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Research Article

Activity of Diclofenac Sodium (I) *In Vitro* Susceptibility of Selected Pathogens and, (II) *In Vivo* Evaluation against *Staphylococcus epidermidis* in Surgical Wound Infection Model Antibacterial

Muhammad UmerAnwer[1]*, Saima Aslam[2], Muhammad Bilal Anwar[3], Asad Manzoor[2] Misbah Ijaz[2] and Faizan Saleem[1]

[1]*BahauddinZakariyya University, Multan, Pakistan*

[2]*University of Agriculture, Faisalabad, Pakistan*

[3]*University of Sargodha, Pakistan*

***Corresponding Author:** Muhammad UmerAnwer, BahauddinZakariyya University, Multan, Pakistan.

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Abstract

The increasing resistance against antimicrobials has encouraged the alertness in the innovation and preparation of various non-antibiotics antimicrobials. The resistance against different antibiotics in bacteria is a vital process usually happening and crucial apprehension in whole of the ecosphere. The principal collective nosocomial pollutant comprises of *Staphylococcus* (S.) *epidermidis-contamination*. It is manifestly obvious from the ancient times of the establishment of pharmaceutical agents, that to some extent, a medicine may have diverse functions and has appreciated belongings in exclusively transformed zones of medication. Resistance of medicines against microorganisms documents analysis of firsthand antibiotics which were operational against resistant microorganisms. Subsequently, the simultaneous investigation was programmed to analyze the antimicrobial belongings of a non-antibiotic antimicrobial medicine, diclofenac sodium against *S. epidermidis* isolate of bacteria. The research comprised of two sections i.e., In vitro susceptibility examination and minimum inhibitory concentration (MIC) of diclofenac sodium was assessed beside *S. epidermidis* during the early section of the investigation and *in vivo* susceptibility examination by topical application of diclofenac sodium over the surgical wounds. Diclofenac sodium generates no area of growth inhibition at 2, 5 and 10µg/disc concentration against all three isolates while creates zone of growth inhibition computing 16, 18 and 20 mm at concentration of 25, 50 and 100µg/disc against *S. epidermidis* correspondingly, however for judgment sulphamethoxazole discs were applied as standard, which created a region of growth inhibition computing 22 mm against the similar isolate. The standard medicine formed a region of growth retardation measuring 24 mm. Minimum inhibitory concentration values was measured by agar dilution method was 25µg/ml for *S. epidermidis.* When diclofenac sodium topically applied to the wounds then the cuff/gr determined was varied from 4.86 ± 0.04 X 10^6 while 5.68 ± 0.07 x 10^6 colony forming units/gram was determined in normal saline cured wounds.

Keywords: Surgical Wounds; Antibacterial Activity; Diclofenac; Staphylococcus; *Invivo*; *Invitro*,

Introduction

Any infection which occurs on the surgical site after the performance of a surgical procedure is known as surgical site infection (SSI). The severe form of surgical site infection occurs in various organs and the skin [1]. These infections are known as nosocomial infections if occurred within thirty days of surgery. In surgical patients the important source of illness are the postoperative surgical site infections. The most common isolated pathogen in these infections is *Staphylococcus epidermidis*. Due to resistance of *Pseudomonas aeruginosa* to broad spectrum antibiotics it is considered as the great threat in SSI causing delayed in the wound healing. Pathogens and body commensals may also contaminate the surgical wound [3].

In humans the most common nosocomial infection is the surgical site infection (SSI), accounting for 16% of such infections in all patients and 38% of nosocomial infections among surgical patients in the United States. Nosocomial infections particularly SSI's, play major role in prognosis and patient survival in veterinary practice too. In small animal surgery the SSI's describe the complication of 0.8% to 18.1%, with vast variation related with surgical procedure [12].

Surgical site infections are mainly caused by microorganisms like bacteria. These bacteria are either useful or injurious for the animals and human health. In case of human, there are almost 50 bacterial infections which are caused by harmful pathogens. To control these harmful pathogens in order to prevent the surgical site infection, antibacterial agents are used. Antibiotic resistance is the major drawback of these agents when used extensively [16]. Some important examples of antibiotic resistance are methicillin and fluoroquinolone which are resistant against *Streptococcus aureus*, *Streptococcus pneumonia* and *Streptococcus pyogenes* [14] and vancomycin which is resistant against Enterococci [7].

Antibiotics act against the pathogen either by inhibition of protein synthesis or by causing damage to cell wall and DNA. Antibiotic resistance occurs when microbes limit antibiotic action by adopting different mechanisms. Microbes may change the permeability of antibiotic or produce some enzymes which cause inactivation of drug. The use of non-antibiotic drugs is the solution that shows antibacterial action through different methods. Previous studies have revealed that variety of compounds, which are used in the management of non-infectious pathological conditions, have broad spectrum *in vitro* and *in vivo* antimicrobial activity against different types of Gram-positive and Gram-negative bacteria. Such types of compounds are known as "non-antibiotics" [19].

Non antibiotic antibacterial have temperate to potent antimicrobial action. One of these drugs which also have antibacterial action is NSAID (non-steroidal anti-inflammatory drugs). Diclofenac is awell-known anti-inflammatory agent and also shows antibacterial activities against both gram-positive and gram-negative bacteria. It is used as a pain reducing and anti-inflammatory agent. It is also effective in many other disorders like gout, osteoarthritis; dysmenorrheal pain etc. Diclofenac works by blocking the action of cyclooxygenase enzyme which is necessary for the production of prostaglandin and is responsible for the sensation of pain and inflammation [10]. Molecular formula and molecular weight of diclofenac is C14H11C12NO2 and 318.13 respectively. It is available in white powder form having crystals. It is easily soluble in organic solutions and cautiously soluble in water. It remains stable at 288-290^{0}C [16].

Diclofenac sodium shows bactericidal effect against both gram-positive and gram-negative bacteria through inhibition of DNA synthesis. Diclofenac sodium has antimicrobial action against different strains of *Mycoplasma*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa, Candida* albicans, *Bacillus subtilis* and *Staphylococcus aureus* [17].

Now a day's antibiotic resistance is an emerging issue leading to the use of non-antibiotic antimicrobials to inhibit microbial infections as an alternative. Therefore, the current study was designed to estimate *in vitro* susceptibility and minimum inhibitory concentration of diclofenac sodium against *Staphylococcus epidermidis; a*nd to estimate *in vivo* antimicrobial action of diclofenac sodium in induced surgical site infections by *Staphylococcus epidermidis*.

Methodology

In vitro antibacterial action of diclofenac sodium

The antimicrobial property of diclofenac sodium was studied *in vitro* against Methicillin resistant *S. epidermidis* by disc diffusion technique [6]; and this evaluation of minimum inhibitory concentration (MIC) was done through agar dilution technique in order to evaluateits antibacterial susceptibility.

Disc diffusion technique

Non-antibiotic antibacterial *viz.,* diclofenac sodium was used as test antibacterial agent and Whatman® filter paper was used to make filter paper discs (Coe-Parmer, USA) which soaked with Diclofenac sodium (2, 5, 10, 25, 50 and 100 µg/ml) and Sulphathiazole/trimethoprim as a standard drug. Different dilutions of each non-antibiotic agent and its 10-fold dilutions were prepared and kept at -20°C until used. For preparation of Petriplates Mueller-Hinton medium was used (NCCLS, 1994).The inoculum was formed via taking, four to five clean colonies with the help of sterile inoculation loop from an overnight growth. Then in sterile normal saline these colonies were emulsified. Solution was prepared until its turbidity matched with turbidity of 0.5 percent McFarland.

For every test organism, a sterile disposable swab was saturated into uniform liquid inoculum and pressed gently by moving the swab along the inner side of the glass tube above the fluid, for the withdrawal of excess fluid. The whole surface agar plate was pasted with swab by whizzing the swab in zig zag fashion. The plates were permitted to dehydrate at 35 to 37^{0}Cfor 5-10 minutes and then placed in the incubator for 28 hours.

In the center of disc, a needle of syringe was stabbed which upheld the disc vertically. Appropriate amount of test non-antibiotic antimicrobial was taken by a micro-dispenser and placed gently on the disc until the disc was saturatedcompletely. Then discs were transferred to the medium of agar plate. This process is repeated multiple times by changing needles and micro dispenser. Distances of discs from edges of the plates and from each other were kept same.Plates were kept in incubator for 18 hours at 35^0C. After incubation, these plates were examined with black wallpaper and brightened with light to determine zone of growth inhibitionwith Kirby Bauer ruler method.

Agar dilution technique

Mueller-Hinton agar medium was used for the evaluation of minimum inhibitory concentration (NCCLS, 1996). Refined isolates of methicillin resistant*S. epidermidis* were taken as experimental organisms anddiclofenac sodium was taken as experimental antimicrobial agent, with specified potency, suggested storage and expire circumstances.The number of antimicrobial concentrations depends upon the microorganism and antimicrobial agent to be evaluated. The concentrations in stock solution were retained at level, 20 times greater as that chosen in the final tests in petri dishes. As one milliliter of antimicrobial agent was needed for 19 milliliters of molten agar, each 9 cm petri dish had a volume of 20 milliliter. A design of conventional pattern of diluting stock solutions was implemented.For the preparation of petri plates Mueller-Hinton agar medium was developed according to manufacturer instructions. In water bath the sterilized molten agar flasks were placed to cool to 50^0C. In a separate container the antimicrobial solution was mixed with molten agar, transferred into petri plates and then kept undisturbed to solidify. Drugs less petri plates were contained as controls for each dilution.The inoculum was formed from each isolate by taking four or five wholesome colonies from an overnight growth of the isolate. The colonies were gradually diluted. The 0.5 McFarland turbidity standard was used for visual contrast till the turbidity was equal to about 10^8cfu/ml. After inoculation, the plates were placed in the incubator at 35^0C in aerobic condition for 18 hours and observed for results.

In vivo antimicrobial property of diclofenac sodium

A total of twenty active, adult and healthy rabbits were obtained from native market and housed indoor homogenously throughout the research period. The animals were grouped into two identical groups. A complete clinical and laboratory inspection was performed throughout the study period to find out the health situations of all animals. All animals were kept and maintained for 2-weeks at Laboratory Animal Facility of the Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad. Animal were provided with 8-12 hours light and 14-hour dark period in a comfortable, temperature controlled ~25-28^0C room.

Preparation of inoculum

One day before inoculation, five purified isolated colonies of S. *epidermidis* were collected and shifted to a test tube having 5 mL of sterile Mueller-Hinton broth. The bacteria and broth were then placed in the incubator at 35^0C for 18 hours. The inoculum was then diluted 1:20 in Mueller-Hinton broth, incubated at 35^0C for four hours, and rotated at 75 rpm to let bacteria to grow and attain a proliferative mid-log phase of action. After four hours, the bacterial concentration of the inoculum was determined and compared to a 0.5-McFarlane standard conforming 10^8 organisms per ml. This inoculum was then diluted to the recommended concentration and kept in cold storage before being transported to the animal laboratory facility.

Induction of Surgical Site Infection (SSI)

12 hours before operation the animals were kept off feed. Before surgery, area was shaved and sanitized properly with antiseptics. Injection of ketamine hydrochloride intramuscular was injected (Ketarol®, Global pharmaceuticals, Pakistan) with dose rate of 14-31mg/kg body weight to anesthetize the rabbits. 4cm clean vertical surgical incisions were made on the flank region. Each wound was administered with 10^8 CFU/ml of *S. epidermidis* and openings were then sutured. Topically infected Group Wounds were managed with diclofenac sodium with dose more than MIC.

Determination of Antibacterial efficacy

1 cm^2tissue was collected from the incision site 24 days after rabbits were sacrificed for microbiological test. The weighed tissue was homogenized for 15 to 30 seconds. Dilutions of each serial homogenate were performed after homogenization. Weighed sample of tissues shifted to the tissue homogenizer and creased into an interruption by using coated pestle with Teflon (Black and Decker, Towson, Md.). The mixture of tissue and broth was then used for two serial dilutions (1 to 10) in Mueller-Hinton broth and shifted to germ-free tubes at 4^0C. Every sample (100 x 10^6 L) having serial dilutions, was then shifted to agar plates with tryptic soy and placed in incubator at 35^0C for 24 hours. Totals were considered precise for number ranging from 30 to 300 and were matched with their serial dilutions and duplicate plates for reproducibility. Colony counts were then interpreted to $cfug^{-1}$ by following formula;

$$\text{Cfu/gm} = \frac{\text{No. of colonies X Factor of Dilution X 50}}{\text{Tissues weight (gm)}}$$

Statistical Analysis

In statistical analysis mediated sites with control sites were computed by measuring of cfu ± SE of the mean and their average.

Results

S. epidermidis growth inhibition

Regarding growth inhibition of *S. epidermidis* by diclofenac sodium, results showed no zone of growth inhibition with concentration of 2µg, 5µg and 10µg per disc. However, disc with 25µg of drug revealed a zone of 16 mm diameter. Disc with 50µg concentration of diclofenac sodium resulted in a zone of inhibition of 18 mm in size. A 20 mm zone was produced by the disc having 100µg of diclofenac sodium (Figure 1). In comparison, standard drug produced a zone of 22 mm diameter (Table 1).

Figure 1: Zones of growth inhibition alongside *S. epidermidis* at 2,50,100 µg/dics diclofenac.

Test Drug	Isolate	Disc Potency µg/disc	Zone of growth inhibition (mm)
Diclofenac sodium	*Staphylococcus epidermidis*	2	No zone
		5	No zone
		10	No zone
		25	16
		50	18
		100	20
Standard	*Staphylococcus epidermidis*	100	22

Table 1: Zones of growth inhibition evaluation of diclofenac sodium in contradiction of *S. epidermidis*.

Minimum Inhibitory Concentration (MIC)

Regarding MIC of diclofenac sodium against. *epidermidis*, technique of agar gel dilution was used. Once seasoned in contradiction of *S. epidermidis*, MIC related to diclofenac were logged by means of 25µg/ml, and this application were abundant to stop the progression of *S. epidermidis* (Figure 2), despite the fact MIC beside MRSA were noted as 50 micro gram per ml.

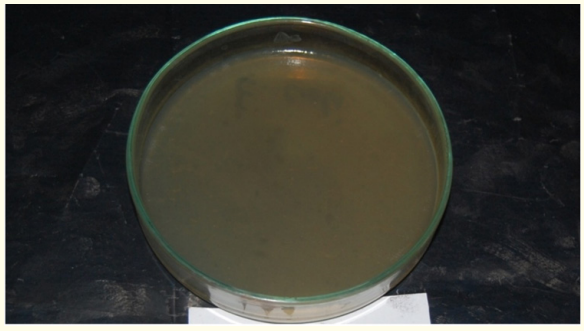

Figure 2: Determination of minimum inhibitory concentration against *S. epidermidis*

In vivo antimicrobial efficacy of diclofenac sodium

After *in vitro* trials, *in vivo* antimicrobial efficacy of diclofenac sodium was measured. Tissue obtained from the contaminated wound region was measured for microbiological determination and sum of colony forming units per gram (cfu/g) of the soft tissue was determined.

A noteworthy difference was witnessed in bacterial population between the diclofenac sodium treated wounds and normal saline applied control group (Table 2). Computable wound culture amounts demonstrated a meaningful decline in cfu/g in healed group with diclofenac sodium. From diclofenac sodium smeared wounds the cfu/g value measured up to $1.43 \pm 0.05 \times 10^6$, however around $4.21 \pm 0.07 \times 10^6$ cfu/g were observed in normal saline treated control group.

Groups	Medicine Used	Average bacteriological count
Treated	Diclofenac sodium ointment	$4.86 \pm 0.04 \times 10^6$
Control/untreated	Normal saline	$5.68 \pm 0.07 \times 10^6$

Table 2: Outcomes after 48 hours medication with superficially smeared diclofenac sodium (NSAID) on experimental constructed persuaded infection with *S. epidermidis*.

Discussion

SSI after surgical procedure precedes a foremost foundation of disease in patients fronting the surgical approach. SSI is a domineering consequence among the traditional side effects that take place successively when an anguish individual experiences a surgical approach [21]. SSI may lead toward the delayed wound restoration leading to increase in hospital visits succeeding in greater expenditures than earlier [20]. It causes delay in the length of post

operative recovery due to destructive pathogens and through commensals of body [2]. Ostensible cuts are characteristically poisoned through commensals which are generally the endogenous bacteriological vegetation such as *S. Epidermidis* [8].

Drug resistance against antibiotics is a matter of concern when dealing with the management of bacterial, viral, fungal and parasitic infections after surgery [3,4]. The competence of currently available antibiotics is falling rapidly due to the development of resistance mechanisms by the pathogens [11]. Resistance has already been witnessed in *Pseudomonas aeruginosa* and *Enterobacteriaceae* against variety of antibiotics such as penicillin, tetracycline and cephalosporines. In case of *Haemophilus, Neisseria, Enterobacteriaceae* and *Pseudomonas* spp., the resistance against beta-lactamase has additionally been noticed [13,15]. As a widespread apprehension, drug resistance in microbes stimulates the inventors for pioneering antibacterial mixtures. Certain satisfying mixtures, correlated to abundant pharmacological collections, and accomplished through antibacterial efficiency in further addition to their preceding pharmacological accomplishments are nominated as "non-antibiotic antibacterials" [18].

Due to the prevailing issue of antibiotic resistance, the current study was proposed to administrate *in vitro* vulnerability and lowest inhibitory concentration of diclofenac beside methicillin resistant *S. epidermidis* along with the assessment of *in vivo* antimicrobial stuff of diclofenac in induced surgical spot contamination by *S. epidermidis*. The research comprised of two sections i.e., ***In vitro*** susceptibility examination and minimum inhibitory concentration (MIC) of diclofenac sodium and *in vivo* susceptibility examination by topical application of diclofenac sodium over the surgical wounds.

When virtually applied beside three transformed segregates quantified earlier, diclofenac showed no section of growth retardation at concentration 2, 5 and 10µg per disc but it showed 16-, 18- and 20-mm zone of inhibition at 25, 50 and 100 µg per disc against *S. epidermidis*. 100µg of sulphamethoxazole/disc were applied as a control/standard and it showed 22 mm zone of inhibition. These findings are in line with Padma and Yalavarthy, (2015), Dutta., *et al.*, (2007) and Mazumdar., *et al.*, (2006), who reported a growth inhibitory effect (MIC 25-200µg/ml) of diclofenac sodium against *E. coli* and *Staph aureus*.

Concerning Minimum Inhibitory Concentration (MIC), the experimental drug fashioned fallouts at a dose rate of 25µg/ml beside *S. epidermidis*. When diclofenac sodium topically applied to the wounds then the colony forming units/gram was determined as $4.86 \pm 0.04 \times 10^6$ while $5.68 \pm 0.07 \times 10^6$ colony forming units/gram was determined in normal saline cured wounds. It represents the antibacterial efficacy of diclofenac sodium as described by Padma and Yalavarthy, (2015) who described these actions of diclofenac sodium against *E. coli* and many other bacteria.

Conclusion

Diclofenac sodium is as effective as topical corticosteroid and can be used as a prophylactic drug against post-operative wound impurity or as a hypothetical satisfying agent in the management of diseased wound caused by *S. epidermidis*. In this manner, resistance to antibiotics in microorganisms could be diminished.

Author's Contribution

MUA and SA conducted the research trials. MUA, SA and FS write the main manuscript. MBA and FS proof read the manuscript and finalized the results. FS and MBA helped in conduction of research trial and also helped in manuscript writing. AM and MI supervised the research.

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