such as infertility etc., was approximately INR 7165.51 crore per annum. As it is a multifactorial disease, critical assessment of various factors involved in causation should be ascertained for effective control of the disease. Apart from infectious causes, various host and environmental factors were associated with bovine mastitis (Cheng and Han, 2020; Sinha *et al*., 2021). In India, cross-bred cattle, non-descript cattle, indigenous breeds of cattle, non-descript buffalo, indigenous breeds of buffalo account for 29.81, 9.51, 10.73, 12.87 and 31.94 per cent of milk production respectively(Basic Animal Husbandry & Fisheries Statistics, 2023). According to breeding policy, Jersey is the breed of choice for cross-breeding of non-descript cattle in Chennai, Tamilnadu. However, due to high milk yield when compared to Jersey farmers prefers Holstein-Friesian breeding. Further, exotic inheritance should be restricted to 50 per cent, for better adaptation of cross-bred cattle in India. Hence, dairy cattle exposed to various stress factors, which eventually predisposes to mastitis. Pathogens including bacteria, fungus often found to be major infectious causes involved in bovine mastitis. Earliest identification of causative agent could pave way for implementation of effective therapeutic measures for the treatment of mastitis. Inappropriate use of antimicrobials in the absence of identification of causative agent can lead to both treatment failure and development of resistance.

Emergence of antimicrobial resistance among mastitis pathogens lead to failure of antimicrobial therapies. This increases economic burden among the dairy farmers in developing countries like India. Lacuna in knowledge on various environmental and animal risk factors associated with bovine mastitis further complicate the situation. In addition, AMR profile of pathogens is ever changing due to selection pressure created by inappropriate use of these agents. Hence, this present study was aimed to evaluate the role of various risk factors associated with bovine mastitis and to assess the AMR pattern of pathogens associated with bovine mastitis.

Materials and Methods

Samples collection

The present cohort study was conducted during the year February,2021 to January, 2022 in the Madras Veterinary College Clinical Complex. Total of 174 milk samples were collected from cattle with clinical or subclinical mastitis. Presence of subclinical mastitis was tested using California Mastitis Test (CMT) kit. Milk samples collected aseptically from the affected quarter(s) of udder after discarding initial 2-3 strips. Details of risk factors associated with mastitis such as breed of cattle (Holstein-Friesian cross-bred, Jersey Crossbred, Non-descript cattle), quarter affected, number of calving and environmental risk factors such as month of collection were documented.

Isolation and identification of bacterial pathogens

Milk samples were directly streaked into brain heart infusion agar or else if the animal undergone treatment before the sample collection milk samples were inoculated into BHI broth to dilute the antibiotic residues in the milk and into enhance the possibility of isolating the bacteria. Further, the colonies were stained with Gram staining and sub-cultured in to selective mediums, MacConkey agar, Mannitol salt agar, Edward's media, Eosin-Methylene Blue agar. Pure cultures were further characterized using battery of biochemical tests, oxidase, catalase, Indole, Methyl Red, Voges Proskauer, Citrate utilization tests.

Antibiotic sensitivity test

Assessment of antimicrobial susceptibility of bacterial isolates was performed, as per the Clinical Laboratory Standards Institute (CLSI) guidelines in Muller-Hinton Agar using Kirby-Bauer Disk Diffusion method (CLSI, 2018). Total of seven antimicrobials, Amoxicillin/ Clavulanic acid (20/10µg, AMC), Ampicillin/ Sulbactam (10/10µg, A/S), Cefotaxime (30µg, CTX), Ceftriaxone Tazobactam (80/10µg, CIT), Ciprofloxacin (5µg, CIP), Gentamicin (10µg, GEN), and Moxifloxacin (5µg, MO) belonged to three antibiotic classes *i.e* β-lactam, fluoroquinolones and aminoglycosides were tested.

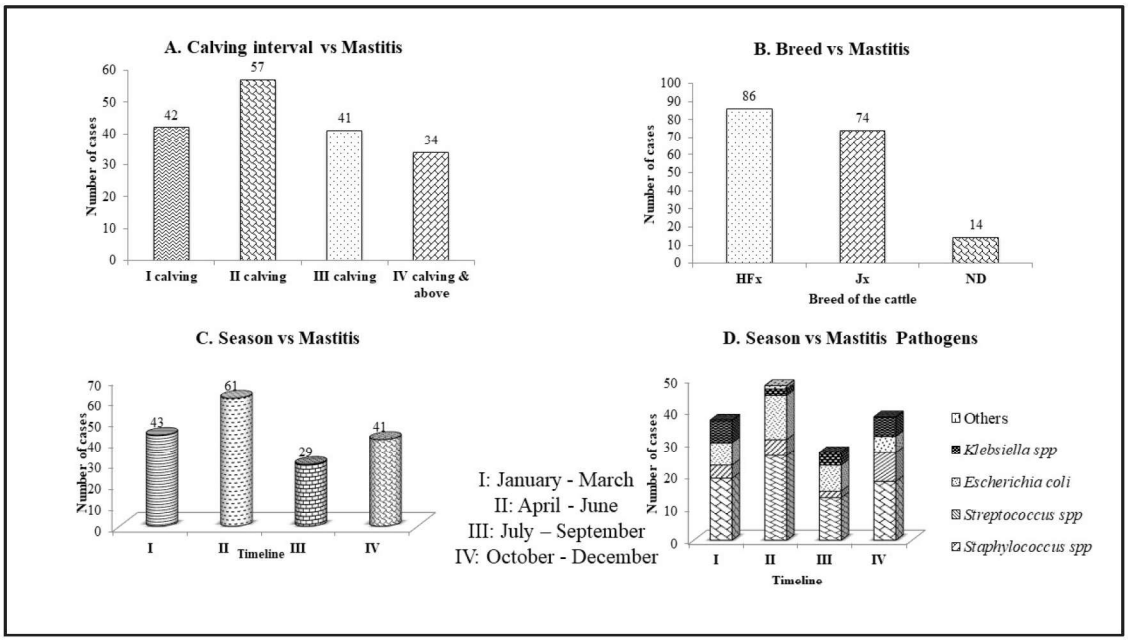

Fig 1: Association of host and environmental risk factors with bovine mastitis and prevalence of bacterial agents causing bovine mastitis in Chennai, Tamilnadu

Results and Discussion

Bovine mastitis is a global concern, potential threat to food security and public health. As the cost involved in the treatment of mastitis and substantial reduction in milk yield post recovery incur huge economic loss to the dairy farming. Studies have reported association of mastitis with host (parity of animal, stage of lactation, breed) and environmental risk factors including method of milking, sanitary measures followed during the milking process and environmental factors including season, method of housing etc. The present study was conducted in Chennai district, Tamilnadu, a metropolitan citywhere, animals are maintained often in very minimal space and un hygienic environment. In this cohort study, data was collected during a span of one year, including all seasons indicated that maximum number of cases were presented during the summer season *i.e.* April to June, followed by rainy (October to December) and winter (January to March). Higher incidence of mastitis during the summer season might be due to poor adaptation of cross-bred dairy cows especially Holstein-Friesian cross-bred cattle in the tropical climate of Chennai (13.04°N 80.17°E), where average temperature during summer in the range of 40-43°C (Moosavi *et al.*, 2014; Oliveira *et al.*, 2015). Breed of choice for crossbreeding in Chennai region is Jersey, however more number cases presented in clinics were of HF crossbred, indicate the poor compliance of breed policies. Poor adoptability of HF crossbred to these climatic conditions might have predisposed them to mastitis, therefore more number mastitis cases were reported in these animals. However, indigenous breeds such as Sahiwal, Gir are well adapted to arid climatic conditions had lesser incidence of subclinical mastitis when compared to rainy and winter seasons and they have better innate immune response (Sinha *et al.*, 2021).Our results were also indicated considerable number of mastitis cases during rainy and winter seasons, which might be due to poor housing conditions and other managemental practices followed by the dairy farmers in the study area.

High yielding breeds are more susceptible to mastitis due to their udder anatomy and comparatively less efficient immune cells. Further, increased requirement of energy and nutrients during the lactation can lead to

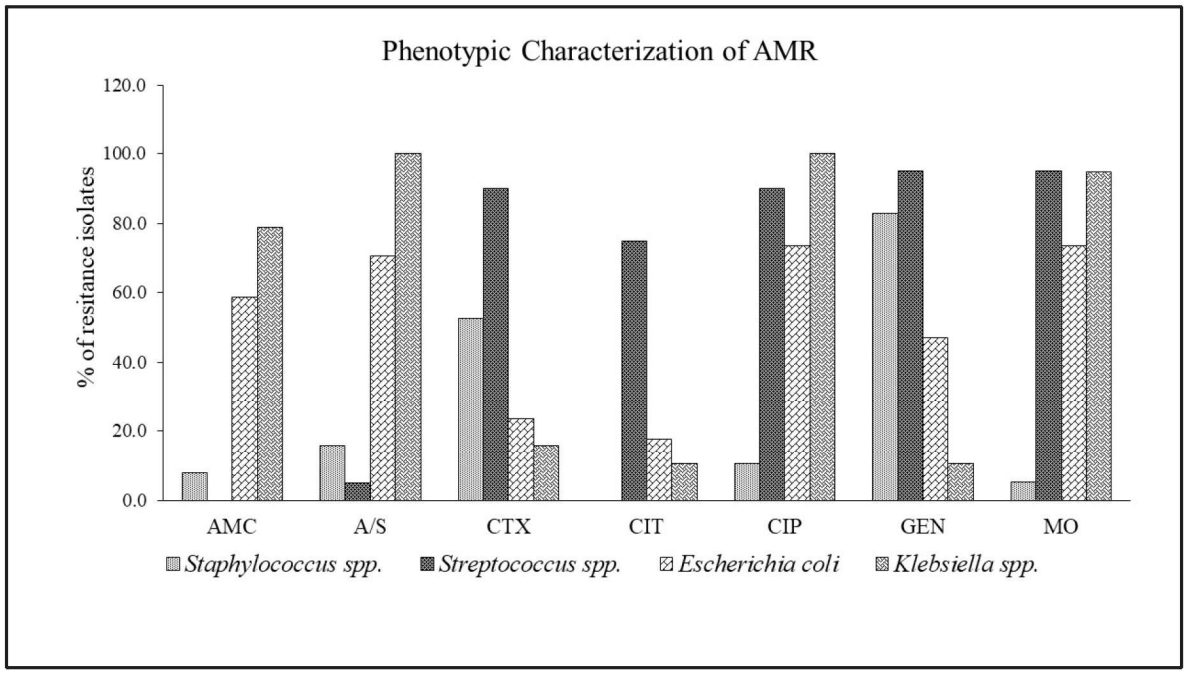

Fig 2: Phenotypic characterization of antimicrobial resistance among bacterial agents causing bovine mastitis.
AMC: Amoxicillin and Clavulanic acid, A/S: Ampicillin and Sulbactam, CTX: Cefotaxime, CIT: Ceftriaxone Tazobactam, CIP: Ciprofloxacin, GEN: Gentamicin, MO: Moxifloxacin

negative energy balance. In addition, inadequate supplementation of essential minerals and vitamins can lead to immunosuppression (Cheng and Han, 2020).Our data also in accordance with previous findings in India and other parts of the world that high yielding Holstein-Friesian and Jersey cross-bred cattle were more commonly affected with mastitis than indigenous breeds (Fig.1).

Analysis of influence of parity on the incidence of mastitis revealed that 1/3rd of the cases was in second lactation (Fig.1). Mastitis incidence was equally distributed in animals in first and third lactation and together they contributed half of the total cases studied. Even though peak milk yield observed during third and fourth lactation, due to inadequate nutrition resulting prolonged calving intervals farmers prefers to cull or sell cross-bred dairy animals after third lactation. This might be a possible reason for the decrease in mastitis after second lactation but not due to negative correlation of parity with mastitis. Moreover, pathogens involved also can influence incidence of mastitis, as Streptococcal infections more common in multiparous than primiparous animals and coliform infections were common during dry period (Oliveira *et al.*, 2015).

Anatomical location of hind quarters of udder predisposes them to mastitis when compared to fore quarters. Our results were in accordance with earlier study conducted in Tamilnadu, India that hind quarter are prone to mastitis when compared to the fore quarters (Ramya and Madhanmohan, 2021) Further, manual milking has significant impact as milkers' practices milking from left side of animals, could hinder complete milking in right hind quarters of udder. Further, certain pathogens predominately affect particular quarter, however, non-significant association of mastitis and quarters of udder was reported in studies where machine milking is practiced (Ramírez *et al.*, 2014; Zhang *et al.*, 2016).

Out of 174 milk samples cultured for isolation of bacterial pathogens, 150 isolated were obtained belong to Gram positive and negative bacteria. Among the isolates recovered,

50.7 % of mastitis cases had *Staphylococcus* spp. followed by *E. coli* (22.7%). Apart from these two pathogens 13 and 12.7 % animals were affected with *Streptococcus* spp. and *Klebsiella* spp. respectively and one of the animals had *Pseudomonas* species (Fig.1). Similar prevalence pattern also reported by Ramasamy *et al*. (2020), in the same geographical area. In a study conducted by Chandrasekaran *et al*. (2014) in Chennai region also revealed similar prevalence of *Staphylococcus* spp. and *E. coli*. In contrary, Priyadharshini *et al*. (2022) reported higher prevalence of *Staphylococcus* spp. (80 %) in Chennai and its adjacent areas. Various factors including season, managemental and host factors have influence on incidence of mastitis caused by different pathogens (Fávero *et al*., 2015). This signifies importance of continuous surveillance programs in bovine mastitis and analysis of data over a period of time will indicate the trends in prevalence of pathogens.

Phenotypic characterization of antimicrobial resistance was carried by Kirby-Bauer, disk diffusion method. Our results revealed that both *Staphylococcus* spp. and *Streptococcus* spp. were highly sensitive to potentiated penicillin *i.e* amoxicillin clavulanate (AMC) and ampicillin sulbactam (A/S). Higher per cent of Streptococcal isolates exhibited resistance to third generation cephalosporins when compared to Staphylococcal isolates. However, 50 % Staphylococcal isolates were resistant to cefotaxime (Fig.2). Our results indicated that fluoroquinolones were effective against *Staphylococcus* spp., but ineffective against *Streptococcus* spp. Deepak *et al*. (2023) and Joshi *et al*. (2014) also reported similar findings that Staphylococcal isolates were sensitive against different classes of antimicrobials such as cephalosporins and fluoroquinolones. Ramasamy *et al*. (2020) also reported a similar susceptibility pattern in Staphylococcal isolates with respect to fluoroquinolones. However, in contrary to our findings, they observed higher resistance per cent to beta-lactam group of antibiotics.

Antimicrobial susceptibility testing revealed that third generation cephalosporins were effective against both the Gram-negative bacterial genera. Among other classes of antimicrobials tested resistance to fluoroquinolones were found in 2/3rd of *E. coli* and *Klebsiella* spp. isolates. Aminoglycoside, gentamicin found to be more effective against Gram negative than Gram positivebacterial species. Saravanajayam *et al*. (2015)also reported that Gram-negative bacterial species (*E. coli* and *Klebsiella* spp.) isolated from bovine mastitis were resistant to fluoroquinolones and shown lesser resistance to gentamicin and penicillin.

Summary

Analysis of animal and environmental risk factors associated with bovine mastitis indicated that poor adaptation of Holstein-Friesian cross-bred cattle to the arid climate of Chennai, Tamilnadu. Among various bacterial pathogens, *Staphylococcus* spp. was more commonly found to associated with bovine mastitis in the study area. Antimicrobial resistance pattern of bacterial pathogens isolated were found to be varying considerably with earlier in this region, signifies the assessment of susceptibility is essential for effective control of bacterial agents in the era of antimicrobial resistance.

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Conflict of interest:

Authors have no conflict of interests.

Ethical approval:

As per the norms of IAEC, approval is not required for the collection of milk samples for diagnostic purposes.

Authors' contributions:

EA conceived, designed the experiment and wrote the manuscript; EA and SM conducted culture studies; RM and JC collected data on risk factors; SMRB edited manuscript. All authors read and approved the manuscript.

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