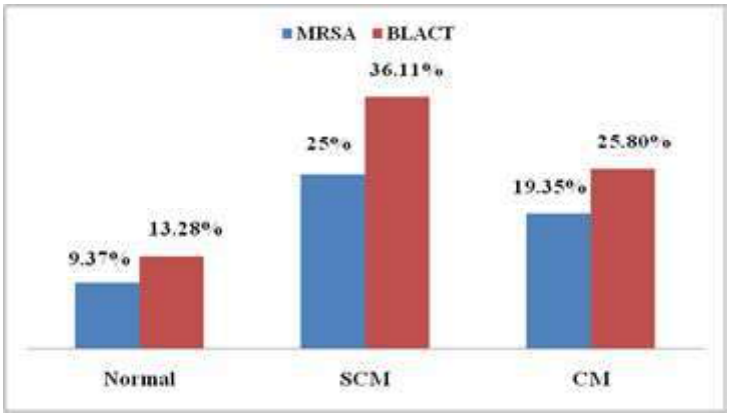

Fig. (4b): Resistance pattern of S. aureus isolates at different stages of mastitis

control the usage of antimicrobials and resistant development in dairy pathogens.

Acknowledgement

We sincerely thank Department of Biotechnology, Government of India and Economic and Social Research Council, grant UK for the financial support to undertake this project as "Diagnostic for One health and User driven solutions for AMR (DOSA) Sanc. No: BT/IN/ Indo-UK/AMR.03/RKE/2018-19; 04th September, 2018).We also acknowledge Indian Council of Agricultural Research (ICAR), New Delhi and Director and staffs of DM Division, ICAR- National Dairy Research Institute (NDRI), Karnal, Haryana, India conducting this study under DBT funded project .

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