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Study of antimicrobial effect of ozone against bovine uterine infection

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Abstract
In the present study a total 30 numbers of sample were collected from December 2019 to December 2021 from various case presented with a history of uterine infection to Teaching Veterinary Clinical Complex, College of Veterinary Science & Animal Husbandry, Bhubaneswar. All the cows were showing various sign of uterine infection like infertility, repeat breeding, endometrial discharge and abortion. All the thirty samples were processed as per routine microbial procedure and *E. coli* (31.94%) was found to be the predominant isolates followed by *Staphylococcus* (26.39%), *Streptococcus* (20.00%), *Corynebacterium* (11.11%), *Klebsiella* (4.17%) and *Pseudomonas* (6.00%). Ozonated water with various concentration of 0.3 ppm, 0.5ppm, 0.6 ppm after 20 mints, 30 mints and 60 mints interval was prepared with ozone water dispenser as provided by Embeeson Controls Pvt. Ltd., Patia, Bhubaneswar, Odisha. All the above microbial isolates were tested against various concentrations of ozonated water as per the method of Kirby Bauer disc diffusion method.

Keywords: Antimicrobial, minimum inhibitory concentration, ozonated water, bovine uterine infection

Introduction
Ozone is the naturally occurring gas and most powerful oxidising agent, also called as nature's self-cleaner. Ozone can be produced from anode of electrochemical cell for different uses. For the therapeutic usage of ozone, it is produced by medicinal generator which through high voltage electrical discharges is capable to capture oxygen from a cylinder and splits it, so that molecules are reorganised to produce ozone (Bocci 2006) [4]. The half-life of ozone is around 40 mins at 20 °C and 140 mins at 0 °C (Manjunath *et al*. 2021) [13]. Ozone has multifaceted functions namely, antiseptic, germicide and for sterilisation of air and water and also acts as dying agent for feeding oils, starch etc. It is used for disinfecting the equipment to maintain proper hygiene and sanitation, preservation for improving the self-life of products. Clinically, it has helped in treating infectious diseases, healing of wounds, whitening of teeth and helps in improving circulation (Manjunath *et al*. 2021) [13].
Researches suggests the efficacy of ozone gas in augmenting the uterine environment, oxygenation of tissue and blood flow towards the uterus thereby improving the conception rate by reducing the microbial load in the uterus (Mali *et al*. 2020) [11]. A dose of about 20 µg of ozone/ml in a gaseous O_3 or O_2 mixture (1% O_3 or 99% O_2) in a single topical application by nebulisation for 5 mints under atmospheric pressure has shown to be efficiently hindering the growth of all the probable bacteriological strains with known antimicrobial resistance (Fontes *et al*. 2012) [9].
Ozone is therapeutically available in dispensaries in many forms like foams, boluses, creams and in gaseous forms (Đuričić *et al.* 2015) [6]. Ozone is effective against bacterial isolates of *E. coli, Staphylococcus* spp., *Pseudomonas* spp., *Corynebacterium* spp. and mixed bacterium isolated from uterine lavage. (Mali *et al.* 2020) [11]. Ozone can be used as prophylaxis to reduce uterine infection and improve in fertility of cows. (Zobel *et al.* 2014) [17].
Scanty literatures are available on effectiveness of ozone on reproductive infections. Keeping in view all the facts the recent study was aimed at studying the antimicrobial effect of ozone against bovine uterine infection in terms of measuring Minimum zone of inhibition.

Materials and Methods
Sample Collection
Thirty numbers of uterine samples were collected aseptically from Indigenous and cross breed cows present in and around Bhubaneswar, Odisha with a history of repeat breeding, infertility

and endometritis. The samples were then placed in an ice box and were brought to microbiological laboratory for bacterial isolation. Bacterial isolates were cultured, identified by Gram's Method and standard procedure of biochemical tests in the laboratory as per the method of Malik (1967) [12]. All the media used in the present study were obtained from HI-Media Laboratories, Mumbai.

Preparation of Ozonated Water

Ozonated water was produced with the help of ozone water dispenser (Embeeson Controls Pvt. Ltd., Patia, Bhubaneswar) by following the manufacturer's protocol. Around 300-350 ml of cool distilled water was taken in the cylindrical tube of ozone water dispenser. When the electricity was applied the energy splitted the oxygen molecules into 2 oxygen atoms. The loose oxygen atoms then recombined with ordinary oxygen molecules to form ozone. The ozone formed was then bubbled into water. The concentration of dissolved ozone in the water was measured using comparator cell. The ozonated water was utilised within 20 minutes of time.

Preparation of the antimicrobial disc from Ozonated water

Grade 1 Whatman filter paper was taken for the preparation of 6 mm antimicrobial discs and impregnated with varied concentrations of ozonated water. The MHA plates were streaked with the help of swab collected from dairy cattle which were diagnosed with uterine infections. After the empty discs were placed over the MHA plate, the disc was charged with 10 µl of volume using a single pure culture of bacterial isolated with 3 different concentrations of ozonated water of 0.3 ppm, 0.5 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 Uterine isolates

The Minimum Inhibitory Concentration of Ozonated water was determined against uterine isolates was conducted as per the method of Sadatullah *et al*. (2012) [14]. The minimum concentration of the antimicrobial treatment which inhibits the microbial growth after incubation was called as the MIC. The MIC of the ozonated water differs from 0.3 ppm to 0.6 ppm for different microorganisms. The diameter of the zone of inhibition on the MHA plate was used to measure antimicrobial activity. The test organisms used for the above experiment were *Escherichia coli*, *Staphylococcus* spp., *Streptococcus* spp., *Corynebacterium*, *Klebsiella* spp. and *Pseudomonas* spp.

Results and Discussion

In this present study a total of 74 bacterial isolates were recovered from 30 numbers of uterine samples with a clinical history of uterine infections. Out of which 42 (56.76%) were found to be gram positive and remaining 32 (43.24%) were found to be gram negative. The recovered bacterial isolates were as *E. coli* (31.94%), *Staphylococcus* (26.39%), *Streptococcus* (20.00%), *Corynebacterium* (11.11%), *Klebsiella* (4.17%) and *Pseudomonas* (6.00%), (Table No-1). In this current work *E. coli* was most predominantly occurring microorganism in uterine infected discharges of peri-parturient dairy cows with uterine infection which was followed by *Staphylococcus species*, *Streptococcus* etc. These results are in agreement with Sheldon *et al.* (2006) [16], Barman *et al.* (2013) [3], Azizunnesa and Faruk (2011) [1], Bajaj *et al.* (2018) [2].

Table 1: Bacterial Isolates from the cows with Uterine Infections

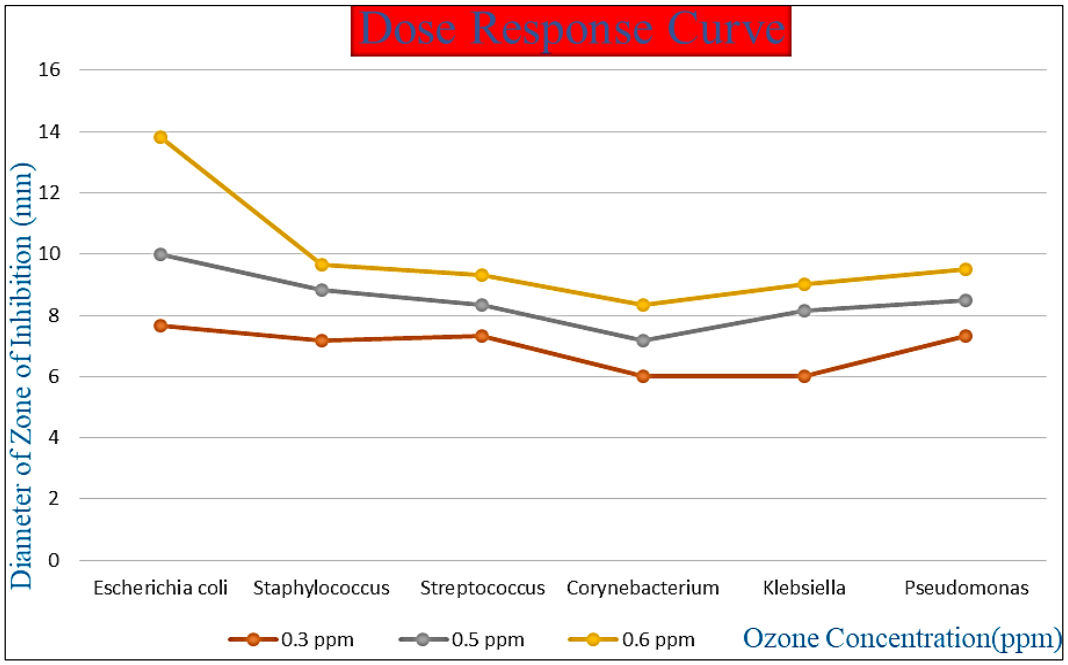
SL. No.	Name of Bacterial Isolates	No. of Bacterial Isolates	Single Isolate	% of Isolates
1.	*Escherichia coli*	23	3 (4.16%)	31.94
2.	*Staphylococcus*	19	2 (2.77%)	26.39
3.	*Streptococcus*	14	0 (0%)	20.00
4.	*Corynebacterium*	9	0 (0%)	11.11
5.	*Klebsiella*	5	0 (0%)	4.17
6.	*Pseudomonas*	4	1 (1.50%)	6.00

The data obtained from this study was tabulated and statistically analysed using ANOVA and their mean zone of inhibitions was recorded. The results showed that there was significant (p<0.05) difference between the zone of inhibition diameters among 0.3 ppm, 0.5 ppm and 0.6 ppm against different uterine isolates (Table No-2).

Table 2: Minimum Inhibitory Concentration of Ozone against Uterine Isolates

Ozone Concentration	Diameter of Zone of Inhibition (mm) of different Uterine Isolates						
	Escherichia coli	***Staphylococcus***	***Streptococcus***	***Corynebacterium***	***Klebsiella***	***Pseudomonas***	**P value**
0.3 ppm	7.67 ± 0.33^{bA}	7.17 ± 0.31^{bA}	7.33 ± 0.21^{bB}	6.00 ± 0.00^{aA}	6.00 ± 0.00^{aA}	7.33 ± 0.21^{bB}	<0.01
0.5 ppm	10.00 ± 0.51^{cB}	8.83 ± 0.30^{bcB}	8.33 ± 0.21^{abC}	7.16 ± 0.16^{aB}	8.16 ± 0.30^{abB}	8.50 ± 0.22^{abcC}	<0.01
0.6 ppm	13.83 ± 0.60^{bC}	9.66 ± 0.49^{aB}	9.33 ± 0.21^{aD}	8.33 ± 0.21^{aC}	9.00 ± 0.25^{aB}	9.50 ± 0.22^{aD}	<0.01
P Value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	

Each value is the mean of 6 Replicates (n=6) except for *Klebsiella* (n=5) and *Pseudomonas* (n=4).
$(Mean \pm SE)^{abc}$ with different superscripts in small letter varies significantly along the row.
$(Mean \pm SE)^{ABCD}$ with different superscripts in capital letter varies significantly along the column.



Graph No. 1: Dose Response Curve of diameter of zone of inhibition of different uterine isolates with respect to different ozone concentrations

In the current study the efficacy of ozone was measured in terms of Minimum Inhibitory Concentration (MIC) where the diameter of zone of inhibition is counted as the efficiency of its antimicrobial effect.

Ozone was produced *in vitro* with the help of Ozone water dispenser. The concentration of ozone ware found to be 0.1 ppm, 0.3 ppm, 0.5 ppm, 0.6 ppm at the time interval of 2 mints, 20 mints, 30 mints and 01 hour respectively.

Bacterial isolates were tested with different concentrations of ozonised water. The diameter of zone of inhibition of filter paper discs varied according to different concentrations. The concentration of ozone is directly proportional to the zone of inhibition up to certain level i.e., higher the concentration of ozone higher is its antimicrobial property. This result indicated that ozonised water is having better efficiency against *E. coli* as compared to other isolates of uterine infection samples like *Staphylococcus, Klebsiella, Corynebacterium* spp. Which is in agreement with the result of Đuričić *et al.* (2012) [7]. So, this can be presumed that as *E. coli* is more frequent cause of different uterine infections in dairy cattle use of ozone can be an alternative to treat different uterine infections.

At 0.1 ppm, there was no zone of inhibition in all the tested microorganisms but when the concentration was increased to 0.3 ppm there was increase in diameter of inhibition zone. For instance, in *E. coli* at 0.3 ppm of ozonised water the diameter was increased to 7.67 ± 0.33 mm whereas in case of Staphylococcus it was 7.17 ± 0.31 mm. Further, when the concentration was increased to 0.6 ppm there was further increase in the level of diameter of inhibition zone of microorganisms. The most noticeable activity of inhibition was exhibited by *E. coli* which was 13.83 ± 0.60 mm at 0.6 ppm while *Staphylococcus* it was 9.66 ± 0.49 mm at 0.6 ppm.

Antimicrobial activities of ozone was reported by (Cho *et al.* 2010) [5], (Elvis and Ekter 2001) [8] which is with agreement of this present study.

The dose response to different concentration of ozonated water when compared column wise to *Escherichia coli* revealed that at 0.6 ppm concentration is significantly higher (p<0.01) when compared to other concentrations. For *Staphylococcus* and *Klebsiella* spp. at 0.5 and 0.6 ppm concentration of ozonated water is significantly higher (p<0.01) than 0.1 ppm and 0.3 ppm concentration. The zone of inhibition is significantly higher (p<0.01) at 0.6 ppm concentration of ozonated water for *Streptococcus* spp., *Corynebacterium* and *Pseudomonas* spp. (Graph No-1).

Fig 1: Zone of inhibition at 0.3 ppm against *E Coli*

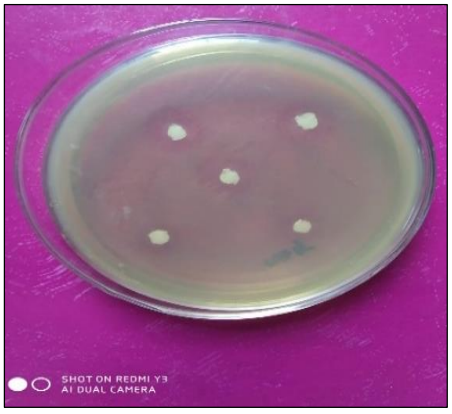


Fig 2: Zone of inhibition at 0.6 ppm against *E Coli*

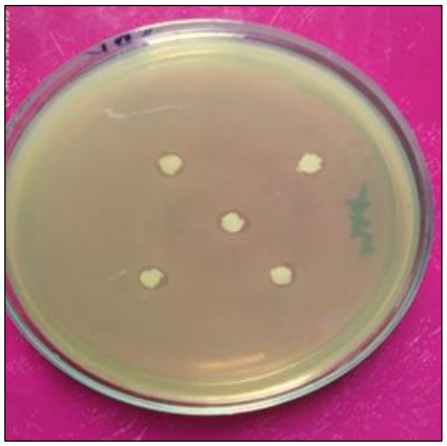


Fig 3: Control charged with distilled water

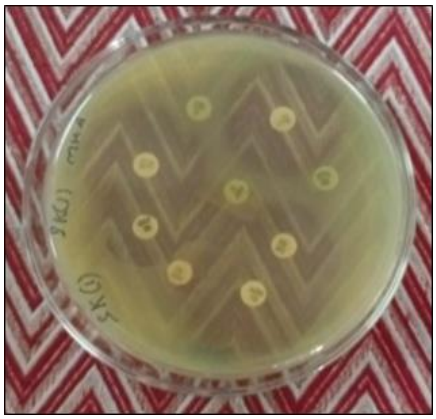


Fig 4: Antibiotic Sensitivity test of uterine sample

Seo *et al.* (2007) [15] documented that when chitosan craw fish samples at 0.5-1% concentration was treated with ozone against *E. coli* the diameter of zone of inhibition was found to be between 8.50±0.58 mm to 9.67±0.82 mm and for *Staphylococcus spp.* it varied from 7.00±0.00 to 10.30±1.21 mm. Similar experiments were conducted with chitosan samples exposed to ozone by Guirguis *et al.* (2016) [10] in which it was revealed that the diameter of zone of inhibition for *E. coli* varied between 13.67±0.47-17.00±0.82 mm when the duration of exposure with ozone ranged between 55 min to 220 mints. Findings of this present study for *E. coli* and *Staphylococcus* spp. were close and ranged between 6.00 ± 0.00 -13.83±0.60 mm and 6.00 ± 0.00-9.66±0.49 mm respectively at different concentrations of ozonated water. This Implies that ozonated water has the possibility of antimicrobial activity against both gram positive and gram-negative bacteria.

Conclusion

From the present study this can be concluded that Ozone has antimicrobial effect against the pathogens isolated from infected uterine discharges of cattle at 0.3 ppm level onwards and its antimicrobial effect increases proportionally up to a level of 0.6 ppm. Hence use of ozone can be advocated in combating the uterine infections in dairy cows. Moreover, ozone can be used as disinfectants and surfactants to reduce the microbial load in uterus for treating uterine infections. The use of ozonated water can further limit the invasion of microbes and reduce the recurrence rate of uterine infections. However, further *in vivo* studies are warranted to standardise the dose and mode of application of ozone.

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