

# Varying levels of disinfectant resistance among invasive *Klebsiella pneumoniae* isolates

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## SUMMARY

Disinfectants are essential agents for pathogen/infection control in medical settings. As antibiotic resistance accelerates, disinfectants become more crucial for pathogen control. *Klebsiella pneumoniae*, a gram-negative bacterium with extensive antibiotic resistance, is a common cause of devastating infections that are mostly acquired in hospitals where disinfectants are regularly applied. Hospital-acquired or nosocomial infections are among the healthcare system's most significant challenges. We hypothesized that nosocomial pathogens could survive regular disinfection since these bacteria are commonly identified in medical settings despite the use of disinfectants. We tested this hypothesis by treating 11 invasive *K. pneumoniae* strains isolated from the human bloodstream with trichloroisocyanuric acid (TCCA), a widely used antimicrobial agent in medical settings. Temporary treatment of *K. pneumoniae* with 32 mg/L TCCA (as would occur in a hospital setting) for 1 hr did not completely eradicate 10 of the 11 isolates. Cultivation for 24 hr in the presence of TCCA at 700 mg/L, more than 20 times the typical concentration for the sanitation purpose, revealed two invasive *K. pneumoniae* isolates with significant resistance. Subsequent polymerase chain reaction (PCR) amplification found no close association between TCCA resistance and previously identified disinfectant-resistant genes. Our work has thus indicated that *K. pneumoniae* resistance to TCCA might be attributed to a new mechanism(s) other than the known disinfectant-resistant genes.

## INTRODUCTION

Hospital-acquired or nosocomial infections are defined as infections developed in patients after 48 hours of hospitalization or medical care absent during admission (1). This type of infection is caused by passing pathogenic microorganisms to susceptible individuals in hospitals or other healthcare facilities from healthcare workers, nonliving objects (e.g., respiratory machines), and invasive medical devices (e.g., catheters) (2). Susceptible patients are primarily individuals with compromised immune systems, such as organ transplantation recipients and cancer patients

receiving radio-/chemotherapy. Nosocomial infections often lead to severe pneumonia and the spread of pathogens in the bloodstream, leading to bacterial sepsis (3). For example, there are approximately 1.7 million cases of nosocomial infections and nearly 100,000 associated deaths each year in the United States, which makes them the country's sixth leading cause of death (4). Nosocomial infections are estimated to cost the United States \$28-45 billion annually (4).

The most common pathogens responsible for nosocomial infections include *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (2). A significant challenge in treating nosocomial infections is antibiotic resistance among these pathogens. Approximately 16% of the 1.7 million cases of nosocomial infections in the United States yearly are caused by antibiotic-resistant pathogens (4). Hospitals have increasingly relied on disinfectants to control nosocomial infections for this and other pathogens. The selection pressure imposed by disinfectants has also resulted in genetic resistance to disinfectants among common pathogens of nosocomial infections (5). The genes conferring disinfectant resistance are often carried by mobile elements, which renders the resistance transferable among microorganisms by lateral gene acquisition (1). In some cases, the disinfectant-resistant genes also confer cross-resistance to certain antimicrobials (5).

*K. pneumoniae*, a gram-negative bacterium, is one of the leading causes of hospital-acquired infections in the United States and the second leading cause of bloodstream infections among gram-negative bacteria (6). *K. pneumoniae* alone causes 20–30% of fatal bloodstream infections (6). The bacterium is ubiquitously found in environmental sources, such as water, soil, and plants. In hospitals, potential sources for spreading *K. pneumoniae* include contaminated surfaces and instruments as well as person-to-person contact. In humans and animals, *K. pneumoniae* typically resides in the gastrointestinal tract and oropharynx (7). Diseases commonly associated with *K. pneumoniae* include noninvasive pneumonia and invasive diseases (i.e., when bacteria are detected in normally sterile body fluids), such as bacteremia and meningitis. *K. pneumoniae* has become increasingly problematic in the last two decades due to multidrug resistance (8–10).

Trichloroisocyanuric acid (TCCA) is a disinfectant commonly used in hospitals and other settings (e.g., swimming pools and

restaurants) (11). TCCA targets almost all microorganisms (fungi, bacteria, viruses, and microbial spores). Given its strong oxidization capability, it is believed that TCCA controls microbial growth by reacting with essential biomolecules for growth and viability such as nucleotides, fatty acid, and proteins, thus damaging cellular structures and inactivating cellular processes including DNA replication and cell division (11). *K. pneumoniae* strains with genetic resistance to TCCA and other disinfectants have been reported (12–14). While the precise genetic basis of *K. pneumoniae* resistance to TCCA remains to be defined, the *qacED1* and *cepA* genes have been detected in some TCCA-resistant strains (12). While *qacED1* and its full-length *qacE* gene encode proton-driven efflux pumps of quaternary ammonium compounds, *cepA* encodes a cation efflux pump that is associated with the resistance to another broad-spectrum disinfectant chlorhexidine (13, 15). In this study, we hypothesized that invasive *K. pneumoniae* strains isolated from human blood in Chinese hospitals could survive commonly used disinfectant TCCA, which could be a significant infection threat in medical settings. We treated 11 *K. pneumoniae* isolates with TCCA and found that the resistant levels of different strains varied, which is not correlated with the reported disinfectant-resistant genes.

## RESULTS

### Invasive *K. pneumoniae* isolates survived disinfectant treatment

We used TCCA and invasive isolates of *K. pneumoniae* to assess the susceptibility of invasive bacterial pathogens to routine disinfectants. We obtained *K. pneumoniae* isolates from blood samples of human patients in Beijing Tsinghua Changguang Hospital, Beijing Tongren Hospital, and Beijing Renmin Hospital in Beijing, China (Table 1). NTUH K-2044, a standard *K. pneumoniae* strain, was used as a control

Strain ID	Capsule serotype	Hospital
NTUH K-2044	K1	National Taiwan University Hospital
TH12878	K20	Beijing Tsinghua Changguang Hospital
TH12880	K7	Beijing Tsinghua Changguang Hospital
TH12908	K1	Beijing Tsinghua Changguang Hospital
TH12971	K3	Beijing Tongren Hospital
TH12981	K20	Beijing Tongren Hospital
TH13011	K2	Beijing Tsinghua Changguang Hospital
TH13083	K1	Beijing Tongren Hospital
TH13091	K2	Beijing Tongren Hospital
TH13095	K17	Beijing Tongren Hospital
TH13098	K27	Beijing Tongren Hospital
TH13139	K19	Beijing Renmin Hospital

Table 1: *K. pneumoniae* strains used.

(16). Since we could not find any published data on TCCA concentration used for hospital sanitation, we designed our experiment based on the TCCA concentration recommended for drinking water sanitation (30 mg/L, 129.1 μM) (17). We treated *K. pneumoniae* with either 32 mg/L or 64 mg/L TCCA (in water) at room temperature for 1 hr (Figure 1). After serial dilutions of the treated bacterial suspensions mentioned above, we plated the dilutions on LB agar dishes for 12-hr incubation at 37°C and measured enumeration of colony forming units (CFUs) to determine bacterial viability after TCCA treatment. Lastly, we determined survival rate by dividing the CFUs of the untreated bacterial strain with the CFUs of the matching treated bacterial strain.

As compared with the untreated control, none of the 12 *K. pneumoniae* isolate strains treated with 64 mg/L TCCA formed colonies (data not shown). This result showed that this TCCA concentration effectively killed all tested *K.*

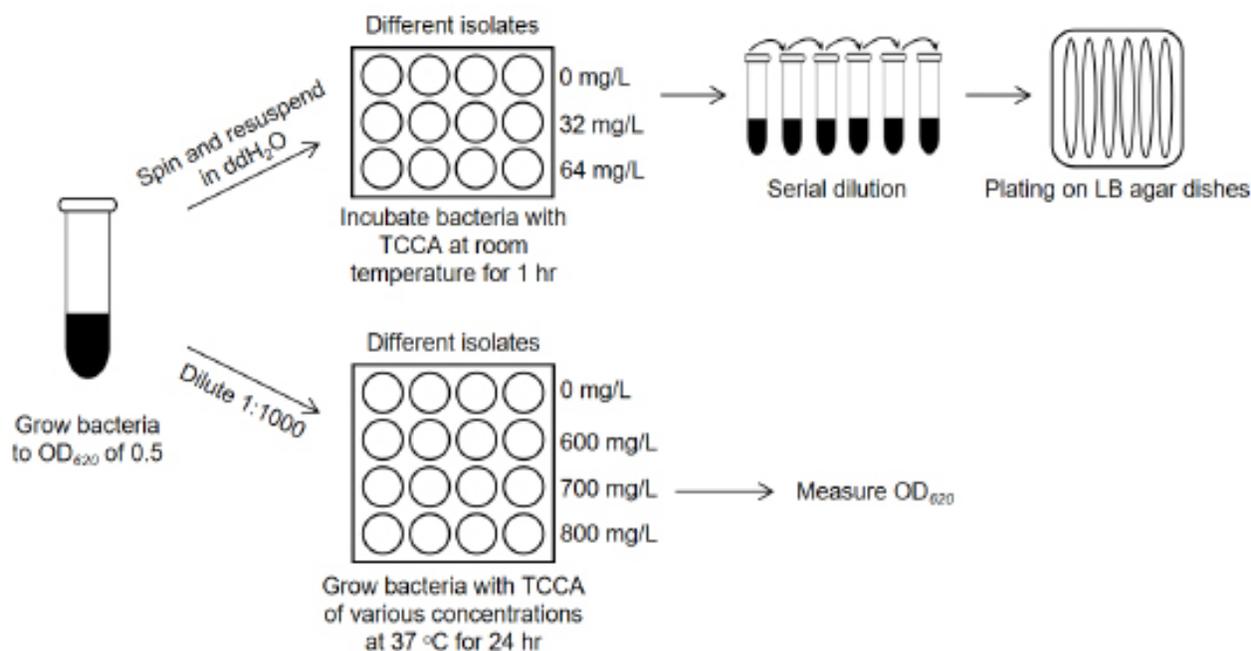
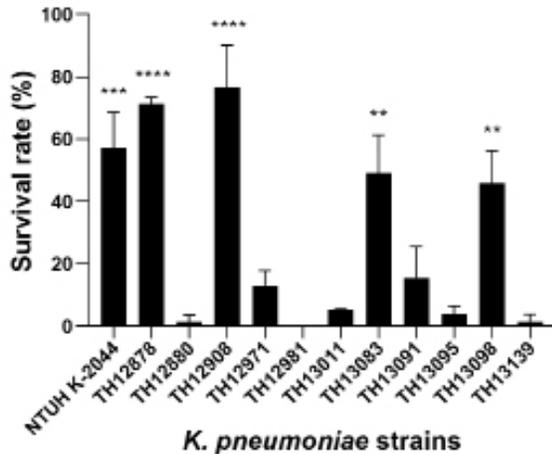


Figure 1: Experimental process of assessing resistance levels of *K. pneumoniae* isolates to TCCA. Bacteria were cultured to an  $OD_{620}$  of 0.5 before examining survival rate after being temporarily treated with TCCA (upper panel) and determining  $OD_{620}$  growing in TCCA of various concentrations the entire time (lower panel) as described in the Materials and Methods.



**Figure 2: Bacterial survival after temporary exposure to TCCA.** *K. pneumoniae* strains were treated with 32 mg/L TCCA (in water) at room temperature for 1 hr before being plated on LB agar dishes for CFU enumeration. The data are presented as percentages of the CFU values for the TCCA-treated group versus untreated controls. The data are presented as Mean  $\pm$  Standard deviation. Bacterial survival in the presence of TCCA with certain concentrations was analyzed by comparing survival rate with the most susceptible group (TH12981) using One-Way ANOVA followed by Tukey's test. \* p-value < 0.05, \*\* p-value < 0.01, \*\*\* p-value < 0.001, \*\*\*\*p-value < 0.0001.

*pneumoniae* isolates. By contrast, all of the isolate strains except for TH12981 produced colonies on LB agar plates when the disinfectant concentration was reduced to 32 mg/L. Further analysis of viable bacteria revealed variable levels of survival. While six strains (TH12880, TH12971, TH13011, TH13091, TH13095, and TH13139) showed modest survival below 15%, the remaining five strains (TH12878, TH12908, TH13083, and TH13098, along with NTUH K-2044) displayed statistically significant levels of survival, when these strains

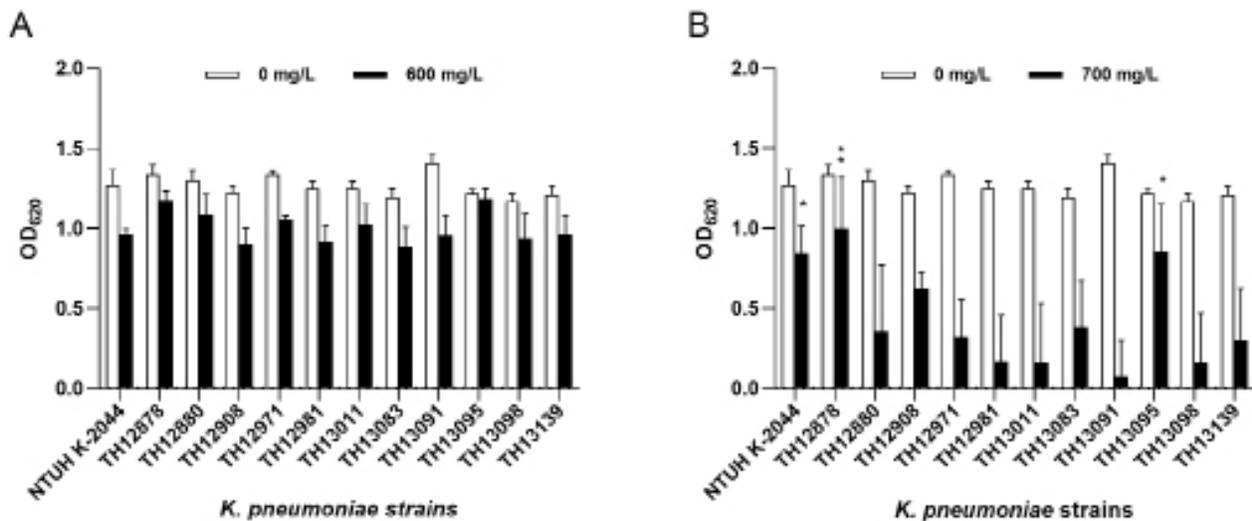
were compared with the non-resistant strain TH12981 (Figure 2, p-value < 0.05). This result indicated that certain invasive *K. pneumoniae* isolates are able to survive temporary treatment with TCCA at the concentration that is typically used to sanitize drinking water.

### Invasive *K. pneumoniae* isolates are able to grow in the presence of TCCA

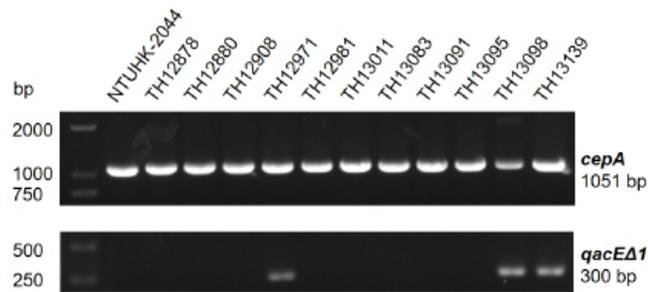
To determine if any of the invasive *K. pneumoniae* isolates were able to grow in the presence of TCCA, we cultivated the strains in LB broth to OD<sub>620</sub> of 0.5 (OD represents optical density). The 0.5 inoculum suspensions were further diluted at 1:1000 in fresh LB, and cultivated in the presence of TCCA at various concentrations at 37°C for 24 hr. A previous study showed a minimal inhibitory concentration (MIC) of 64-128 mg/L for TCCA against *K. pneumoniae* (12). However, our pilot experiments revealed growth of all of the 11 invasive *K. pneumoniae* isolates in LB broth in the presence of 128 mg/L TCCA, quantified by an increase in optical density as a proxy for bacterial growth. All the 12 *K. pneumoniae* isolate strains showed comparable growth in the presence of 600 mg/L TCCA in LB broth (Figure 3A). Significant differences in growth emerged at 700 mg/L TCCA (Figure 3B). Three strains reached statistically significant differences in growth (TH12878, TH13095, and NTUH K-2044) when their OD<sub>620</sub> values were compared with the most susceptible strain TH13091. None of the 12 *K. pneumoniae* strains showed significant growth in LB broth when the disinfectant concentration was increased to 800 mg/L (data not shown). Together with the bacterial survival data, this experiment showed that certain invasive *K. pneumoniae* isolates from human blood employed in this study were able to grow in the presence of TCCA, suggesting a resistance phenotype.

### Known disinfectant-resistant genes are not responsible for *K. pneumoniae* resistance to TCCA

Chen *et al.* have detected the *qacEΔ1* and *cepA*



**Figure 3: Assessment of bacterial resistance to TCCA.** *K. pneumoniae* strains were cultivated in 96-well plates in the presence of (A) 600 mg/L and (B) 700 mg/L TCCA at 37°C for 24 hr. The wells containing TCCA-free LB broth (0 mg/L) were included as a normal growth control. The data represent the Mean  $\pm$  Standard deviation. Bacterial growth in the presence of TCCA with certain concentrations was analyzed by comparing OD<sub>620</sub> values with the most susceptible group (TH13091) using One-Way ANOVA followed by Tukey's test. \* p-value < 0.05, \*\* p-value < 0.01. Each experiment was repeated at least three times with n = 4.



**Figure 4. PCR amplification of known disinfectant-resistant genes in *K. pneumoniae* isolates.** The *cepA* and *qacED1* genes in the 12 *K. pneumoniae* strains were detected by PCR using primers specific for the two genes as listed (Table 2). Agarose gel electrophoresis was applied to detect the amplicons. Sizes of the molecular weight markers are indicated on the left side in base pair (bp).

disinfectant-associated genes in certain *K. pneumoniae* strains, although no close relationship was observed between these two genes and TCCA resistance (12). We thus tested whether these genes are responsible for the aforementioned *K. pneumoniae* resistance to TCCA by PCR amplification of these genes followed by detection of the PCR products with agarose gel electrophoresis. PCR amplification yielded an expected 1,051-bp amplicon of *cepA* for each of the 12 strains (Figure 4). Among these, TCCA-sensitive strains such as TH12880, TH12971, and TH12981 showed the *cepA* amplicon. This result indicated that *cepA* is not responsible for *K. pneumoniae*'s resistance to TCCA, because it was found in all strains regardless of tested TCCA resistance. Electrophoresis of the *qacED1* PCR reactions identified a small PCR product for TH12971, TH13098, and TH13139, consistent with the calculated size of the 300-bp *qacED1* amplicon. However, these *qacED1*-positive strains grew marginally in the presence of TCCA. Together, the PCR amplification experiment suggested that the presence of the *qacED1* and *cepA* genes is not associated with the TCCA resistance capacity of the invasive *K. pneumoniae* strains we observed in this study.

## DISCUSSION

It has been well-documented that *K. pneumoniae* and other nosocomial pathogens are resistant to disinfectants (5). However, it is not clear whether the pathogens of invasive infections (e.g., blood-borne infections), one of the more dangerous forms of bacterial infections, are particularly resistant to common disinfectants used in healthcare settings. While TCCA is commonly used for various sanitation purposes, we found that the majority of the invasive *K. pneumoniae* isolates tested here (10 out of 11) were able to tolerate temporary treatment with TCCA at the concentration of 30 mg/L, as would occur in hospital settings. This finding raises concerns that common sanitation practices with TCCA in hospital settings may not fully eradicate environmental *K. pneumoniae* as intended. The high frequency of TCCA tolerance in the invasive isolates tested might be due to the fact that these strains were the "survivors" of TCCA selection in medical settings before they were spread into human blood.

Among the 11 tested invasive *K. pneumoniae* isolates from human blood, we have found that two strains are resistant

to the common disinfectant TCCA, indicating the increasing prevalence of disinfectant-resistant nosocomial pathogens (1). It is reasonable to believe that these strain's ability to tolerate TCCA means they remain present in the hospital environment after disinfection, thus serving as a possible source for hospital-acquired infections. This finding has important implications for the future use of TCCA and other disinfectants. It is worthwhile to mention that all of the *K. pneumoniae* isolates tested in this work grew in the presence of 600 mg/L TCCA, which is substantially higher than the MIC values of TCCA towards *K. pneumoniae* clinical strains (64–128 mg/L) reported previously (12). This discrepancy might be caused by potential differences in the chemical purity of the TCCA reagents used in the two studies. The TCCA reagents used in this work and the previous study were commercial products for the general purpose of sanitation; the chemical purity of either product was clearly stated by the manufacturers.

Although *qacED1* and *cepA* have been implicated in conferring bacterial resistance to various disinfectants, our results suggested that the presence of these genes may not be the cause of TCCA resistance in the *K. pneumoniae* clinical strains used in this study (1). *cepA* was identified in both TCCA-susceptible and -resistant strains, while *qacED1* was not identified in most of the TCCA-resistant strains. Our finding is consistent with the observation of a previous study where *qacED1* and *cepA* were detected in fractions of antibiotic resistant *K. pneumoniae* strains, but no close relationship was observed between the two genes and TCCA resistance (12). Together, this work may indicate that the invasive *K. pneumoniae* isolates resisted TCCA via an uncharacterized mechanism. Therefore, our findings highlight the need to define the genetic or non-genetic basis of *K. pneumoniae* resistance to TCCA for the sake of understanding the mechanisms of bacterial resistance to the current disinfectants and developing novel approaches to pathogen control in healthcare settings.

## MATERIALS AND METHODS

### Bacteria isolates and cultivation

The *K. pneumoniae* isolates used in this work were originally obtained from blood samples of patients in Tongren, Renmin, and Tsinghua Changguang Hospitals in Beijing, China and kindly provided to our lab (Table 1). Bacteria were grown in LB broth and preserved as low-passage stocks at -70°C as described previously (18).

### Preparation of primary TCCA solution

We prepared a primary TCCA solution by dissolving a TCCA tablet (0.3 g, effective chlorine concentration of 45–55%) (Fengming Disinfection Technology Co. LTD) in 100 mL sterilized H<sub>2</sub>O to a final concentration of 3 g/L according to the supplier's instructions. The solution was used for further dilutions in H<sub>2</sub>O for disinfectant resistance experiments.

### Measurement of *K. pneumoniae* resistance to TCCA

Bacterial resistance to TCCA was assessed by bacterial survival following temporary disinfectant treatment (CFU counting) and bacterial growth in the presence of the disinfectant (culture turbidity or OD<sub>620</sub>), with TCCA-untreated groups as control. *K. pneumoniae* survival of temporary disinfectant treatment was determined principally according to the previously described method (12). In brief, bacteria from frozen stocks were streaked on LB agar plates; single

colonies were cultured in LB broth at 37°C to reach OD<sub>620</sub> of 0.5. Bacterial cultures (1 mL) were pelleted by centrifugation and resuspended in 1 mL of distilled H<sub>2</sub>O. The suspensions (50 µL) were mixed with 100 µL of TCCA solution to final concentrations of 32 and 64 mg/L and incubated at room temperature for 1 hr. Serial dilution was performed by 10-time dilution for three times and 5-time dilution for four times successively, and 10 µL of each dilution was plated onto LB agar dishes. After 12-hr incubation at 37°C, dilutions proper for accurate counting were counted. CFUs equal to the counting numbers times the corresponding dilution. Survival rate was the ratio of CFUs of TCCA-treated samples and the matching non-treated samples.

*K. pneumoniae* growth in the presence of TCCA was assessed by monitoring the turbidity or OD<sub>620</sub> of cultures containing various concentrations of TCCA (12). Briefly, *K. pneumoniae* strains were grown in TCCA-free LB broth to OD<sub>620</sub> of 0.5 before being diluted at 1:1000 into fresh LB broth. The diluted cultures were aliquoted to the wells of 96-well plates (100 µL/well) and mixed with 100 µL TCCA solution of various concentrations that were prepared from the primary solution. The OD<sub>620</sub> value of each well was determined after incubation at 37°C for 24 hr.

#### PCR Amplification

The PCR reactions were performed using the primer pairs for *qacED1* and *cepA* as described previously (Table 2) (12). We obtained the template DNA by boiling bacteria to achieve lysis. The cultures were adjusted to a similar OD<sub>620</sub> to balance bacterial input and resuspended by sterile water before being heated at 100°C for 10 minutes. The *cepA* gene was used as a positive control for DNA template since our preliminary trial showed this gene was present in all the *K. pneumoniae* strains. The volumes of water, master mix (Vazyme, 2 x Rapid Taq Master Mix), primers (10 µM), and template DNA were 20 µL, 25 µL, 2 µL, and 1 µL, respectively.

We performed PCR using the Dongsheng ETC811 PCR machine. The thermal cycle was programmed for 3 minutes at 95°C for initial denaturation, followed by 35 cycles of the following: 15 seconds at 95°C for denaturation, 15 seconds at 55°C for annealing, 60 seconds/kb at 72°C for extension, and 5 minutes at 72°C for the final extension. Three µL of PCR mixtures were loaded into an agarose gel to detect the PCR products along with a 100-5,000-bp DNA ladder. Electrophoresis was carried out at 130 V for 10 minutes in a TAE buffer before the gels were examined in Gel Imager (ChemiScope 6100).

#### Statistical analysis

Statistical analysis was carried out using GraphPad Prism software version 8.0.0. All data are presented as Mean ± Standard deviation unless otherwise indicated. Bacterial survival and growth in the presence of TCCA with certain concentrations were analyzed by One-Way ANOVA followed by Tukey's test. Differences with a p-value of < 0.05 is

Gene	Primer sequence (5'-3')	Product Size (bp)	Reference
<i>qacED1</i>	F: TAGCGAGGGCTTTACTAAGC R: ATTCAGAAATGCCGAACACCG	300	(12)
<i>cepA</i>	F: CAACTCCTTCGCCTATCCCG R: TCAGGTACAGACCAACCGCG	1,051	(12)

Table 2. Primer sequences of the target genes

considered to be statistically significant.

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