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Correlation Analysis of Antimicrobial Resistance Coexistence among Bacterial Pathogens Associated with Bovine Mastitis

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Abstract

In the era of AMR,it is essential to understand and correlate AMR among pathogens of clinical importance. Analysis of AMR pattern among bovine mastitis pathogens revealed that isolates of *Staphylococcus* spp., *Streptococcus* spp. and *Klebsiella* spp. were either multior extensively drug-resistant, and none of the *E. coli* were resistant to multiple antimicrobials. Further, pMAR index revealed a7.89 – 15.13 % possibility that an isolate mightnot be amenable to first-line antimicrobials. Correlation of coexisting resistanceindicated a weak correlation among β-lactams and a moderate to weak correlation between β-lactams and gentamicin. This analysis will shed more light onto AMR coexistence and might help clinicians in choosing correct antimicrobials.

Keywords: Bovine mastitis, Antimicrobial resistance, Correlation of AMR

Globally, the livestock sector accounts for more than 70 % of antimicrobial use and India accounts for 3 % of antimicrobial used in food animals (Van Boeckel *et al.*, 2015, Mutua *et al.*, 2020). Highest priority critically important antimicrobials (HPCIA) such as 3rdgeneration cephalosporins, fluoroquinolones used in human medicine were also classified as veterinary critically important agents (VCIA) which cannot be used as first-line treatment, and bacteriological tests are mandatory when used in second-line treatment. However, recent findings suggest that in certain pockets of India, 52.42 % of dairy herds were using HPCIA and VCIA drugs to treat mastitis (Vijay *et al.*, 2023).

Inappropriate use of antimicrobials can increase selection pressure among pathogens, leading to the emergence of multidrug resistance. The multidrug-resistant microbes pose a serious threat to clinicians in selecting a suitable antimicrobial agent (Gajdacs *et al.*, 2021;Velazquez-Meza *et al.*, 2022). Global agencies like US Centres for Disease Control and Prevention (CDC) andEuropean Centre for Disease Control and Prevention (ECDC) made efforts to classify these multidrug-resistant organisms into multidrugresistance (MDR), extensive drug resistance (XDR) and pan-drug resistance (PDR) for better understanding resistance pattern in a given epidemiological condition (Magiorakos *et al.*, 2012). These categorisations may help clinicians in choosing suitable antimicrobials; however,ever changing pattern of AMR among pathogens warrants correlation of AMR and clinical outcomes might be useful in choose suitable antimicrobials.

Bovine mastitis, a multifactorial disease, requires precise identification of causative agent and their susceptibility to antimicrobials. Therapeutic options in the hands of clinicians became limited when multidrug resistance isolates were involved.

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Even though evidence of co-resistance among bovine mastitis pathogens were reported (Cheng *et al*., 2019; Awandkar *et al*., 2022; Naranjo-Lucena and Slowey, 2023), studies on the correlation of co-resistance is limited (Kovačević *et al*., 2022). Moreover, pathogens' resistance patterns vary spatially and temporally (Fuhrmeister and Jones, 2019). Hence, the present study was aimed to analyse the phenotypic antimicrobial resistance expressed in 149 bacterial isolates of four genera isolated from bovine mastitis cases in the Chennai region.

Materials and Methods

Antimicrobial susceptibility testing of bacterial pathogens (n=149) isolated from bovine mastitis cases (n=174) presented to the Clinical Complex, Madras Veterinary College, Chennai during 2021-22, was carried out per the guidelines of Clinical and Laboratory Standards Institute (Elamurugan *et al*., 2024). Susceptibility and resistance of four different bacterial pathogens [*Staphylococcus* spp. (n=76), *Streptococcus* spp. (n=20), *E. coli*(n=34), *Klebsiella* spp. (n=19)] against different classes of antimicrobials, beta-lactam (Amoxicillin/ clavulanic acid, Ampicillin sulbactam, Cefotaxime, Ceftriaxone tazobactam), aminoglycosides (Gentamicin), and fluoroquinolones (Enrofloxacin, Ciprofloxacin, Moxifloxacin) were studied.

Resistant categories of isolates

Isolates were classified into different categories:multidrug-resistant (MDR), extensively drug-resistant (XDR) and pan-drug-resistant (PDR) based on their acquired resistance but not intrinsic to different antimicrobial categories. As per the CDC/ECDC recommendations, these categories were defined as follows:MDR: resistant to at least one antimicrobial in three or more classes; XDR: susceptible to one or two antimicrobial classes; PDR: resistant to all antimicrobial classes (Magiorakos *et al*., 2012).

A predictive composite score (pMAR), an enhanced multiple antibiotic resistance index, was determined based on the following formula,

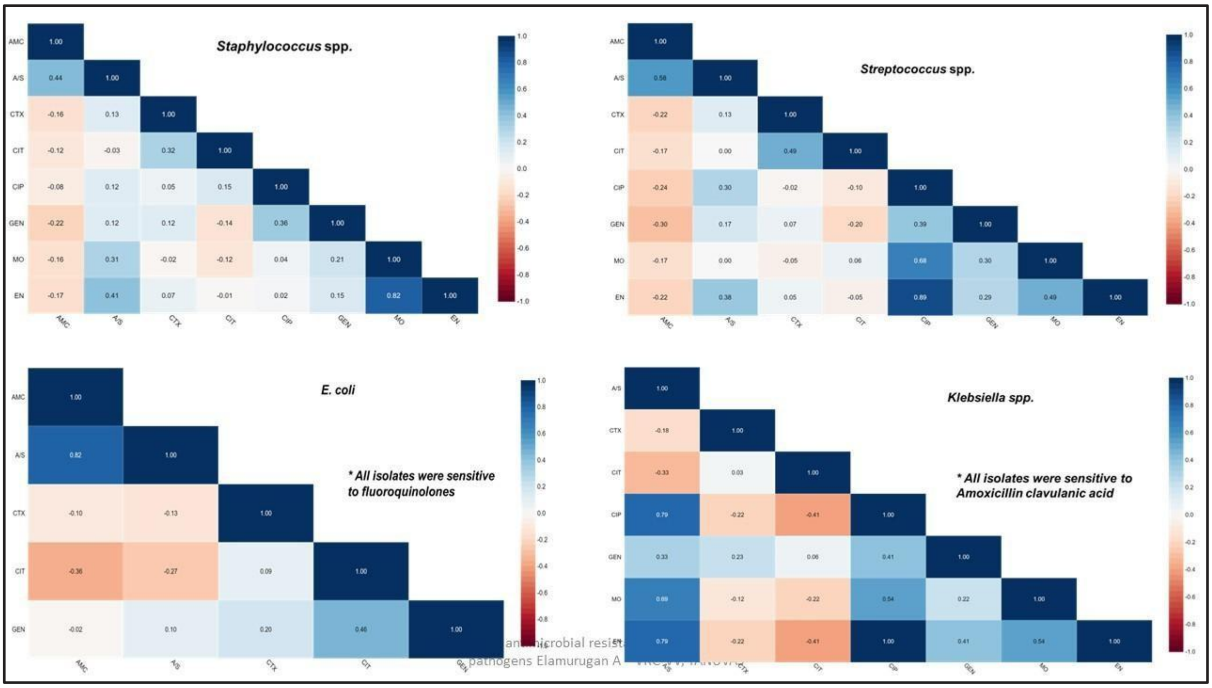

Fig 1: Correlation of antimicrobial resistance among bacterial agents causing bovine mastitis.
AMC: Amoxicillin and Clavulanic acid, A/S: Ampicillin and Sulbactam, CTX: Cefotaxime, CIT: Ceftriaxone Tazobactam, CIP: Ciprofloxacin, GEN: Gentamicin, MO: Moxifloxacin, EN: Enrofloxacin

Table I: Classification of bacterial isolates into resistant categories

Classification of resistance	Percentage of resistant isolates (Number of isolates in parenthesis)				
	***Staphylococcus* spp.**	***Streptococcus* spp.**	***E. coli***	***Klebsiella* spp.**	**Total**
MDR	30.26 (23)	30 (6)	-	15.79 (3)	21.48 (32)
XDR	21.05 (16)	10 (2)	-	5.26 (1)	12.75 (19)
PDR	-	-	-	-	-
Total	51.32 (39)	40 (8)	-	21.05 (4)	34.23 (51)
pMAR	**15.13 %**	**15.0 %**	**0**	**7.89 %**	

$$pMAR = \frac{n_{MDR} \times AB_{MDR}}{n_{all} \times AB_{all}} \times 100$$

Where n_{MDR} is the number of MDR isolates in each genus of bacteria, AB_{MDR} is the minimum number of antimicrobials required in a particular genus of bacteria to consider them as MDR, n_{all} is the total isolates in a particular genus of bacteria, AB_{all} is the total number of antimicrobials used. The pMAR value (0-100 %) indicates that a particular isolate in the present clinical condition is not amenable to front-line antimicrobials under the epidemiological conditions involved (Gajdacs *et al.*, 2020).

Correlation of phenotypic AMR

Isolates showed intermediate values for antimicrobials, which were considered resistant for calculating the correlation coefficient, and data was converted into binary values (1, resistant, and 0, sensitive). Paerson-correlation coefficient (r) was calculated, and the relationship between the variables was determined as weak correlation ($0.1 < r > 0.3$), moderate correlation ($0.3 < r > 0.5$), strong correlation ($0.5 < r > 0.85$) and very strong correlation ($r > 0.85$) as described by Mukaka (2012). A correlation matrix was created using Displayr, an online tool, for the r values obtained.

Results and Discussion

Antibiotic susceptibility test was performed against eightantimicrobial agents (Amoxicillin/ clavulanic acid, Ampicillin sulbactam, Cefotaxime, Ceftriaxone tazobactam, Enrofloxacin, Ciprofloxacin, Moxifloxacin, Gentamicin), belonged to three classes of antimicrobials. The resistance profiles of isolates (n=149) collected over a year showed that the susceptibility pattern of common bovine mastitis pathogens varies considerbly.

Resistance categories of bovine mastitis pathogens

Analysis of phenotypic AMR of isolates obtained from clinical mastitis cases in the Chennai region revealed that Gram-positive isolates had a higher level of resistance when compared to Gram-negative isolates. Out of 149 isolates, 32 (21.48 %)were categorized as multiple drug-resistant and 19 (12.75 %) were classified as extensively drug-resistant (Table I). In the current study, under the prevailing epidemiological conditions, none of the isolates were resistant to all the antimicrobials tested. Among the four genera of bacteria prevalent in the region, *Staphylococcus* spp. (51.32 %) were categorized as either MDR or XDR.Haq *et al.* (2024) also reported that 50 % of Staphylococcal isolated from bovine mastitis in Pakistan were MDR; however, lesser prevalence was reported in other regions worldwide. Interestingly, we found that all the *E. coli* isolates (n=34) were neither MDR nor XDR and were amenable to at least one of the antimicrobials tested in each class.

Determination of the predictive score for multiple antimicrobial resistance index revealed that 7.89 to 15.13 % isolates might be non-amenable to first-line antimicrobials in the Chennai region. The pMAR values for *Staphylococcus* spp., *Streptococcus* spp. and *Klebsiella* spp. were 15.13 %, 15 % and 7.89 %, respectively. None of the *E. coli* isolates showed resistance to multiple antibiotic class pMAR score was not determined.

Correlation of AMR

Pearson correlation efficient analysis of phenotypic antimicrobial resistance against aminopenicillins, cephalosporins, aminoglycosides, and fluoroquinolones indicated that mastitis pathogens show varying degrees of correlations (Fig. 1). Both Gram-positive bacterial pathogens exhibited moderate correlation ($0.3 < r > 0.5$) with respect to aminopenicillins. In contrast, a negative correlation was found between aminopenicillin and cephalosporins. A moderate correlation between fluoroquinolones and ampicillin sulbactam in Gram-positive isolates indicated the existence of co-resistance. *Klebsiella* spp. showed a strong correlation ($0.5 < r > 0.85$) in fluoroquinolones and ampicillin sulbactam co-resistance. Interestingly, isolates of both Gram-negative bacterial genera showed a weak to moderate correlation in the resistance between cephalosporins and gentamicin. Contrary to our findings, Osman *et al*. (2017) reported a negative correlation of resistance between amoxicillin and gentamicin in *E.coli*. Gajdacs *et al*. (2021) reported that isolates of *E. coli* were resistant to ciprofloxacin and gentamicin, as indicated by a strong positive correlation. Our data indicated that a strong correlation ($r > 0.85$) existed among fluoroquinolones across Gram-positive and Gram-negative bacterial agents tested. However, moxifloxacin, a fourth-generation fluoroquinolone antimicrobial agent, had a moderate correlation ($0.3 < r > 0.5$) with enrofloxacin and ciprofloxacin.

Summary

Bovine mastitis is an economically important disease of dairy cattle, accounting for the majority of antimicrobial use.Several pathogens are implicated to bovine mastitis, therefore it is critical to choose correct antimicrobials to curb both infection and spread of AMR.. In the present study, we found the presence of MDR, XDR amoung the isolates of *Staphylococcus* spp., *Streptococcus* spp., and*Klebsiella* spp. associated with bovine mastitis in the Chennai region. Further, determining the pMAR index might helptoassess the usefulness of antimicrobials in the given epidemiological situations. The correlation analysis of AMR indicated a significant level of co-resistance among the bovine mastitis pathogens, vindicatingthemeticulous use of antimicrobials.

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