Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2023.060

RESEARCH ARTICLE

Annotating Susceptibility Potential of Single, Double, Tri and Tetra Mixed Infection Bacteria against Non-beta Lactam Antibiotics

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ARTICLE HISTORY (23-067)

Received:	February 28, 2023
Revised:	July 5, 2023
Accepted:	July 12, 2023
Published online:	August 03, 2023

Key words:
Bovine mastitis
E. coli
S. aureus
Klebsiella pneumoniae
Streptococcus agalactiae
Antibiotics
Mixed bacteria

ABSTRACT

The expression of mastitis and its treatment varies based on type and number of pathogens involved. The studies on therapeutics are thus needed for mixed culture in addition to single bacterial etiology. The current study was aimed to find prevalence and drug susceptibility of single and mixed culture of *E. coli*, *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* against non-beta lactam antibiotics. Total n=200 milk samples from dairy animals were collected and processed for subclinical mastitis and subsequently put to isolation of selected bacteria. A total of n=6 non-beta lactam antibiotics (clindamycin, erythromycin, levofloxacin, vancomycin, teicoplanin and gentamicin) were tested against *E. coli*, *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* alone, double, triple and tetra combinations using disc diffusion method. Highest prevalence of single bacterial mastitis was observed in case of *E. coli* while for mixed infection both *E. coli* plus *S. aureus* stood at the top. The mixed culture of *S. aureus* with others in double combination showed higher zones than that of the single against clindamycin. Similarly, *E. coli* in double and triple combination showed higher sensitivity responses against levofloxacin. Vancomycin showed least efficacy against most of the double, triple and tetra combinations compared to that of single bacteria. Gentamicin on the other hand remained comparatively effective against single and mixed cultures of bacteria. The study thus concluded highly unpredictable responses of bacteria when tested in combination compared to the one used alone which invites further trials to be conducted on underlying complex mechanisms.

To Cite This Article: Kirn N, Lu S, Aqib AI, Akram K, Majeed H, Muneer A, Murtaza M, Prince K, Li K, 2023. Annotating susceptibility potential of single, double, tri and tetra mixed infection bacteria against non-beta lactam antibiotics. Pak Vet J, 43(3): 596-600. http://dx.doi.org/10.29261/pakvetj/2023.060

INTRODUCTION

The presence of mastitis results in significant problems with respect to animal welfare, food safety and profitability of milk production. It is estimated that the worldwide loss caused by the mastitis is ranging from €61 to €97 per cow per year on a farm (Hogeveen *et al*., 2011). In addition to causing damage to udder tissues and milk production, clinical and subclinical mastitis can also have a serious impact on human health because it adversely affects nutritional quality of milk (Gurjar *et al*., 2012). It has also been observed that subclinical mastitis negatively affects dairy animal reproductive performance. A variety of contagious and environmental microorganisms can cause mastitis, including those that survive and multiply on the skin and the teat wounds. *E. coli*, *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* are the most common bacteria that cause mastitis (Kabelitz *et al*, 2021). The most associated pathogens with bovine mastitis are *Streptococcus* species and *Staphylococcus* species. Globally, of all the *Staphylococci* species, *S. aureus* is the pathogen most associated with the occurrence of this disease, and it has been regarded as a major threat to veterinary medicine for many years (Javed *et al*., 2021;

Sarwar et al., 2021). Most of the cattle suffer from subclinical mastitis which is characterized by an increase in somatic cell counts (SCC) without visible changes in milking or mammary gland. It is important to note that taking right decisions in case of subclinical mastitis can significantly stop shifting cases into clinical form.

Studies have reported poly-bacterial mastitis with variable percentages of pathogens. For example, 62.07% of subclinical mastitis was found to be because of *Enterobacteriaceae* while among these 25.86% were *E. coli*. Similarly, 87.93% of *Staphylococci* based mastitis enumerated 36.20% as *S. aureus* while 51.72% were coagulase-negative *Staphylococcus* strains. In another study, 66% poly-bacterial etiology was noted (Abdennebi *et al*., 2020). In some of studies, up to 11.10% poly-bacterial mastitis was observed (Bradley, 2002). Variations may be because of many other factors while it has become evident that mastitis is more a poly-bacterial rather than the single bacterial infection. The studies on antibiotic susceptibility of these pathogens revolve around single bacteria. This might be one reason that efficacy of antibiotics reduces while resistance goes high. It is admitted fact that use of antibiotic against a particular bacterium may bring resistance to the bacteria which had been overlooked. For an example, vancomycin resistance against other bacteria is thought to be due to overuse of this antibiotic against *Pseudomembranous colitis*, and *Clostridium difficile* (Goudah and Abo-El-Sooud, 2008). Thus, the current study was planned to investigate prevalence and antibiotic susceptibility of single and mixed culture of salient mastitis pathogens (*E. coli*, *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*).

MATERIALS AND METHODS

Sample collection: Milk samples (n=200) were collected from different dairy farms (n=10) located in and around district Bahawalpur following convenient sampling technique. The dairy farms were selected based on >40 animals in active milking. The milk samples were screened for subclinical mastitis using SFTM (surf field mastitis test) and subclinical positive samples were shipped to Cholistan University of Veterinary and Animal Sciences, Bahawalpur, Pakistan. Any sample showing at least +1 of category of subclinical mastitis (Muhammad *et al*., 2010) was included in the "collected sample category". Moreover, sample from single animal showing two or more than two teats positive for subclinical mastitis were pooled together and was considered as one sample.

Isolation of bacteria: Samples were incubated overnight in sterile nutrient broth at 37°C for 24hrs and put to centrifugation for 15 minutes at 6000rpm. Sterile swabs were dipped in sedimentation and spread over blood agar for further incubation at 37°C for 24hr. The characteristic colonies were further streaked on different selective culture media for *E. coli* (MacConkey agar), *Klebsiella pneumoniae* (Eosin methylene blue agar), *Streptococcus agalactiae* (blood agar), and *Staphylococcus aureus* (mannitol salt agar). A series of biochemical tests like Gram staining, catalase test, coagulase test, indole test, methyl red test, Voges Proskauer test, citrate test, triple sugar acid/acid gas test, Kligler iron agar test, CAMP test (Christie–Atkins–Munch-Peterson), and urease test were performed keeping positive control of each bacterial along with this protocol. Pooled information of growth and biochemical tests were analyzed for confirmation of targeted bacteria as per directions of Bergey's manual of determinative bacteriology (Holt, 1994).

Antibiotic susceptibility testing by disk diffusion method: Total of Six non-beta lactam antibiotics (clindamycin 10µg, erythromycin 15µg, levofloxacin 5µg, vancomycin 30µg, teicoplanin 30µg, and gentamicin 10µg of Oxoid™ {Oxoid Limited United Kingdom}) were tested against *E. coli* (E), *Klebsiella pneumoniae* (K), *Streptococcus agalactiae* (St), *Staphylococcus aureus* (S.a) separately and in a combination of two, three, and four bacteria. The selection of antibiotics was based on their use in both veterinary and public health because the pathogens are equally important for animals and humans. Also, the availability of antibiotics in the market was considered as selection criterion for these antibiotics. Kirby Bauer's disc diffusion method was applied based on guidelines provided by the Clinical and Laboratory Standard Institute (CLSI, 2021). Briefly, fresh growth of different bacteria adjusted at 1-1:5 × 10^8 CFU/mL were spread over sterile Mueller Hinton agar. Each antibiotic disc was gently placed at equal distances in an aseptic manner. The zones of inhibitions were measured after incubation for 20-24 hours at 37°C (CLSI, 2021) and compared with standards provided by CLSI.

NB: The change in position of abbreviations of bacteria in combination should not be considered new combination e.g both E+K and K+E are describing combination of *E. coli* and *Klebsiella pneumoniae*.

Statistical analysis: The prevalence of subclinical mastitis and bacteria in subclinical mastitis samples were calculated as per formula given below (Thrusfield, 2018). Comparisons of zones of inhibitions of single bacteria with double combination bacteria and similarly comparison of single bacteria with triple combination bacteria were analyzed by ANOVA with Tukey test as post hoc test. Independent *t*-test was applied to compare zones of inhibitions of single bacteria with tetra combination of bacteria. The statistical software SPSS was selected for analysis of data at 5% probability. The SPSS is considered as an efficient, user friendly, powerful data management, and continued improved version. Where needed, Minitab software was also used to cross check results.

$$\text{Prevalence (\%)} = \frac{\text{Number of positive samples (n)}}{\text{total number suspected (N)}} \times 100$$

RESULTS

Prevalence: Overall subclinical mastitis was 24.5% (49/200) from dairy animals. Out of subclinical mastitis milk samples, 93.88% (46/49) were positive for bacteria while 6.12% were negative for any growth of bacteria.

Prevalence of bacterial type and number from mastitis: Higher prevalence among single bacterial mastitis was found in case of *E. coli* (6.12%) followed by *S. aureus* (4.08%), *Streptococcus agalactiae* (2.04%), and

Table 1: Comparison of zone of inhibitions (mm) among single and mixed bacteria against clindamycin

Type of mixed bacteria	*E. coli*		*S. aureus*		*Streptococcus*		*Klebsiella*	
	Combination	Mean± SD	Combination	Mean± SD	Combination	Mean± SD	Combination	Mean± SD
Alone	E	34.33±1.53^{a}	*S.a*	33.33±3.06^{a}	St	39.00±1.00^{a}	K	42.33±2.52^{a}
	E+*S.a*	33.33±3.06^{a}	*S.a*+E	33.33±3.06^{a}	St+E	12.61.15^{b}	K+E	26.00±2.00^{c}
Double	E+St	12.67±1.15^{c}	*S.a*+K	34.67±2.31^{a}	St+K	39.67± 0.577^{a}	K+*S.a*	39.67±0.57^{ab}
	E+K	26.00±2.00^{b}	*S.a*+St	38.00±2.00^{a}	St+*S.a*	38.00± 2.00^{a}	K+St	34.67±2.31^{b}
Triple	E	34.33±1.53^{a}	*S.a*	33.33±3.06^{a}	St	39.00±1.00^{a}	K	42.33±2.52^{a}
	E+*S.a*+St	21.00±1.00^{b}	*S.a*+E+St	21.00±1.00^{b}	St+*S.a*+E	21.00±1.00^{b}	K+E+*S.a*	6.00±0.00^{b}
	E+*S.a*+K	6.00±0.00^{c}	*S.a*+E+K	6.00±0.00^{c}	St+E+K	6.00±0.00^{c}	K+E+St	6.00±0.00^{b}
	E+ST+K	6.00±0.00^{c}						
Tetra	E	34.33±1.53 ^{a}	*S.a*	33.33±3.055 ^{a}	St	39.00±1.00 ^{a}	K	42.33±2.52 ^{a}
	E+*S.a*+K+St	6.00±0.00 ^{b}	E+*S.a*+K+St	6.00±0.00 ^{b}	E+*S.a*+K+St	6.00±0.00 ^{b}	E+*S.a*+K+St	6.00±0.00 ^{b}

Different superscripts within column for each of double, triple, and tetra combination of each of bacteria show significant difference (P<0.05).

Table 2: Comparison of zone of inhibitions (mm) among single and mixed bacteria against levofloxacin

Type of mixed bacteria	*E. coli*		*S. aureus*		*Streptococcus*		*Klebsiella*	
	Combination	Mean± SD	Combination	Mean± SD	Combination	Mean± SD	Combination	Mean± SD
Double	E	40.33±0.57^{a}	*S.a*	29.33±1.15^{b}	St	28.33±1.538^{b}	K	30.00±1.00^{b}
	E+*S.a*	41.00±1.00^{a}	*S.a*+E	41.00±1.00^{a}	St+E	40.33±1.538^{a}	K+E	41.33±1.15^{a}
	E+St	40.33±1.53^{a}	*S.a*+K	29.33±1.15^{a}	St+K	28.00±2.00^{b}	K+*S.a*	28.00±2.00^{b}
	E+K	41.33±1.15^{a}	*S.a*+St	30.00±2.00^{a}	St+*S.a*	30.00±2.00^{b}	K+St	29.33±1.15^{b}
Triple	E	40.33±0.57^{b}	*S.a*	29.33±1.15^{c}	St	28.33±1.538^{b}	K	30.00±1.00^{b}
	E+*S.a*+St	47.00±1.00^{a}	*S.a*+E+St	47.00±1.00^{a}	St+*S.a*+E	47.00±1.00^{a}	K+E+*S.a*	42.00±2.00^{a}
	E+*S.a*+K	42.00±2.00^{b}	*S.a*+E+K	42.00±2.00^{b}	St+E+K	43.33±1.15^{b}	K+E+St	43.33±1.15^{a}
	E+ST+K	43.33±1.15^{b}						
Tetra	E	40.33±0.57 ^{a}	*S.a*	29.33±1.15 ^{a}	St	28.33±1.538^{b}	K	30.00±1.00^{b}
	E+*S.a*+K+St	41.33±1.15 ^{a}	E+*S.a*+K+St	41.33±1.15 ^{b}	E+*S.a*+K+St	41.33±1.15 ^{b}	E+*S.a*+K+St	41.33±1.15 ^{b}

Different superscripts within column for each of double, triple, and tetra combination of each of bacteria show significant difference (P<0.05).

Table 3: Comparison of zone of inhibitions (mm) among single and mixed bacteria against erythromycin

Type of mixed bacteria	*E. coli*		*S. aureus*		*Streptococcus*		*Klebsiella*	
	Combination	Mean± SD	Combination	Mean± SD	Combination	Mean± SD	Combination	Mean± SD
Double	E	31.00±1.00^{a}	*S.a*	26.00±2.00^{b}	St	12.67±1.15^{c}	K	16.67±0.58^{c}
	E+*S.a*	19.67±0.58^{b}	*S.a*+E	19.67±0.58^{b}	St+E	12.00±1.00^{c}	K+E	13.67±0.58^{c}
	E+St	12.00±1.00^{c}	*S.a*+K	34.67±2.31^{a}	St+K	39.33±1.15^{a}	K+*S.a*	39.33±1.15^{a}
	E+K	13.67±0.58^{c}	*S.a*+St	23.33±5.77^{b}	St+*S.a*	23.33±5.77^{b}	K+St	34.67±2.31^{b}
Triple	E	31.00±1.00^{a}	*S.a*	26.00±2.00^{b}	St	12.67±1.15^{c}	K	16.67±0.58^{b}
	E+*S.a*+St	29.67±0.58^{a}	*S.a*+E+St	29.67±0.58^{ab}	St+*S.a*+E	29.67±0.58^{a}	K+E+*S.a*	32.67±2.31^{a}
	E+*S.a*+K	32.67±2.31^{a}	*S.a*+E+K	32.67±2.31^{a}	St+E+K	9.33±0.58^{c}	K+E+St	9.33±0.58^{c}
	E+ST+K	9.33±0.58^{b}	-	-	-	-	-	-
Tetra	E	31.00±1.00^{a}	*S.a*	26.00±2.00 ^{a}	St	12.67±1.15 ^{a}	K	16.67±0.58 ^{a}
	E+*S.a*+K+St	27.33±0.58 ^{b}	E+*S.a*+K+St	27.33±0.58 ^{b}	E+*S.a*+K+St	27.33±0.58 ^{b}	E+*S.a*+K+St	27.33±0.58 ^{b}

Different superscripts within column for each of double, triple, and tetra combination of each of bacteria show significant difference (P<0.05).

Klebsiella pneumoniae (2.04%). Among double bacterial mastitis, *S.a+E* showed higher prevalence (20.41%) followed by S.a+St (10.20%), S.a+K (4.08%), St+K (4.08%), and St+E (2.04%). S.a+E+St and St+E+K among triple bacterial mastitis showed prevalence of 6.12% followed by *S.a*+E+K (4.08%), and S.a+St+K (4.08%). Tetra bacterial mastitis (*S.a+E+St+K*) presented 8.14% while other unidentified bacterial mastitis was 10.20%. It was also noteworthy that 6.12% of subclinical mastitis samples did not show any bacterial growth.

Response of single and combination of bacteria against antibiotics: The study showed some of cases, there was significant higher susceptibility of mixed bacteria than to single bacteria against drugs and vice versa at other instances.

Double combination of bacteria: Double combination of bacteria showed significantly (P<0.05) lower sensitivity compared to that of single bacteria (Table 1-6). E+St showed least ZOI (12.67±1.15 mm) while *K. pneumoniae* showed highest ZOI (42.33±2.52 mm) against clindamycin. In case of levofloxacin, except K+S.a and K+St, none of the double combinations showed lower ZOI compared to the single bacteria. Vancomycin, in comparison to its application on single E, proved to be least effective against double bacteria except E+*S.a*. In case of teicoplanin, more than 3 times higher ZOI were observed for St+K and St+*S.a* compared to that of St. An interesting finding of efficacy of gentamicin in that double combination of *S.a* and St showed reduction in ZOI while that of E and K showed significantly higher ZOI compared to the alone bacteria.

Triple and tetra combination of bacteria: A significant reduction in ZOI was observed in case of triple combination of bacteria compared to that of single bacteria against clindamycin. E+St+K expressed nearly six times lower ZOI compared to E, while tetra combination of bacteria showed range of 6-7 times lower ZOI compared to that of single bacteria against clindamycin (Table 1). The responses from triple combinations of bacteria showed reduced ZOIs compared to that of single bacteria against levofloxacin. The significant lower (P<0.05) ZOI was observed between E+*S.a*+St and E while all other comparisons of E with triple and tetra combinations were non-significant (p>0.05) (Table 2). Erythromycin showed variable efficacy against triple combinations of bacteria and tetra combinations of bacteria compared to the single bacteria (Table 3).

Vancomycin's efficacy against triple combinations of bacteria was considerably different to that of the other antibiotics used in this study. E+ST+K showed no zones while E+*S.a*+St showed significantly higher (P<0.05) ZOI

Table 4: Comparison of zone of inhibitions (mm) among single and mixed bacteria against vancomycin

Type of mixed bacteria	*E. coli*		*S. aureus*		*Streptococcus*		*Klebsiella*	
	Combination	Mean± SD	Combination	Mean± SD	Combination	Mean± SD	Combination	Mean± SD
Double	E	10.67±1.15^{b}	*S.a*	22.00±2.00^{a}	St	20.33±0.58^{a}	K	21.000±1.000^{a}
	E+*S.a*	19.34±0.58^{a}	*S.a*+E	19.33±0.58^{a}	St+E	0.00±0.00^{b}	K+E	0.000±0.000^{c}
	E+St	0.00±0.00^{c}	*S.a*+K	19.00±1.00^{a}	St+K	19.67±0.58^{a}	K+*S.a*	19.667±0.577^{ab}
	E+K	0.00±0.00^{c}	*S.a*+St	19.00±1.00^{a}	St+*S.a*	19.00±1.00^{a}	K+St	19.000±1.000^{b}
Triple	E	10.67±1.15^{c}	*S.a*	22.00±2.00^{b}	St	20.33±0.58^{b}	K	21.000±1.000^{a}
	E+*S.a*+St	30.33±1.53^{a}	*S.a*+E+St	30.33±1.53^{a}	St+*S.a*+E	30.33±1.53^{a}	K+E+*S.a*	20.00±0.00^{a}
	E+*S.a*+K	20.00±0.00^{b}	*S.a*+E+K	20.00±0.00^{b}	St+E+K	0.00±0.00^{c}	K+E+St	0.000±0.000^{b}
	E+ST+K	0.00±0.00^{d}						
Tetra	E	10.67±1.15^{a}	*S.a*	22.00±2.00 ^{a}	St	20.33±0.58^{a}	K	21.00±1.00^{a}
	E+*S.a*+K+St	23.00±3.00^{b}	E+*S.a*+K+St	23.00±3.00^{a}	E+*S.a*+K+St	23.00±3.00^{a}	E+*S.a*+K+St	23.00±3.00^{b}

Different superscripts within column for each of double, triple, and tetra combination of each of bacteria show significant difference (P<0.05).

Table 5: Comparison of zone of inhibitions (mm) among single and mixed bacteria against teicoplanin

Type of mixed bacteria	*E. coli*		*S. aureus*		*Streptococcus*		*Klebsiella*	
	Combination	Mean± SD	Combination	Mean± SD	Combination	Mean± SD	Combination	Mean± SD
Double	E	21.33±2.31^{a}	*S.a*	21.0±1.00^{a}	St	6.0±0.00^{b}	K	21.00±1.00^{a}
	E+*S.a*	22.33±2.08^{a}	*S.a*+E	22.33±2.08^{a}	St+E	6.0±0.00^{b}	K+E	19.67±0.58^{a}
	E+St	6.00±0.00^{b}	*S.a*+K	22.00±2.00^{a}	St+K	21.33±1.1^{a}	K+*S.a*	22.00±2.00^{a}
	E+K	19.67±0.58^{a}	*S.a*+St	20.66±1.15^{a}	St+*S.a*	20.66±1.15^{a}	K+St	21.333±1.155^{a}
Triple	E	21.33±2.31^{ab}	*S.a*	21.00±1.00^{ab}	St	6.0±0.0^{b}	K	21.00±1.00^{b}
	E+*S.a*+St	18.67±1.53^{b}	*S.a*+E+St	18.67±1.53^{b}	St+*S.a*+E	18.6±1.53^{a}	K+E+*S.a*	24.00±2.00^{a}
	E+*S.a*+K	24.00±2.00^{a}	*S.a*+E+K	24.00±2.00^{a}	St+E+K	0.00±0.00^{c}	K+E+St	0.00±0.00^{c}
	E+ST+K	0.00±0.00^{c}						
Tetra	E	21.33±2.31^{a}	*S.a*	21.00± 1.00^{a}	St	6.0±0.00^{a}	K	21.00±1.00^{a}
	E+*S.a*+K+St	0.00±0.00^{b}	E+*S.a*+K+St	0.00± 0.00^{a}	E+*S.a*+K+St	0.00±0.00^{b}	E+*S.a*+K+St	0.00±0.00^{b}

Different superscripts within column for each of double, triple, and tetra combination of each of bacteria show significant difference (P<0.05).

Table 6: Comparison of zone of inhibitions (mm) among single and mixed bacteria against gentamicin

Type of mixed bacteria	*E. coli*		*S. aureus*		*Streptococcus*		*Klebsiella*	
	Combination	Mean± SD	Combination	Mean± SD	Combination	Mean± SD	Combination	Mean± SD
Double	E	20.33±0.58^{b}	*S.a*	32.00±0.00^{a}	St	32.67±2.31^{a}	K	25.00±2.65^{c}
	E+*S.a*	26.33±0.58^{a}	*S.a*+E	26.33±0.58^{b}	St+E	24.67±1.15^{b}	K+E	26.67±2.31^{bc}
	E+St	24.67±1.15^{a}	*S.a*+K	32.00±1.00^{a}	St+K	30.33±0.58^{a}	K+*S.a*	32.0±1.00^{a}
	E+K	26.67±2.31^{a}	*S.a*+St	31.33±1.15^{a}	St+*S.a*	31.33±1.15^{a}	K+St	30.3±0.58^{ab}
Triple	E	20.33±0.58^{b}	*S.a*	32.00±0.00^{a}	St	32.67±2.31^{a}	K	25.00±2.65^{a}
	E+*S.a*+St	24.33±0.58^{a}	*S.a*+E+St	24.33±0.58^{b}	St+*S.a*+E	24.33±0.58^{b}	K+E+*S.a*	25.67±0.58^{a}
	E+*S.a*+K	25.67±0.58^{a}	*S.a*+E+K	25.6687±0.58^{b}	St+E+K	24.67±0.58^{b}	K+E+St	24.67±2.31^{a}
	E+ST+K	24.67±2.31^{a}						
Tetra	E	20.33±0.58^{a}	*S.a*	32.00± 0.00^{a}	St	32.67± 2.31^{a}	K	25.00±2.65^{a}
	E+*S.a*+K+St	29.00±1.00^{b}	E+*S.a*+K+St	29.00±1.00^{a}	E+*S.a*+K+St	29.00±1.00^{a}	E+*S.a*+K+St	29.00±1.00^{a}

Different superscripts within column for each of double, triple, and tetra combination of each of bacteria show significant difference (P<0.05)

Vancomycin's efficacy against triple combinations of bacteria was considerably different to that of the other antibiotics used in this study. E+ST+K showed no zones while E+*S.a*+St showed significantly higher (P<0.05) ZOI compared to that of E, St and S.a (Table 4). Testing teicoplanin against E+St+K revealed no ZOI while E+*S.a*+St showed significantly lower ZOI compared to S.a and E (Table 5). Gentamicin showed significantly higher (P<0.05) efficacy in terms of ZOI when tested against triple combinations compared to that of E while tetra combinations of bacteria showed significantly lower (P<0.05) ZOI compared to all single bacteria except E in this study (Table 6).

DISCUSSION

The bacteria studied are now emerged as major pathogens of animal and public health. For example, *S. aureus* has become a ubiquitous pathogen not only from milk of cattle (Javed et al., 2021) but also from goat (Altaf et al., 2020) and other animals (Sarwar et al., 2021). Contrary to the findings of current study, Abakar *et al*. (2022) observed 40%, and 30% prevalence of *S. aureus* and *E. coli* while Botrel *et al*. (2010) and El-Mohandes *et al*. (2022) reported prevalence of *E. coli* as up to 85.7% in clinical mastitis milk. Earlier subclinical bovine mastitis from district Muzaffargarh, Lahore, and other regions of Punjab had been reported as 35, 30, and 40%, respectively (Ali *et al*., 2011; Mustafa *et al*., 2011a, b; Gao *et al*., 2017a, b). The variations in prevalence within country might be due to hygienic conditions, seasons, age, parity, health status of animals, physiological status, and presence of other diseases. This high prevalence of pathogens may be due to environmental factors and unhygienic conditions. The pathogens build several protective measures to keep them saved from any chemical, physical or immunological factors. *S. aureus* produces glycocalyx which is exopolysaccharide that helps adherence to epithelial cells rendering antibiotics and immunity ineffective. Similarly, *Streptococcus agalactiae* adhere to mammary glands for persistent infection leading to significant resistance to host defense and therapeutics (Rosini *et al*., 2015). *E. coli* is an environmental pathogen of bovine mastitis that plays with host immunity through endotoxin known as lipopolysaccharide. Through a series of actions, inflammatory response is initiated both at local and systemic level and meanwhile leukocytes are also activated to clear the infection. Here the pivotal roles go to the strength of host defense making the concept of *E. coli* mastitis more inclined towards host than to that of pathogenesis of *E. coli* (Gilbert *et al*., 2013). Other

factors like host nutritional status, transition period of cow, and some of environmental factors play important roles in disease development (Cheng *et al*., 2020).

E. coli and *Klebsiella* showed 83.3 and 100% sensitivity against gentamicin (Nam *et al*., 2009) while in contrast, the *S. aureus* showed many folds lower sensitivity (20% sensitive to gentamicin). Levofloxacin appears as highly distributed antibiotic in body fluids and accordingly its uptake by phagocytes makes it suitable for better candidate against intracellular pathogens (Goudah and Abo-El-Sooud 2008). Vancomycin was introduced as a drug of choice in case of MRSA treatment while increased use of this antibiotic against coagulase-negative staphylococcal, *Pseudomembranous colitis*, and *Clostridium difficile* has resulted in resistance to other pathogens as well (Goudah and Abo-El-Sooud, 2008). Teicoplanin on the other hand is regarded as more potentiated compared to vancomycin against gram-positive anaerobic bacteria and streptococci. The other factor includes reduction in intracellular concentration due to induction of efflux pump (Sun *et al*., 2021). Clindamycin, a lincosamide antibiotic, has been reported as drug of choice against *Streptococcal*, *Staphylococcal*, and gram-positive anaerobic bacterial infections (Armengol Álvarez *et al*., 2022). However, the mixed cultures may influence growth of bacteria based on their interaction with each other and several alterations may also be seen in the presence of antibiotics. The studies should investigate non-conventional approaches like response of mixed infections, testing of non-conventional antibiotics, investigations of underlying mechanism, pharmacokinetics studies of drugs when used against mixed infections, and evaluation of safety parameters.

Conclusions: The study highlighted increasing prevalence of mixed infection compared to single bacterial etiology. *Klebsiella pneumoniae* and *Streptococcus agalactiae* were found to be competing with prevalence of *S. aureus* and *E. coli*. The *in vitro* susceptibility of isolates from mastitis milk revealed levofloxacin and gentamicin as effective drugs against single and mixed culture bacteria. Vancomycin was among the least effective antibiotics against the most double, triple and tetra combinations. In comparison to single bacteria, mixed bacteria at some instances showed higher sensitivity to antibiotics while at other instances these responses were many folds lesser than to that of single bacteria. Molecular studies to find reasons of such discrepancies coupled with *in vivo* and field trials are required to develop effective dose regimens against mixed infection.

Author contribution: Nimra Kirn did research and compile data; Sijia Lu and Amjad Islam Aqib conceived idea, did research work, analyzed data, and finalized manuscript; Kashif Akram, Hamid Majeed, supervised work, edited draft, and prepared final manuscript; Afshan Muneer, Maheen Murtaza, and Kun Li prepared initial draft and did analysis; Kashif Prince revised manuscript.

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