The use of quaternary ammonium disinfectants selects for persisters at high frequency from some species of non-tuberculous mycobacteria and may be associated with outbreaks of soft tissue infections

Claudia Cortesia, Gustavo J. Lopez, Jacobus H. de Waard and Howard E. Takiff

Laboratorio de Genética Molecular, CMBC, IVIC, Km. 11, Carr. Panamericana, Caracas 1020A, Venezuela; Laboratorio de Tuberculosis, Instituto de Biomedicina, Hospital Vargas, San José, Caracas, Venezuela

Corresponding author. Centro de Microbiología y Biología Celular, Instituto Venezolano de Investigaciones Científicas, Km. 11, Carr. Panamericana, Caracas 1020A, Venezuela. Tel: +58-212-504-1439; Fax: +58-212-504-1499; E-mail: htokiff@ivic.gob.ve

Received 24 May 2010; returned 19 July 2010; revised 28 August 2010; accepted 30 August 2010

Background: Non-tuberculous mycobacteria (NTM) are increasingly important as opportunistic infections after major and minor surgical procedures, likely because they are ubiquitous and not effectively killed by many commonly used disinfectants. Outbreaks of soft tissue infections with NTM appeared related to the use of commercial disinfectants based on quaternary ammonium compounds (QACs).

Methods: We studied the survival of clinical and environmental isolates of Mycobacterium abscessus, Mycobacterium massiliense, Mycobacterium chelonae and Mycobacterium fortuitum after 20 min, 60 min or 24 h exposures to different QACs, and the surviving bacteria were then re-exposed to QACs to see if the percentage of surviving bacteria had increased. The bacteria were labelled with a dnaA–gfp fusion and their level of QAC resistance monitored as increasing fluorescence. The QAC-resistant bacteria were then serially restreaked onto non-selective medium and retested for QAC survival.

Results: The frequency of survivors was 1 in 100 with Mycobacterium smegmatis, but 1 in 100 with the other mycobacteria studied. Different environmental and clinical isolates had similar QAC MICs, but QAC survivors of each strain were resistant. The QAC-surviving strains reverted to the original, non-resistant phenotype after several passages on non-selective medium.

Conclusions: QACs should not be used in settings where even minimally invasive procedures are performed, as they select for a non-genetically determined reversible resistant phenotype that appears at high frequency with several rapidly growing mycobacterial species associated with healthcare-related infections. M. smegmatis behaves differently and is not an adequate model for testing the activity of disinfectants against NTM.

Keywords: quaternary ammonium compounds, disinfectants, NTM, resistance, tolerance, persistence

Introduction

The recent popularity of conventional and non-conventional cosmetic procedures, often performed by diverse practitioners in less than optimal settings, has been associated with outbreaks of non-tuberculous mycobacteria (NTM) infections of the skin and soft tissues.\textsuperscript{1,2} Infections with the Mycobacterium abscessus–chelonae group and Mycobacterium fortuitum are among the most common and most difficult to treat.\textsuperscript{3} While some of these cases were associated with the injection of contaminated local anaesthetics and mixtures purported to reduce adiposity, many cases were apparently related to the use of surgical instruments that were inadequately sterilized or incomplete disinfection of the skin prior to the procedure.\textsuperscript{4} Some outbreaks appeared to be associated with the use of disinfectants whose active ingredient is a quaternary ammonium compound (QAC), a class of disinfectants regarded as having only mycobacteriostatic activity.\textsuperscript{5} In order to explain more precisely how the use of a disinfectant might be associated with infections,\textsuperscript{6} we investigated the activity of QACs against several species of NTM.

Materials and methods

Bacterial strains and plasmids

Mycobacterium smegmatis mc²155 and Mycobacterium bovis BCG are strains commonly used in the laboratory (Laboratorio de Genética...
Molecular, IVIC). Mycobacterium terrae was obtained from the ATCC (ATCC 15755). The other mycobacterial strains were isolated in Caracas (Lab. TB, Inst. Biomedicina) from patient material or environmental sources (Table 1). The PRA method was used for initial strain identification (polymerase chain reaction–restriction enzyme analysis) (http://app.chuv.ch/prasite/index.html), but definitive species assignment was obtained by sequencing a fragment of the rpoB gene amplified with primers MycoF (5′-GGCAAGGTACCCGAAGGG-3′) and MycoR (5′-AGCGGCTGCTGTTGATC-3′).

Resistant survivors and revertants were obtained as noted in Table 1. The strains were grown at 37°C in Middlebrook 7H9 liquid medium supplemented with 10% OAD (oleic acid, albumin, dextrose), 0.2% glycerol and 0.05% Tween 80 (7H9-OAD-Tw) or on Petri dishes containing Middlebrook 7H10 supplemented with 10% OAD and 0.2% glycerol. Plasmids pFPV279 and pYUB41210 were gifts from Dr Lalita Ramakrishnan (University of Washington, Seattle, WA, USA) and Drs Martin Pavelka (University of Rochester, NY, USA) and William R. Jacobs Jr (Albert Einstein College of Medicine, Bronx, NY, USA), respectively.

### Disinfectants

The disinfectants evaluated were: hexadecyl trimethyl ammonium bromide (centrimonium bromide; C19H42NBr; CTAB); dodecyl trimethyl ammonium bromide (C17H38NBr; CPC); tetradecyl trimethyl ammonium bromide (C15H34NBr; DTAB); cetylpyridinium chloride (C21H38NCl; CPC); and dimethyl benzyl lauryl ammonium bromide [C13H27NBr; TTAB] in 50 mL volumes of 7H9-OAD-Tw to obtain 4% or 10% solutions of each. For the marketed disinfectant, the 10% commercial product solution was used directly in some studies or diluted to 4% in 7H9-OAD-Tw as the starting concentration in the 96-well plate assay. All disinfectant solutions were kept at 4°C until used. The highest concentrations tested were 9%, made up of 9 parts of the 10% solution and one part of bacterial inoculum.

### Determination of mycobactericidal activity and selection of resistant survivors

The mycobacterial strains were grown in 50 mL tubes to an OD600 of 1.0 (×10^9 cfu/mL). Ten 1.0 mm glass beads were added and the tube was vortexed for 20 s and then left to stand for 10 min to allow for clumps to sediment. When necessary, the resulting suspension was diluted serially, 1:10, in 7H9. An aliquot of 1 mL of the final suspension was then added to 9 mL of 10% DBLAB (the undiluted commercial preparation) or 10% CTAB and left to incubate with agitation for 20 min, 1 h or 24 h. Subsequently, after vortexing for 1 min, 1 mL of the disinfectant containing bacterial suspension was added to 9 mL of neutralizing solution (10% Tween 80, 0.5% sodium thiosulphate, 0.1% histidine in 0.25 M phosphate buffer, pH 7.0), which was then centrifuged at 3000 rpm for 10 min. The supernatant was discarded and the pellet resuspended in ~300 μL of the residual medium. Three ~100 μL aliquots of this pellet were then spread onto three 7H10 OAD plates without detergent and incubated at 37°C for 4–8 days, at which point surviving colonies were counted. Assays were performed in triplicate. Controls were treated identically, but incubated without disinfectant.

The selection for glutaraldehyde-resistant colonies was slightly different. After vortexing and allowing for clumps to settle, as above, the resultant supernatant was then transferred to a new 50 mL tube that was placed into a bath sonicator for 5 min. An aliquot of 100 μL of this suspension was added to 900 μL of 2% glutaraldehyde. After 5, 20 and 60 min,

### Table 1. Strains used in this study: mycobacterial isolates, their resistant derivatives selected after exposure to the indicated disinfectants and revertants obtained by restreaking the resistant strains on medium without disinfectant

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. massiliense WT 1.0</td>
<td>M. massiliense clinical isolate LTE3550</td>
</tr>
<tr>
<td>M. massiliense 1.2</td>
<td>M. massiliense WT 1.0 selected in 9% CTAB</td>
</tr>
<tr>
<td>M. abscessus WT 2.0</td>
<td>M. abscessus clinical isolate LTE3514</td>
</tr>
<tr>
<td>M. abscessus 2.1</td>
<td>M. abscessus WT 2.0 selected in 9% CTAB</td>
</tr>
<tr>
<td>M. abscessus 2.1.3</td>
<td>M. abscessus 2.1 restreaked 3 times</td>
</tr>
<tr>
<td>M. abscessus 2.1.6</td>
<td>M. abscessus 2.1 restreaked 6 times</td>
</tr>
<tr>
<td>M. abscessus 2.2</td>
<td>M. abscessus WT 2.0 selected in 9% DBLAB</td>
</tr>
<tr>
<td>M. abscessus 2.2.3</td>
<td>M. abscessus 2.2 restreaked 3 times</td>
</tr>
<tr>
<td>M. abscessus 2.2.6</td>
<td>M. abscessus 2.2 restreaked 6 times</td>
</tr>
<tr>
<td>M. abscessus 2.3</td>
<td>M. abscessus WT 2.0 selected in glutaraldehyde 2% for 5 min</td>
</tr>
<tr>
<td>M. abscessus 2.3.3</td>
<td>M. abscessus 2.3 restreaked 3 times</td>
</tr>
<tr>
<td>M. abscessus 2.3.6</td>
<td>M. abscessus 2.3 restreaked 6 times</td>
</tr>
<tr>
<td>M. fortuitum WT 1.0</td>
<td>M. fortuitum clinical isolate LTE1428</td>
</tr>
<tr>
<td>M. fortuitum 1.2</td>
<td>M. fortuitum WT 1.0 selected in 9% DBLAB</td>
</tr>
<tr>
<td>M. chelonae WT 2.0</td>
<td>M. chelonae clinical isolate LTE2714</td>
</tr>
<tr>
<td>M. chelonae 2.1</td>
<td>M. chelonae WT 2.0 selected in 9% CTAB</td>
</tr>
<tr>
<td>M. chelonae 2.2</td>
<td>M. chelonae WT 2.0 selected in 9% DBLAB</td>
</tr>
<tr>
<td>M. fortuitum WT 3.0</td>
<td>M. fortuitum WT 3.0 selected in 9% DBLAB</td>
</tr>
<tr>
<td>M. fortuitum 3.2</td>
<td>M. fortuitum environmental isolate LTE2297</td>
</tr>
<tr>
<td>M. terrae</td>
<td>ATCC 15755</td>
</tr>
<tr>
<td>M. smegmatis mc²155</td>
<td>laboratory strain (Laboratorio de Genética Molecular, IVIC)</td>
</tr>
<tr>
<td>M. bovis BCG</td>
<td>laboratory strain (Laboratorio de Genética Molecular, IVIC)</td>
</tr>
</tbody>
</table>
100 μL of the glutaraldehyde suspension was added to 900 μL of neutralizing solution (6% Tween 80, 1% sodium thioussphate, 0.5% sodium thiglycollate and 0.15% cysteine). From the neutralized suspension, three 100 μL aliquots were plated onto 7H10 plates without OAD and incubated at 37°C for 4–8 days, when surviving colonies were counted. Negative controls were performed with sterile water in place of glutaraldehyde.

Cloning of the dnaA–gfp fusion

Reagents for DNA manipulations were obtained from commercial sources and used according to routine protocols. For the construction of plasmid pDNAA–gfp, the dnaA promoter region from M. tuberculosis (582 bp) was amplified from a cosmid containing the rpmH–dnaA region, using primers GLRA3 (5′-CTA TGT CTG GCA ACA T) and GLRA4 (5′-TAT CTC CCT GGT TCT CGT TA), and ligated into the BamHI and EcoRV sites upstream of gfp on plasmid pFPV27. The chromosomal integrating version of the dnaA–gfp fusion, on plasmid pINDA–gfp, was obtained by cloning a BamHI–BclI fragment from pDNAA–gfp into the BclI site of pYUB412.10 Plasmids pDNAA–gfp and pINDA–gfp were electroporated into the mycobacterial strains and plated onto medium containing kanamycin (pDNAA–gfp) or hygromycin (pINDA–gfp). GFP-expressing mycobacteria were visualized for fluorescence using a Nikon Eclipse 600 fluorescence microscope.

Monitoring bacterial growth by fluorescence

A colony of mycobacteria from solid medium was inoculated into 5 mL of 7H9-OAD-Tw (with kanamycin when the strains contained plasmid pDNAA–gfp or hygromycin for strains with integrating plasmid pINDA–gfp) in a 50 mL conical tube and grown with agitation at 37°C until reaching exponential phase. The colony was then transferred to 50 mL of the same medium and grown in 500 mL flasks. For the initial studies comparing fluorescence versus growth, samples were taken periodically to measure the optical density (OD) and fluorescence: every 3 h for M. smegmatis; every 6 h for M. abscessus; and every 24 h for M. chelonae. ODmax was measured in a 96-well spectrophotometer (Molecular Devices SpectraMax 340) and fluorescence (485 nm excitation and 535 nm emission) was measured in a 96-well plate fluorimeter (Molecular Devices SpectraMax, GeminiXS).

Assay of disinfectant activity using fluorescent mycobacteria

The different bacteria were grown in 50 mL tubes to an OD600 of 1.0 (~108 cfu/mL). Then, 5 mL of each was transferred to a new 50 mL conical tube, 10 glass beads (1.0 mm) were added, and the tube was vortexed for 20 s and left to stand for 10 min to allow for clumps to settle. An aliquot of 100 μL of the suspension was added to 9.9 mL of 7H9-OAD-Tw and, then, 1 mL of this was added to 9 mL of the same medium, which was then mixed and used as the inoculum in the 96-well plate assay. For some assays, a second 1:10 dilution in medium was performed, so that 106 or 107 bacteria, in 100 μL, were added to each well. The assay was performed using black 96-well plates, one disinfectant per plate. An aliquot of 200 μL of the highest concentration of the disinfectant to be tested was placed into the first column and 100 μL of 7H9-OAD-Tw was added to the other columns. Using a multichannel pipette, 100 μL from the wells with the highest disinfectant concentration was diluted into the 100 μL of 7H9-OAD-Tw in the next column, and the dilution process serially repeated to obtain final concentrations of 4%, 2%, 1%, 0.5%, 0.25%, 0.125% and 0.06%. Each concentration was evaluated in triplicate. An aliquot of 100 μL of the mycobacterial suspension was added into each well, and positive controls without disinfectants and negative controls with medium alone were included on each plate. Peripheral wells were filled with 200 μL of sterile distilled water. The plates were incubated with agitation at 37°C and the fluorescence read periodically over 4 days in a Molecular Dynamics Spectra- max GeminiXS Fluorimeter/Luminometer.

Results

QACs kill most mycobacteria, but survivors appear at high frequencies in M. chelonae and M. abscessus

Although the QAC disinfectants are cidal for most bacteria, they have been generally regarded as only mycobacteriostatic. To test this, M. smegmatis, M. chelonae and M. abscessus were exposed to CTAB or to a commercial disinfectant commonly used in Venezuela, which will be referred to as DLBLAB, for dimethyl benzyl lauryl ammonium bromide (C13H27-NBr), its active QAC ingredient. Both CTAB and DLBLAB were used as 9% concentrations, close to the 10% commercial presentation of DLBLAB.

The mycobacteria were exposed to the QACs for 20 min, 60 min or 24 h, after which the QACs were neutralized and the mycobacteria plated on non-selective medium. After 20 or 60 min exposures, both QACs appeared to effectively kill M. smegmatis, with resistant colonies appearing at a frequency of <1 in 105 (Table 2). The disinfectants also appeared to kill most M. abscessus and M. chelonae, but surviving colonies appeared at frequencies of >5 in 100 after 20 and 60 min exposures, with lower frequencies of survival after a 24 h exposure.

The surviving colonies were then selected, grown in liquid medium without QACs and again subjected to 20 min, 60 min or 24 h exposures to 9% CTAB or 9% DLBLAB. The survival rates were now ~50% after 20 and 60 min exposures to both agents, but remained at <1 in 100 after 24 h exposures (Table 2). This was true even for those strains obtained from colonies surviving a 24 h exposure. Strains obtained from colonies surviving a 5 min or 20 min exposure to 2% glutaraldehyde behaved similarly, but were only minimally more resistant to glutaraldehyde than the parent strain. No survivors were obtained with longer exposures to 2% glutaraldehyde.

Equivalent results were obtained when the experiment was repeated with clinical or environmental isolates of M. massilicense and M. fortuitum. Some of these original, parental isolates displayed the rough colony morphology, while others had smooth colonies. The original colony morphotype tended to persist in the QAC surviving colonies, but some smooth QAC surviving strains began to produce a minority of rough colonies when restreaked. Smooth strains showed greater motility (data not shown), but neither motility nor colony morphology appeared to be correlated with QAC resistance.

Susceptibility to QACs determined by fluorescence

To assess QAC susceptibility in another format, the mycobacteria were labelled with a dnaA–gfp fusion, which permitted bacterial growth to be monitored as fluorescence in a 96-well plate assay (Figure S1, available as Supplementary data at JAC Online). Using this method, clinical and environmental isolates of M. abscessus and M. massilicense were less susceptible to DLBLAB than M. chelonae and M. fortuitum, but the strains grown from colonies surviving exposure to DLBLAB and CTAB were resistant to all concentrations tested (Figure 1).
To learn more about the innate susceptibility of different mycobacteria to QACs, we compared the growth of *M. chelonae*, *M. abscessus*, *M. bovis* BCG and *M. terrae* in the presence of increasing concentrations of CTAB, DLBLAB and three other QACs (Figure S2, available as Supplementary data at JAC Online). The susceptibilities varied somewhat with the different QACs, but *M. abscessus* tended to be the most resistant and *M. smegmatis* the most susceptible. Notably though, the susceptibility of *M. smegmatis* to DLBLAB was very similar to that of *M. chelonae* and *M. fortuitum* (Figure 1b).

### Reversibility of QAC resistance

The very high frequency of QAC-resistant survivors of *M. abscessus*, *M. massiliense*, *M. chelonae* and *M. fortuitum* seemed inconsistent with a genetic alteration, so we tested whether the resistant phenotype was reversible. Three *M. abscessus* survivor strains, derived from the same parent strain after exposure to CTAB (2.1), DLBLAB (2.2) or a 5 min exposure to glutaraldehyde (2.3), were serially restreaked to non-selective medium. After three and six passages, they were again subjected to 20 or 60 min exposures to CTAB and DLBLAB. After three passages, the survival of all three strains had fallen to ≏30% and after six passages only ≏5% of the plated bacteria survived, similar to the original parent strain (Figure 2). Resistant *M. chelonae* survivor strains showed similar reductions in QAC survival after serial restreaking on non-selective medium (data not shown).

### Discussion

Provoked by the occurrence of NTM infections that appeared to be associated with the use of commercial QAC disinfectants,12,13 we investigated the effectiveness of QACs against mycobacteria. QACs are described in the literature as only mycobacteriostatic, so we were surprised to find that a 20 min exposure to QACs appeared to be cidal against *M. smegmatis*, with at least 5 logs of killing;14 although their effectiveness would likely be less if assayed in a carrier test in the presence of organic material.15 Against *M. fortuitum*, *M. chelonae*, *M. massiliense* and *M. abscessus*, however, even with the simple suspension test employed here, we found that QACs kill only ≏90%–95% of bacteria. This suggests that the use of QACs will not prevent, and may even promote, the occurrence of infections with *M. fortuitum* and the *M. chelonae–abscessus* group of NTM.

From these results, the QACs cannot accurately be called mycobacteriostatic, because when the agent is removed, the majority of the bacteria are not viable. Neither can they be termed effectively mycobactericidal, at least for *M. fortuitum* and the *M. chelonae–abscessus* group of NTM. From these results, the QACs cannot accurately be called mycobacteriostatic, because when the agent is removed, the majority of the bacteria are not viable. Neither can they be termed effectively mycobactericidal, at least for *M. fortuitum* and the *M. chelonae–abscessus* group of NTM. From these results, the QACs cannot accurately be called mycobacteriostatic, because when the agent is removed, the majority of the bacteria are not viable. Neither can they be termed effectively mycobactericidal, at least for *M. fortuitum* and the *M. chelonae–abscessus* group of NTM.

The number of colonies of different strains surviving exposures to CTMB, DLBLAB or glutaraldehyde is assayed in a carrier test in the presence of organic material.15 The strains tested were the parental isolates and their resistant derivatives, selected as survivors of previous exposures to the indicated disinfectants. NT, not tested; GTA, glutaraldehyde; Select., the strain was selected as a survivor after exposure to the indicated disinfectant.

### Table 2. The number of colonies of different strains surviving exposures to CTMB, DLBLAB or glutaraldehyde

<table>
<thead>
<tr>
<th>Exposure</th>
<th>M. abscessus</th>
<th>M. chelonae</th>
<th>M. smegmatis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.0 WT</td>
<td>2.1 Select.</td>
<td>2.2 Select.</td>
</tr>
<tr>
<td></td>
<td>~10² cfu</td>
<td>CTAB</td>
<td>DLBLAB</td>
</tr>
<tr>
<td>plated</td>
<td>~10² cfu</td>
<td>plated</td>
<td>plated</td>
</tr>
<tr>
<td></td>
<td>~10² cfu</td>
<td>~10² cfu</td>
<td>~10³ cfu</td>
</tr>
<tr>
<td></td>
<td>plated</td>
<td>plated</td>
<td>plated</td>
</tr>
</tbody>
</table>

5 min
- 2% GTA: 0 0 0 0 1 4 0 0 0 2 0
- 9% CTAB: 12 72 61 78 NT NT 14 86 76 NT 0
- 9% DLBLAB: 15 68 58 92 NT NT 4 NT NT NT 0
- 2% GTA: 0 0 0 0 0 0 0 NT NT 0 0

20 min
- 9% CTAB: 6 47 57 52 NT NT 8 53 73 NT 0
- 9% DLBLAB: 6 63 48 70 NT NT 3 NT NT NT 0
- 2% GTA: 0 0 0 0 0 0 0 NT NT 0 0

60 min
- 9% CTAB: 1 0 0 0 NT NT 0 0 0 NT 0
- 9% DLBLAB: 1 0 0 0 NT NT 0 0 0 NT 0

Discussion

To learn more about the innate susceptibility of different mycobacteria to QACs, we compared the growth of *M. smegmatis*, *M. chelonae*, *M. abscessus*, *M. bovis* BCG and *M. terrae* in the presence of increasing concentrations of CTAB, DLBLAB and three other QACs (Figure S2, available as Supplementary data at JAC Online). The susceptibilities varied somewhat with the different QACs, but *M. abscessus* tended to be the most resistant and *M. smegmatis* the most susceptible. Notably though, the susceptibility of *M. smegmatis* to DLBLAB was very similar to that of *M. chelonae* and *M. fortuitum* (Figure 1b).
The QAC survivors of *M. fortuitum* and the *M. chelonae–abscessus* group appeared at frequencies between 1% and 10%, which seems too high to be caused by genomic mutations. Although these survivors showed QAC resistance, when they were serially restreaked in the absence of QACs, the frequency of QAC survivors returned to that of the original strains. This reversible resistance, although it persists for several generations, suggests a phenotype dependent upon non-genetic changes in gene expression. After a 5 min or 20 min exposure to 2% glutaraldehyde, there also appeared, at a lower frequency,

![Graph](https://academic.oup.com/jac/article-abstract/65/12/2574/753925)

**Figure 1.** Different isolates of the *M. abscessus–chelonae* group and *M. fortuitum* show similar susceptibilities to DBLAB, while their derivatives, selected after surviving a previous exposure, are resistant to at least 2% DBLAB. The strains were labelled with plasmid *p dnaA–gfp* and grown in 96-well plates in medium containing DBLAB at the indicated concentrations. Mycobacterial growth was measured as fluorescence after 4 days of incubation at 37°C. All results are from representative experiments that were repeated at least three times. 

(a) Comparison of the growth in DBLAB of *M. massiliense* (*M. mass. WT 1*), *M. abscessus* (*M. abs. WT 2*), derivatives of each that were selected as survivors of a previous exposure of DBLAB (*M. mass. 1.2* and *M. abs. 2.2*), *M. smegmatis* and *M. chelonae*. (b) Comparison of the growth in DBLAB of *M. chelonae* (*M. chel. WT 2*), two isolates of *M. fortuitum* (*M. fort. WT 1* and *M. fort. WT 2*), derivatives of each that were selected as survivors of a previous exposure to DBLAB (*M. fort. 1.2*, *M. chel. 2.2*, and *M. fort. 3.2*), *M. smegmatis* and *M. abscessus*. 

The QAC survivors of *M. fortuitum* and the *M. chelonae–abscessus* group appeared at frequencies between 1% and 10%, which seems too high to be caused by genomic mutations. Although these survivors showed QAC resistance, when they were serially restreaked in the absence of QACs, the frequency of QAC survivors returned to that of the original strains. This reversible resistance, although it persists for several generations, suggests a phenotype dependent upon non-genetic changes in gene expression. After a 5 min or 20 min exposure to 2% glutaraldehyde, there also appeared, at a lower frequency,
glutaraldehyde-tolerant survivors that showed only minimally increased resistance to glutaraldehyde, but had 60% QAC survival, suggesting that they had also acquired the QAC-resistant phenotype. This phenotype could therefore reflect some sort of general stress or global resistance response.\textsuperscript{16,17} Escherichia coli tolerant to QACs have been described, but were selected by adaptation to serially increasing concentrations of the disinfectant,\textsuperscript{18} much different from the one-step selection in high QAC concentrations described here.

The QAC resistance seen in the survivors is not complete, as there was no meaningful increase in survival after 24 h exposures to the QACs. The \textit{M. abscessus} and \textit{M. chelonae} bacteria that survived 24 h exposures to QACs appear to represent a second, additional form of QAC persisters. While they survive a 1 h exposure to QACs, they are no more resistant to a 24 h QAC exposure than the parent strains, which is similar to the previously described antibiotic persistence of non-replicating bacteria.\textsuperscript{19,20} However, the frequency of the 24 h survivors seen here (\textasciitilde1/100--1/1000) is higher than has been reported for non-replicating persisters and suggests that mycobacteria could perhaps survive in QAC disinfectant stocks.

Like many other NTM, the \textit{M. chelonae-abscessus} group have been observed in two morphotypes, smooth and rough. While the rough morphotype is more virulent in pulmonary infection models,\textsuperscript{21} the smooth morphotype has a higher production of glycopeptidolipids\textsuperscript{12,23} that is thought to cause their increased motility and the increased biofilm formation that has been associated with decreased drug susceptibility.\textsuperscript{24} We found no association of QAC resistance and colony morphotype, as QAC-resistant strains were selected from both rough and smooth isolates, and tended to maintain their morphotype (data not shown).

\textit{M. abscessus} and \textit{M. massiliense} belong to a family of closely related mycobacteria.\textsuperscript{25} They are among the NTM most commonly isolated from healthcare-associated skin and soft-tissue infections,\textsuperscript{26} although \textit{M. chelonae} is closely related and also frequently encountered. \textit{M. fortuitum} is more phylogenetically distant, but is also found in healthcare-related infections. The dramatic recent increase in articles describing human infections with NTM, especially with the \textit{M. abscessus} family, suggests that their incidence is increasing, and \textit{M. abscessus} may have greater pathogenic capacity than other NTM.\textsuperscript{27} The innocuous \textit{M. smegmatis} would seem an inadequate model to study the effectiveness of disinfectants against mycobacteria.

We are currently investigating possible mechanisms for the QAC-resistant phenotype, such as decreased cell wall permeability\textsuperscript{28} or the extrusion of the QACs\textsuperscript{7,29,30} by efflux pumps,\textsuperscript{31} similar to LfrA\textsuperscript{12} or Tap.\textsuperscript{33} However, while the molecular mechanisms responsible for the resistant phenotype may yield insights into the pathogenic potential of these bacteria, the principal conclusion from this work is that QAC disinfectants are insufficient to prevent, and may actually promote, healthcare-associated infections with \textit{M. fortuitum} and the \textit{M. abscessus-chelonae} group of NTM.

Acknowledgements

We thank Donald Court for helpful discussions and Drs Sylvia Leao, Elba Guerrero, Glenda Vilchez, Donald Court and Stewart Cole for critically reading the manuscript.
Funding
This work was funded by FONACIT (Fondo Nacional de Ciencia, Tecnología y Innovación) Project G-2005000393 and by Helmerich & Paine de Venezuela, C.A. through LOCTI (Ley Orgánica de Ciencia, Tecnología y Innovación) project ‘Las Cepas de Tuberculosis Mas Virulentas de Venezuela’. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Transparency declarations
None to declare.

Author contributions
C. C. performed the experiments, analysed results and helped write the manuscript, G. J. L. constructed reagents and developed methods employed, J. H. de W. helped to conceive the study, provided strains and analysed the results, and H. E. T. conceived the study, analysed the results and wrote the manuscript.

Supplementary data
Figures S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References
23 Howard ST, Rhoades E, Rech J et al. Spontaneous reversion of Mycobacterium abscessus from a smooth to a rough morphotype is associated with reduced expression of glycopeptidolipid and reacquisition of an invasive phenotype. Microbiology 2006; 152: 1581–90.

