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Original Article

In vitro and *in vivo* evaluation of some antimicrobials and disinfectants against bacterial pathogens from hoof lesions in dairy cattle

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Abstract

Background: Lameness in dairy cattle is prevalent worldwide and has serious economic and welfare implications. Nevertheless, it is an overlooked and least studied dairy problem in Pakistan. **Aims:** This study was executed for *in vivo* and *in vitro* evaluation of antimicrobials and disinfectants against bacterial pathogens from hoof lesions of commercial dairy cattle. **Methods:** For *in vitro* studies, 23 bacterial isolates (n=10 *Staphylococcus aureus*, n=8 *Fusobacterium necrophorum*, and n=5 *Bacteroides*) from hoof lesions were used for antimicrobial and disinfectants susceptibility testing. *In vivo* trials were carried out among 4 groups of dairy cows suffering from hoof lesions using different combinations of antimicrobials, non-steroidal anti-inflammatory drugs (NSAIDs), and disinfectants either parenterally or topically. **Results:** Results indicated that most of the isolates of *S. aureus*, *F. necrophorum*, and *Bacteroides* were resistant to penicillin, amoxicillin, trimethoprim + sulphamethoxazole, oxytetracycline, and tylosin. Ciprofloxacin and gentamicin were the most effective antimicrobials (*in vitro*) against all three bacterial pathogens. Comparison of *in vitro* efficacy of disinfectants showed that copper sulfate was the most effective disinfectant against the three pathogens followed by povidone-iodine and chloroxylenol. *In vivo* trials revealed that ciprofloxacin at 5 mg/kg/day intramuscular (IM) for 7 days, flunixin meglumine at 2.2 mg/kg/day IM for 7 days, and copper sulfate (5% solution) as foot-bath twice daily for 21 days was the most effective treatment regimen to treat lameness in commercial dairy cows. **Conclusion:** It was concluded that *in vitro* antibiogram and disinfectant studies were useful tools to assess the effectiveness of routinely used antimicrobials and disinfectants for the treatment of lameness.

Key words: *Bacteroides*, Copper sulfate, *Fusobacterium necrophorum*, Lameness, Phenol

Introduction

Lameness, a very common disorder seen in dairy cows, is prevalent worldwide and has serious economic and welfare implications (Cook *et al*., 2004; Von Keyserlingk *et al*., 2009). The economic losses associated with lameness include lower milk production (Amory *et al*., 2008), high mortality and culling rates (Booth *et al*., 2004; Bicalho *et al*., 2007), and a decline in reproductive performance (Hernandez *et al*., 2001; Melendez *et al*., 2003).

Many factors are involved in the onset of lameness, including infectious agents, laminitis and conformational lesions; however, the most important ones are hoof and claw lesions such as sole ulcer (SU), sole hemorrhage (SH), white line disease (WLD), heel ulcers (HU), and interdigital dermatitis (IDD). The cause of hoof and claw lesions is not completely understood, however, a number of risk factors affect their development (Kumar *et al*., 2019). Hoof lesions have also been reported in the subclinical form of lameness (Tadich *et al*., 2010) which caused a reduction in milk production (Green *et al*., 2014).

Different types of bacteria have been isolated from foot lesions. These include *Fusobacterium* spp., *Bacteroides* spp., *Peptococcus* spp., and *Campylobacter* spp., (Ohya *et al*., 1999; Demirkan *et al*., 2000). Spirochaetes are the most common bacterial cause of digital dermatitis (DD) (Stamm *et al*., 2002).

It is believed that timely detection and treatment of lameness helps in a better cure rate, decreased prevalence, and duration of lameness in dairy herds

(Hernandez *et al*., 2005). Improving lameness detection by farm workers is likely to significantly reduce the interval between reduced mobility and lameness treatment (Alawneh *et al*., 2012). Available products for the treatment of lameness range from systemic antibiotics to topical organic acids. There is evidence to support the notion that systemic antibiotics are effective in treating lameness (Read *et al*., 1992). Lame cows were healed when treated with intramuscular (IM) ceftiofur or IM procaine penicillin in the USA. Similarly, in the UK, several non-antibiotic products are available for topical use with positive results in the treatment and control of DD. These products include benzalkonium chlorides like antiseptics, acidified copper salts, essential oils with organic acids, effective enzymes, and specific trace minerals (Read and Walker, 1998).

Earlier work has described some contrary findings regarding the systemic use of antibiotics. The studies by Blowey and Sharp (1988), and Watson (2007) have reported it as ineffective, while other studies support that systemic antibiotics are effective in controlling lameness (Read *et al*., 1992). The topical application of oxytetracycline spray is the best treatment (Blowey and Sharp, 1988), particularly for treating individual cases. Larson and Morton (1991) reported that antiseptics and disinfectants are important and essential for controlling and minimizing the infection. However, the problem is the selection of antiseptics and disinfectants, as the response of these agents is different against different causes. Furthermore, due to the rising resistance of microorganisms to new antiseptics and disinfectants, none of these are effective singly against the pathogens causing the lameness. In the backdrop of the existing contradictory information, this study was aimed at *in vitro* and *in vivo* evaluation of different antibiotics and disinfectants against bacterial pathogens isolated from the lameness cases of commercial dairy cows in Punjab, Pakistan.

Materials and Methods

Bacterial isolates

Twenty-three bacterial isolates (n=10 *Staphylococcus aureus*, n=8 *Fusobacterium necrophorum*, and n=5 *Bacteroides*) were used in this study. These isolates were obtained from hoof lesions of exotic and crossbred dairy cows in Southern and Central Punjab province of Pakistan during 2018-2019. All the isolates were cultured and identified by standard microbiological procedures described in Bergey's manual of systemic bacteriology (Bergey *et al*., 1984) and stored in Microbank (Pro-Lab Diagnostics, USA) at Dairy Health Research Laboratory, Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore, Pakistan.

Antibiotic susceptibility testing

The bacterial isolates (*S. aureus*, *F. necrophorum*, and *Bacteroides*) were suspended in nutrient broth in separate tubes and the concentrations of the suspensions were adjusted to a turbidity equivalent to 0.5 McFarland standard. The antibiotic susceptibility test of *S. aureus* isolates was performed on Muller-Hinton agar whereas brain heart infusion agar was used to test antimicrobial susceptibility of *F. necrophorum* and *Bacteroides* isolates by disk diffusion method as described by the Clinical Laboratory Standard Institute (CLSI, 2002). Briefly, separate sterile cotton swabs were soaked in bacterial culture tubes, squeezed gently to ward off the excessive inoculum and uniformly swabbed on Muller-Hinton agar (*S. aureus*) and brain heart infusion agar (*F. necrophorum* and *Bacteroides*) plates. The lawns were allowed to dry for 3 min. Using sterilized forceps, antibiotic discs were placed on agar plates and were gently pressed to embed in the agar. Antibiotic disc embedded Muller-Hinton agar plates were incubated aerobically at 37°C for 24 h and brain heart infusion agar plates were incubated in anaerobic jars at 37°C for 48 h. After incubation, zones of inhibition (ZI) were measured and isolates were categorized as sensitive, intermediate or resistant based on ZI as per standards of CLSI. Antimicrobial agents used were: penicillin (10 IU), norfloxacin (5 µg), gentamycin (10 µg), ciprofloxacin (5 µg), oxytetracycline (30 µg), tylosin (30 µg), metronidazole (50 µg) (against *F. necrophorum* and *Bacteroides* only), amoxicillin (20 µg), and trimethoprim plus sulfamethoxazole (25 µg).

In vitro disinfectants testing

In vitro testing of bacterial isolates (*S. aureus*, *F. necrophorum*, and *Bacteroides*) for disinfectants was performed using the Phenol Co-efficient test (Cappuccino and Sherman, 2005). Three disinfectants namely 5% copper sulfate, 10% povidone-iodine, and 4.8% Chloroxylenol were tested. In brief, nutrient broth tubes were labeled with the names of the disinfectants and time interval of sub-culturing for individual bacteria separately. In a test tube rack, 5 serial dilutions (1:50, 1:100, 1:150, 1:200, and 1:250) of each disinfectant were prepared using normal saline. In separate dilution tubes of disinfectants, 100 µL of *S. aureus*, *F. necrophorum*, and *Bacteroides* broth cultures were added and agitated to mix thoroughly. Using sterile technique, at intervals of 5, 10, and 15 min a loopful of the material from each tube was transferred to separately labeled nutrient broth tubes. Nutrient broth tubes were incubated aerobically at 37°C for 24 h for *S. aureus* and 48 h for *F. necrophorum* and *Bacteroides* at 37°C in airtight anaerobic jar. The value of the phenol coefficient was computed using the following formula.

$$\text{Phenol coefficien t} = \frac{\text{Highest dilution of disinfectant that kills microorganism in 10 min}}{\text{Highest dilution of phenol that kills microorganism in 10 min}}$$

In vivo trials

For *in vivo* evaluation, a total of 28 lame cows were selected and divided into 4 groups of 7 viz. A, B, C, and D. This sample size was based on convenient sampling technique (non-probability sampling) as described by

(Thrusfield, 2005). Cows in group A were treated with ciprofloxacin at 5 mg/kg/day IM for 7 days, flunixin maglumine at 2.2 mg/kg/day IM for 7 days, and copper sulfate (5% solution) as foot-bath twice daily for 21 days. Similarly, animals in group B were treated with gentamycin at 5 mg/kg/day IM for 7 days, flunixin maglumine at 2.2 mg/kg/day IM for 7 days and copper sulfate (5% solution) as foot-bath twice daily for 21 days. Cows in group C were treated with ciprofloxacin and copper sulfate (5% solution) as foot-bath twice daily for 21 days, and flunixin maglumine at 2.2 mg/kg/day IM for 7 days, whereas cows in group D were treated with gentamycin and copper sulfate (5% solution) as foot-bath twice daily for 21 days, and flunixin maglumine at 2.2 mg/kg/day IM for 7 days. In each group, effected hoof was dipped in foot-bath for 15 min each time. It was ensured that whole hoof up to coronary band was properly dipped in the bath. The antimicrobials and disinfectant used *in vivo* trials were those which proved most effective during *in vitro* trials. All animals in each group were closely examined and assessed for recovery and improvements from lameness on daily basis. Finally, the efficacies of the treatment regimen were measured and compared based on recovery rate at various time intervals.

Statistical analysis

Data originated from the trials were statistically analyzed using the Chi-square test, and confidence interval (95%) was calculated. All the statistical analyses were performed in IBM SPSS Statistics for Windows, version x 22 (IBM Corp., Armonk, NY, USA). The probability level ($P<0.05$) was considered statistically significant.

Results

In vitro antibiotic sensitivity test

Among *S. aureus* isolates, 90% (9/10) were resistant to penicillin and amoxicillin whereas 80% (8/10) and 70% (7/10) were resistant to trimethoprim + sulphamethoxazole and to oxytetracycline, respectively. On the other hand, ciprofloxacin was the most effective antimicrobial for which 70% (7/10) isolates of *S. aureus* were susceptible, and 50% (5/10) *S. aureus* isolates were sensitive to gentamicin, making gentamicin the second most effective antimicrobial (Table 1). The *in vitro* antimicrobial susceptibility data for *F. necrophorum* are presented in Table 2. Out of 8 *F. necrophorum* isolates, 87.5% (7/8) isolates were sensitive to ciprofloxacin while 50% (4/8) isolates were sensitive to gentamicin and norfloxacin. In contrast to this, 100% (8/8) isolates of *F. necrophorum* were resistant to penicillin, amoxicillin, tylosin and trimethoprim + sulfamethoxazole. Similarly, 87.5% (7/8) of the *F. necrophorum* isolates were resistant to metronidazole. Among the *Bacteroides*, 80% (4/5) and 60% (3/5) isolates were sensitive to ciprofloxacin and norfloxacin, respectively. Likewise, 40% (2/5) isolates of *Bacteroides* were sensitive to gentamicin. On the other hand, 100% (5/5) isolates of *Bacteroides* were resistant to penicillin, amoxicillin, tylosin and trimethoprim + sulphamethoxazole. Similarly, 80% (4/5) *Bacteroides* isolates were resistant to metronidazole (Table 3). These antibiogram results indicated that selected bacterial isolates from the lesions of dairy cattle hooves were resistant to the antibiotics most commonly used on dairy farms.

Table 1: Results of *in vitro* antimicrobial susceptibility test against *S. aureus* isolates from hoof lesions of dairy cows

Antibiotics	Conc.	No. of isolates tested	Level of sensitivity					
			Sensitive		Intermediate		Resistant	
			No. of isolates	Percent	No. of isolates	Percent	No. of isolates	Percent
Ciprofloxacin	5 µg	10	07	70.0%	03	30.0%	0	0.0%
Oxytetracycline	30 µg	10	01	10.0%	02	20.0%	07	70.0%
Trimethoprim + sulfamethoxazole	25 µg	10	01	10.0%	01	10.0%	08	80.0%
Penicillin	10 IU	10	01	10.0%	0	0.0%	09	90.0%
Gentamycin	10 µg	10	05	50.0%	02	20.0%	03	30.0%
Norfloxacin	5 µg	10	04	40.0%	02	20.0%	04	40.0%
Tylosin	30 µg	10	02	20.0%	01	10.0%	07	70.0%
Amoxicillin	20 µg	10	01	10.0%	0	0.0%	09	90.0%

Table 2: Results of *in vitro* antimicrobial susceptibility test against *F. necrophorum* isolates from hoof lesions of dairy cows

Antibiotics	Conc.	No. of isolates tested	Level of sensitivity					
			Sensitive		Intermediate		Resistance	
			No. of isolates	Percent	No. of isolates	Percent	No. of isolates	Percent
Ciprofloxacin	5 µg	8	7	97.5%	01	12.5%	0	0.0%
Trimethoprim + sulfamethoxazole	25 µg	8	0	0.0%	0	0.0%	8	100%
Penicillin	10 IU	8	0	0.0%	0	0.0%	8	100%
Gentamycin	10 µg	8	4	50.0%	4	50.0%	0	0.0%
Norfloxacin	5 µg	8	4	50.0%	4	50.0%	0	0.0%
Tylosin	30 µg	8	0	0.0%	0	0.0%	8	100%
Amoxicillin	20 µg	8	0	0.0%	0	0.0%	8	100%
Metronidazole	50 µg	8	1	12.5%	0	0.0%	7	87.5%

Table 3: Results of *in vitro* antimicrobial susceptibility test against *Bacteroides* isolates from hoof lesions of dairy cows

Antibiotics	Conc.	No. of isolates tested	Level of sensitivity					
			Sensitive		Intermediate		Resistance	
			No. of isolates	Percent	No. of isolates	Percent	No. of isolates	Percent
Ciprofloxacin	5 µg	5	4	80%	01	20%	0	0.0%
Trimethoprim + sulfamethoxazole	25 µg	5	0	0.0%	0	0.0%	5	100%
Penicillin	10 IU	5	0	0.0%	0	0.0%	5	100%
Gentamycin	10 µg	5	2	40%	3	60%	0	0.0%
Norfloxacin	5 µg	5	3	60%	2	40%	0	0.0%
Tylosin	30 µg	5	0	0.0%	0	0.0%	5	100%
Amoxicillin	20 µg	5	0	0.0%	0	0.0%	5	100%
Metronidazole	50 µg	5	1	20%	0	0.0%	4	80%

Table 4: Comparisons of different disinfectants against various test microorganisms isolated from hoof lesions of dairy cows

Test organism	Disinfectant	Highest dilution of disinfectant	Highest dilution of Phenol	Phenol coefficient (Pc)	Remarks
Staphylococcus aureus	Copper sulfate (5%)	1:200	1:150	200/150=1.33[a]	Very good
	Povidone iodine (10%)	1:100	1:150	100/150=0.66[a]	Good
	Chloroxylenol (4.8%)	1:100	1:150	100/150=0.66[a]	Good
Fusobacterium necrophorum	Copper sulfate (5%)	1:100	1:100	100/100=1[a]	Very good
	Povidone iodine (10%)	1:50	1:100	50/100=0.5[a]	Satisfactory
	Chloroxylenol (4.8%)	1:50	1:100	50/100=0.5[a]	Satisfactory
Bacteroides	Copper sulfate (5%)	1:150	1:150	150/150=1[a]	Very good
	Povidone iodine (10%)	1:100	1:150	100/150=0.66[a]	Good
	Chloroxylenol (4.8%)	1:100	1:150	100/150=0.66[a]	Good

Values in the column bearing same superscript letter are statistically non-significantly different (P>0.05)

Table 5: *In vivo* efficacy of antimicrobials and disinfectants selected from *in vitro* trials to treat lameness in dairy cows at commercial dairy herds of Punjab during 2018-2019

Group	Recovery at day				95% CI at day 21
	5	10	15	21	
A (n=7)	4 (57.14%)	6 (85.7%)	6 (85.7%)	6 (85.7%)	48.68-97.43
B (n=7)	1 (14.28%)	3 (42.8%)	5 (71.42%)	5 (71.42%)	35.89-91.78
C (n=7)	0	1 (14.3 %)	3 (42.85%)	3 (42.85%)	15.82-74.96
D (n=7)	0	1 (14.3 %)	2 (28.57%)	2 (28.57%)	8.22-64.11

A: Ciprofloxacin at 5 mg/kg/day IM for 7 days, flunixin maglumine at 2.2 mg/kg/day IM for 7 days and copper sulfate (5% solution) as foot bath twice daily for 21 days, B: Gentamycin at 5 mg/kg/day IM for 7 days, flunixin maglumine at 2.2 mg/kg/day IM for 7 days and copper sulfate (5% solution) as foot dip twice daily, C: Ciprofloxacin and copper sulfate (5% solution) foot bath twice daily for 21 days and flunixin maglumine at 2.2 mg/kg/day IM for 7 days, and D: Gentamycin and copper sulfate (5% solution) foot bath twice daily for 21 days and flunixin maglumine at 2.2 mg/kg/day IM for 7 days

In vitro disinfectant trials

When the disinfectants were compared for the efficacy against *S. aureus* using phenol coefficient, copper sulfate (5%) was the most effective with the highest phenol coefficient (1.33) than povidone iodine (10%), and chloroxylenol (4.8%). In contrast, against *F. necrophorum,* copper sulfate (5%) was as effective as phenol with phenol coefficient value of 1, whereas povidone iodine (10%) and Chloroxylenol (4.8%) showed the least effective with phenol coefficient value of 0.5 each. Similarly, when the efficacy of disinfectants was tested against *Bacteroides* using phenol coefficient, copper sulfate (5%) was the most effective (phenol coefficient=1) compared to povidone iodine (10%), and chloroxylenol (4.8%) with phenol coefficient 0.66 each (Table 4). However, statistical analysis revealed a non-significant difference (P>0.05) among efficacies of different disinfectants.

In vivo trials

For final *in vivo* efficacy trials, a total of 28 lame cows were selected and divided into 4 treatment groups. Results demonstrated ciprofloxacin IM with copper sulfate foot-bath and flunixin maglumine IM as the most effective treatment regimen (recovery rate 85.7%) for the lameness. The next effective regimen was gentamycin IM along with copper sulfate (5%) foot-bath and flunixin maglumine IM (recovery rate 71.42%). Ciprofloxacin plus 5% copper sulfate as foot-bath with IM flunixin maglumine (recovery rate 42.85%) or gentamycin plus 5% copper sulfate as foot-bath with IM flunixin maglumine (recovery rate 28.57%) were the least effective treatment regimens for the lameness (Table 5).

Discussion

Lameness, a very common disorder seen in dairy cows, is prevalent worldwide and has serious economic and welfare implications. This study was executed for *in vivo* and *in vitro* evaluation of antimicrobials and disinfectants against bacterial pathogens isolated from hoof lesions of commercial dairy cattle. Results of the

antimicrobial susceptibility revealed that *S. aureus*, *F. necrophorum*, and *Bacteroides* isolates were the most sensitive to ciprofloxacin followed by gentamycin and norfloxacine, respectively. These isolates were resistant to penicillin, amoxicillin, tylosin, oxytetracycline, and trimethoprim + sulphamethoxazole. This resistance to antimicrobials may be attributed to the frequent indiscriminate use of these antimicrobials for the treatment of different ailments. Wani *et al*. (2003) observed that resistance of different bacterial isolates to ampicillin/cloxacillin was similar to the resistance observed with bacterial isolates of other pyogenic infections in different animal species. Khan (2019) reported that *F. necrophorum* from foot rot of dairy cattle was sensitive to norfloxacin and ciprofloxacin, and showed intermediate activity against gentamycin while *F. necrophorum* was resistant to penicillin, metronidazole, amoxicillin, sulfamethoxazole + trimethoprim, and tylosin. These observations are congruent with the results of our study. It has been shown that *F. necrophorum* from liver abscess in cow, was sensitive to penicillin G, clindamycin, lincomycin, and tylosin and was resistant to erythromycin, gentamycin, ampicillin, doxycycline, and nalidixic acid (El-Shorbagy *et al*., 2008).

From *in vitro* disinfectant study, it was evident that copper sulfate (5%) was the most effective disinfectant against *S. aureus*, *F. necrophorum*, and *Bacteroides* followed by povidone-iodine (10%) and chloroxylenol (4.8%). Saha *et al*. (2009) reported variable response of antiseptics and disinfectants against different pathogens and response was also concentration-dependent. They observed formalin and hydrogen peroxide as the most effective against all pathogens while phenyl and iodine showed the least effective. The antibacterial effects of chloroxylenol and cetrimide plus chlorhexidine digluconate were moderate. Okore *et al*. (2014) found chloroxylenol the most effective against *Escherichia coli*, *S. aureus,* and *Streptococcus* spp.

From *in vivo* trials, it was found that antibiotic therapy with ciprofloxacin intramuscularly along with flunixin meglumine intramuscularly and 5% copper sulfate foot-bath were the most effective treatments of lameness in commercial dairy herds in Punjab. Three approaches have been used to treat lameness cases in dairy cows: (1) systemic antibiotics, (2) a topical treatment for individual cows, and (3) group foot-bath treatments (Laven *et al*., 2006). In the USA, foot lesions in cows were successfully treated with procaine penicillin or ceftiofur when given intramuscularly. Likewise, parenteral use of cefquinome has been effective for the treatment of DD in the UK (Read and Walker, 1998). According to Fjeldaas *et al*. (2014), copper sulfate foot-baths were effective in curing the cows suffering from IDD and hoof horn erosion (HHE). Moreover, hooves were much harder with copper sulfate after a trial period than other treatments (Fjeldaas *et al*., 2014). Laven and Proven (2000) studied the usage of antibiotics in foot-baths with erythromycin in 111 dairy cows with variable results. Likewise, Solano *et al*. (2017) found foot dipping as the most common practice to control IDD at herd level and Copper sulfate foot-bath at dairy farms significantly reduced the cases of DD. Speijers *et al*. (2010) found 5% copper sulfate the most effective treatment of foot lesions. Dragonhyde®, a commercial dust dissolvable foot-bath, performed better than formalin and there was no difference between copper sulfate and Dragonhyde® in treating the DD lesions (Teixeira *et al*., 2010).

This is ostensibly the very first study on lameness treatment in commercial dairy cows in Punjab, Pakistan. *In vitro* antimicrobial and disinfectant studies are useful tools to assess the effectiveness of routinely used antimicrobials and disinfectants for the treatment of lameness cases. From this study, it is concluded that the bacterial isolates were resistant to antibiotics which were most commonly used on dairy farms. Ciprofloxacin at 5 mg/kg/day IM for 7 days, flunixin meglumine at 2.2 mg/kg/day IM for 7 days and copper sulfate (5% solution) as foot bath twice daily for 21 days is the most effective treatment regimen for lameness cases in commercial dairy cows. The knowledge and information generated through this investigation will be highly beneficial to veterinary practitioners and dairy farmers.

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Conflict of interest

The authors declare that they have no conflicting interest.

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