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Research Article

EFFECT OF CHLORINE (DISINFECTANT) ON VIABILITY OF PATHOGENIC FREE LIVING AMOEBAE

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ABSTRACT
Chlorine is a disinfectant which can kill most harmful micro-organisms; this is the reason that chlorine is used in water treatment. Chlorination has become almost the only method used for the active disinfection of potable water supplies due to its effectiveness as a germicide. During *In vitro* action chlorine, the most common disinfectant used for treating drinking water and also swimming pools. Species of pathogenic free-living amoebae isolated from different water samples were used in the present study. The purpose was to ascertain the difference in chlorine sensitivity among different species of pathogenic small free-living amoebae and also to find out their occurrence is chlorine treated water. The experiment was conducted on pathogenic free-living amoebae (*Naegleria fowleri* and *Acanthamoeba culbertsoni* and *A.rhysodes*). The strains of amoebae selected for the experiment were PP-2 and HS-1 of *N. fowleri*; strains HP-1 and DS-1 of *A. culbersoni*; strains MS-3 and Us-2 of *A.rhysodes*. The concentrations of chlorine used in this study wee 0.25mg/ml, 0.50mg/ml,2.0mg/ml, 3.0mg/ml and 3.5mg/ml. From the present study, it was concluded that cysts and trophozoites of *Naegleria fowleri* strains can be destroyed in with application of free chlorine. But the strains of *Acanthamoeba culbertsoni* and *A. rhysodes* can only be checked in drinking water by the use of prolonged super chlorination, this condition is possible only when preparing the water for use and if there is no further contamination possible in the distribution network.

Key Words: *Chlorine, Naegleria Fowleri and Acanthamoeba Culbertsoni and A.rhysodes, Microorganism.*

INTRODUCTION
Disinfection defined as "the killing of the large portion (but not necessarily all) of the harmful and objectional micro-organism in or on a medium by means of chemicals, heat or ultraviolet light etc." (Wang and Pek, 1975; Cursons *et al.*, 1980).
Chlorine is a disinfectant which kills most harmful micro-organisms. This is the reason that chlorine is used in water treatment. It has become almost the only method used to its effectiveness as a germicide. It is extremely effective for bacteria, viruses and cyst forming protozoan which are more resistant to chlorine disinfection (Rubin *et al.*, 1983).The mode of action of chlorine, especially HOCl,against bacteria and protozoan cysts has been previously studied. This powerful non selective oxidant reacts with a variety of sub cellular compounds. It has been shown that chlorine and HOCl mainly targets the cell envelop, inhibits ATP,electron and metabolite transports (Barrette *et al.*,1989) and also affects membrane permeability (Small *et al.*,2007).Obviously, these effects may depend on chlorine concentration.
Duma *et al.*, (1969) reported that *N. fowleri* is more resistant to chlorine than *E. histolytica* and mentioned 4µg/ml as an effective concentration. Heavy chlorination to 10ppm failed to eradicate *N. fowleri* from a swimming pool, but salination to 0.7% resulted in negative culture for a period of 5 months (Shin and IM, 2004).
Cursons *et al.*, (1980) examined the amoebicidal capacity in axenic condition of disinfectants, chlorine, chlorine dioxide, ozone and Deciquam 222. They found that *Naegleria sp.* were sensitive than *Acanthamoeba sp.* to chlorine and chlorine dioxide.

Derreumaux *et al.,* (1974) reported that trophozoites of pathogenic *Naegleria* and *Acanthamoeba* were destroyed when concentrations of 0.5 to 1.0µg of free, active chlorine per ml are maintained in water. However Jonckheere and Voorde, (1976) reported that the cysts of pathogenic *Naegleria* and *Acanthamoeba* were more resistant to chlorination than were their trophozoites. Thus close attention should be give to effective chlorination in water purification stations, especially if they are associated with a body of water that may contain pathogenic amoebae. An appreciation of the nature of sub lethal injury and repair is therefore important in devising chlorination strategies and in developing combination treatments with synergistic actions against the target microorganisms. The mechanisms of action of chlorine on microorganisms have been widely investigated. Nevertheless, the mechanism by which chlorine exerts its lethal effect has never been fully elucidated. Moreover, the occurrence of sub lethal injury caused by chlorine treatments is scarcely known (Gottardi and Nagl, 2005).

MATERIALS AND METHODS

Amphizoic amoebae were isolated from all the collected water samples of different sources in Lucknow city. For isolation of amoebae, 15-20 ml of sterilized (2.5% W/V) non nutrient agar (Hi- Media lab, Mumbai, India) with NaCl and pH 6.6 to 6.8 is poured into pre-sterilized Petri dish (Borosil 9-10 cm in diameter) and allowed to set for 24 hours. *Escherichia coli* culture of 24 hour old, grown on the surface of nutrient agar slants (pH 7.2) was used as food for amoebae. Bacterial culture was then scraped with nicrome wire loop and spread as thick suspension on the solidified non-nutrient agar surface in the form of circular patch or bacterial circle of about 20-25 mm in diameter aseptically. Two to three such bacterial circles, well separated from each other, were made in each Petri dish before making it ready for inoculation of substrate for the growth of amoebae.

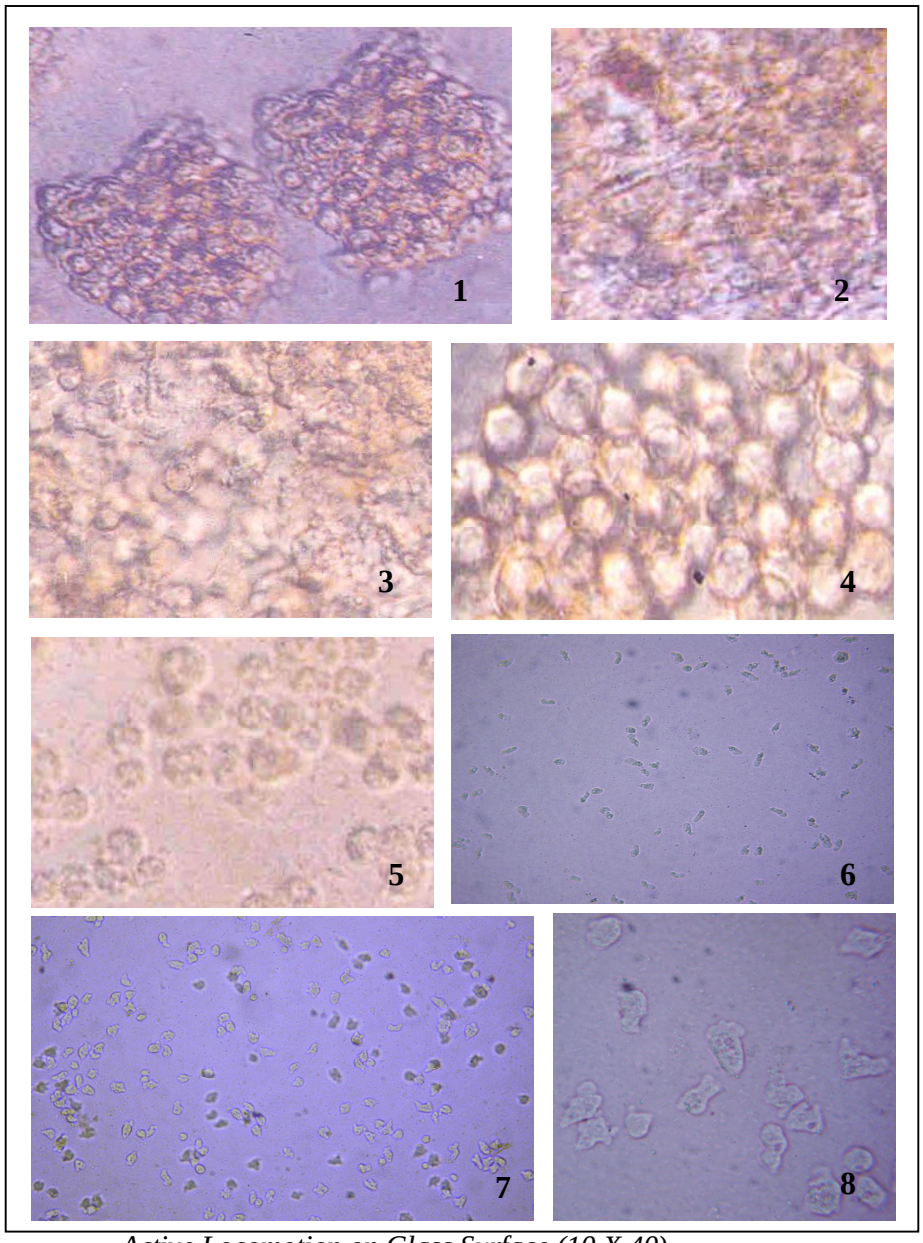
About two liters of water from each source was collected aseptically in sterilized bottles. This was then filtered through sterile filter paper (Whatmann No.1), using conical funnel and after filtration; the inner narrow end of the filter paper with collected sediments was aseptically cut into 3-4 small pieces and these were placed in the centre of each bacterial patch in a Petri dish ready for inoculation. These plates were incubated at 28°C and 37°C for 10 to 12 days for the amoebae to grow and form cyst. The plates were examined daily under low power of microscope for the presence of trophic and cystic stage of amoebae.

RESULTS AND DISCUSSION

Chlorine is a disinfectant which can kill most harmful micro-organisms. This is the reason that chlorine is used in water treatment. It has become almost the only method used for the active disinfection of potable water supplies due to its effectiveness as a germicide. During *In vitro* action chlorine, is the most common disinfectant used for treating drinking water and swimming pools. Species of pathogenic amphizoic amoebae isolated from different samples was investigated in the present study .The purpose was to ascertain the difference in chlorine sensitivity between different species of amphizoic amoebae, to understand their occurrence in treated water and to enable in preventing amoebic contamination of drinking water.

The experiment was conducted on the species of pathogenic free-living amoebae such as *Naegleria sp*. and *Acanthamoeba sp.* The strains of amoebae selected for the experiment were PP-2 and HS-1 of *N. fowleri*, strain HP-1 and DS-1of *A. culbertsoni*, strain MC-3 and US-2 of *A. rhysodes*. The concentrations of chlorine used in this study were 0.25mg/l, 0.50mg/l, 2.0 mg/l, 2.5 mg/l, 3.0 mg/l and 3.5 mg/l. (Table-1&Fig-1). The picture shown in Plate-1 (Fig.1-8) illustrates that high chlorine concentration may induce important modifications. Most of these cells looked like ghosts and their cell organelles were deformed, moreover, the nucleus was no longer visible. The chlorine treatment shows led to loss of pseudopodia, as cells become smoother. The phenomenon may be due to the direct deleterious effect of chlorine leading to pseudopodia stripping or to an active mechanism involving actin mediated retraction. The fact that the reduction of cell size accured in a manner concomitant with loss of pseudopodia suggests that cytoskeleton dynamics is a primary target for chlorine (Moga *et al.*, 2010).

Antiamoebic activity of the agents was expressed as the minimal inhibitory concentration (MIC). MIC was defined as the lowest concentration of antimicrobial agents at which the growth of amoebic population was 70% less than the control. MAC was the minimal concentration at which complete destruction of the amoebic population was recorded (Ferrante *et al.*, 1984).



Active Locomotion on Glass Surface (10 X 40)

Plate-1: Photographs showing *in vitro* effect of chlorine (as disinfectant) on amphizoic amoebae

Figure 1: Very large clumps are formed due to effect of Chlorine, showing Minimal Amoebicidal Concentration (MAC)

Figure 2: Stage during the formation of large clumps, with some cells showing lysis.

Figure 3: Smaller clumps formed due to effect of chlorine.

Figure 4: All the trophozoites in rounded condition, similar to the cystic stage.

Figure 5: Trophozoites are transforming into rounded condition, showing lysis.

Figue 6-7: Trophozoites under controlled condition, during active locomotion on glass surface (10 x 10)
Figure 8: Trophozoites under controlled condition, during active locomotion (10x40).

Table 1: *In vitro* effect of Chlorine on amphizoic amoebae showing MIC and MAC concentrations

S. No.	Amoebae	Strain	MIC mg/L	MAC mg/L	Pathogenicity
1	*N. fowleri*	PP-2	0.25	1.0	P
2	*N. fowleri*	HS-1	0.25	1.0	P
3	*A. culbertsoni*	HP-1	0.50	1.5	P
4	*A. culbertsoni*	DS-1	2.0	2.5	P
5	*A. rhysodes*	MC-3	2.5	4.0	P
6	*A. rhysodes*	US-2	3.0	8.0	P
7	*N. gruberi*	HP-3	3.5	8.5	NP
8	*A. polyphaga*	US-1	3.5	8.5	NP

***MIC**= Minimal Inhibitory Concentration*
***MAC**= Minimal Amoebicidal Concentration*
***P**= Pathogenic*
***NP**= Non-pathogenic*

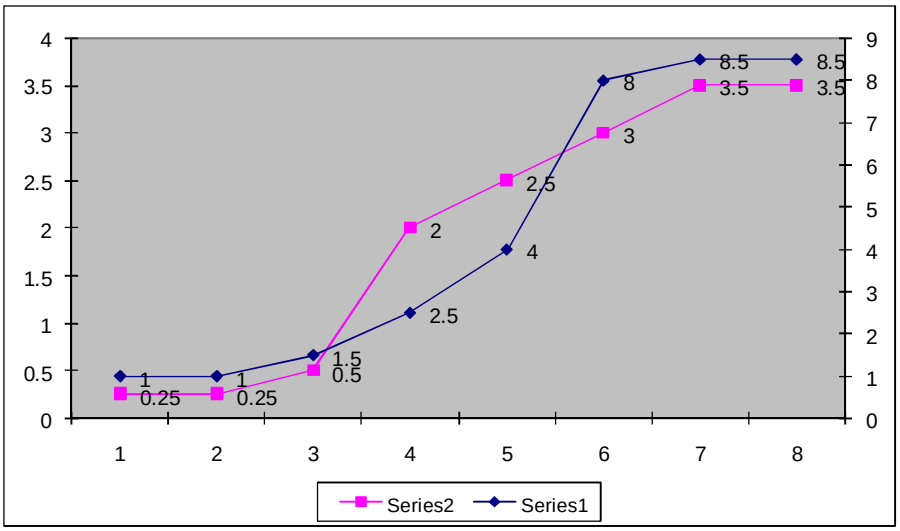


Figure 9: Showing MIC and MAC Concentration of Chlorine

According to the result of the present study on *Naegleria sp*., it was seen that pathogenic *N. fowleri* was more sensitive to the effect of chlorine than that of *N. gruberi* which was non-pathogenic. MIC for both the strains (PP-2 and HS-1) of *N. fowleri* was 0.25 mg/l and MAC was 1.0 mg/l of free chlorine. Where as strain of *N. gruberi* (HP-3) was more resistant and its MIC value was 3.5 mg/l and concentration for MAC was 8.5 mg/l of free chlorine. It has been reported that sensitivity of *Naegleria sp*. to chlorine concentration is dependent on the strain variation (Jonckheere and Voorde, 1976).
Chlorine treatment appears to induce drastic cellular changes in *Acanthamoeba culbertsoni* trophozoites, such as cell condensation, loss of pseudopodia and organelles modification.
On the other hand, *Acanthamoeba* needs more attentions it was much more resistant against free chlorine than *Naegleria sp*. Both the strains of *A. culbertsoni* (HP-1 and DS-1) had MIC of about 2.0mg/l of free

chlorine. MAC of the pathogenic strain (DS-1) was greater than that of other non-pathogenic strain of *Acanthamoeba sp*.

In, addition, our results suggest that chlorination affects cultivability of cells that may still be viable. It is also indicated that *Acanthamoeba* population in water treatment by chlorine might be underestimated by standard culture methods. Effect of chlorine for *A. rhysodes* was different for both the strains (MC-3 and DS-2) pathogenic as well as non-pathogenic. Both the strains had MIC and MAC of 2.5mg/l, 3.0mg/l, 4.0mg/l and 8.0mg/l respectively.

The result of the present study were in conformity with Jonckheere and Voorde, (1976) who reported that pathogenic *N. fowleri* was more sensitive to chlorine than those of the non-pathogenic *N. gruberi* and cyst were destroyed at concentration of 0.5µg/ml of the free chlorine. Cursons *et al.*, (1980) reported that a concentration of 0.79 ppm was more effective. In addition 1.05ppm of free chlorine could destroy cyst of. *N. fowleri* (White, 1972). They have also stated that *Acanthamoeba sp*. is more resistant to free chlorine than *Naegleria sp.* and the virulent *A. culbertsoni* is more resistant to chlorine.

Derreumaux *et al.*, (1974) demonstrated that 0.5mg/l of HOCl, the active disinfecting compound of chlorine treatment, was able to destroy both *Naegleria* and *Acanthamoeba sp*. It was demonstrated by Venkobacher *et al.*, (1977) that chlorine damages the cell membrane of bacteria, which results in leakage of macromolecules and the complete cessation of oxidation phosphorylation. It is therefore, possible for chlorine to exert a double effect on amoebae, destroying both cell membrane and metabolism. Anderson and Jamieson, (1972) failed to eradicate *Naegleria sp*. from a swimming pool by super chlorination upto 10 µg/ml.

This study has shown that chlorine and chlorine dioxide to be amoebicidal to pathogenic free-living amoebae when used in lightly polluted water such as likely to be found in swimming pool. Thus, close attention should be given to effective chlorination in water purification stations, especially if they are associated with a body of water that may contain pathogenic amoebae.

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