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RESEARCH

Herpes Simplex virus type 1 persistence in water from different sources

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ABSTRACT

Background*:* Herpes Simplex virus type 1 (HSV-1) is a widely spread virus. Primary infection occurs via direct contact of the virus with host's mucous membranes especially of the mouth and eyes. It is one of the most common human viral infections with a life-time latency period; therefore,most of the infected persons are not aware of their infection. Complications among adults are blindness, encephalitis and aseptic meningitis. Stability of HSV-1 in water, saliva droplets as well as wet surfaces was reported.
Objectives*:* 1) to study HSV-1 stability and infectivity in collected water samples from Red Sea (RS), Nile River (NR) and pool (P) which have different physiochemical properties, 2) to determine the chlorine concentration needed to inactivate the virus.
Methods: physio-chemical properties and heavy metals contents of water samples were analyzed according to the standard methods recommended by the American Public Health Association (APHA). Plaque infectivity assay was used to test changes in viral count (infectivity) upon being inoculated into different types of water or at different chlorine concentrations at the chosen time points.
Results: the virus remained infectious for up to 1 week both in the RS and NR water although the very high salt concentration in RS compared to NR which was not the case with the P water that was attributed to its chlorine content. Also, exogenous addition of chlorine at concentrations 0.5, 1 and 2 ppm highly reduced the percentage of the recorded viral counts, yet, it did not completely abrogate viral infectivity as demonstrated by the still detected viral plaques. Further increasing chlorine concentration to 3 or 4 ppm completely arrested viral infectivity.
Conclusion*:* 1) both RS and RN water can support transmission of HSV-1 during the first week of its shedding into water irrespective of their different physical properties and salt concentrations, 2)Chlorine should be exogenously added to pool water at concentration 3-4 ppm to ensure complete disinfection of HSV-1 particles.

Keywords: River Nile water, Sea water, Pool water, Chlorine concentration, and HSV-1 infection.

BACKGROUND

Herpes Simplex Virus (HSV) is a double-stranded DNA virus which is classified as an α-member of the family Herpesviridae (Liesegang *et al.*, 1989, Farooq and Shukla; 2012). Two kinds of HSV infection were identified including HSV-1 (The studied virus in the present work) and HSV-2 (Sauerbrei *et al.*, 2011). It is a widely spread virus where primary infection occur via direct contact of the virus with mucous membranes of the host (Liesegang *et al.*, 1989, Farooq and Shukla; 2012). The most common sites of infection are the mouth and eyes (Darougar *et al.*, 1985). Also it can enter through wounds anywhere in the body (Corey and Spear; 1986). In very young children, and rarely in adults, the brain may also become infected (Darougar *et al.*, 1985).

HSV infection is increasing worldwide (Karad and Khade; 2007). It is one of the most common human infections with a life-time latency period (Sauerbrei *et al.*, 2011). Most infected persons have no knowledge about their infection. Complications among adults are blindness, encephalitis and aseptic meningitis (Bradley *et al.*, 2014).In developed countries, HSV infections of the eye are the leading cause of infectious corneal blindness (Darougar *et al.*,

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1985). In the US around 500,000 are currently infected with ocular HSV (Farooq and Shukla; 2012, Lairson *et al.*, 2003).

Herpes simplex encephalitis is a potentially fatal disease (Whitley *et al*., 1977). The mortality and morbidity rates remain high, even when an early diagnosis is made based on the detection of the HSV in the cerebrospinal fluid by PCR, and despite the advent of acyclovir therapy (Raschilas *et al.*, 2002). HSV-1 initially infects epithelial cells as a lytic infection, and then enters peripheral neurons where it establishes latency (Knipe and Cliffe; 2008, Nicoll *et al.*, 2012).

HSV-1 seroprevalence has been reported to be more than 90% in many countries (Bernstein *et al.*, 2013). During the last two decades, this infection affected from 50->85% of German, Spanish and Norwegian population (Sauerbrei *et al.*, 2006). However, based on the National Health and Nutrition Examination Survey (NHANES), there was a reduction in the seroprevalence of HSV-1 (53.9%) during 2005-2010 (Bradley *et al.*, 2014).

Water surfaces can serve as reservoirs for different types of viruses. What directly come in mind are the enteric viruses for the facts that over 100 types of pathogenic viruses are excreted in human and animal wastes (Melnick; 1984). These viruses can be transported in the environment through groundwater, seawater, rivers, insufficiently treated water or drinking water (Bitton *et al.*, 1984, Sobsey *et al.*, 1986, Lee *et al.*, 2002, Lipp *et al.*, 2002). But different reports observed presence of viruses other than enteric viruses in water such as avian influenza viruses (Justin *et al*.**,** 2009**).** A very few reports studied HSV stability in water. A group of researchers examined the stability of HSV both in saliva and in tap water and showed that there is no loss of virus infectivity in both types of liquids so far as the saliva or water drops did not dry within, 2 hours, the experiment duration (Bardell; 1993). Others showed that the HSV can survive up to 4 hours in tap water and 24 hours in distilled water and correlated it to the low chlorine percentage present in tap water (Nerurkar *et al.*, 1983).

Since Herpes simplex viruses are transmitted during close personal contact through the exchange of virus-containing secretions like vesicle fluid from active lesions or saliva, it can also be shed into the environment from infected swimmers or people working in contact with different water types.

Therefore the present work aimed to study HSV-1 stability and infectivity in collected water samples from Red Sea (RS), Nile River (NR) and pool (P) which have different physiochemical properties, 2) to determine the chlorine concentration needed to inactivate the virus.

MATERIALS AND METHODS

Sample collection

Water samples were collected from the Red Sea shore in Sharm El-Sheikh, South Sinai Governorate, a swimming pool located in a resort in the same area and the Nile River in Imbaba District, Giza Governorate. The used dry containers were prewashed with water from each water source before being filled with 2 liters of each water type.

Virus

Stock of the HSV-1 was prepared by propagating the virus to a final viral count of $1x10^7$ plaque forming unit (PFU)/ ml.

Cells

Green monkey kidney cells (Vero; ATCC) was used as host cells for viral propagation. The cells were propagated in DMEM medium (Lonza, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% antibiotic–antimycotic mixture (Lonza).

Physico-chemical Water analysis

This included determinations of pH, turbidity, electric conductivity (EC), total dissolved solids (TDS), total hardness, calcium and magnesium hardness, calcium, magnesium, chloride, sulfate, ammonia, nitrate, nitrite, total phosphorus, silica, iron and manganese using the standard methods recommended by the American Public Health Association (APHA) in 2005.

Heavy metals analysis

Presence of heavy metals was recorded in Red sea, River Nile and Pool water as they might reach different water types from any source of environmental pollution. The reason of studying their levels in various types of water is that we wanted to check or to exclude if they play any role in HSV-1 persitance. Atomic absorption spectrometer Varian SpectrAA (220) with a graphite furnace SpectrAA GTA (110) was used for the determination of trace levels of environmentally sensitive elements including metals. All samples were digested with the analytical method (APHA; 2012). For each series of measurements intensity calibration curve was constructed composed of a blank and three or more standards from Merck (Germany). Accuracy and precision of metals measurements were confirmed using external reference standards from Merck, a standard reference material and quality control sample from the National Institute of Standards and Technology (NIST) to confirm the instrument reading.

Persistence of HSV-1 in Red Sea, River Nile and Pool water

A volume of 10 ml of the three water types was sterilized by filtration through sterile 0.2 µm nitrocellulose membrane. A volume of 100 µl of 10^{-2} dilution of the virus stock was inoculated in each water type or in cell culture medium that was used as a control followed by continuous shaking for 30 seconds. Tubes were kept 4 weeks at room temperature and a volume of 100 µl was taken from the diluted virus in each water type at 15, 30 min, 1, 3, 6, 24 hours, 4 days, 1, 2, 3, 4 weeks and mixed with 900 µl of the complete DMEM medium.

Determination of optimum chlorine concentration

Stock chlorine was diluted in sterile filtered de-ionized water to final concentrations of 0.5, 1, 2, 3, 4 ppm. A volume of 100 µl of 10^{-2} dilution of the HSV-1 stock was inoculated into individual water tubes at each chlorine concentration followed by continuous shaking for 30 seconds. Tubes were left at room temperature and 100 µl was taken at 15, 30 min, 1, 3, 6, 24 hours from each tube and added to 900 µl of complete DMEM.

Monitoring viral infectivity using plaque count assay

Plaque count assay (Tebas *et al.,* 1995) is a sensitive method to monitor changes in viral infectivity which lead to change in number of plaques caused by the virus in mammalian cells. The assay was performed in a 12 Well plate where Vero cells (10^5 cells/ ml) were seeded for 1 day at 37^{o}C prior to infection. Growth medium was removed from the cell culture plates and cells were washed once with 1X PBS before being inoculated with 50µl of media containing virus. After 2 h contact time for virus adsorption, 1 ml of DMEM supplemented with 2% agarose was added onto the cell monolayer in each well and plates were left to solidify at room temperature then incubated at 37^{o}C till formation of viral plaques. For visualization of the formed plaques, infected cells were fixed by 10% formaldehyde for 1 h, stained with crystal violet for 20 minutes and destained with water till clear plaques could be seen in contrast to a dark blue background. Control wells were included where untreated virus was used to infect Vero cells and finally plaques were developed and counted as above mentioned.

Data management and statistical analysis

Statistical analysis and plots were done using the GraphPad PRISM version 5 software. In each plot the columns represent the means of three experimental replicates and the error bars represent the standard deviation between the results of the three experiments. Means, standard deviations and degree of significance were calculated using the unpaired data comparison application of the student t test included in the software. Differences were considered significant when p-values were <0.05.

The number of asterisks represents the degree of significance where * means P<0.05, ** means P<0.005 while *** P<0.0005 while "ns" means none significant.

RESULTS

Both physico-chemical properties and presence of heavy metals were analyzed in the RS, NR and P water to investigate if difference in composition of the three types of water might influence HSV-1 infectivity as measured by plaque infectivity assay. In addition, plaque reduction assay was used to determine the optimum concentration of chlorine to be added to pool water to completely suppress HSV-1 infectivity.

Table 1 showed the physico-chemical properties of the RS, NR, and P water in terms of pH, turbidity, color, odor, color, conductivity and inorganic salts were conducted according to APHA 2015. The pH values of the three types of water are slightly alkaline. pH of River and Nile water were nearly the same a little higher than pool water. Turbidity values revealed that River Nile water have highest value (due to the suspended matter) than Red Sea water and Pool water. The main difference between the three types of water was detected in total dissolved solids. The Red sea water had the highest total dissolved solids (due to the chloride ion).

Turbidity appears slightly higher in Nile water for the presence of sediments, total hardness, calcium hardness and magnesium hardness together with calcium, magnesium, chloride and sulphate concentration were obviously higher in sea water followed by pool then Nile water

Table 2 showed that all heavy metals (Mn, As, Zn, Cd, Pb, Ni, Cr, and Cu) are in the lowest detectable limits except for iron in River Nile water sample (0.82mg/L), also sodium was found with high concentration in sea water followed by pool water and Nile water was the third.

The kinetics of the HSV-1 persistence in the RS, NR and P water is presented in Figure 1. Results showed that upon co-incubating the virus with the pool water for periods ranging from 15-60 minutes the obtained plaque counts were comparable to the counts of the primary inoculums (Figure 1). In addition, incubating the virus with pool water for 3-6 hours resulted in ~50% drop in the plaque counts compared to the control counts (Figure 1). However, starting from 1 day co-incubation time of the virus with the pool water a sharp drop in the plaque counts was recorded. Yet, detectable virus persisted until 1 week co-incubation time (Figure 1). Moreover, two weeks co-incubation of the virus with the pool water resulted in complete abrogation of the virus infectivity (Figure 1). Of note, co-incubation of the virus with water from either the Red Sea or the River Nile for different time points till 4 days did not result in significant drop in the virus count in comparison to the primary virus inoculums (Figure 1). At 1 week co-incubation period with either the sea or the river water a significant drop in the viral count was recorded. Yet, the recorded viral counts in both cases were significantly higher in the case of co-incubation with the pool water (Figure 1). This is due to the traces of chlorine still present in pool water. At later co-incubation of the virus with the 3 water sources for 2, 3 and 4

weeks no virus plaques could be visualized (results of week 2 was shown in Figure 1, figures showing results of weeks 3 and 4 are not shown as they are just the same as that of week 2). Each data column represents the mean three experimental replicates and the error bars represent the standard deviations between the three experimental replicates.

Table (1): Physicochemical properties of the water samples collected from the Red Sea, the Nile and a swimming pool

Parameters/	**Unit**			
		Red Sea	**Nile River**	**Pool**
pH		8	8.2	7.5
Turbidity	NTU	1	3.3	0.9
Odor		Odorless	Odorless	Odorless
Color	Co/Pt Unite	Colorless	Colorless	Colorless
Electric Conductivity	μmohs/Cm	54300	445	3780
Total Dissolved Solids	mg/L	28900	288	2030
Total Hardness (as $CaCO_3$)	mg/L	10400	190	830
$CaCO_3$ Hardness	mg/L	1800	110	750
$MgCO_3$ Hardness	mg/L	8600	80	80
Calcium	mg/L	720	44	300
Magnesium	mg/L	2064	10.6	19.2
Chloride	$mgCl_2/L$	24000	40	1400
Sulfate	$mgSO_4/L$	2695	28	42
Ammonia	$mgNH_3/L$	0.0	0.0	0.0
Nitrite	mg NO_2/L	0.0	0.0	0.0
Nitrate	mg NO_3/L	0.12	0.1	0.1
Iron	mg/L	0.37	0.82	0.12
Manganese	mg/L	0.0	0.21	0.0
Residual chlorine	mg/L			0.1

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Table (2): Heavy metals concentrations in the water samples collected from the Red Sea, the Nile and the swimming pool.

Heavy metal	**Red Sea**	**River Nile**	**Pool**
As μg/L	<0.001	<0.001	<0.001
Zn μg/L	<0.005	<0.005	<0.005
Cd μg/L	<0.001	<0.001	<0.001
Pb μg/L	<0.001	<0.001	<0.001
Ni μg/L	<0.001	<0.001	<0.001
Cr μg/L	<0.001	<0.001	<0.001
Cu μg/L	<0.01	<0.01	<0.01
Na mg/L	$2x10^4$	52	700

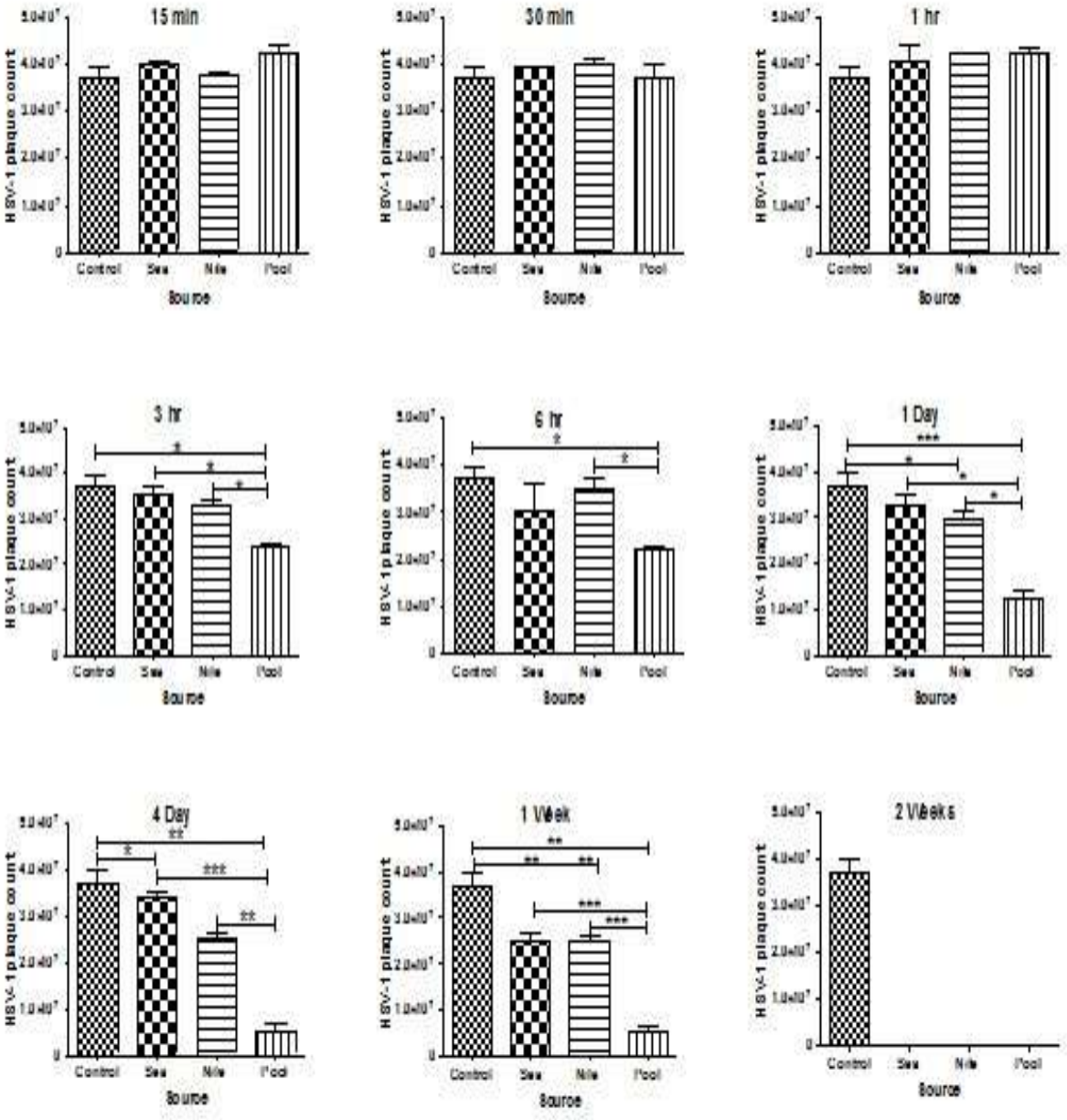

Fig. 1: Persistence of HSV-1 in the RS, RN and P water. The number of asterisks represents the degree of significance were * means P<0.05, ** means P<0.005 while *** P<0.0005.

The kinetics suppression of the viral infectivity using serial concentrations of chlorine is illustrated in Figure 2. Co-incubation of the virus with serial concentration of chlorine water for various time points caused a gradual significant reduction in the recorded viral count in plaque infectivity assay in comparison to control mock-treated virus. Results showed that the top reduction in the plaque counts was recorded at chlorine concentrations 3 and 4 ppm where almost no virus plaques could be visualized starting from 3 hours incubation time. At lower chlorine concentrations (0.5-2 ppm) the virus persisted infectious and caused formation of plaques at all-time points. The top infectivity was recorded at chlorine concentration of 0.5 ppm. The error bars represent means of three experimental replicates.

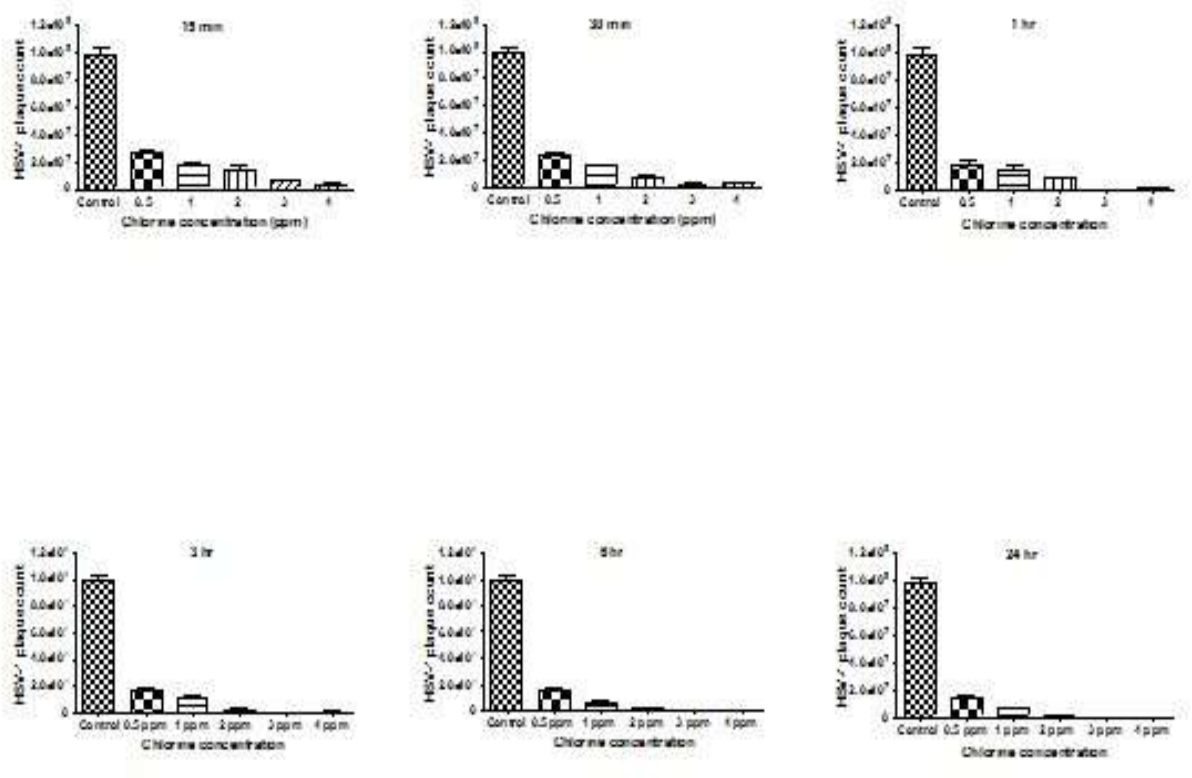

Fig. 2: Effect of chlorine on infectivity of the HSV-1 virus. Co-incubation of the virus with serial concentration of chlorine water for various time points caused a gradual significant reduction in the recorded viral count in plaque infectivity assay in comparison to control mock-treated virus. The top reduction in the plaque counts was recorded at chlorine concentrations 3 and 4 ppm where almost no virus plaques could be visualized starting from 3 hours incubation time, whereas, at lower chlorine concentrations (0.5-2 ppm) the virus persisted infectious and caused formation of plaques at all-time points and the top infectivity was recorded at chlorine concentration of 0.5 ppm. The error bars represent standard deviation between results of three experimental replicates.

DISCUSSION

Testing how long HSV-1 can stay infectious outside the body was recorded by number of scientists. Epstein *et al.,* (1993), were able to recover infectious HSV virions for up to two hours from door handles that were inoculated with HSV-1 in saliva or water. Bardell, (1993) also recorded the stability of HSV-1 in saliva and tap water. They found that the marked drop in titre coincided with drying of the saliva and that there was no decline in titer of infectious HSV-1 in a humid atmosphere in which the saliva remained liquid during the two hours of the experiment. HSV-1 has also been shown to survive in a patient's dental chart for several hours (Thomas *et al.,* 1985). This research is experimenting the stability of HSV-1 in different water types that might be a source of human infection. The capability of the virus to persist being infectious and to resist drop in its titer in comparison to the primary virus inoculums upon co-incubation with water samples from either the Red Sea or the River Nile for up to 4 days. This opens the question of human infection by the virus through both types of water. There is potentiality of the virus to infect aquatic organisms in both habitats. This was further confirmed by the significantly

recorded higher HSV-1 titers at 1 week post co-incubation of the virus with either the sea or the river water samples in comparison to the recorded viral titer in the pool water at the same time point (Figure 1).

Although the different values for the physico-chemical properties in terms of pH, turbidity, color, odor, conductivity and the high inorganic salts concentration in the Red Sea water compared to the low values recorded in that of the River Nile; the HSV-1 virus could persist effectively infectious in both types of water for up to one week suggesting that such physicochemical parameters do not represent rate limiting factor in the virus infectivity (Table 1 and Figure 1).

Chlorine is the widely used disinfectant to pool water. Optimum concentration was thought to be 2ppm to avoid irritation of eyes and skin (King, 1948) and according to U.S environmental protection agency (EPA) it should never be over 4 ppm (USEPA, 1999). Although time needed to get rid of pathogen infectivity in pool water was reported for number of pathogens like E.coli (less than 1 minute), Hepatitis A virus (16 minute), Giardia (45 minute) and Cryptosporidium (10.6 days) by the American National Standard for water quality in public pool and spa (ANS, 2009), the same was not considered for HSV-1. Moreover Orgradi and his colleagues were able to detect infectious HSV-1 from dental instruments after disinfection (Ongradi *et al.*, 1993) and so the optimum conditions required to get rid of the HSV-1 need to be further studied.

The persistence of virus infectivity at post co-incubation of the HSV-1 with chlorine water at concentrations 0.5-2 ppm reflects the necessity to use higher chlorine concentrations to guarantee appropriate disinfection of water against this viral infection. This was confirmed by the complete abrogation of the viral infectivity upon co-incubating the virus at chlorine concentrations 3-4 ppm for longer periods than 3 hours.

Although the significant drop in the HSV-1 count upon 1 day co-incubation with the pool water, nevertheless, the virus persisted infectious until 1 week co-incubation time which represents an alarming ring regarding the possibility of the virus to persist for longer time and to be transmitted among swimmers.

CONCLUSION

We can say that Sea and Nile water can play a role in viral transmission whether among swimmers or people working in direct contact with water. Pool water can do the same if chlorine was not added in the optimum concentration. Contact with water should be avoided in case you have HSV-1 infection and in case of having wounds anywhere in the body in order not to get infection.

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