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Bactericidal activity of Chlorine Dioxide on *Escherichia coli* in water ponds at animal farms

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ABSTRACT

Escherichia coli (*E. coli*) belongs to the bacterial species and causes hazardous effects on health of humans, animal and even on plants that need to be eliminated. Due to chlorine dioxide's effectiveness in disinfecting water and acting as a powerful oxidizer, it has taken the place of chlorine in many applications. This study aims to assess the chlorine dioxide (CLO_2) disinfectant's bactericidal activity against *E. coli* in water ponds at animal farms. To isolate the *E. coli*, water samples were gathered from several locations and cultivated on the MacConkey agar. Purified colonies were taken to enrich the bacteria and preserve the bacteria in 50% glycerol. Stock solution was used to make dilutions to check the efficacy of chlorine dioxide. Different concentrations of ClO_2 were tested at different exposure time ranges from 3-14 min. The decreased ratio of viable cells of bacteria in the suspension at different amount of chlorine dioxide was determined. Values were calculated to determine how much the *E. coli* population was decreased at each sanitizer concentration in aqueous solution. Additionally, under the same experimental settings, linear functions of the log of bacteria inactivation (log 90 percent killing time vs. log concentration of disinfectant) were plotted to test the effectiveness of ClO2. The outcomes were manually recorded.

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Introduction

Water is an important substance for life and therefore the accessibility of water should be safe and hygienic for all purposes of life to everyone. The water contaminated with fecal material contains high microbial load such as, pathogenic bacteria, protozoa, helminths and viruses and intake of such water causes diseases in humans and animals. Microbial safety is mainly concerned with fecal derived pathogens because they are perquisites of waterborne diseases (Anonymous 2004). The most commonly found pathogens which cause infections are *Escherichia coli* and present in fecal matter. *Escherichia coli*, group of gram negative bacteria that belongs to 1% of bacterial biomass mostly resides in the intestinal flora (mucous layer of the colon) of healthy humans and warm blooded animals (Nataro et al. 1998). Few strains of *E. coli* are dangerous and pathogenic which cause certain diseases in food and water such as pathogenic serotypes *E. coli* O157:H7 (Rasmussen et al. 2001). Like other mucosal pathogens, pathogenic *E. coli* microbes practice a various-step pattern of pathogenesis i.e. mucosal wall transplanting, host resistances dodging, duplication of cells and destruction to host (Kaper et al. 2004.) All the strains of *E. coli*, either enteropathogenic strains or other *E. coli* strains, showed no difference to water treatment and disinfection procedure (Water and WHO 2004).

Disinfection of water resources is essential part of our routine and for this purpose, the basic method of coagulation, flocculation filtration was used which initially purifies water up to 99% from bacteria but the remaining 1% bacteria can cause serious illness (Somani et al. 2011) Reactive chemicals are used preferably (i.e. chlorine) that provide barriers to certain pathogens more commonly to bacteria (Anonymous, 2004). Moreover, different types of chemical and physical techniques are brought such as chlorination, ozonation and germicidal lamps (UV) (Dadjour et al. 2005).

Chlorine is an ancient technique mainly applied for the sterilization of water on commercial basis as a disinfectant (Han et al. 2000). But during this, the traces of organic substances existing in the raw water combine with Cl_2 that produce byproducts and halogenated products which may be toxic (Somani et al. 2011). Moreover, chlorination is proved to become less effective for many other pathogens. Therefore, it must be necessity of time to develop new techniques and chemicals to kill the bacteria in drinking water. Chlorine dioxide (ClO_2), another chemical, which effectively replaces the chlorine due to its efficacy in many aspects as it is a strong oxidant and has a great bactericidal activity i.e. inhibits or destroys the microbes (Sanekata et al. 2010). Oxidation capability of chlorine is 2-3 spells less than the oxidation capacity of ClO_2. Furthermore, ClO_2 is highly preferable at supplies with low temperature and high pH (Gray 2014). ClO_2 is a USDA Food Safety Inspection Service approved chemical which can be utilized to disinfect surfaces, wash water and decontaminate the food items as well as used in poultry processing (Berrang et al. 2011). Different food spoilage microorganisms can be reduced up to 6-log fold present on the surface of juice storage tanks by ClO_2 gas treatment (Han et al. 1999). Moreover, this treatment can successfully reduce 3-log fold *E. coli* O157:H7 and 5.5-logCFU *L. monocytogenes* from surface of green pepper, skin surface of apples and stem & calyx cavities (Han et al. 2001).

The purpose of this study was to find effectiveness and efficacy of chlorine dioxide (ClO_2), bactericidal disinfectant, to inactivate *E. coli* present in aqua by using different experiments as well as techniques. Influence of several quantities of ClO_2 and its exposure time (different contact time) to bacterial cell also considered to fully understand the role of both of these factors in eliminating *E. coli* in aqueous solution or suspension.

Materials and Methods

Study Setting

An experimental study was conducted at the cell culture laboratory of the Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan. Water samples were obtained from multiple sites water ponds at different animal farms and Brooke Equine Hospital, University of Agriculture, Faisalabad during February to May 2022 and analyzed.

Cultivation and Bacterial Isolation

Culture media (MacConkey agar) sterilization was done in autoclave at temperature 121°C for 15-20 min/15lb and petri plates were prepared by pouring media into them.

Sample inoculation

0.1ml of water samples, collected in sterilized test tubes was spread over the prepared petri plates of MacConkey agar with the help of spreader. The petri plates were kept in incubator for 24 hours at the temperature of 37°C. After the incubation of 24 hours, all the plates were examined for bacterial growth.

Gram Staining

Gram staining techniques were executed to find gram positive and gram negative bacteria and their differentiation as well as the presence of *E .coli*. After gram staining the slides were observed with 100X, 400X, 1000 X and oil immersion lens of microscope.

Biochemical Characterization

Biochemical characterization was done by IMVC series i.e. Indol test, methyl red test, Voges Proskauer test, citrate utilization test, catalase test, oxidase test and. In indol test, the color development was observed (positive test showed red color production and negative result showed no color change). In methyl red test, capability of *E. coli* to use glucose and convert it into acid resulted in change in color from yellow to red i.e. acidic fermentation products were detected. In Voges Proskauer test, results were recorded for glucose fermentation and production of acetyl methyl carbinol. In Citrate Utilization Test, Simmons citrate agar was used for pH indication and slant was observed for positive or negative result. Catalase test was conducted to see quick bubbling while oxidase test was performed for the detection of cytochrome oxidase.

Enumeration of Bacteria

Nutrient broth was used for the enrichment of bacterial growth by adding about 4ml of it in two test tubes, one for control and other for test. The control group and other tube results were compared.

Culture Storage

600 microliters of 50% glycerol and 600 microliters of bacterial culture were poured into the Eppendorf tube and stored at -4°C refrigerator.

Preparation of Disinfectant

Sodium chlorite (261 mg, 80% grade) and Acetic anhydride (6481Jl) were mixed for the preparation of 200-ml ClO_2 stock solution (372 mg/L).

Titration of Disinfectant

Iodometric titration was used to check stock solution concentration while working solutions concentrations were determined by spectrophotometric measurements of absorbance.

Suspension Test

Serial dilutions of 4 sets of 5 test tubes, labeled as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}, were made by 9ml of distilled water and 1ml of bacterial broth. 2 sets of dilutions were examined without giving chlorine dioxide treatment while treatment of different concentrations of chlorine dioxide at different time exposure was executed in increasing pattern at other

two sets. In one set, chlorine dioxide was added 9µl, 15µl, 24µl, 38µl and 60µl and then in other set concentrations were changed by two points to check the bactericidal efficacy of this disinfectant. The tested concentrations were 9µl, 15µl, 24µl, 38µl and 60µl in 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} respectively. The results were computed by comparing and noted the values of colony forming units before and after giving the treatment of chlorine dioxide.

Results

Isolation and Identification

Bacterial colonies found in 10 samples out of 12 samples on the nutrient agar while 7 samples showed bacterial growth on MacConkey agar. The *E. coli* grew on the MacConkey agar shown in the form of pink to dark pink growth and some was like dry and donut shaped. Gram staining technique showed, out of 7 samples, 5 samples were *E. coli*. These 5 samples were further streaked over the MacConkey agar for 24hrs. Later on, the gram staining technique was employed and observed under microscope. The results of gram staining showed pink road shaped *E. coli* as observed under 100X, 400X and oil immersed 1000X lens of microscope.

Biochemical Characterization

The biochemical tests justified the presence of *E. coli* as Indole test showed positive results for *E. coli*. Pink colored ring formation was observed after adding the Kovac's reagent which indicated the positive results and confirmed the presence of *E. coli*. When Methyl red indicator was put into the broth medium the colour changed from yellow to red color which confirmed the presence of *E. coli*. However, Voges Proskeur showed negative results for *E. coli*. Because upon addition of alcoholic KOH and α-naphthol and incubating the color was not changed from yellow to pink. In citrate utilization test, after incubation, the color of bromothymol blue was not changed from green to blue which indicated the negative results and confirmed the presence of *E. coli*. Catalase test showed positive result for *E. coli* because bacteria showed rapid bubbling when hydrogen peroxide H_2O_2 converted into oxygen and water. While Oxidase test showed negative results for *E. coli*. p-Aminodimethylaniline oxalate reagent (light pink reagent) converted into blackish reagent by donating electrons and further no color change was observed due to the presence of cytochrome oxidase enzyme.

Table 1: Efficacy of different concentrations of chlorine dioxide

Dilution factor	Time (min)	Concentration of chlorine dioxide	Viable Count (CFU/ml)		*Log reduction	**Percentage reduction %
			Before treatment	After treatment		
10^{-1}	14	9 µl	TNTC	1.27×10^4	ND	ND
10^{-2}	10	15 µl	3.52×10^5	1.08×10^5	0.513	69.32 %
10^{-3}	8	24 µl	3.15×10^6	2.2×10^5	1.156	93.02 %
10^{-4}	5	38 µl	3.06×10^7	9.0×10^5	1.531	97.06 %
10^{-5}	3	60 µl	2.96×10^8	No colony	ND	ND

Table 2: Efficacy of chlorine dioxide by increasing concentrations by two points

Dilution factor	Time (min)	Concentration of chlorine dioxide	Viable Count (CFU/ml)		*Log reduction	**Percentage reduction %
			Before treatment	After treatment		
10^{-1}	14	11 µl	TNTC	1.32×10^{4}	ND	ND
10^{-2}	10	17 µl	3.62×10^{5}	1.03×10^{5}	0.546	71.55 %
10^{-3}	8	26 µl	3.32×10^{6}	2.0×10^{5}	1.220	94.28 %
10^{-4}	5	40 µl	3.12×10^{7}	6.0×10^{5}	1.716	98.08 %
10^{-5}	3	62 µl	2.61×10^{8}	No colony	ND	ND

*Log reduction = log10 (Initial CFU / Final CFU)
**Percentage reduction = (Initial CFU – Final CFU) / Initial CFU × 100
TNTC = Too numerous to count
ND = Not determined

Gram staining technique and biochemical characterization showed 4 samples provided true *E. coli* growth and 1 provided pseudo effects out of 5 samples which were further streaked over the MacConkey agar. The purified growth obtained by the streaking was enriched in the nutrient broth and turbidity of solution was achieved after 24 hours due to the enumeration of bacteria while in control group there was no turbidity. The stock solution of bacterial colony was preserved and used to make 4 sets of 10-fold serial dilutions up to the 10^{-4} for the quantitative estimation of viable microorganisms. The set 1 and set 3 remained as same as control group while set 2 and set 4 treated with different concentrations of ClO_2 and cultured on the MacConkey agar. After 24 hours of incubation at 37°C, these provided the colony forming units. Thus the comparison of viable bacterial count was done before and after the treatment which provided the significant results (Table 1). It was found that the untreated sample provided non-significant results and merely reduced the bacterial load.

Different concentrations of chlorine dioxide provided the slope of inactivation of *E. coli* in serial dilutions. It was noted that the increasing ClO_2 pattern provided significantly efficient results expectedly, because the serial dilutions gradually reduced the viable cell count, earlier and the addition of increasing quantity of ClO_2 reduced bacterial colonies. On the other hand, at dilution 10^{-4} bacterial colonies were too few to count and at dilution 10^{-5} no colony was observed. Inactivation of *E. coli* with varying ClO_2 exposure time was also observed in aqueous solution. The results were compiled into two columns; after treatment column and before treatment column (Table 2). It showed that after the implementation of disinfectant, viable cells at a given time provided same outcomes as in the control test. Following formula was used to calculate bactericidal effect at *E. coli* reduction in percentage.

$$\frac{\text{Initial CFU} - \text{Final CFU}}{\text{Initial CFU}} \text{x} 100$$

Despite of 9µl ClO_2 treatment for 8 min, the organisms were reduced by only 1-log cycle thus cells of *E. coli* remained tolerant. As the amount of disinfectant increased, the count of viable cells decreased rapidly by 2-fold within 3 min, always true for all the initial bacterial concentrations. Viable colonies of bacteria were also found even after 10 min contact time with 17µl. However, concentration of 60 and 62µl of ClO_2 applied for 3min provided no colony. Moreover, 90% killing time or 90% reduction of viable counts with respect to time for each concentration of ClO_2 were also calculated.

Discussion

The outcomes of this research indicated that the concentration of chlorine dioxide gas and the time of exposure were the major factors to obtain 90% reduction of *E. coli* in aqueous solution. By increasing the concentration of ClO_2, more reduction of *E. coli* was obtained at even little disclosure of time. Han et al. (2000) described that the concentration of ClO_2 gas, exposure time, relative humidity and temperature were the significant factors (in order of most to least) for the reduction of *E. coli*. They reported that at 75% RH and 15°C as the ClO_2 gas quantity increased from 0.1-0.5 mgL^{-1} the reduction of *E. coli* O157:H7 increased by 0.8-5.5 log, linearly, within 31 min. While at concentration of 0.3 mgL^{-1} of ClO_2 gas asymptotical increased of log reduction was obtained by increasing RH from 55 to 95% however, the temperature and time of exposure kept same (i.e. 15°C and 31min, respectively) (Han et al. 2001).

This postulate could be supported by other studies conducted by the Han et al. (2000) and Jeng et al. (1990) who postulated that during the treatment of

ClO_2 gas for the reduction of microbes; the concentration, time and RH were significant (Jeng et al. 1990). The criteria and the method employed in this study could be helpful to find the antimicrobial efficacy of a disinfectant for the inactivation of microorganisms under same experimental conditions as well as different test conditions fagainst different microbes. The efficacious sanitizer caused 5-log elimination of viable organisms within 30s and the ClO_2 concentration of 3.4mg/L was needed to prove it efficacious according to the criteria described by the AOAC definition (Foschino et al. 1998) but 0.9mg/L concentration of ClO_2 could cause 10^5-fold reduction of *E. coli* at 5min in aqueous solution hence, considered efficacious according to the European standards.

It was noted that the results of reduction of gram-negative *E. coli* by ClO_2 in aqueous solution obtained in this study was correlated to the previous studies. According to the report accompanied by the Roller et al. (1980), in distilled water 3.5-log reductions of *E. coli* obtained with 2.0mg/L concentration of ClO_2 after 30s and in our study 2.5 mgL^{-1} of chlorine dioxide required for obtaining same reduction. Furthermore, 1.0 mgL^{-1} of chlorine dioxide caused 5-log inactivation of *E. coli* after 5min, correlated with the research data of Harakeh et al. (1998) i.e. 5-log elimination of *E. coli*, *Klebisella pnemoniae*, *Pseudomonas aeruginosa* and *Yersinia enterocolitica* in saline solution obtained by 5min treatment of 0.75mg/L of ClO_2.

Junli et al. (1997) found that at 7.0pH, 2.5 mg/L of ClO_2 caused 98% reduction of *E. coli* in aqueous solution after 1min and findings of our study showed that 1.4 mg/L of ClO_2 at 4.5 pH produced the same rate of inactivation of germs (germicidal effect). The another research conducted by the Rovito et al. (1985) described that 5-log reduction of *Proteus vulgaris* was obtained in 3min just by 0.5 milligram per liter of chlorine dioxide, however, it was not preferred to use any method to nullify the sanitizing action after the duration of contact. It was reported by the Notably, that ClO_2 could reduce the population of both gram positive and negative bacteria as 0.05ppm of ClO_2 could incapacitate *Staphylococcus aureus* and *E. coli* present on the crystal surface (Morino et al. 2011).

Foschino et al. (1998) concluded that 1.4ppm ClO_2 solution for 30s caused 5-log elimination of *E. coli* ATCC 11229 when put in water. The biocidal activity of ClO_2 could be supported by another report conducted by the Tanner (1989) who tested eleven disinfectant against 2 bacteria included *Staphylococcus aureus* and *Pseudomonas aeruginosa* and a yeast and concluded that ClO_2 had a higher microbial efficacy. The state of cells during the treatment period had a significant impact on the antimicrobial activity of ClO_2 contrary to *E. coli* as increasing the log value of ClO_2 concentration by 0.642 units resulted in decrease killing time of planktonic bacteria up to 85% by 1-log cycle. Therefore, the vulnerability of microbes in authentic experimental circumstances was not well described by the suspension tests as reported by other authors too (Brouillaud-Delattre et al. 1994). The attached cells were merely sensitive to the chlorine sanitizers than unattached gram-negative bacteria in water as they are highly sensitive, reported by the Le Chevallier et al. (1988). So the conclusions obtained by the suspension test was not applicable to define the effectiveness as well as to specify the killing time of certain disinfectant for the hard surfaces (Lechevallier et al. 1988). ClO_2 could inactivate the microbe by denaturing and oxidation of proteins, by modifying the tryptophan and tyrosine residues, as Ogata et al. (2012) evaluated the antimicrobial mechanism of ClO_2 by the oxidation of tryptophan residual of viral protein hemagglutinin.

Bernarde et al. (1967) also reported that the bactericidal activity of ClO_2 was due to the blocking of biosynthesis of proteins (Benarde et al. 1967) while Cho et al. (2010) declared that the oxidation of lipids by ClO_2 increased the permeability of the bacterial membrane that released proteins from the microorganisms (Cho et al. 2010). But according to another study, the impairment of mechanism of the penetrability of K^+ and nonspecific oxidative destruction to the outer sheath of *E. coli* by ClO_2 bring about the damage of the trans-membrane ionic gradient as well as destruction of respiration, but it was observed that the intracellular macromolecules from the bacterial cells did not leaked considerably (Berg et al. 1986). Young and Setlow(2003) reported that the ClO2 could destruct the integrity of cell membrane and germination inhibition in bacterial spores due to strong oxidation.

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Author Contribution: All authors contributed to the study conception, design and analysis.

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