Environmental stability of *V. cholerae*: Implications in terms of its recovery, detection, and control

Public Health England

Samiksha Venkatesan[1,2], Dr Ginny Moore[1], Susan Paton[1]

[1]Biosafety, Air and Water Microbiology, PHE, Porton Down, UK

[2]Department of Biology and Biochemistry, University of Bath, Bath, UK

UNIVERSITY OF BATH

A) Introduction

V. cholerae causes the water-borne diarrheal disease cholera. It kills millions annually worldwide despite only the severest cases requiring antibiotics. This is because WASH (water, sanitation, hygiene) is compromised in endemic nations.

Its survival in water has been extensively studied, as well as its survival and mode of infection in the small intestine. However, its survival on fomites (contaminated surfaces) and their ability to act as a vector for this disease is poorly elucidated. Furthermore, there is even less literature on surface disinfection of *V. cholerae,* especially when the viable-but-non-culturable (VBNC) state is considered.

This state is induced when environmental conditions are unfavourable[1] and is problematic as standard techniques used to assess the efficacy of a disinfectant rely on culturable methods. Thus, they will pick up viable and culturable cells that were not killed by the disinfectant but one does not know if VBNC cells were killed too.

Unfortunately, VBNC cells can produce virulence factors like cholera toxin and toxin co-regulated pili[2], and a human trial has shown the potential to develop symptoms from them[3]. Moreover, VBNC cells of other *Vibrio* species have shown to have higher chemical resistances[4][5]. Thus, it is of utmost importance to establish whether household disinfectants work against VBNC cells of *V. cholerae*.

B) Aims

To study the survival of three toxigenic strains on household materials found in endemic nations such as stainless steel, plastic, and cloth, while mimicking their environmental conditions.

To optimise current methodology used to recover and detect *V. cholerae* from these surfaces post-disinfection, as the VBNC state has rarely been accounted for in previous literature.

C) Methods

The three toxigenic strains

They were purchased from the National Collection of Type Cultures (NCTC). O1 and O139 (seen in Table 1) are the two main disease-causing serogroups and have disseminated worldwide.

NCTC	serogroup	serotype	biotype
8021	O1	Ogawa	-
10256	O1	Ogawa	El Tor
12946	O139	-	-

Table 1: The three strains *of V. cholerae* used in this project. They have been distinguished by their serogroup, serotype within the serogroup, and finally by biotype, based on information obtained from NCTC.

Minimum biocidal concentration (MBC) assay using free available chlorine (FAC)

The purpose of this assay was to determine the lowest effective concentration of FAC against the three strains in LB broth (high-nutrient, mimicking faeces) and PBS (low nutrient). HazTabs containing sodium dichloroisocyanurate (NaDCC) were used as the FAC source.

Two-fold serial dilutions of the disinfectant were performed along the length of two 96-well microplates, each containing a different medium.

A wide range of concentrations were covered, from 20,000 ppm (2%) down to 5 ppm ($5x10^{-4}$%). The final volume of disinfectant in each well was 100 µL.

Each test well was inoculated with 20 µL overnight culture of a particular strain **(around 1-4 $x10^7$ cfu)**. The microplates were incubated at 37ºC for 24h.

The following day, 10 µL droplets from the wells were dropped onto TSA plates and incubated overnight at 37ºC. **The last concentration to exhibit no growth was deemed the lowest biocidal concentration of FAC (in that medium)**.

Stainless steel culturability assay

Three stainless steel coupons (see Figure 1) per strain and time-point **(t= 0, 0.5, 1, 2, and 4h)** were inoculated with 10 µL overnight culture in LB broth **(around 3.0-5.5 $x10^6$ cfu)** and placed in the climate control chamber set to **28ºC and 82% RH**. At t_0, the coupons were recovered immediately into 10 mL Universal tubes of PBS; they weren't placed in the climate control chamber.

Regardless of time-point, after recovery, the tubes were vortexed then the solutions serially diluted ten-fold in PBS. 100 µL aliquots of particular dilutions were streaked in duplicate onto TSA plates and incubated overnight at 37ºC.

All plates were read the following day and the number of recovered culturable bacteria at each time-point per strain calculated. This was used to plot a graph of average recovered cfu over time.

Figure 1: The stainless steel coupon used for the culturability assay. Bacteria was inoculated onto the centre of the coupon.

D) Results

MBC assay

All three strains has the same MBC as each other, regardless of incubation medium. This was **156 ppm in LB broth** and **78 ppm in PBS**.

Since there were around 10^7 cfu per well, this meant that all effective FAC concentrations displayed a **>7log_{10} reduction in viable and culturable bacteria**.

Stainless steel culturability assay

Both O1 strains (NCTC 8021 and 10256) **lost culturability by 2h**, with a sizeable drop between hours 1 and 2 (>6log_{10}), as seen in Figure 2.

However, the O139 strain (NCTC 12946) **lost culturability later by 4h**, with the biggest drop between hours 2 and 4 (>5log_{10}).

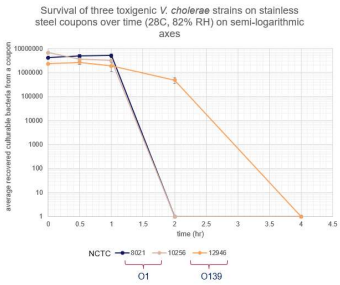

Figure 2: Survival of three toxigenic *V. cholerae* strains on stainless steel coupons over time at 28°C and 82 % relative humidity. Semi-logarithmic axes were chosen to visualise the magnitude of the decreases in culturability over time for the strains. Since zero values cannot be plotted on a logarithmic scale, they were replaced with one. The standard deviations were calculated for each strain at each time point and plotted as error bars.

E) Conclusions

MBC assay

When NaDCC dissolves in water, it releases hypochlorous acid (HOCl), an active antimicrobial compound. This dissociates into OCl^-, a powerful oxidising agent that reacts heavily with organic matter[6][7].

The lowest effective concentration of FAC was higher in LB broth due to some OCl^- being sequestered by the organic compounds in the broth. Thus, there was less HOCl to kill the cells. Both 78 and 156 ppm FAC were still lower than the concentrations employed in cholera treatment centres for disinfection of various surfaces, with the lowest being 500 ppm[8].

Stainless steel culturability assay

Data was compared to that obtained by Farhana et al in their 2016 paper[9].

Their O1 strain also lost culturability by 2h on stainless steel despite being suspended in PBS and not a high-nutrient medium like LB broth. Therefore, another factor was hypothesised to cause the drop in culturability.

This was suspected to be the desiccation of the bacteria on the material- other porous materials they had tested like cloth and paper retained culturability for longer, possibly due to greater moisture retention.

F) Other techniques used in this project

-Determining the best high-nutrient broth to culture *V. cholerae*
-The effects of low nutrient levels and salinity on growth of *V. cholerae*
-Growth curves in different media at different temperatures
-Biofilm assays (in different media)
-Quantitative suspension tests (QSTs) to test the efficacy of lab-based disinfectants on *V. cholerae*
-Neutraliser validation for QSTs

Survival in liquid media

Disinfection in liquid media

G) Future work

Study the survival and culturability of the three toxigenic strains on other materials.

Test for the presence of VBNC cells on these materials using live/dead fluorescent microscopy (and maybe other techniques)[1].

Disinfect these surfaces with free available chlorine and examine them for viable cells (culturable or not) post-disinfection.

H) References

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sv407@bath.ac.uk