Inactivation of rotavirus in water by copper pot

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ABSTRACT

The objective of this study was to evaluate the use of copper pots for inactivation of rotavirus present in water. Distilled water was inoculated with rotavirus (2.2×10^7 plaque-forming units (PFU)/mL) and stored overnight (16 hours) at room temperature in copper pots (test) and in glass bottles (controls). The viable count of infectious virus was tested using a plaque assay on MA 104 monkey kidney cell lines. No plaques were recovered from the water stored in copper pots. On the other hand, over 10^6 PFU/mL of virus was recovered from water stored in controls. The copper leached into the water was at a concentration of 447.25 ± 4.78 ppb, which is well within the safety limits prescribed by the World Health Organization (2,000 ppb). The copper pot has the potential to be used as a point-of-use household water purification system, especially against waterborne pathogens such as rotavirus, which is the cause of 22% of diarrhoea hospitalizations in children less than 5 years of age in developing countries.

Key words | ayurveda, copper, diarrhoea, rotavirus, water

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INTRODUCTION

Diarrhoea caused by rotavirus accounts for about 22% of hospitalizations in children worldwide (Parashar *et al.* 2006). Rotavirus is an important cause of morbidity in developed countries accounting for about 55,000 hospitalizations in the USA (Malek *et al.* 2010) and more than 10,000 deaths in children under 5 years of age each year in the World Health Organization (WHO) European Region (www.euro.who.int/en/what-we-do/health-topics/diseases-and-conditions/rotavirus). However, the incidence of rotaviral infection is highest (80%) in developing countries of Asia and Africa. Annually, approximately, 527,000 diarrhoeal deaths are caused by rotavirus and over 23% of these occur in India (Parashar *et al.* 2009). Added to this is the economic burden that is associated with the disease which is a major challenge for developing countries such as India where up to US$72 million is spent annually in medical costs to treat rotavirus diarrhoea (Tate *et al.* 2009).

Infection with rotavirus causes fever, diarrhoea and emesis leading to rapid dehydration (Rose *et al.* 2009). Oral rehydration therapy, which is recommended to be used to replace loss of body fluids, is difficult to administer in children with severe vomiting and there are no specific drugs to treat rotavirus infection (Parashar *et al.* 2006).

Many diarrhoeal diseases in developing countries are transmitted through contaminated drinking water and through contact with infected persons (Ashbolt 2004). In developing countries such as India, drinking water is commonly obtained from sources such as open wells and bore wells, and can easily be contaminated by unhygienic practices, socio-cultural factors and during transport (Banda *et al.* 2007). Simple, cost-effective, point-of-use methods are needed to treat drinking water to prevent waterborne disease (Mintz *et al.* 2001).

Ayurveda, the ancient Indian medical system, recommends the use of storage containers made of metals such as gold, copper and silver. It advises that water stored in such containers overnight will impart health benefits (Sharma 2004). We have previously reported the

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use of copper pots and a copper device for storing and improving the microbial quality of water. Enteric pathogenic bacteria such as *Vibrio cholerae*, *Salmonella* Typhi and *Escherichia coli* were not recovered from water after storing in a copper pot (Sudha *et al.* 2009). In this study we report the effect of copper pot on rotavirus present in water.

MATERIALS AND METHODS

Virus strains and cells

Simian rotavirus strain SA11 adapted to culture on African green monkey kidney epithelial cells (MA104 cells) at the Wellcome Trust Research Laboratory, CMC, Vellore, was used for all experiments in this study. MA104 cells were used for plaque titration experiments.

MA104 cell culture for plaque assay

Seven-day-old culture of MA104 cells in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) in 150 cm^2 tissue culture flasks were examined microscopically to verify the healthy condition of the cells. The medium was decanted from the flasks and the monolayer was rinsed twice with 20 mL of trypsin diluent. After decanting the last wash, 3 mL of 0.25% trypsin EDTA (Gibco, USA) was added and flasks were incubated at 37 °C in the presence of 5% CO_2 for 3–4 min. The flask was checked to determine if the monolayer was beginning to peel from the plastic and gently tapped to allow the cells to flow to the bottom of the flask. The cells were resuspended in 7 mL of growth medium to obtain a final volume of 10 mL. The suspension was mixed thoroughly to ensure that no clumps of cells were visible. An additional 150 mL of growth medium was then added to the flask and the suspension was mixed. Cluster 6 plates (Corning, USA) were used for plaque titration. About 3 mL of the cell suspension was added to each well for preparation of the monolayer for infection. Each tissue culture flask (150 cm^2) could be used for the preparation of six Cluster 6 plates using approximately 20 mL of cells per plate. The plates were incubated at 37 °C, 5% CO_2 for about 3 days till a confluent monolayer could be seen on microscopic examination.

Preparation of virus stock

Four-to-five-day-old 150 cm^2 culture flasks with confluent monolayers of MA104 cells were selected for culturing rotavirus strain SA11. The flasks were washed twice with Earle's Balanced Salt Solution with calcium (Sigma, USA) using 10 mL per wash per flask. The last wash was decanted and the flasks were infected with 3 mL of SA11 that had been trypsinized with 10 μg/mL of trypsin (Worthington, USA) for 30 minutes. The flasks were placed on a rocker for 1–2 h at 37 °C to absorb virus. Each flask was then overlaid with 15–20 mL supplemented media containing 10 μg/mL trypsin and incubated at 37 °C in the presence of 5% CO_2. The flasks were observed each day microscopically for cytopathic effect. Once the cytopathic effect was evident, lysates from all flasks were pooled and frozen at −20 °C. This was used as stock SA11 for this experiment.

Study design

Copper pots of 2 L capacity with a surface area of approximately 750 cm^2 (for tests) were purchased from a kitchenware shop in Bangalore, Karnataka, India. The copper pots were thoroughly cleaned each time before use with tamarind and salt as traditionally done in Indian homes. After cleaning, the pots were thoroughly rinsed with distilled water before autoclaving to ensure that there was no remnant of tamarind or salt in the rinsed pot. Glass bottles (Schott Duran, Mainz, Germany) of 1 L capacity with a surface area of approximately 518 cm^2 were used as controls. The test and control containers were sterilized by autoclaving before use.

One litre water samples were seeded with 10 mL of stock virus to obtain a concentration of 2.25×10^7 plaque-forming units (PFU)/mL of rotavirus SA11. Virus was concentrated from the water sample using granular activated charcoal (GAC) prior to treatment and at the end of 16 hours. The viral count in the water samples was estimated by plaque titration as described below. All experiments were carried out in duplicate and the results averaged.

Virus concentration from water

The virus in the water samples was concentrated using GAC according to Jothikumar *et al.* (1995) prior to experiment (0 hour) and at the end of 16 hours after exposure to copper pots. A 500 mL water sample was concentrated at each point. Briefly, water seeded with the virus was passed through a GAC column. The collected water was discarded and 50 mL of urea arginine phosphate buffer (UAPB) was then passed through the column. The eluate was collected and 0.5 mL of 1 M $MgCl_2$ was added, mixed well and centrifuged for 30 minutes at room temperature at 5,000 *g*. After centrifugation, the supernatant was discarded and the pellet resuspended in 4 mL of McIlvaine's buffer. The pH was neutralized with 8% sodium bicarbonate. Aliquots of the eluate thus obtained were frozen at −70 °C. This process was repeated for each water sample used in the study.

A series of 10-fold dilutions of the concentrated samples were made in supplemented media containing 1 mL of antibiotic-antimycotic solution (Gibco) and 1 mL of glutamine solution (Gibco) per 100 mL of DMEM for plaque titration.

Plaque titration of rotavirus

The virus was allowed to adsorb to the cells for 60 minutes at 37 °C, 5% CO_2. The plates were rocked once every 15 minutes to allow uniform spread of virus. At the end of 1 h, the inoculum was discarded into a tray containing disinfectant solution (Dakin's solution) and 2.5 mL of high antibiotic agarose overlay (SeaKem LE Agarose, lonza) was added to each well along the walls of the well. The overlay was allowed to set before incubating the plates at 37 °C and 5% CO_2 for 2–3 days. Inoculation of cells was used for inoculation with 200 μL of supplemented media serving as negative control.

After 2–3 days incubation, the plates were carefully removed from the incubator and examined for plaques. The agarose overlay was removed from each plate by shaking off into a pan containing hypochlorite disinfectant solution. The cells were stained with 1 mL of crystal violet (Sigma) stain (0.5%) such that the monolayer is completely covered with a thin layer of stain. The plates were kept at room temperature for about 6–7 min. The stain was removed and the wells were gently rinsed with 500 μL of distilled water. The water was discarded and the number of plaques was counted over a white light box (Bangalore Genei, Bangalore).

The assay was considered valid if the monolayer was intact in negative control wells and distinct plaques could be seen in infected wells. Wells with 5–50 plaques were counted for calculation of virus titre. Since 200 μL of inoculum was used in the experiments, the number of infectious virus particles/mL was calculated as five times the number of plaques multiplied with the dilution factor.

Estimation of copper content and pH of the water sample

The pH and copper content of the water samples were measured before and after storing water in a copper pot. The pH was determined using a pH meter (DI 707; Digisun Electronics, Hyderabad, India) while copper was measured using a commercially available, ready to use kit (Spectroquant™, Merck, Darmstadt, Germany) according to manufacturer's instructions. Distilled water was used as negative control and copper solutions prepared using copper turnings as per Bureau of Indian Standards (BIS 2005) were used as positive controls.

OBSERVATION AND RESULTS

The initial viral count in inoculated water at 0 hour was 2.25×10^7 PFU/mL. The pH of the water was 7.02 ± 0 and no copper could be detected initially. After treatment of water for 16 h in the copper pot, no virus could be recovered in the plaque assay which indicated a more than 7 log reduction of the viral count (Table 1).

Virus detected from the controls (glass bottles) was 8×10^6 PFU/mL. The results indicate that treatment of water for 16 hours in a copper pot could result in complete

Table 1 | Effect of overnight storage of water inoculated with rotavirus in copper pot and in control glass bottle

Time (hours)	Viral counts (PFU/mL) Control	Test
0 hours	2.25×10^7	2.25×10^7
16 hours	8×10^6	Not detected

Table 2 | Average copper content and pH of water stored overnight in copper pots

Before storage		After 16 h of storage in copper pot		After 16 h of storage in glass bottle	
pH	Copper content (ppb)	pH	Copper content (ppb)	pH	Copper content (ppb)
7.02	Not detected	8.76 ± 0.03	447.25 ± 4.78	8.5 ± 0.2	Not detected

inactivation of rotavirus. Copper content and pH of the water in the tests after 16 hours was 447.25 ± 4.78 ppb and 8.76 ± 0.03 respectively. There was no copper detected in the control samples and the pH after 16 hours storage in the control glass bottle was 8.5 ± 0.2 (Table 2).

CONCLUSION

Our study has demonstrated for the first time that copper inactivates rotavirus present in water. Copper which leached into the water in tests (~448 ppb) was well within WHO (1993) safety limits (2,000 ppb). We chose to test the copper container in reagent grade test water to know if the effect is only due to copper or other factors/chemicals present in water. This would give conclusive results on the effect of copper alone. However, the effect of copper on bacteria in natural waters, possibly containing chelating agents still needs to be tested.

Copper has been shown to be active on a number of bacteria and viruses (Borkow & Gabbay 2004). Polio virus showed more than 4 log reduction in the presence of copper and silver ions in combination with free chlorine (Abad *et al*. 1994). Copper has been shown to inactivate Junin virus (Sagripanti 1992), HIV (Sagripanti & Lightfoote 1996), herpes simplex virus (Sagripanti *et al*. 1997), and influenza A virus (Noyce *et al*. 2007).

As preventive measures, vaccines have been developed for rotaviral infection but there are several problems associated with this, such as cost and the emergence of new strains (Gentsch *et al*. 2009). Many earlier vaccines, such as live oral vaccines for polio and cholera, have performed less well than expected in developing countries (Lahariya 2009; Rose *et al*. 2009).Therefore preventive measures against diarrhoea are essential and more feasible.

In India, approximately, 72.2% of rural populations consume untreated water (Gopal *et al*. 2009) owing to various reasons including taste (in the case of chlorine) or cost (Banda *et al*. 2007). Studies have shown that point-of-use (household) water purification is best suited for reduction of waterborne diarrhoea (Mintz *et al*. 2001). Copper pots can be used as household microbial water purifiers that are easy to use, simple and do not need fuel or electricity.

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