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Antimicrobial Effects of Oxygen Against Bovine Mastitogenic Isolates

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ABSTRACT

In the present study oxygen in the form of ozonated water has been used to tests its antimicrobial activity against the pathogens isolated from bovine mastitis. Ozone is a well-known oxidant that can be commercially manufactured using basic ozonizer equipment. Ozone dissolves quickly in water and keeps its antibacterial properties even when dissolved as reported by several researchers. In this study, the effect of 0.1 ppm,0.3 ppm, 0.4 ppm and 0.6 ppm ozonated water charging after of time 20 minutes was analyzed on different microbial isolates isolated from mastitis. The antimicrobial effect of ozonated water was evaluated using the disc diffusion method following routine procedure. The 0.6 ppm ozonated water was found to be highly sensitive against *S. aureus*. As its action was studied with single charging, multiple charging or using ozonated water in 20 minutes interval may reduce the microbial load as an ideal antiseptic. Ozonated water at different concentration was effective in reducing the bacterial load, but it did not eliminate them completely. It may be advocated as a surfactant before milking like microbial antiseptics.

HIGHLIGHTS

- A concentration of 0.6 ppm ozonated water was found to be highly sensitive against *S. aureus*.
- Ozonated water at different concentrations was effective in reducing the bacterial load.

Keywords: Bacteriocidal, Ozone, Cow, Mastitis

Due to low cytotoxicity and presence of antimicrobial power ozone can be used as substitute for antiseptics. But little investigation has been carried out regarding the time and concentration of its use. In the present study, different concentration of ozonated water were tested against a wide spectrum of microbial isolates from bovine mastitis within 20 minutes. As per the reports available the half-life of ozonated water is 20 minutes (Zainuddin *et al*., 2017).

Ozone has been found to be a consistent and potent antibacterial agent against bacteria, virus, fungi, and protozoa in both aqueous and gaseous forms (Naveenaa *et al.*, 2021). Three oxygen atoms make up ozone (O_3), a highly reactive gas. It is a natural and man-made substance that exists in the high (stratosphere) and lower (troposphere) atmospheres of the Earth. It is not a stable gas and has the

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capacity to oxidize by any biological organism. Ozone's biocidal activity stems from its capacity to oxidize organic material in bacterial membranes, weakening the cell wall and inducing cell rupture, resulting in cell death. When ozone is added to water, it dissolves quickly and generates ozonated water. Ozonated water has antibacterial and oxidizing properties (Azuma *et al.*, 2014).

Mastitis is typically due to contagious pathogens which transmits from animals to the herdsman. Under one health programme transmission of antibiotic resistance through milk is of serious concern. Therefore, use of antimicrobials must be lessened for betterment of health status of the stakeholders. Taking into consideration the present work has been envisaged with the following objectives. It has been reported that *Staphylococci* occurrence is nearly 70% in the mastitis and it is the major mastitis causing bacteria which is highly resistance to common antibiotics. So, periodical use of oxygen in the superficial area of udder limits the invasion of microbial growth. In the present study, attempt was taken to determine the Minimum Inhibitory Concentration (MIC) of oxygen against major microbial bovine mastitis isolates for periodical use to prevent the invagination of microorganisms into the teat canal of udder. So that the colonization can be restricted. Further attempt was taken to determine the dose response curve of oxygen.

MATERIALS AND METHODS

The present study was carried out to study the antimicrobial effects of oxygen against bovine mastitogenic isolates. The experiments were conducted in the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Odisha University of Agriculture and Technology, Bhubaneswar. A total of 25 milk samples were collected from the cows presented to the Teaching Veterinary Clinical Complex, College of Veterinary Science and Animal Husbandry, Bhubaneswar, with a history of clinical mastitis. Primary screening was carried out by California Mastitis Test as per routine procedure.

Preparation of ozonated water

The ozonated water is freshly prepared by using ozonated water dispenser machine. Ozone gas produced from the ozonizer was added into 250 ml of sterile distilled water for 2 minutes, 10 minutes, 30 minutes and 60 minutes for getting ozone concentration of 0.1 ppm, 0.3 ppm, 0.4 ppm and 0.6 ppm respectively. The concentration of dissolved ozone in the water was measured using comparator cell. After preparing the ozonated water, it was utilized within 20 minutes of time.

Preparation of antimicrobial disc from ozonated water

HiMedia filter paper number 6 was taken and it was punched with paper punching machine to obtain small circles of 6 mm. These sterile filter paper disks were impregnated with different concentration of ozonated water. After air drying,they were placed on the surface of each petri dish. Petri dish containing Muller-Hinton agar was swab streaked with mastitogenic pathogens. The empty disks were placed over the Agar plate and the disc were charged with 10 micro liters of volume with various concentration using a single pure culture of bacterial isolated with 3 different concentrations of ozonated water treat in 0.3 ppm, 0.4 ppm and 0.6 ppm. The lowest concentration of ozonated water that generated an inhibition zone around a disc, followed by 24-hour incubation, was used to measure antibacterial activity as MIC.

Determination of MIC against various mastitogenic isolates

Determination of Minimum Inhibitory Concentration of ozonated water against the microorganisms as conducted as per the method of Sadatullah *et al*. (2012). The minimum concentration of the antimicrobial treatment which inhibits the pathogen growth after incubation was called as the MIC. The MIC of the ozonated water varies from 0.3 ppm to 0.4 ppm for different bacteria. The diameter of the zone of inhibition (mm) around bacterial growth was used to measure antibacterial activity. The test bacteria used for the the above experiment are *Staphylococcus* species, *Escherichia coli*, *Streptococcus* species, *Pseudomonas* species, *proteus* species and *Klebsiella* species.

RESULTS AND DISCUSSION

The results were tabulated and statistically analyzed using analysis of variance (ANOVA). Mean zone of inhibitions was recorded. The statistical analysis with ANOVA showed that there was significant ($p<0.05$) difference between the

zone of inhibition diameters among 0.3 ppm, 0.4 ppm and 0.6 ppm against different mastitogenic pathogens (Table 1).

The facts presented here demonstrate the effectiveness of oxygen in the form of ozonated water. Several researchers have claimed that oxygen possesses antibacterial properties (Li *et al.,* 2012). The methodology of this study was almost similar to performed by (Sadatullah *et al*., 2012) with some modifications. This study was performed by taking different concentration of ozonated water from 0.1 ppm to 0.6 ppm on mastitogenic isolates instead of only 0.1 ppm. Because 0.1 ppm concentration shows no zone of inhibition. The ozonated water is impregnated with 6 mm filter paper to prepare antibiotic disc. Then the antibiotic discs are used to find out the zone of inhibition and all the experimental inoculation was completed within the stipulated time keeping in mind the half-life of ozonated water is 20 minutes.

The results of this study can be compared with the previous studies on Comparative Evaluation of Antimicrobial Efficacy of 2% Sodium Hypochlorite, 2% Chlorhexidine, Ozonated Water, and Turmeric Extract against *Enterococcus faecalis* (Lokhande *et al*., 2018). When the concentration of ozonated water increased the

Table 1: Mean diameter of zone of inhibition at various concentration of ozonated water

Sl. No.	Test organisms	Diameter of inhibition zone (mm) of different concentration of ozonated water			SEM	P Values
		0.3 ppm	0.4 ppm	0.6 ppm		
1	*Staphylococcus sp.* (n=23)	8.00^{a}±0.22	10.87^{b}±0.22	12.13^{c}±0.19	0.24	<0.001
2	*Streptococcus sp.* (n=4)	7.75^{a}±0.48	9.50^{b}±0.29	11.75^{c}±0.48	0.54	<0.001
3	*Bacillus sp.* (n=3)	0.00^{a}±0.00	8.33^{b}±0.33	9.33^{c}±0.33	1.49	<0.001
4	*Corynebacterium sp.* (n=3)	0.00^{a}±0.00	7.33^{b}±0.33	10.67^{c}±0.33	1.58	<0.001
5	*Pseudomonas sp.* (n=2)	0.00^{a}±0.00	6.50^{b}±0.50	7.00^{b}±0.00	1.43	0.001
6	*Escherichia coli* (n=5)	7.20^{a}±0.37	8.60^{a}±0.51	10.80^{b}±0.49	0.47	<0.001
7	*Klebsiella sp.* (n=3)	0.00^{a}±0.00	6.33^{b}±0.33	7.67^{c}±0.33	1.19	<0.001
8	*Proteus sp.* (n=2)	0.00^{a}±0.00	6.50^{b}±0.50	8.00^{c}±0.00	1.56	0.001

Values bearing different superscripts in a row differ significantly ($p<0.05$).

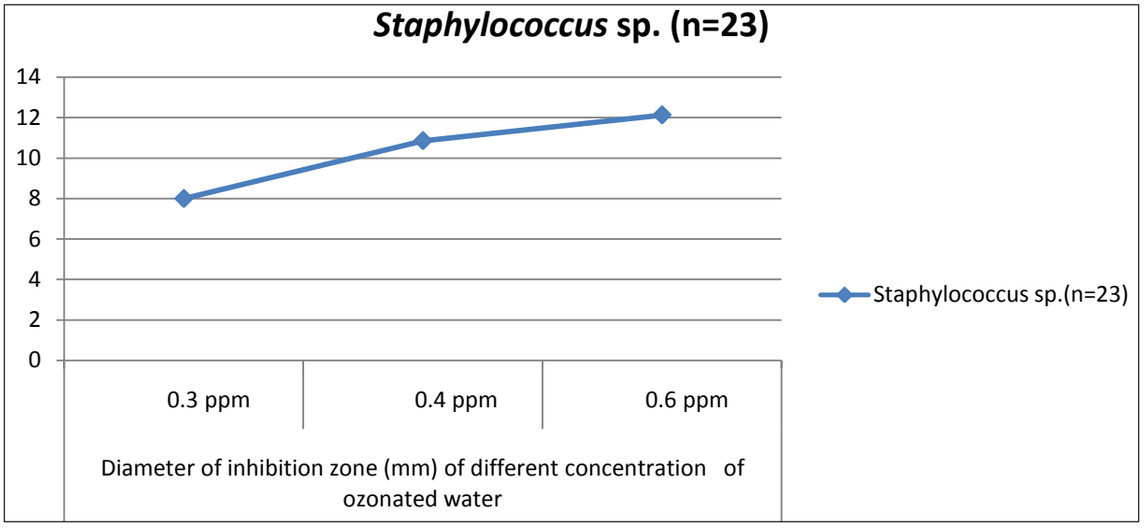

Fig. 1: Dose response curve of different concentration of ozonated water against Staphylococcus species

Table 2: Comparative analysis of mean diameter of zone of inhibition of antibiotics with various concentration of ozonated water

Name of the organisms	Concentration of ozonated water	Antibiotics used	Zone of inhibition (Mean ± SE)
Staphylococcus sp. (n=23)	0.3 ppm	Cefotaxime	22.43±0.43
		Vancomycin	20.34±0.41
	0.4 ppm	Cefotaxime	23.43±0.45
		Vancomycin	22.13±0.32
	0.6 ppm	Cefotaxime	25.53±0.46
		Vancomycin	22.87±0.41
Streptococcus sp. (n=4)	0.3 ppm	Penicillin	20.25±0.48
		Amoxicillin	20.00±0.91
	0.4 ppm	Penicillin	22.25±0.48
		Amoxicillin	21.50±0.64
	0.6 ppm	Penicillin	25.25±0.63
		Amoxicillin	22.00±0.41
Bacillus sp. (n=3)	0.3 ppm	Vancomycin	20.66±0.33
		Neomycin	18.66±0.33
	0.4 ppm	Vancomycin	22.33±0.33
		Neomycin	19.00±0.58
	0.6 ppm	Vancomycin	23.66±0.89
		Neomycin	21.33±0.33
Corynebacterium sp. (n=3)	0.3 ppm	Tetracycline	19.33±0.33
		Erythromycin	17.67±0.33
	0.4 ppm	Tetracycline	20.33±0.33
		Erythromycin	19.67±0.33
	0.6 ppm	Tetracycline	21.67±0.33
		Erythromycin	20.00±0.58
Pseudomonas sp. (n=2)	0.3 ppm	Penicillin	27.50±0.50
		Ciprofloxacin	29.00±1.00
	0.4 ppm	Penicillin	29.00±0.00
		Ciprofloxacin	31.50±0.50
	0.6 ppm	Penicillin	30.50±0.50
		Ciprofloxacin	32.50±0.50
Escherichia coli (n=5)	0.3 ppm	Gentamicin	30.20±0.86
		Cefotaxime	21.40±0.40
	0.4 ppm	Gentamicin	32.00±0.32
		Cefotaxime	22.40±0.40
	0.6 ppm	Gentamicin	33.40±0.40
		Cefotaxime	25.00±0.32
Klebsiella sp. (n=3)	0.3 ppm	Gentamicin	18.67±0.33
		Ciprofloxacin	31.33±0.33
	0.4 ppm	Gentamicin	20.33±0.33
		Ciprofloxacin	31.66±0.33
	0.6 ppm	Gentamicin	22.00±0.00
		Ciprofloxacin	33.33±0.33
Proteus sp.(n=2)	0.3 ppm	Amoxicillin	18.00±0.00
		Ciprofloxacin	20.50±0.50
	0.4 ppm	Amoxicillin	20.00±0.00
		Ciprofloxacin	21.50±0.50
	0.6 ppm	Amoxicillin	21.50±0.50
		Ciprofloxacin	24.50±0.50

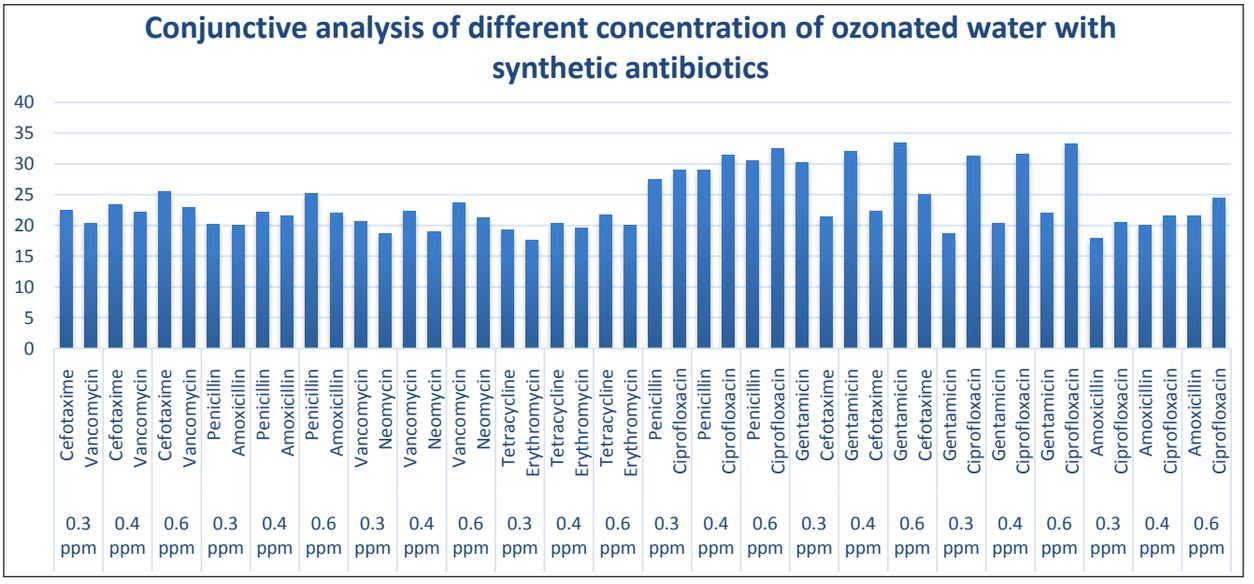

Fig. 2: Conjunctive analysis of different concentration of ozonated water with synthetic antibiotics

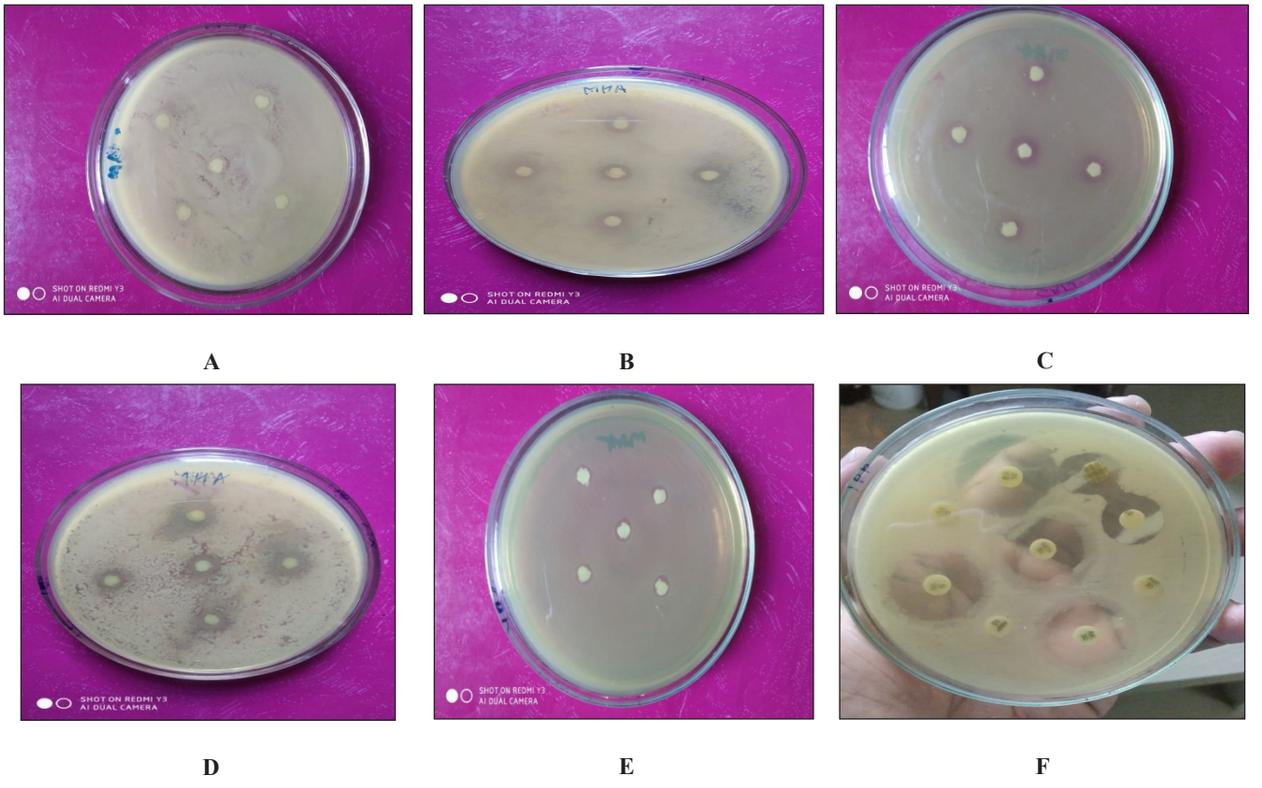

Fig. 3: Plates showing *E. coli* inhibition zone against various concentration of ozonated water **(A)** 0.1 PPM, **(B)** 0.3 PPM, **(C)** 0.4 PPM, **(D)** 0.6 PPM, **(E)** Control charged with distilled water, **(F)** Antibiotic disk

zone of inhibition also increased. At 0.6 ppm the maximum inhibition zone of (12.13+0.19) mm was observed against *Staphylococcus aureus,* and the minimum of (7.00+0.00) mm is for *Pseudomonas*. Whereas they found 16.9 mm of inhibition zone for *E. faecalis* because of use of different concentration of ozonated water. This suggests that ozonated water has a broad spectrum of antibacterial action against both Gram positive and Gram negative microorganisms. However, it was observed that the extent of the inhibitory zone varies depending on the type of bacteria isolated and the ozonated water concentration.

CONCLUSION

In the present study it is revealed that ozone at 0.6 ppm concentration was turned to be effective in reducing *Staphylococcus* spp. population but does not eliminate completely. The prevention of mastitogenic pathogens from the udder surface and other innate object is more important than complete elimination. Therefore, ozonated water can be used as a potent antiseptic to apply on the udder surface and other innate object to reduce occurance of mastitis in cows. 850 oxygen plants being set up in various districts of country by the government. So, now a days as it is very easy to get oxygen for preparation of ozonated water all over the country. Periodical use of ozonated water in the superficial area of udder leads to prevent the danger of mastitis in milch cows by limiting the invasion of microorganisms. A more comprehensive investigation of mastitogenic isolates from various geographical locations is required to confirm the aforementioned conclusion.

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