Traditional copper water storage vessels and sub-lethal injury of *Salmonella enterica* serovar Typhi and *Vibrio cholerae*

Riti Sharan, Sanjay Chhibber and Robert H. Reed

ABSTRACT

Recent studies on *Escherichia coli* have demonstrated sub-lethal injury–sensitivity to oxygen and selective agents prior to irreversible inactivation when kept in water in a brass vessel. The present study was carried out to investigate whether equivalent responses occur in copper vessels using the pathogens *Salmonella enterica* serovar Typhi and *Vibrio cholerae*. Bacterial suspensions were stored in water in a traditional copper vessel for up to 24 h at 30 °C. Samples were withdrawn and plated on selective and non-selective media, then incubated under (a) aerobic conditions and (b) conditions where reactive oxygen species were neutralized to enumerate injured bacteria. Short-term incubation in water kept in a copper vessel caused a greater decrease in counts for both pathogens on selective media, compared to non-selective media with greater differences between aerobic and reactive oxygen species-neutralized counts using selective media compared to non-selective nutrient agar. These findings have practical implications for the short-term storage of water samples in copper storage vessel as the possibility of bacterial injury is high, hence enumeration under conventional aerobic conditions may not be sufficient to give a count of all viable bacteria.

Key words | copper, reactive oxygen species, *Salmonella enterica* serovar Typhi, sub-lethal injury, *Vibrio cholerae*

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INTRODUCTION

Over 21 million episodes of illness and more than 200,000 deaths occurred in 2,000 due to typhoid fever (Crump *et al.* 2004). The burden of typhoid has been underestimated in developing nations owing to poor disease reporting in private health care units (DeRoeck *et al.* 2007). In 2006, over 200,000 cholera cases including more than 6,000 deaths were reported from 52 countries by the World Health Organization (WHO 2007) with the incidence being most common in those less developed nations that are unable to sustain the infrastructure required to provide a clean, safe water supply. Provision of basic sanitation and hygiene has led to a decline in the incidence of these two diseases in the USA and Europe (Olsen *et al.* 2003) but they continue to be a serious health concern in developing nations.

Cholera, caused by *Vibrio cholerae*, is one of the major waterborne diseases prevalent in parts of the world such as Jakarta, Indonesia (Agtini *et al.* 2005), Kolkata, India (Sur *et al.* 2005) and Beira, Mozambique (Lucas *et al.* 2007). Cholera outbreaks have been reported from rural areas in India with unhygienic environmental conditions, inadequate sanitation and unsafe water supplies (Alam *et al.* 2007). Some experts consider West Bengal in India with neighbouring Bangladesh as the 'homeland of cholera' with the detection of the disease from March to November each year (National Institute of Cholera and Enteric Diseases 2002). In India, among the waterborne pathogenic bacteria prevalent in developing nations, *Salmonella* is of considerable significance, with *Salmonella* Typhi being the most common aetiological agent of enteric fever (Sur *et al.* 2007). A typhoid incidence rate of 274 per 100,000 people in Asia was reported in 2000 (Crump *et al.* 2004) and approximately 104 per 100,000 people in West Bengal,

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India (Sharma 2007). The prevalence of these pathogens in developing nations such as India makes it imperative to study their inactivation and sub-lethal injury upon storage in a traditional copper water storage vessel.

Boiling (Angulo *et al*. 1997), chlorination (Arnold & Colford 2007), solar disinfection (Khaengraeng & Reed 2005) and use of biosand filters (Clasen & Bastable 2003) are some of the commonly practised household-level water treatment methods. Besides point-of-use water treatment, water storage in traditional water storage vessels is also effective in reducing the incidence of diarrhoeal disease, and recently complete inactivation of *S.* Typhi and *V. cholerae* was reported from South India upon overnight storage of water in a copper pitcher (Sudha *et al*. 2009). Copper ions, either alone or in copper complexes, have been used to disinfect liquids, solids and human tissue (Airey & Verran 2007). Today copper is used as a water purifier (Huang *et al*. 2008), algaecide (Villada *et al*. 2004), fungicide (Gharieb 2002), nematocide (McIntosh *et al*. 2007), molluscicide (Reddy *et al*. 2004), and as an anti-bacterial (Neel *et al*. 2005) and anti-fouling agent (Jones & Bolam 2007). Copper also displays potent anti-viral activity (Borkow & Gabbay 2009). Water storage in brass vessels caused a complete inactivation of the faecal indicator bacterium *Escherichia coli* within 48 h with lower counts on selective media than on non-selective media during shorter-term incubation (Tandon *et al*. 2005). Additionally, a recent study has also demonstrated that this effect is dependent on temperature and pH (Sharan *et al*. 2010).

There are several reports of *V. cholerae* entering a dormant or a viable-but-non-culturable state due to nutrient deprivation and/or stressful environmental conditions (e.g. Wong *et al*. 2004; Oliver 2005). However, an alternative explanation involves sub-lethal injury, as demonstrated for *Salmonella typhimurium* following thermal and non-thermal food preservation procedures, where injury was demonstrated by plating on different selective media such as tryptic soy agar and violet red bile glucose agar, with the latter giving lower counts (Wuytack *et al*. 2003). It has also been shown that a burst of intracellular free radicals produced by sub-lethally injured *E. coli* growing by respiratory metabolism under aerobic conditions causes irreversible inactivation of such cells, preventing their enumeration under conventional aerobic conditions (Dodd *et al*. 2007). Consequently, the use of conditions in which reactive-oxygen species are neutralized together with non-selective media, rather than standard aerobic incubation conditions and conventional selective media, has proved beneficial in the enumeration of sub-lethally injured *E. coli* and *Enterococcus faecalis* (Tandon *et al*. 2005, 2007a).

The present study was conducted to investigate the dynamics of inactivation of the commonly prevalent waterborne pathogens *S.* Typhi and *V. cholerae* during storage in water kept in a copper vessel, since any sub-lethal injury would have significance in relation to conventional enumeration procedures. Studies on inactivation and sub-lethal injury of *E. coli* and *E. faecalis* have been conducted previously (Tandon *et al*. 2005, 2007a). The authors have looked for similar effects in the more potent pathogens of the developing nations, such as India, in this study.

MATERIALS AND METHODS

Bacterial cultures

S. Typhi 733 and *V. cholerae* 3,906 strains were procured from Institute of Microbial Technology, Chandigarh. *V. cholerae* O1 and *S.* Typhi Ty2 strains were obtained from the Central Research Institute, Kasauli, India. The bacterial strains used in the present study were attenuated to negate the risk of transmission of disease. The study was conducted at Punjab University, India. Bacterial cultures for experiments were prepared by loop inoculation of a single colony of the strain to be studied into 100 ml of nutrient broth. This was followed by overnight incubation at 37 °C without shaking. The broth culture was centrifuged at $5{,}300 \times g$ for 5 min to pellet the cells. The centrifuge used was designed to prevent leakage and placed in a well-ventilated room with exhaust filters. The supernatant was discarded and the pellet was washed twice with sterile 0.85% NaCl to remove any traces of the broth. The pellet was finally suspended in sterile distilled water (adjusted to pH 7.0 for *S.* Typhi, 7.5 for *V. cholerae* using 0.01 mol l^{-1} HEPES/NaOH) to give a final dilution of 1:100. One hundred millilitres of broth was added to 10 l of distilled water. This was added to a single storage vessel to give an initial count of approximately 6×10^6 CFU (colony-forming units) ml^{-1}.

Hand gloves, face masks and head caps were used at all times while handling the bacterial strains. Additionally, all the experimental procedures were conducted in a biosafety cabinet containing high efficiency particulate air filters.

Storage vessel and water source

All experiments were conducted using sterile distilled water adjusted to appropriate pH for different organisms. Copper storage vessels were obtained locally in Chandigarh; they were narrow-mouthed, and 12 l in capacity with a surface area of 1,327.7 cm^2. The experimental vessels were washed with sterile distilled water followed by thorough scrubbing with a non-abrasive surface to remove any biofilm formation or adherent dust/contamination from the inner surface. The vessels were then disinfected with 2% phenol (Qualigens, GlaxoSmithKline Pharmaceuticals Limited, Mumbai, India) for 3 h and again washed thoroughly three times with several litres of sterile distilled water each time before use. To prevent airborne contamination, the mouths of all the vessels were kept covered with sterile paper throughout the course of the experiment. Glass flasks of equivalent volume were used as controls.

Experimental procedures

Bacterial suspensions of each strain were stored in the copper and glass vessels at 30 °C for up to 24 h. Water samples were withdrawn from each vessel at 0, 2, 4, 6, 8, 10, 12 and 24 h, serially diluted to cover the dilution range 10^0–10^{-4}. Each dilution was surface plated in triplicate onto (i) deoxycholate citrate agar (DCA) with and without 0.05% sodium pyruvate (Stephens *et al*. 2000) for *S.* Typhi, (ii) thiosulphate citrate bile salt agar (TCBS) with and without 0.05% sodium pyruvate for *V. cholerae* and onto (iii) nutrient agar with and without 0.05% sodium pyruvate for *V. cholerae* and *S.* Typhi. Pyruvate-containing plates were incubated overnight in an anaerobic jar (Anaerobic System Mark II, HiMedia, Mumbai, India) containing an anaerobic gas pack (HiMedia, India) at 37 °C, to provide reactive oxygen species-neutralized (ROS-n) conditions during the first day of culture, followed by an additional 24 h period of aerobic incubation to allow all colonies to become large enough to be counted. Plates without sodium pyruvate were incubated overnight under conventional aerobic conditions at 37 °C. The colony count was expressed as CFU ml^{-1} by adjusting for volume and dilution. The minimum count in all the experiments was 1 CFU 60 μl^{-1} of undiluted sample, which is equivalent to 16.7 CFU ml^{-1}. The results were statistically compared and analysed using *t*-tests. Each experiment was repeated three times with each strain.

While the graphs represent a time scale of 0–12 h, it is important to realize that all experiments were conducted up to 24 h for water stored in copper and glass vessels. A 12 h time scale was used because there were no counts detected after 12 h in any of the samples from the copper (experimental) vessel under aerobic and ROS-n conditions. All such counts fell below the minimum detection limit indicated in all the graphs as the dotted trend line by 24 h and cannot be shown directly on the log graph. The aerobic and ROS-n counts of samples from the glass vessel remained consistent and did not show any significant change up to 24 h.

RESULTS

Figure 1 demonstrates the inactivation and sub-lethal injury of *S.* Typhi strains 733 and Ty2 upon storage in copper vessel and enumerated on nutrient agar (Figure 1(a) and (c)) and DCA (Figure 1(b) and (d)) at 30 °C, pH 7.0 under aerobic and ROS-n conditions. A visibly faster inactivation was observed on selective media (DCA) as compared to non-selective media (nutrient agar) for both strains of *S.* Typhi. While it took on an average 10 h for the aerobic and ROS-n counts to fall below the minimum detection limit when enumerated on nutrient agar, an apparent inactivation was observed after an average of 4 h on DCA with significantly higher differences between the aerobic and ROS-n counts on DCA ($p = 0.0001$). No significant change was observed in aerobic and ROS-n counts of either of the strains of *S.* Typhi on both selective and non-selective media when stored in glass vessels (Figure 1).

Both *V. cholerae* 3,906 and O1 demonstrated an apparent inactivation under aerobic and ROS-n conditions after 10 h of storage (i.e. counts were observed at 10 h but not at 12 h or at any longer time) when enumerated on nutrient agar (Figure 2(a) and (c)) but took only 6 h to fall below minimum

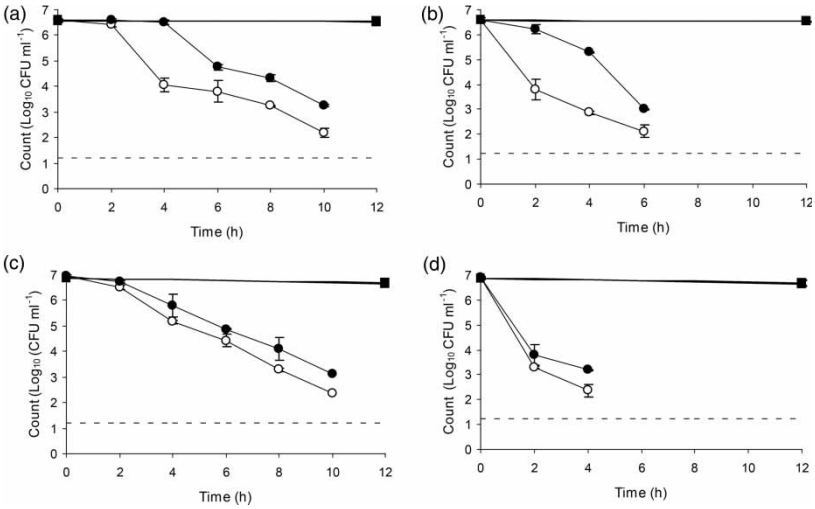

Figure 1 | Plate counts of *Salmonella* Typhi 733 on (a) nutrient agar (b) DCA and *Salmonella* Typhi Ty2 on (c) nutrient agar (d) DCA in distilled water at pH 7.0 stored in a copper vessel (circles) and a glass vessel (squares) at 30 °C and enumerated under aerobic conditions (open symbols) and ROS-n conditions (closed symbols). No counts were observed after 10 h of storage in copper vessel. Error bars represent 95% confidence limits ($n = 3$). The dotted horizontal line shows the detection limit ($\log_{10}$ CFU ml^{-1} = 1.22).

detection limit on TCBS agar (Figure 2(b) and (d)). A highly significant difference between aerobic and ROS-n counts was observed on nutrient agar plates at 10 h ($p = 0.001$ for *V. cholerae* 3,906; $p = 0.001$ for *V. cholerae* O1). On TCBS plates, the difference between aerobic and ROS-n counts became significant by 2 h ($p = 0.0001$ for *V. cholerae* 3,906; $p = 0.0001$ for *V. cholerae* O1). When compared to *S.* Typhi, *V. cholerae* demonstrated a larger difference between aerobic and ROS-n counts on both selective and non-selective media (Figure 2). No significant change was observed in both aerobic and ROS-n counts upon storage of these strains of *V. cholerae* in a glass vessel (Figure 2).

DISCUSSION

Brass and copper vessels have been used for storage of water and milk in India since ancient times (Tanner *et al.* 1983;

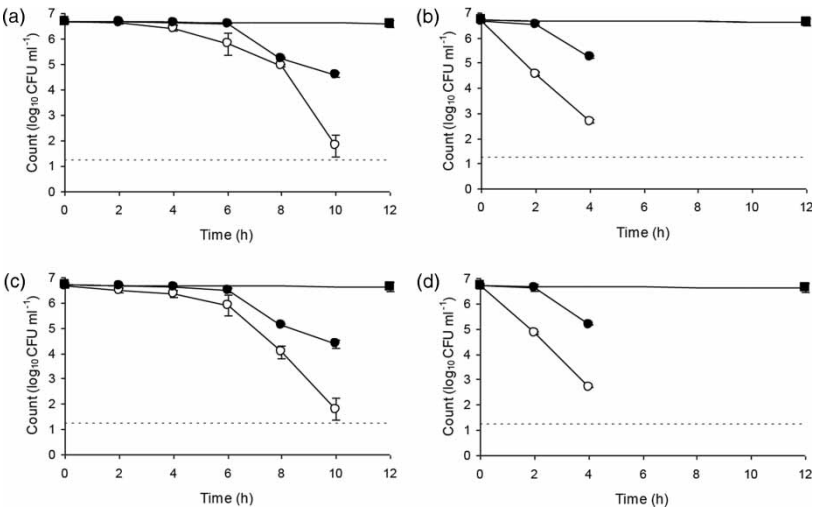

Figure 2 | Plate counts of *V. cholerae* 3,906 on (a) nutrient agar (b) TCBS agar and *V. cholerae* O1 on (c) nutrient agar (d) TCBS agar in distilled water at pH 7.5 stored in a copper vessel (circles) and a glass vessel (squares) at 30 °C and enumerated under aerobic conditions (open symbols) and ROS-n conditions (closed symbols). No counts were observed after 10 h of storage in copper vessel. Error bars represent 95% confidence limits ($n = 3$). The dotted horizontal line shows the detection limit ($\log_{10}$ CFU ml^{-1} = 1.22).

Bhave *et al*. 1987; Radhika & Balasubramanian 1990). Recent studies have clearly demonstrated the antibacterial effects of brass vessels in inactivating bacteria such as *E. coli* and *E. faecalis*, while also showing the damaging effects of reactive oxygen species (ROS) on enumeration of sub-lethally injured bacteria (Tandon *et al*. 2005, 2007a). The present study has demonstrated the negative effects of using conventional aerobic conditions and selective media for the enumeration of copper-stressed *S.* Typhi and *V. cholerae* and has shown the positive effect of using ROS-n conditions and non-selective media to enumerate sub-lethally injured cells.

Routine enumeration of copper-exposed bacteria is carried out under normal aerobic conditions in laboratories. However, there is a possibility of inactivation of sub-lethally injured bacteria due to the generation of ROS as a result of aerobic metabolism that may interfere with the enumeration of injured bacteria (Tandon *et al*. 2005). In their study, Tandon *et al*. (2007a) demonstrated that enumeration of *E. faecalis* stored in a brass vessel for up to 48 h on selective media under aerobic conditions was not sufficient to count all the injured bacteria, due to their oxygen sensitivity. There are two main sources of oxidative stress during enumeration: (i) reactive components of the growth medium, such as oxidation of medium nutrients during autoclaving and photo-oxidation of growth medium components after autoclaving (Spiegeleer *et al*. 2004; Gurtler 2009); (ii) cellular respiration of the growing bacteria. It is this cellular respiration that results in self-destruction of sub-lethally damaged bacteria under aerobic conditions (Stephens *et al*. 2000). Hence, the bacterial count obtained on an agar-based medium incubated under standard aerobic conditions constitutes those healthy cells that have the ability to cope with the ROS generated by the medium used for their growth. To obtain the ROS-sensitive bacterial count, the inhibitory effects of ROS from all sources need to be neutralized. This can be achieved by the use of (i) a pyruvate supplemented medium containing 0.05% sodium pyruvate as a specific ROS quencher, to neutralize the peroxides of the medium and (ii) anaerobic conditions for incubation, to prevent respiratory ROS, since the organisms will be required to grow by fermentation (Tandon *et al*. 2007b).

Though a number of studies have discussed the use of enrichment and/or selective media for accurate enumeration of *V. cholerae* (Rennels *et al*. 1980; Rahman *et al*. 2006), none have directly described the damaging effects of ROS on sub-lethally injured bacteria. Similarly heat-injured *Salmonella* have been shown to be inhibited on certain selective media (Qing-mei *et al*. 2009), while other studies have demonstrated the existence of an oxygen-sensitive sub-lethally injured state for *Salmonella* cells in stressful environmental conditions, following exposure to various stressors, such as pH (Tiganitas *et al*. 2009), heat (Gurtler & Kornacki 2009) and oxygen (Wesche *et al*. 2009).

The frequency of isolation of *V. cholerae* and *Vibrio vulnificus* is affected by environmental conditions (Constantin de Magny *et al*. 2009), and it has been proposed that such organisms may enter a viable but non-culturable state (Chaiyanan *et al*. 2001). However, such an interpretation is typically based on conventional aerobic counts, and the ROS-sensitivity of sub-lethally injured cells of *V. vulnificus* (Bogosian *et al*. 2000) provides an alternative possibility. Thus the resuscitation of starved *V. vulnificus* (Bang *et al*. 2007) and *Vibrio parahaemolyticus* (Mizunoe *et al*. 2000) through supplementation of the growth medium with pyruvate- and/or catalase reinforces the need to consider ROS-n conditions for bacterial enumeration. The present study extends such observations to *V. cholerae*, which showed a greater sensitivity to the use of standard aerobic growth conditions and the use of a selective medium than *S.* Typhi (c.f. Figures 1 and 2), suggesting a high degree of sub-lethal injury of cells of this pathogen and making it imperative to use conditions that will successfully enumerate such bacteria, especially from environmental samples where sub-lethal injury might be expected.

CONCLUSIONS

Accurate enumeration of sub-lethally injured bacteria plays a pivotal role in any monitoring programme designed to detect the threat of such bacteria through culture-based counts. Providing appropriate ROS-n conditions to overcome oxygen sensitivity through the combination of the peroxide quencher sodium pyruvate in the growth medium, together with incubation of pyruvate-supplemented plates in anaerobic conditions allows the sub-lethally injured cells to be

counted, providing a clearer picture of the inactivation process and the role of sub-lethal injury. The experiments show promise in the use of copper storage as a means of contributing to the reduction in risk of waterborne diseases. It should be noted that the results are for household-level pitchers, and should not be extrapolated to vessels of other sizes.

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