WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.074
Volume 7, Issue 4, 121-133. Conference Article ISSN 2277– 7105

TREATMENT OF CHLORINE RESISTANT BACTERIA PRESENT IN DRINKING WATER SYSTEM BY USING OZONE

Gauri S. Devasthale*, Vaishali S. Prabhune, Amrita M. Bhatia, Heena Momin and Batul Kachwala

Department of Microbiology, Abeda Inamdar Senior College, Azam Campus, Pune-411001, Maharashtra, India.

Article Received on
17 Jan. 2018,
Revised on 07 Jan. 2018,
Accepted on 28 Feb. 2018,
DOI: 10.20959/wjpr20184-10768

***Corresponding Author**
Gauri S. Devasthale
Department of Microbiology, Abeda Inamdar Senior College, Azam Campus, Pune-411001, Maharashtra, India.

ABSTRACT

Life as we know could not have evolved without water and we would have died without it. Water consists of two hydrogen atoms attached to an oxygen atom which ideally fit into the requirements for carbon-based life. The quality of water equally matters for survival, consumption and health related problems. If water quality is poor it can transmit several water borne diseases like cholera, diarrhoea, typhoid and filariasis. The water system needs to be monitored for presence of wide variety of contaminants and water borne bacterial pathogens like *Escherichia coli, Salmonella, Shigella, Campylobacter jejuni*. Viruses like hepatitis A, enteroviruses, polio virus, rotavirus and parasites such as *Giardia lamblia* and *Entamoeba* are also common contaminants of water. Contaminated water can be treated using chlorine which is a conventional method of disinfecting drinking water. However the problem is with eliminating the chlorine resistant pathogenic microorganisms like *Staphylococci, Enterococci, Bacillus,* etc which are present in drinking water. The aim was to eliminate the chlorine resistant bacteria present in drinking water system. Fifty drinking water samples were collected from in and around Pune district to check the presence of chlorine resistant bacteria. Bacteriological tests (MPN test) for potability of drinking water were performed. The isolated microorganisms were characterized by colony morphology, Gram staining, motility and biochemical tests. Those isolates which showed resistance to chlorine were then treated with alternative water disinfection method i.e. Ozone (using OTEEN-4E Ozonator). Ozone treatment showed more effective results by inhibiting the growth of chlorine resistant bacteria.

KEYWORDS: Pathogens, Chlorine resistant, Ozonator.

INTRODUCTION

The quality of drinking water is a powerful environmental determinant of health. Water is an essential part of life but it can also transmit various diseases in countries all over the world – from the poorest to the wealthiest. The most predominant waterborne disease, diarrhea has been estimated to have an annual incidence of 1.5 billion and causes 4 million deaths every year (Martha Vargas *et. al*., 2003) [1] There are several variants of the faecal-oral pathway of water-borne disease transmission. These include contamination of drinking water by human or animal faeces at different sources through leaky pipes or obsolete infrastructure within the distribution system and unhygienic handling of stored household water (J. T Macy *et. al*., *2005*).[2]In Maharashtra areas like Sholapur, Sangli, Satara, have registered maximum demand of water tankers (144 tankers) for drinking and daily life use. (Permeshwar Udmale *et. al.*, 2016).[3]The demand for tankers has gone up significantly this year.[4]This may be linked to lack of proper management of tankers in rural and urban area, potentially giving rise to long term exposure to pollutants, which can have a range of serious health implications.[5]Chlorine disinfection method is carried out all over India which is the cheapest and effective method to treat the drinking water.[6] Most of the bacteria which were susceptible to chlorine have become resistant after few generations because of frequent exposure to chlorine in drinking water. e.g., *Staphylococci, Enterococci, E.coli, etc.* (Mohammad I. Al-Berfkani*et. al., 2014*)[7]. The development of resistance has led to their survival in presence of chlorine at higher concentrations. This problem can now be solved by using an alternative like ozone treatment. Ozone is a very strong disinfectant and oxidizer. Most of the pathogens or contaminants can be disinfected by oxidation process. Molecular ozone or its decomposition products (for example, hydroxyl radical) inactivate microorganisms rapidly by reacting with intracellular enzymes, nuclear material and components of their cell envelope, spore coats, or viral capsid. [8]

MATERIALS AND METHODS

Collection of samples

Fifty different water samples were collected from overhead tanks of residential buildings, schoolsand colleges; bore wells, bus stands, and few were directly collected from PMC supply.

Bacteriological tests

The technique of enumerating Coliforms by means of most probable number (MPN) had been used to check potability of water. A series of test tubes containing MacConkey's broth (HiMedia Laboratories Limited, Mumbai, India) with inverted Durham tube were inoculated with appropriate decimal dilutions of the water sample for 24 hrs at 37°C and were observed for acid and gas production or abundant growth formation which constitutes a positive presumptive test. Brilliant green lactose bile broth was used as presumptive broth. But(Rompré, A,*et. al*.,*2002*)had obtained interference, with high numbers of non-coliforms bacteria using lactose broth.[9] All tubes with positive presumptive reaction were subsequently subjected to confirmatory test. Production of gas within 24 hrs at 37°C in Brilliant Green Lactose Bile (BGLB) broth (HiMedia Laboratories Limited, Mumbai, India) fermentation tube was constituted as positive confirmation test. The tubes showing gas and turbidity were considered as positive. They were further cultured on Eosin Methylene Blue (EMB) agar (HiMedia) for faecal coliforms isolation.[10]Isolated organisms were confirmed by biochemical test results.[11]This MPN number is a statistical estimation of the mean of coliforms in the sample. Consequently, this technique offers a semi-quantitative enumeration of coliforms.

Characterization of bacteria

Bacteria were isolated from different water samples. Isolated microorganisms were subjected to bacteriological analysis i.e., Enzyme detection and Metabolic tests. Characterization was done in terms of colony characteristics, Gram staining and motility. Enzyme profile of the isolates was determined by enzyme detection tests like oxidase, catalase, amylase, and gelatinase test. Metabolic tests included sugar fermentation test, nitrate reduction test and IMViC.

Disk assay method

All isolated bacteria were subcultured in different batches of 100 ml of sterile Nutrient broth and were incubated for 24 hrs at room temperature on shaker at 200 rpm. Small aliquot of each culture was diluted into fresh nutrient broth. This corresponds to 0.1-0.2 O.D at 580 nm containing 10^6-10^8 CFU/ml. 20µl of this suspension was spread on Nutrient agar plate. Whatman filter paper discs were used for assay by autoclaving them. The autoclaved discs were dipped in different concentrations of sodium hypochlorite solution i.e. 2ppm - 8ppm. These discs were then placed aseptically on sterile Nutrient Agar plate and SS agar (Hi

Media) plate. Plates were incubated for 24 hrs at 37°C. After incubation zone diameter was measured.

Using Ozone to disinfect drinking water sample

20 liters water sample was treated with ozone using Ozonator for a specific time (10min -40min). Small aliquots of treated sample were taken at an interval of 10 minutes and diluted up to 10^8. Spread plate method was performed. Same procedure was repeated for treated samples for 20min, 30min, and 40min.

Estimation of ozone

Part1- Standardization of $Na_2S_2O_3$ solution (approx. 0.05N)

Standardization of $Na_2S_2O_3$ solution was done to check normality of $Na_2S_2O_3$.For preparation of $Na_2S_2O_3$, 1.24g of $Na_2S_2O_3$ (Sodium thiosulphate) and 0.240g of $K_2Cr_2O_7$ (Potassium dichromate) was dissolved into100 ml distilled water. Burette no 1 was filled by 0.05 N (approx.) $Na_2S_2O_3$ solution and burette no 2was filled by 0.05 N (exact.) $K_2Cr_2O_7$solution. 9 ml of 0.05 N $K_2Cr_2O_7$solution from burette no 2 was taken and filled in a stoppered bottle or in a 100ml conical flask. 3ml of conc. HCl and 10ml of 10% KI solution was added in flask containing 9ml of $K_2Cr_2O_7$solution. The bottle or flask was shaken well and was allowed to mix for 5 minutes. The liberated iodine in the bottle or flask was titrated with $Na_2S_2O_3$solution added by burette no 1 till a pale yellow colour appeared. 1 to 2 ml of starch indicator solution was added. The solution turned blue. The titration was continued and end point of the titration was noted. This burette reading was recorded as X1, X2, and X3.

Part 2 – Determination of Ozone by using potassium Iodide (KI)

10% Potassium Iodide (KI) solution was treated by ozone for specific time interval i.e. 10 min, 20 min, 30min, 40 min respectively. After 10 minutes treated KI solution was filled in burette no 1 and burette no 2 was filled with 0.05 N (approx.) $Na_2S_2O_3$(sodium thiosulphate) solution. 9 ml of 10% KI solution from burette no1 was taken in 100ml conical flask. 2 to 3 drops of starch indicator was added and flask were shaken well and kept for 5min.The liberated iodine in the flask was titrated with $Na_2S_2O_3$ solution added by burette no 2 till the pale yellow colour change to colourless. The end point of the titration was noted when colour of the solution changed from pale yellow to colourless. This burette reading was considered as X1, X2, X3respectively. This procedure was repeated for 20min, 30min and 40min respectively.

RESULT

MPN of Drinking water Sample

Table 1 shows the MPN results of 30 drinking water samples collected from all over Maharashtra state.9 samples showed potability and rest 21 samples showed non-potability of drinking water which was confirmed by MPN test. Five different bacteria *Pseudomonas, Staphylococcus, Micrococcus, Salmonella* and *E.coli* were isolated from different water samples.

Characterization of isolated microorganisms

Isolated microorganisms were characterized by Gram staining and motility (shown in Fig 1a)and then were subjected to biochemical tests(Fig 1b) which was followed by enzyme detection and metabolic tests. (Fig 1c)(Table 2, 3, 4)

Estimation of ozone produced by Ozonator

In this method, a fixed volume of $K_2Cr_2O_7$ (potassium dichromate) was added to a water sample. The organic matter present in the water sample was first oxidized with a known volume of $K_2Cr_2O_7$and then excess of oxygen was allowed to react with 10% KI (Potassium Iodide) to liberate iodine in amount equal to excess oxygen. This was further estimated by titration with 0.05N $Na_2S_2O_3$ as an indicator.

Step 1: Testing of Ozonator

1ml of India ink was added to 800 ml of water well. The Ozonator tube was dipped in water and ozone was allowed to react with the mixture. If the water becomes colourless within 40-50 seconds, it indicates that sufficient amount of ozone was produced by the machine (OTEEN-4E Ozonator).

Step 2: Estimation of ozone by Iodometric titration:

10% KI solution was prepared and then ozone was allowed to react with 200ml of 10% KI solution for 10, 20, and 30minutes. (Fig 2a,b, c). The solution was then titrated with $Na_2S_2O_3$ using 1% starch indicator. The amount of ozone was then estimated by the given formula below. Thus, 6.8mg of ozone was found to be produced in 30 minutes. After titrating the solution against $Na_2S_2O_3$ the following red colour solution changed to colourless.

Formula:

$$\frac{48 \times N \times \text{Reading}}{2 \times 1000}$$

Reaction of Ozonation treatment

$2KI+O_3 + 2H_2O \rightarrow I_2 + O_2 + 2KOH$

$2Na_2S_2O_3 + I_2 \rightarrow Na_2S_4O_6 + 2NaI$

Minimum Inhibitory Concentration

The concentration of sodium hypochlorite solution (Sigma) on ATCC 9017 *Pseudomonas aeruginosa* culture and stock culture of *Salmonella sp* showed the zone of inhibition from lowest concentration i.e. 2ppm to 8ppm(Fig 3a &Table 5)and (Fig 4a&Table 6) while the isolated *Pseudomonas sp* and *Salmonella sp* showed no zone of inhibition against the sodium hypochlorite solution (Sigma). Therefore it was concluded that the isolated *Pseudomonas sp* and *Salmonella sp* were resistant to sodium hypochlorite solution from 2ppm to 8ppm (Fig 3b&Table 5) and (Fig 4b &Table6).

Disk assay

After performing disk assay method it was concluded that the zone diameter differed in size as per the different concentrations of sodium hypochlorite solution. (Fig 3a& Fig 4a)

Treatment of chlorine resistant bacteria using OTEEN-4E Ozonator

Table7 shows effect of ozone against chlorine resistant bacteria in water sample. Water sample with MPN more than 100 coliforms/100ml were collected in 20 litre jar and treated with ozone using OTEEN-4E Ozonator. MPN was performed at intervals of0, 5, 10, 15 minutes.

It was found that the MPN count showed drastic reduction in 15 minutes (from 1000 coliforms/100 ml to 3 coliforms/100 ml).Three such experiments were run with different water samples.

TVC of Microorganisms

The total viable count of the isolated *Pseudomonas sp* and *Salmonella sp* drastically decreases as the time interval increases with ozone treatment. (Fig 5 a &Table 8)

Table 1: Results of Water Analysis.

Sr. No.	Sample	MPN Count	BGLB (Faecal Coliforms)	EMB agar (*E.coli*)	Remark
1.	Manohar Mangal karyalay (PMC water)	00	-	-	Potable
2.	Manohar Mangal Karyalay (Bore water)	250	+	--	Non-Potable
3.	Chohotto Bazar (Tank water)	35	+	+	Non-Potable
4.	Akola Bus stand (Tank water)	1800	+	+	Non-Potable
5.	Khamgaon Bus stand (tank water)	1800	+	-	Non-Potable
6.	Jalna Bus stand (can water)	11	+	-	Non-Potable
7.	Jalna Bus stand (Tank water)	1800	+	+	Non-Potable
8.	Mont Vert (PMC sample)	00	-	-	Potable
9.	Echelon co-operative Pashan	00	-	-	Potable
10.	Ojas Apt, Pashan	09	+	-	Non-Potable
11.	Orange County Pashan	240	+	+	Non-Potable
12.	Mont Vert (Baner)	7	+	+	Non-Potable
13.	Mount Vert Bore Water (0006)	1800	+	+	Non Potable
14.	Bore well water mont real business center (0007)	04	-	+	Non Potable
15.	Mont real business center PMC water (0008)	00	-	-	Potable
16.	Borerwell water Sahakar Nagar (0009)	460	+	+	Non Potable
17.	Borewell water wood land avenue, Kothrud (0010)	1100	+	+	Non Potable
18.	Prafulla Kondhwa Bibvewadi road (0011)	1800	+	+	Non Potable
19.	Treasure Park Borewell (0012)	0	-	-	Potable
20.	Yashwin soc Domestic water(0013)	93	+	+	Non Potable
21.	Yashwin soc Borewell (0014)	1800	+	+	Non Potable
22.	Palladio Tathawade Borewell (0015)	1800	+	+	Non Potable
23.	Sky lounge drinking water (0016)	07	+	-	Non Potable
24.	Sky lounge Borewell water (0017)	0	-	-	Potable
25.	Sky lounge aqua guard water (0018)	0	-	-	Potable
26.	Parampara Masala Wagholi swimming tank water(0019)	0	-	-	Potable
27.	Parampara Masala WagholiBorewell water (0020)	1800	+	-	Non Potable
28.	AISC college overhead tank (021)	1800	+	+	Non Potable
29.	Swargate Bus stand Water (022)	1800	+	+	Non Potable
30.	Mayur colony, Kothrud PMC (023)	00	-	-	Potable

Key: + Present;- Absent

Isolation and Characterization of Microorganisms

Gram staining and Biochemical tests

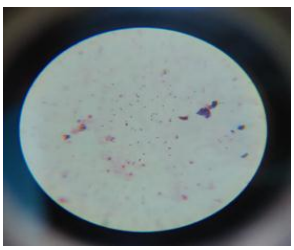

Fig. 1: a Gram negative short rods.

Fig. 1: b Results of IMViC Test.

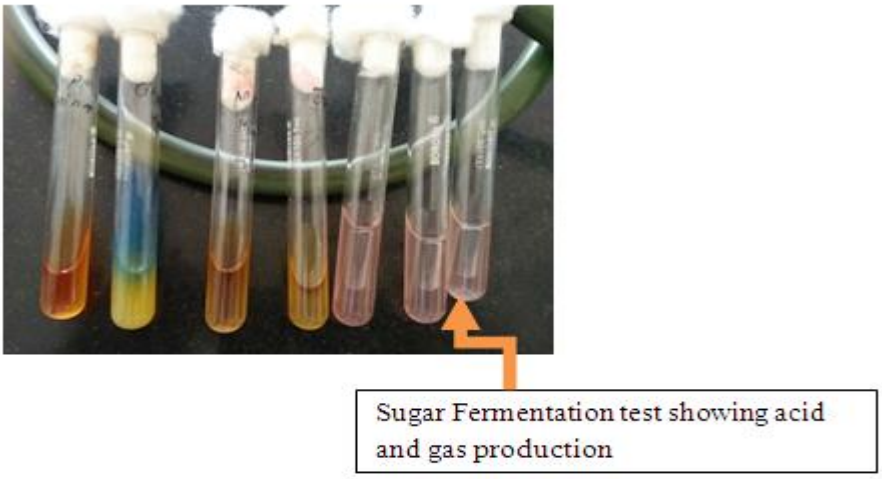

Fig. 1: c Sugar fermentation test.

Table 2: Characterization of Microorganisms.

Character	***E.coli***	***Pseudomonas***	***Staphylococcus***	***Salmonella***	***Micrococcus***
Size	2mm	1mm	2-3mm	1-2mm	3mm
Shape	Circular	Circular	Circular	Circular	Circular
Colour	Cream	Bluish green diffusion	Pale yellow	Cream	yellow
Margin	Entire	Entire	Entire	Entire	Entire
Consistency	Butyrous	Mucoid	Butyrous	Butyrous	Butyrous
Elevation	Raised	Raised	Raised	Flat	Raised
Opacity	Translucent	Translucent	opaque	Translucent	Opaque
Gram Character	Gram negative rods	Gram negative rods	Gram positive cocci	Gram negative rods	Gram positive cocci
Motility	Motile	Motile	Non-motile	Motile	Non-motile

Table 3: Biochemical Tests.

Organism	**Indole test**	**Methyl red**	**Voges-Proskauer**	**Citrate Utilization**
E.coli	+	+	-	-
Pseudomonas	-	-	-	+
Staphylococcus	-	+	+	+
Salmonella	-	+	-	+
Micrococcus	-	+	-	+

Key (+) Positive (-) Negative

Table 4: Sugar Fermentation test.

Organism	**Glucose**	**Lactose**	**Mannitol**	**Sucrose**	**Xylose**
E.coli	G	A	-	A	AG
Pseudomonas	-	-	-	-	-
Staphylococcus	-	-	AG	-	-
Salmonella	G	-	-	-	AG
Micrococcus	-	-	AG	-	-

Key = A-Acid G- Gas

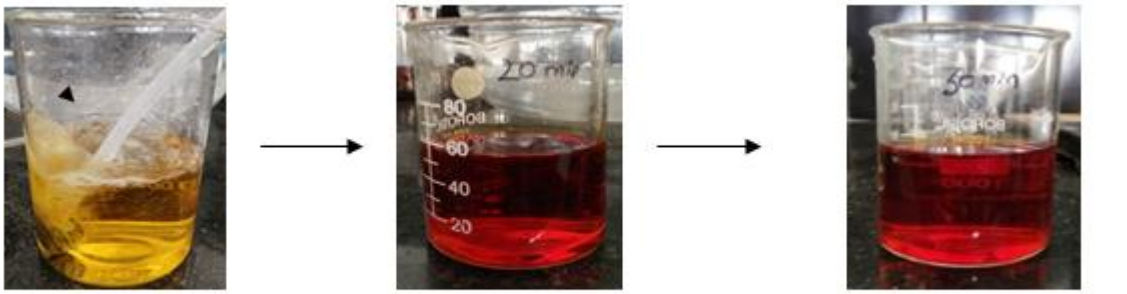

Fig 2.a: 10 minutes | Fig 2.b: 20 minutes | Fig 2.c: 30 minutes

Fig. 2: A: Estimation of ozone produced by Ozonator.

$$2KI + O_3 + 2H_2O \rightarrow I_2 + O_2 + 2KOH$$

$$2Na_2S_2O_3 + I_2 \rightarrow Na_2S_4O_6 + 2NaI$$

Fig. 2: B: Reaction of Ozonation treatment.

Disk Assay

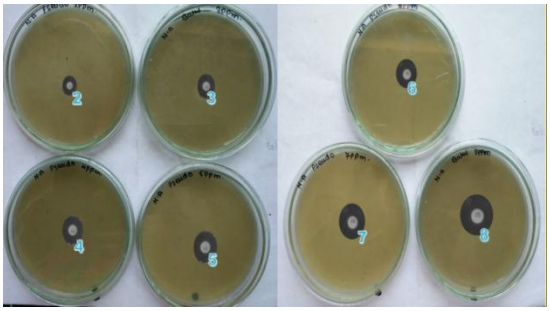

Fig. 3: a) ATCC 9017 *Pseudomonas aeruginosa* with sodium hypochlorite solution of concentrations 2ppm, 3ppm, 4ppm, 5ppm, 6ppm, 7ppm, 8ppm respectively.

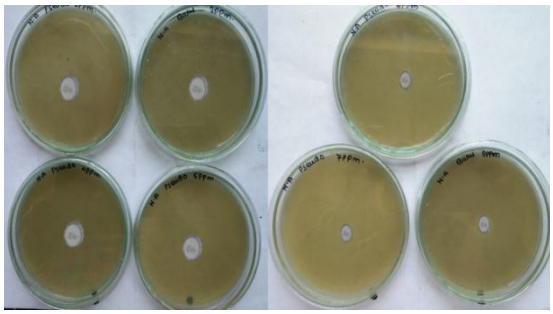

Fig. 3: b) Isolated *Pseudomonas* with sodium hypochlorite solution of concentrations 2ppm, 3ppm, 4ppm, 5ppm, 6ppm, 7ppm, 8ppm respectively.

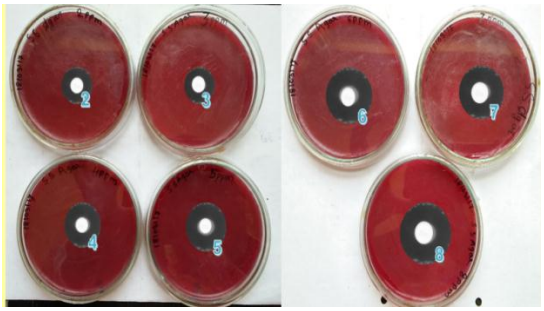

Fig 4: a) Stock culture of *Salmonella sp* with sodium hypochlorite solution of concentrations 2ppm, 3ppm, 4ppm, 5ppm, 6ppm, 7ppm, 8ppm.

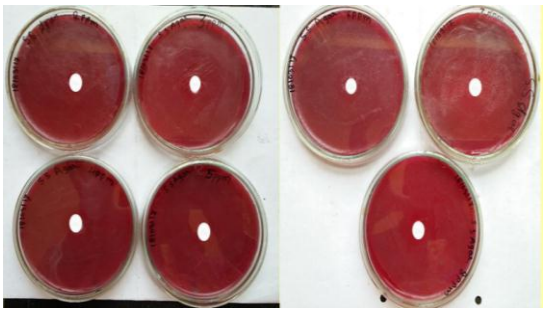

Fig. 4: b) *Salmonella sp* with sodium hypochlorite solution of concentrations 2ppm, 3ppm, 4ppm, 5ppm, 6ppm, 7ppm, 8ppm.

Table 5: Zone of Inhibition against sodium hypochlorite.

Concentration of Sodium hypochlorite (ppm)	Zone of Inhibition in diameter (mm) for ATCC 9017 *Pseudomonas aeruginosa* culture.	Zone of Inhibition in diameter (mm) for isolates. *Pseudomonas sp*
2	1	-
3	1.5	-
4	3	-
5	3.3	-
6	3.6	-
7	3.9	-
8	4	-

Table 6: Zone of Inhibition against Sodium hypochlorite.

Concentration of Sodium hypochlorite (ppm)	Zone of Inhibition in diameter (mm) for stock culture (*Salmonella sp*)	Zone of Inhibition in diameter (mm) for isolates (*Salmonella sp*)
2	2	-
3	2.7	-
4	3.6	-
5	4.2	-
6	4.7	-
7	6.7	-
8	7	-

Table 7: Results with ozone treatment.

Sr. No.	Ozone treatment (Time in minute)	Amount of ozone produced in water (ppm)	MPN/100 ml
1	0	0	1000
2	05	1.4	420
3	10	2.8	180
4	15	4.2	03

Formula

$$\text{Percent Reduction} = \frac{\text{Initial count} - \text{Final count}}{\text{Initial Count}} \times 100$$

Table 8: Total Viable Count.

Ozone treatment (minutes)	Total Viable Count of *Pseudomonas sp.*	Total Viable Count of *Salmonella sp.*
00	Mat growth	Mat growth
10	192	256
20	101	132
30	26	67
40	0	15

TVC of Microorganisms

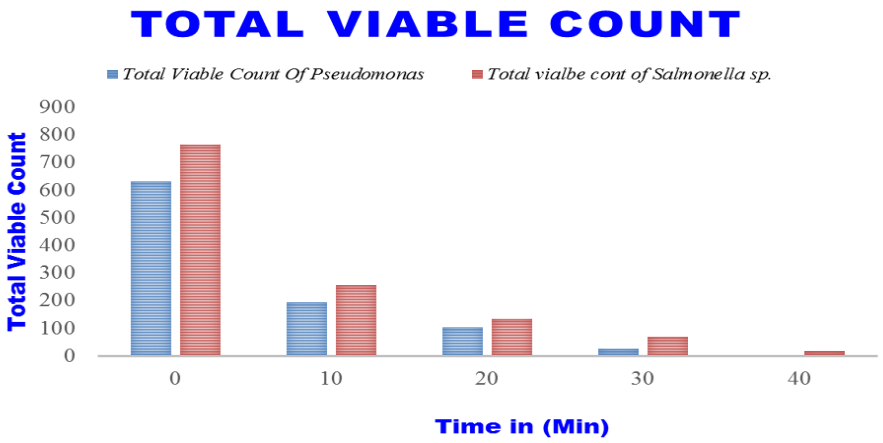

Fig. 5: Graphical representation of TVC of *Pseudomonas sp* and *Salmonella sp.*

DISCUSSION

Sensitivityof microorganisms towards chlorine were performed. In previous research three different isolates *Staphylococcus aureus, Micrococcus varians* and *Aeromonas hydrophila* showed resistance to chlorine, [7] while in our research work we found five different isolates *E. coli, Pseudomonas, Staphylococcus, Salmonella* and *Micrococcus* were resistant to chlorine. It was verified that all five strains were sensitive to 9ppm, only *Pseudomonas sp*

and *Salmonella sp* showed resistant to 2ppm, 3ppm, 4ppm,5ppm,6ppm,7ppm and 8ppm of sodium hypochlorite solution. Ozone treatment was used to disinfect the drinking water. As per the result, ozonation showed 99% reduction in total viable count of chlorine resistant bacteria present in drinking water. This process led to inactivation of bacteria by rapidly reacting with the essential components of cell, such as respiratory enzyme, nuclear material (DNA, RNA), component of their cell envelope, spore coat or viral capsid.[8] Ozone is a potent sanitizer and promising method for disinfecting drinking water. It is also considered as the strongest molecule available for disinfection in water treatment (M.A. Khadare*et al*., 2001).[12]As compared to chlorine or most common water disinfectant, ozone is 50% stronger oxidizer ant act over 3000 times faster.[13]Therefore, chlorine is being replaced by ozone all over the world. The method of ozonation kills all the microbes at 4ppm concentration whereas chlorine treatment killed all the microbes at 10 ppm. The following research was carried out with a single method whereas previous work was performed along with U.V treatment.[14]Previous work showed reduction in MPN count after chlorination.[15]whereas in our research work MPN count was reduced after Ozonation.

CONCLUSION

Isolates were found to be chlorine resistant and were killed by the dose was 4.2ppm of ozone at 30 minutes which was lesser as compared to chlorine. We characterized and demonstrated the resistance and reduction of chlorine resistant bacteria after ozone treatment.

ACKNOWLEDGEMENT

We are thankful to Dr. Mujahid Yusufi, Department of Chemistry, for his guidance and help in understanding the chemistry linked with this project.

FUNDING

We are thankful to Sunbird Medica Company for funding the project.

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