Full Length Article

IN VITRO ANTIMICROBIAL EFFECT OF OXYCLOZANIDE ANTHELMINTIC AGAINST DRUG RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATED FROM BOVINE MASTITIS MILK

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ABSTRACT

This study investigated oxyclozanide, a salicylanilide anthelmintic, as an alternative treatment for Methicillin-resistant Staphylococcus aureus (MRSA) in bovine mastitis. With conventional treatments facing challenges due to antibiotic resistance, oxyclozanide demonstrated promising in vitro antimicrobial effect against MRSA isolates. The minimal inhibitory concentration (MIC) ranged from 0.5 to 1.0 µg/ml, indicating its efficacy. Oxyclozanide's mechanism involved disrupting the transmembrane structure of bacteria, presenting a potential strategy against MRSA. This suggested the repurposing of oxyclozanide as a viable option for combating MRSA in bovine mastitis, prompting further investigation into its precise mechanism and therapeutic potential.

Keywords: Bovine Mastitis, Oxyclozaninde, MRSA, *In vitro,* MIC

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INTRODUCTION

Mastitis is an inflammatory reaction of the mammary gland tissue that is the most prevalent disease in dairy cattle and causes maximum loss to the dairy business (Jingar *et al.,* 2017). The udder is frequently inflamed as a result of germs infiltrating the mammary gland through the teat canal. Clinical mastitis causes problems in the udder and milk, but subclinical mastitis causes no obvious indications of infection and can only

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be diagnosed with the California mastitis test / somatic cell count assay.

Mastitis can sometimes be treated with a long course of antibiotics; however milk from those cows is not marketable until the antibiotic residues have been removed from the udder.

Antibiotics can be given systemically or by intra-mammary infusion therapy, which involves forcing antibiotics into the mammary gland through the teat canal.

Staphylococci are the most commonly isolated causal organisms in the milk samples taken from cows with clinical and subclinical mastitis in numerous countries. *Staphylococcus aureus* is the most common pathogen in this genus, accounting for up to 40% of all mastitis cases in some locations (Hewagama *et al.,* 2016).

Antibiotic therapy has been the mainstay in the treatment and control of mastitis. However, owing to their indiscriminate usage, emergence of resistance against a variety of antibiotics poses significant challenge for the clinicians and scientists alike (Kappeli *et al.,* 2019). Inappropriate usage of antimicrobials such as improper dose, drug or duration contributes the most to the increase in antimicrobial resistance without improving the outcome of treatment (Chandrasekaran *et al.,* 2014).

β-lactam antibiotics are often utilized in intramammary infusion therapy. Methicillin-resistant *S. aureus* (MRSA) on the other hand, is a significant problem. MRSA are resistant against all β-lactam antibiotics except few anti-MRSA β-lactam antibiotic, because the activity of antibiotic-inhibited penicillin-binding proteins is replaced by the function of an acquired penicillin-binding proteins with low affinity (Ramasamy *et al.,* 2021).

An alternative approach to responding to the issue of antimicrobial resistance and promoting good antimicrobial stewardship is repurposing of existing drugs. Repurposing existing medications is one way to address the growing concern in veterinary care about multi-drug resistant bacterial infections. The anthelmintic drug class salicylanilide is used primarily for treating helminths in humans and ruminants.

Oxyclozanide belongs to the anthelmintic medication class salicylanilide, which has mostly been used in ruminants to treat and prevent *Fasciola hepatica* (Rajamuthiah *et al.,* 2015). The anthelmintic mechanism of action (MOA) comprises uncoupling of oxidative phosphorylation and inhibiting anaerobic metabolism. Safety data for salicylanilides has been documented in both humans and animals (Swan, 1999).

Numerous studies have examined the potential of repurposing salicylanilides for antineoplastic effects (Gooyit and Janda, 2016). While research has been conducted on 'the antimicrobial properties of salicylanilides, their effectiveness against MRSA from bovine mastitis milk samples has not been widely investigated in India. With this background, present study has been undertaken to identify

in vitro antimicrobial activity of oxyclozanide against MRSA isolated from bovine milk samples collected at Madras Veterinary College and Veterinary College and Research Institute, Orathanadu hospitals.

MATERIALS AND METHODS

The samples were collected from the Large Animal Medicine Unit, Department of Clinics, Madras Veterinary College Hospital and Veterinary Clinical Complex, VC&RI, Orathanadu. The study was carried out in the Department of Veterinary Pharmacology and Toxicology, Madras Veterinary College, Chennai as well as Veterinary College and Research Institute, Orathanadu.

The milk samples were collected from mastitis affected cows following strict aseptic conditions. California Mastitis Test was used as a screening test for mastitis. The sensitivity of the isolates to various antibiotics was performed using disc diffusion method. The disc diffusion method was performed using the procedure outlined by the Clinical and Laboratory Standards Institute (CLSI, 2010).

Bacteriological examination of the milk samples were carried out within 24 hrs of collection of the milk sample. Nutrient broth was prepared and 10 ml was transferred into sterile test tubes, sealed, autoclaved, cooled and stored after sterility checking. Milk swab was dipped in 10 ml of nutrient broth in test tube, plugged with cotton and incubated at 37°C for 18 hrs. The inoculum was streaked onto mannitol salt agar plates and incubated at 37°C overnight. The growth of yellow colonies signified the presence of *Staphylococcus aureus.* The test was carried out according to the procedure described by Kampf *et al.* (1998), with some modifications.

It was determined by the standardized agar diffusion test on Muller-Hinton agar (Hi Media) using the methicillin discs (10 µg) (Hi Media). Isolates were classified as susceptible, intermediate and resistant based upon interpretative criteria developed by the Clinical and Laboratory Standards Institute (CLSI, 2012). The resistant isolates were identified as Methicillin resistant *Staphylococcus aureus* (MRSA) and stored in 50% glycerol solution at -20 °C for further use.

The disc diffusion test was performed on' Müller-Hinton agar. Discs made from Whatman filter paper using a paper punch were impregnated with 25 µg of test compounds and then air-dried. Three hundred microliters of an' overnight broth culture was spread on an agar plate and allowed to air-dry. The antimicrobial discs were placed on the plate and incubated at' 37°C 'for 20 hours. Antimicrobial susceptibility was assessed by measuring 'the diameter of the inhibition' zone.

Broth dilution and agar dilution are the commonly used methods for the determination of MIC values. The microdilution method is accurate, inexpensive, and easier to perform than the agar dilution method (Ehlshik *et al.,* 2016). Resazurin acts as a redox indicator, in which metabolically active bacterial cells reduce the blue non-fluorescent resazurin

to pink fluorescent resorufin (O Brien *et al*., 2000).

To standardize the inoculum density for a susceptibility test, a barium sulfate 'turbidity equivalent to a 0.5 McFarland standard' was used To prepare 10 ml of 0.5 McFarland standard, 50 µl of 1.17% barium chloride was added to 9550 µl of 1% sulfuric acid.

A concentration of 320 µg/ml of Oxyclozanide was prepared by dissolving 320 µg of Oxyclozanide in dimethyl sulfoxide, mixing thoroughly by vortexing, and then storing it in the refrigerator until use. 0.015 % of resazurin was prepared by dissolving 1.5 mg of resazurin in 10 ml of autoclaved distilled water, vortexed, and stored in the refrigerator until use. *Staphylococcus aureus* isolates from glycerol stock were streaked onto mannitol salt agar and incubated overnight at 37°C. The colony growth was then recorded.

The inoculum was prepared by culturing a single colony from an agar plate in 5 ml of nutrient broth at 37°C. The overnight culture was spun down in a centrifuge at 3380 x g for 5 min (Sarker *et al*., 2007). The supernatant was discarded and the pellet was suspended with normal saline until the turbidity matched with 0.5 McFarland which is used as a source of bacteria in the Microdilution method.

50 µl of nutrient broth was pipetted into 2 to 12 wells of a microtitre plate. In the first well, 100 µl of Oxyclozanide (320 µg/ml) was pipetted, and then 50 µl was transferred from the first well and serially diluted from wells 2 to 12. Finally, 50 µl of the content from the 12th well was discarded. 10 µl of 0.015 % resazurin was added to all wells followed by 30 µl of nutrient broth. Finally, 10 µl of McFarland adjusted bacterial culture was added to all wells. A sterility control well was set up by adding 90 µl of nutrient broth and 10 µl of 0.015% resazurin dye. The microtitre plate was then sealed and incubated overnight at 37°C.

The lowest concentration of antibiotic that resulted in complete inhibition of visible growth and did not produce pink colour (resazurin reduction) was taken as MIC end point.

RESULTS AND DISCUSSION

Between September 2021 and January 2022, analysis was conducted on 34 milk samples collected from cows affected by both clinical and subclinical mastitis. The primary objective of the study was to investigate the resistance of *staphylococci* to Methicillin during this period. To gauge the prevalence of drug resistance, Methicillin-resistant *Staphylococcus aureus* (MRSA) was specifically selected as an indicator. To unravel the resistance patterns comprehensively, we utilized Methicillin discs, allowing us to discern and evaluate the levels of MRSA and understand the resistance profile.

From the table 2, the MIC_{50} of oxyclozanide against the *S. aureus* isolates is

Table 1. Prevalence of MRSA

Organism	No. of samples tested	Culture (MSA) positives	Methicillin resistant *Staphylococcus aureus* in ABST	Prevalence of MRSA (%)
Staphylococcus aureus	34	26	8	30.76%

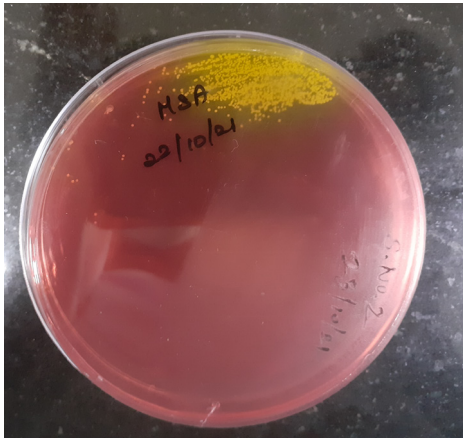

Fig. 1. Isolation of *Staphylococcus aureus* in Mannitol Salt Agar

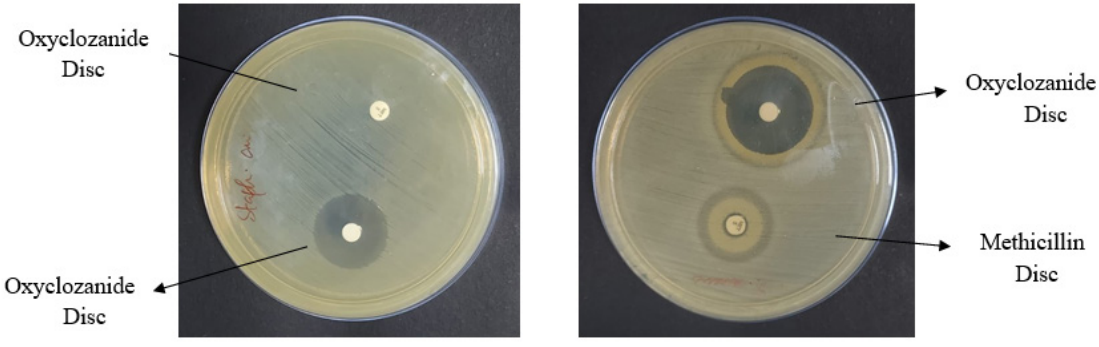

Fig. 2. *Staphylococcus aureus* showing resistant for Methicillin and sensitive for Oxyclozanide

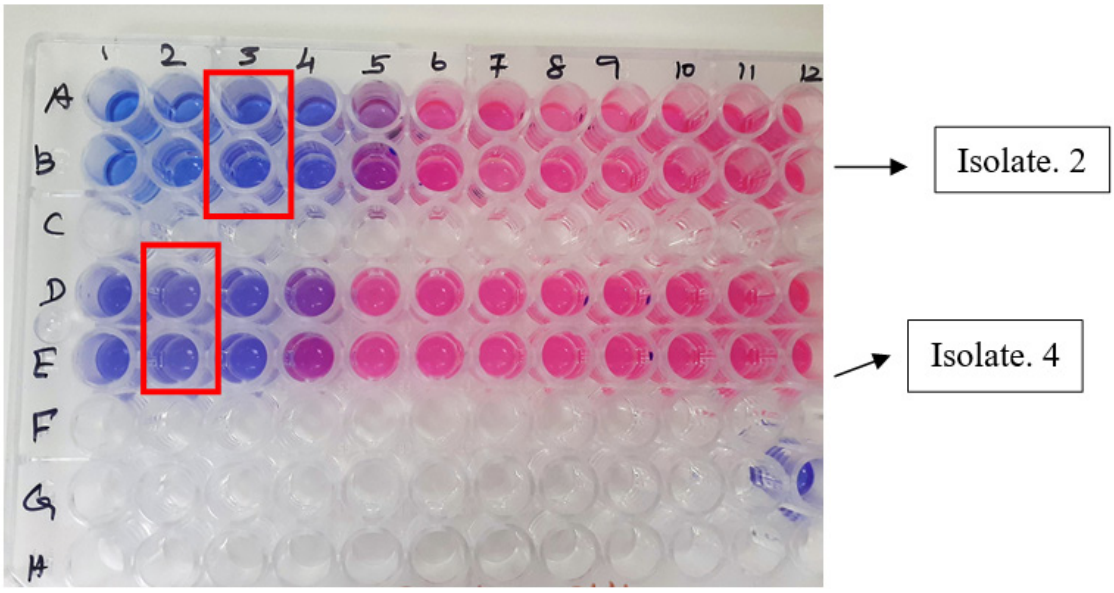

Fig. 3. MIC of Oxyclozanide in MRSA using microdilution method

Table 2. MIC of Oxyclozanide against *MRSA*

Isolate no.	MIC (µg/ ml)
1	0.5
2	0.5
3	0.5
4	1.0
5	0.5
6	0.5
7	1.0
8	0.5

0.5 µg/ml and MIC_{90} of oxyclozanide against the *S. aureus* isolates is 1.0 µg/ml.

The DMSO alone failed to produce growth inhibition at any dilution, for all isolates tested. The antimicrobial MOA of oxyclozanide is not well defined but it is believed that it acts through uncoupling oxidative phosphorylation similar to its known anthelmintic MOA (Martin, 1997). It is also thought to involve the dissipation of transmembrane potential or proton motive force (PMF), which modulates spatial organization of morphogenetic proteins in addition to adenosine triphosphate (ATP), which is vital for bacterial survival.

Targeting the PMF through dissipation of the components involved in maintaining PMF has shown to be a promising strategy in combating MRSA. Through the process of causing a disruption of PMF, salicylanilides can lead directly to a phase shock protein response of the bacterial organism (Kratky *et al.,* 2013).

It could be theorized that oxyclozanide displays poor activity against *E. coli* due to insufficient dissipation of PMF and lack of a phase shock protein response (Froyd *et al.,* 1968).

Further studies are needed to determine oxyclozanide's antimicrobial MOA to understand the role and function it may have in disrupting PMF in bacterial pathogens.

Oxyclozanide swiftly disrupted the MRSA cell membrane after brief exposure, as indicated by a dose-dependent increase in fluorescence due to Sytox Green dye binding with bacterial nucleic acids (Rajamuthiah *et al.,* 2015). These observations suggest that additional research is necessary to determine the specific mechanisms by which salicylanilide anthelmintic drugs act against *S. aureus*. It is plausible that oxyclozanide, beyond targeting the bacterial cell envelope, may also affect other cellular targets, similar to the actions of niclosamide (Ye *et al.,* 2014).

The core aim of this study was to lay the groundwork for the potential repurposing of oxyclozanide as a potent antimicrobial agent against MRSA-infected cases of bovine mastitis. Despite the invaluable insights gained, it's crucial to acknowledge the study's limitations, particularly the constrained number of isolates and concentrations of oxyclozanide examined. However, even within these limitations, the study's findings present a compelling case for further exploration. By delving deeper into the antibacterial mechanisms of salicylanilide anthelmintic drugs, we can unlock their full potential and pave the way for innovative treatments against staphylococcal infections.

CONCLUSION

Oxyclozanide, belonging to the salicylanilide anthelmintic class, exhibits potent growth inhibition against Gram-positive bacteria, notably Methicillin-resistant *S. aureus* derived from cases of bovine mastitis. This efficacy can be attributed to oxyclozanide's adeptness in targeting the bacterial cell wall coupled with its propensity

to minimize the emergence of antimicrobial resistance. However, further research is warranted to meticulously delineate the precise mechanism of action of these compounds, as well as to elucidate their pharmacokinetic and pharmacodynamic profiles.

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