INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
21–0645/2021/26–5–644–650
DOI: 10.17957/IJAB/15.1877
http://www.fspublishers.org

Full Length Article

In vitro Evaluation of Combination of Different NSAIDs and Antibiotics against MDR *Escherichia coli*

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Received 31 May 2021; Accepted 11 September 2021; Published 15 November 2021

Abstract

The current study was designed to examine the MDR *E.coli* in dairy animals, estimation of risk factors and to check the antibacterial activity of different NSAIDs alone and in combination with antibiotics. A total of 250 milk samples from cattle (n = 160) and buffaloes (n = 90) were collected aseptically in different dairy farms, situated in and around the Nankana District. Analysis of acquired data was done at 5% probability with the help of non-probability statistical tools. The study established whole prevalence of subclinical mastitis 42% established on screening by SFMT. While, from positive samples of milk, 16% *E. coli* were isolated whereas, multiple drug resistant *E. coli* was 7.5%. MDR *E. coli* was treated by disc diffusion method using different classes of antibiotics like Cefoxitin and Sulphamethoxasole. The MIC of NSAIDs (ibuprofen, meloxicam) was measured using method of broth micro dilution. FIC shows that combination of trimethoprim and meloxicam were synergistic and combination of gentamicin plus ibuprofen was additive or antagonistic, while combination of gentamicin plus meloxicam and trimethoprim plus ibuprofen was indifferent using checkerboard method. The present findings concluded a highly significant association in ($P \leqslant 0.026$) general body condition score, in ($P \leq 0.022$) antibiotics used in mastitis, in ($P \leq 0.038$) use of teat dipping, in ($P \leq 0.014$) lactation status, in ($P \leq 0.028$) hygienic condition during milking, and in ($P \leq 0.027$) presence of ticks or tick infestation. While non-significant association in ($P \geq 0.572$) teat abnormality and in case of sawdust used for cattle bedding ($P \geq 0.398$) prevalence in buffaloes and cattle. © 2021 Friends Science Publishers

Keywords: NSAIDs; Antibiotics; MDR *Escherichia coli;* Mastitis; Cattle

Introduction

Livestock sector plays a significant role in dairy population in Pakistan. The total population of cattle and buffaloes are 84.9 million numbers including cattle 46.1 million and buffalo 38.8 million numbers. These provide 44,831,000 tons of milk (Pakistan 2018). Quality milk production in modern dairy production is facing many challenges. Salient in them is mastitis which is responsible for decline in milk production, altered milk composition and compromised udder health. The malaise consists of multiple bacterial aetiologies where *E. coli* is being isolated regularly in all epidemiological studies. Mastitis is main disease in dairy animals that may cause economic losses, reduced milk production, degrade the quality of milk and culling of cows (Soest *et al.* 2018).

Resistance of antimicrobial agent has been known as a developing problem of worldwide in both veterinary and human medicine and use of antimicrobial agent is considered the most significant element for developing, selection and distribution of anti-microbial agent (Neu 1992). The treatment of multiple drug resistant (MDR) bacterial infection use combination of two or more antibiotics. There are many *E. coli* isolates that were identified and the resistance percentage is very high from raw meat 13.3% unpasteurized milk 6.7% and chicken 23.3%. The overall resistance was 14.7% of drug incidence in *E. coli*. Antibiotic resistance in *E. coli* isolates is a main distress and *E. coli* is the gram negative bacteria that cause urinary tract infection (Rasheed *et al.* 2014).

Non-steroidal anti-inflammatory drugs which are commonly used to relief the pain and process of inflammation and previous research shown that certain NSAIDs have an antimicrobial activity (Cai *et al.* 2007).

Hence there is need of antibiotic testing sensitivity of *E. coli* isolates against different antibiotics for exact diagnosis of bacteria so that exact etiological treatment of mastitis and effective control can be performed (Malinowski *et al.* 2008). This study was conducted to check the risk factors and prevalence related to *In vitro* evaluation of combination effect of different NSAIDs and antibiotic against MDR *E. coli*.

Materials and Methods

The current study was conducted for period of 2 months in cattle and buffalo population of district Nankana. Nankana district is located at 73.1350° E and 31.4504° N with 604 ft. beyond the level of sea describing the normal temperature of 24.2°C with range 11.9°C and the average annual rainfall is 346 mm. Nankana district were designated based on higher dairy populations 531720, 1608281 in Nankana Sahib, respectively and availability to the dairy animals.

A total of 250 dairy buffaloes and cattle from different lactation stages, different ages and different farms of Nankana Sahib from which milk samples (n = 160 cattle, n = 90 buffaloes) were collected using convenient method of sampling (Thrusfield 2018). Aseptic collection of milk was done as per method of National Mastitis Council (Middleton *et al.* 2014). Milk samples were collected after on-spot screening of clinical mastitis (CM) and subclinical mastitis (SCM) using Surf field mastitis test (SFMT) to check subclinical mastitis (Anju *et al.* 2018) while, more confirmatory tests and trials were conducted in laboratory of Clinical Medicine and Surgery, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan maintaining cold chain at 4°C.

Isolation and Identification of *Escherichia coli*

Milk samples were cultured through swabbing on blood agar. After overnight incubation at 37°C, microbial growth was estimated through haemolytic pattern, staining of the organisms. Gram negative rods (pink in colour) were selected as *E. coli* and more cultured on selective medium (*i.e.*, MacConkey agar) by three way method of streaking. Incubation at 37°C after 24 h, ideal round marginally raised colonies with specific fermentation pattern of MacConkey indicator were visible on agar plates. A pin point colony was chosen with the help of a sterile inoculation loop and secondary culturing was done by three-way method of streaking on the plates of MacConkey agar. Incubated at 37°C after 24 h, separate purified pinpoint colonies of *E. coli* were obtained. The glycerol stocks were prepared for the confirmed isolates and stored at -20°C for use in future (Bayer *et al.* 1966).

Estimation of the activity of antibacterial antibiotics

Antibacterial activity of antibiotics was implemented through modified method of well diffusion following the recommendations of Clinical and Laboratory standards institute (CLSI 2015). In our study we used Chloramphenicol (30 μg), Cefoxitin (30 μg), Trimethoprim (1.25 μg), Sulphamethoxazole (23.75 μg), Amikacin (30 μg), Vancomycin (30 μg), Fuscidic acid (10 μg) and Gentamicin (10 μg).

Firstly, we prepared culture inoculum at concentration of 0.5 McFarland (1.5×10^8 CFU/mL) in sterilized common saline. Then Swabbing of inoculum on prepared Muller Hinton agar plates was performed. Antibiotic discs on pre-swabbed Mueller Hinton agar plates were placed aseptically. Then plates were incubated in inverted position for 24 h at 37°C. Measurement of inhibition zones round the antibiotic discs was performed after 24 h. At the end, measurements were compared with Clinical and Laboratory standards institute for antibiotic efficacy evaluation (CLSI 2015).

Identification of MDR *E. coli*

Escherichia coli isolated in previous experiment were subjected for antibiotic susceptibility following the instructions given by CLSI (2015).

For agar well diffusion method wells of 6 mm were made in Muller Hinton agar, 0.5 McFarland of standard from fresh culture of *E. coli* was made in phosphate buffered saline solution. 0.5 McFarland was swabbed homogeneously on Muller Hinton agar. Different concentrations of antibiotics were poured in each well. Agar plates were incubated at 37°C for 24 h then zones of inhibition were measured in millimeters.

Results

Prevalence of subclinical mastitis and *E. coli* in cattle and buffaloes

Subclinical mastitis in cattle and buffalo remains an un-highlighted issue in Pakistan in spite of its great incidence rate in altered areas of the country and its linked financial losses. To design the treatment plans and appropriate control measures, it is essential to know about causative agents of mastitis and associated risk factors of mastitis. In District Nankana, great number of farmers is connected with cattle and dairy farming and hence, they depend on cattle and buffalo milk production. The present study was performed to know about the prevalence of subclinical mastitis in dairy cattle and buffalo of Nankana and to accomplish associated risk factors of *E. coli* isolates which is a main pathogen of bovine mastitis.

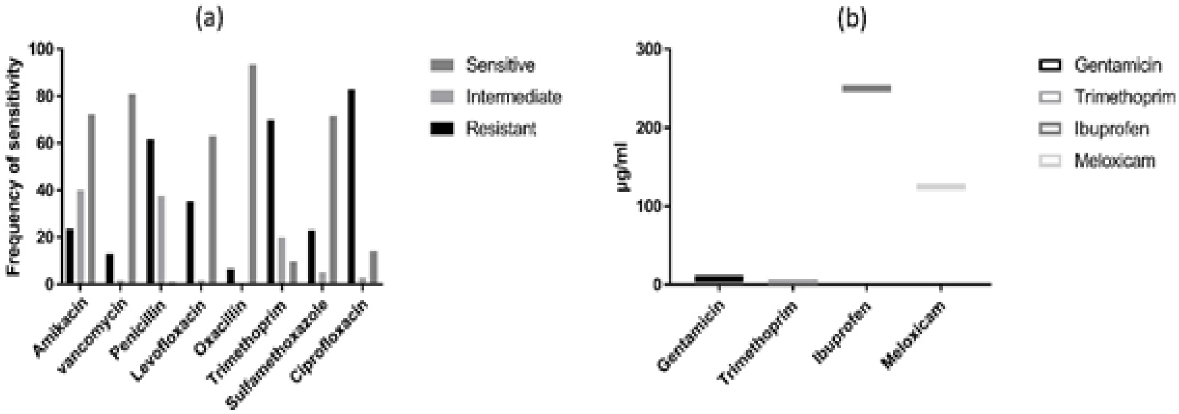

Fig. 1: (**a**) Frequency of sensitivity to different antimicrobial agents. (**b**) Combinations of antibiotic and NSAIDs used

Antibiotic susceptibility against *E. coli* from cattle and buffalo subclinical mastitis

Disc diffusion method for antibiotic sensitivity testing exposed that *E. coli* was resistant to different classes of antibiotics. The present study has reported that *E. coli* bacteria from in and around District Nankana have developed resistance against Ampicillin (83%), Trimethoprim + Sulphamethoxasole (70%) and Penicillin (61.8%). On the other hand, *E. coli* showed resistance to Levofloxacin (35.3%), Amikacin (23.6%), Ciprofloxacin (22.9%), Vancomycin (13%) and Oxacillin (6.5%) while E. coli showed intermediate resistance against some drugs like Amikacin (40%), Penicillin (37.4%), Trimethoprim + Sulphamethoxasole (20%), Ciprofloxacin (5.3%), Vancomycin (1.7%), Levofloxacin (1.5%), Ampicillin (3%) and Oxacillin 0% respectively. While Oxacillin (93.5%) was found highly sensitive against *E. coli* mastitis from District Nankana and more sensitivity was shown by Vancomycin (81.0%), Amikacin (72.4%), Ciprofloxacin (71.8%), Levofloxacin (63.2%), Ampicillin (14%), Trimethoprim + Sulphamethoxasole (10%), and least sensitive drug was Penicillin (0.8%) (Fig. 1).

Risk factor analysis

A complete data capture procedure was used for recording the observations concerning risk factors of subclinical mastitis in District Nankana. In the first step information associated to age of animal, body condition, tick infestation, sawdust used in cattle bedding, use of antibiotics for mastitis, teat dipping, teat abnormality and hygienic condition during milking were recorded. An informed approval of individual farmers was obtained to conduct this study. The recorded material was then entered into an excel sheet. The combination was made for approximate variables to conclude well considerate round the epidemiological factors linked by mastitis in cattle and buffalo's population of the study area. Statistical analysis of expected risk factors indicated significant response ($P \leq 0.05$) in case of bad condition, tick infestation, use of teat dipping, antibiotic used in mastitis, lactation status and hygienic condition during milking with spread of *E. coli* in dairy milk. While on the other hands, teat abnormality and sawdust used for cattle bedding gave indication of non-significant ($P \geq 0.05$) association with the spread of *E. coli* isolated from mastitis milk. All fibrosed udders presented 100% association of *E. coli* while the normal udder exhibiting 57.66% of cases involvement with bacterial spread. Animals having weak body condition, larger number of parities had greater percentage of *E. coli.* The present findings concluded a highly significant association in ($P \leq 0.026$) general body condition score, in ($P < 0.022$) antibiotics used in mastitis, in ($P \leq 0.038$) use of teat dipping, in ($P \leq 0.014$) lactation status, in ($p \leq 0.028$) hygienic condition during milking and in ($P \leq 0.027$) presence of ticks or tick infestation. While non-significant association in ($P \geq 0.572$) teat abnormality and in case of sawdust used for cattle bedding ($P \geq 0.398$) prevalence in buffaloes and cattle (Table 1).

Percentage of *E. coli* resistant to different number of antibiotics

Antibiotics like Ampicillin 83.0%, Trimethoprim + Sulphamethoxazole 70.0%, Penicillin 61.8%, Levofloxacin 35.3%, Amikacin 23.6%, Ciprofloxacin 22.9%, Vancomycin 13% and Oxacillin 6.5% were resistant against *E. coli*, while Amikacin 40%, Penicillin 37.4%, Trimethoprim + Sulphamethoxazole 20%, Ciprofloxacin 5.3, Ampicillin 3.0%, Vancomycin 1.7% and Levofloxacin 1.5% showed intermediate resistance against *E. coli*. Moreover, Oxacillin 93.5%, Vancomycin 81.0%, Amikacin 72.4%, Ciprofloxacin 71.8%, Levofloxacin 63.2%, Ampicillin 14%, Trimethoprim + Sulphamethoxazole 10%, and Penicillin 0.8% were sensitive against *E. coli* (Fig. 2a).

Factorial inhibitory concentration index (by Checkerboard method)

Factorial inhibitory concentration was also found on the basis of minimum inhibitory concentration and furthermore procedure given below.

In the current study we added 50 μL nutrient broth in each well and made two-fold serial dilution except 1st well which was +ve well. Then 0.5 McFarland was prepared and added in each well except –ve well or last well. Optical density value was measured in physiology lab before incubation at 570 nm. Then, incubated it at 37°C for 24 h (Table 2).

Antibiotics were used like gentamicin at 250 μg/mL and trimethoprim at 31.25 μg/mL, while NSAIDs was used at 500 μg/mL.

Minimum inhibitory concentrations of antibiotic and NSAIDs

In our study different concentrations of antibiotics and NSAIDs were used and their value of MIC was taken in μg/mL. MIC value of gentamicin was 7.81 μg/mL and value of trimethoprim was 1.95 μg/mL, while values of NSAIDs and ibuprofen were 250 μg/mL and MIC value of meloxicam was 125 μg/mL (Fig. 1b).

Gentamicin + Ibuprofen showed additive effect and the FICI value of this combination was 0.999, Gentamicin + Meloxicam showed indifferent effect and the FICI value of this combination was 1.0312, while on the other hand Trimethoprim + Ibuprofen also showed indifferent effect and the FICI value of this combination was 1.0156 and in 4th Trimethoprim + Meloxicam showed synergistic effect and the FICI value of this combination was 0.092 this combination indicated good effect for *E. coli* (Table 3).

Factorial inhibitory concentration index (by Checkerboard method)

In current study, 50 μL nutrient broth was added in each well and made two-fold serial dilution except 1st well which was +ve well. Then 0.5 McFarland was prepared and added in each well except –ve well or last well. Optical density value was measured in physiology lab before incubation at 570 nm. Then we incubated it at 37°C for 24 h. Interpretation was done based on optical density value.

Prevalence of multiple drug resistance (MDR) *E. coli*

First of all, swabbing was done on blood agar and incubated for 24 h at 37°C. Pink colour colonies showed *E. coli*. In our study, gram staining was also performed to isolate and identification of MDR *E. coli*. In this study, isolates of MDR *E. coli* were 30.4%. Chloramphenicol, trimethoprim + sulfamethoxazole, ampicillin and tetracycline were found best antibiotics for resistance (Fig. 2b).

Table 3: Interpretation based on these values whether drug effect is synergistic, indifferent or additive

FIC index	Interpretation
0.5 or less	Synergistic
0.5-1.0	Additive
1-4	Indifferent
> 4	Antagonistic

Discussion

Data were documented according to survey regarding risk factors associated in dairy buffaloes. Total 600 buffalo samples were screened out from small holding, organized, and individual holding for mastitis with White Side Test (WST) as defined by Ali *et al.* (2011) and 264 samples of milk were positive for mastitis, and exhibited prevalence of subclinical mastitis (44%) in lactating dairy animals of four Districts in Punjab (Narowal, Lahore, Okara and Sialkot). Total 40 isolates of *E. coli* were examined for Ampicillin, Chloramphenicol, Trimethoprim, Gentamicin, Streptomycin, Tetracycline and Kanamycin resistance with the help of standardized disk diffusion method of seven antibiotic sensitivity testing. Prevalence of MDR *E. coli* in mastitis dairy cattle and buffaloes were observed in 7.5/40 (18.75%) on the basis of resistance to streptomycin (1 μg) disk (Kikuvi *et al.* 2007).

The current study showed 42% (105/250) prevalence of subclinical mastitis in cattle and buffaloes of Nankana on the basis of Surf field mastitis test (SFMT). Our study also described prevalence of *E. coli* 40.1% associated with mastitis in cattle and buffaloes. Finding of mastitis was in line with previous studies such as reported 52.33%, Baloch and colleagues reported 54.29% and Bachaya and colleagues recorded 51.6% incidence rate of subclinical mastitis in cattle and buffaloes (Muhammad *et al.* 1995; Waage *et al.* 2001). Current study showed that 10.2% mastitis cases were due to infection of *E. coli* (Sharma *et al.* 2007), while on the other hand, (Awandkar *et al.* 2009) reported greater incidence of infection with *E. coli* (40.0%). Isolates of *E. coli* from both buffaloes and cows exposed high sensitivity towards amikacin, chloramphenicol and enrofloxacin (Verma *et al.* 2018).

The study found that 40 isolates of *E. coli* were confirmed against eight corporate antibiotics. The results indicated that high sensitivity of these isolates was in ascending order to amikacin, amoxicillin, vancomycin, oxacillin, ciprofloxacin and levofloxacin. Ampicillin and trimethoprim + sulphamethoxazole were found to be maximum effective and oxacillin and vancomycin was also found to be least effective against several mastitis pathogens like *E. coli.* Least efficacy of oxacillin in all isolates may be due to resistance in bacteria due to extensive usage of antibiotics in buffaloes and cattle. Khan and Muhammad (2005) described that resistance of one or more antibiotics in

Table 1: Bivariate analysis of risk factors associated with *E. coli* mastitis in cattle and buffaloes

Variable	Parameter	Total	Positive	Percentage (%)	Relative Risk	*P*-value
Specie	Cattle	160	30	18.75	1.69	0.114
	Buffalo	90	10	11.12	0.56	
Teat Abnormality	Normal	190	29	15.26	0.83	0.572
	Injured	60	11	18.34	1.2	
Body condition score	Normal	180	23	12.78	0.52	0.026
	Weak	70	17	24.28	1.9	
Sawdust used for cattle bedding	Yes	70	9	12.85	0.74	0.398
	No	180	31	17.22	1.34	
Antibiotics used in mastitis	Yes	170	21	12.35	0.52	0.022
	No	80	19	23.75	1.92	
Use of teat dipping	Yes	64	5	7.81	0.41	0.038
	No	186	35	18.81	2.4	
Lactation status	Dry	100	9	9	0.43	0.014
	Lactating	150	31	20.66	2.3	
Hygienic condition during milking	Yes	88	8	9.09	0.46	0.028
	No	162	32	19.75	2.17	
Presence of ticks/tick infestation	Yes	151	31	20.52	2.25	0.027
	No	99	9	9.09	0.45	

$P \leq 0.05$ indicate significant difference

Table 2: Synergy testing of NSAIDs with antibiotics (by Broth micro dilution method)

Sr. No.	Combination	Antibiotic alone	Antibiotic combine	NSAID Alone	NSAID Combine	FICA	FICB	FICI	Remarks
1	Gentamicin + Ibuprofen	7.81	3.9	250	125	0.499	0.5	0.999	Additive
2	Gentamicin + Meloxicam	7.81	7.81	125	3.90	1	0.0312	1.0312	Indifferent
3	Trimethoprim + Ibuprofen	1.95	1.95	250	3.90	1	0.0156	1.0156	Indifferent
4	Trimethoprim + Meloxicam	1.95	0.06	125	7.81	0.030	0.062	0.092	Synergistic

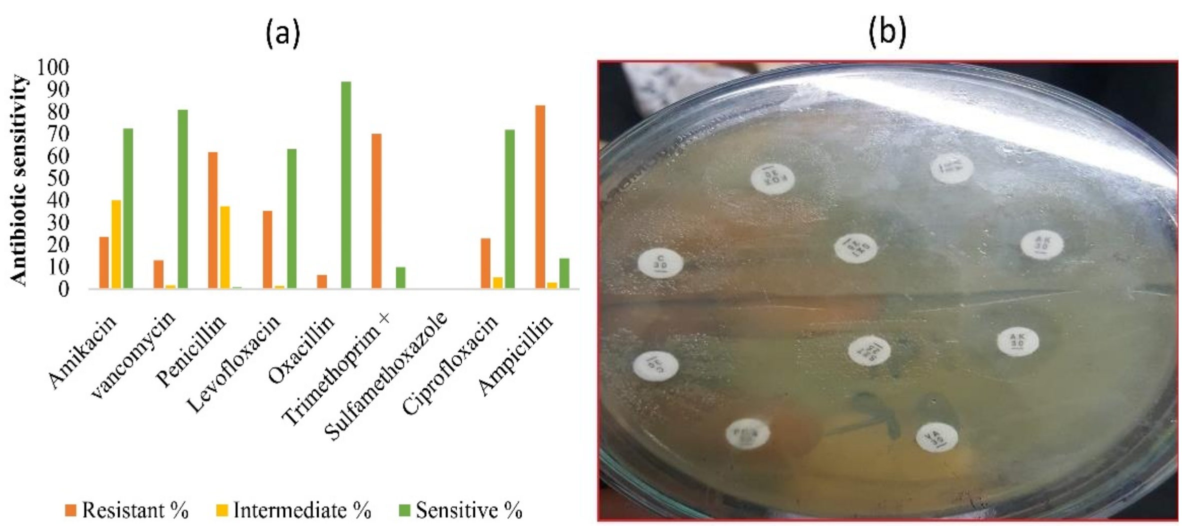

Fig. 2: (**a**) Comparison of resistant, intermediate, sensitive *E. coli* strains of each antibiotic. (**b**) Zones of inhibition of different antibiotics against multiple drug resistance (MDR) *E. coli*, Ciprofloxacin, Amikacin, Vancomycin used as standard effective in several studies, while Sulphamethoxasole is showing comparable zone of inhibition

strains of Shiga toxin producing *E. coli* (STEC) in India is 49.2% with approximate strains revealing multiple drug resistance.

Four strains of *E. coli* were resistant to 11 antimicrobial agents. Strains of *E. coli* from treated drug udders were commonly (> 50%) resistant to sulfonamides, streptomycin, chloramphenicol, tetracycline and trimethoprim, while in the isolates of healthy udder the most collective was (40%) tetracycline resistance. Strains of gentamicin resistance were first isolated from animals untreated (Zdolec *et al.* 2016). The susceptibility of antibiotic tests exposed that maximum number of *E. coli* were least susceptible to 38.46% Pencillin G and susceptible to tetracycline cephalothin 76.92% (Kurjogi and Kaliwal 2011).

In previous study isolates of Finnish, 16% were resistant to cephalexin, related with only 3% isolates of

Israeli; though, only one isolate of Finnish exhibited high value of MIC (>128 μg/mL) (Bishop *et al.* 1980; Trolldenier 1995). In our study different antibiotics and NSAIDs and their value of MIC in μg/mL. MIC value of Gentamicin was 7.81 μg/mL and value of trimethoprim was 1.95 μg/mL, while NSAIDs and their value of Ibuprofen was 250 μg/mL and MIC value of meloxicam was 125 μg/mL.

The prevalence of subclinical mastitis was very high between dairy cows and buffaloes in District Nankana and its control plans should focus on associated risk factors such as teat abnormality, sawdust use for cattle bedding, hygienic condition during milking, lactation status, use of teat dip, and body condition score with management practices monitored by the farmers. *E. coli* which was slightly resistant to most of the regularly used antibiotics. In our findings, we concluded a highly significant association general body condition score, antibiotics used in mastitis, use of teat dipping, lactation status, hygienic condition during milking, and presence of ticks or tick infestation. While non-significant association with teat abnormality and in case of sawdust used for cattle bedding.

Conclusion

As the incidence of drug resistant bacteria increases, there is an urgent need for new ways to combat the infections caused by these bacteria. The use of combination therapies of other drugs (such as antibiotics and NSAIDs) to treat other MDR bacterial infections is an interesting alternative. In conclusion of our study, Factorial inhibitory concentration shows that combination of trimethoprim and meloxicam were synergistic and combination of gentamicin plus ibuprofen was additive or antagonistic, while combination of gentamicin plus meloxicam and trimethoprim plus ibuprofen was indifferent. Our study concluded a highly significant association in general body condition score ($P \leq 0.026$), in antibiotics used in mastitis ($P \leq 0.022$), in use of teat dipping ($P \leq 0.038$), in lactation status ($P \leq 0.014$), in hygienic condition during milking ($P \leq 0.028$), and in presence of ticks or tick infestation ($P \leq 0.027$). While non-significant association in teat was found in abnormality ($P \leq 0.572$) and in case of sawdust used for cattle bedding ($P \leq 0.398$) prevalence in buffaloes and cattle. The findings of the current study of NSAIDs and antibiotic combination therapies may offer alternatives to overcome antibiotic resistance. However, further comprehensive *in vivo* and clinical trials are required to support this recommendation.

Acknowledgements

No acknowledgements to declare

Author Contributions

AT and IR planned the experiments, AT and GM, HJ, MI, AIA interpreted the results, AT, ZAB, MA and IM made the write up and MFAK statistically analyzed the data and made illustrations

Conflicts of Interest

All authors declare no conflicts of interest

Data Availability

Data presented in this study will be available on a fair request to the corresponding author

Ethics Approval

Not applicable in this paper

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