



FULL LENGTH ARTICLE

A novel method to detect bacterial resistance to disinfectants



Xiao-Feng He ^{a,1}, Hui-Jie Zhang ^{d,1}, Jin-Gui Cao ^a, Fang Liu ^a,
Jian-Kang Wang ^b, Wen-Jie Ma ^a, Wen Yin ^{c,*}

^a Department of Infection Control, Air Force General Hospital, Beijing 100142, China

^b Department of Pharmacy, Air Force General Hospital, Beijing 100142, China

^c Department of Transfusion Medicine, Xijing Hospital, The Fourth Military Medical University, Xi'an 710032, China

^d Ministry of Education Key Laboratory of Diagnostic Medicine, College of Laboratory Medicine, Chongqing Medical University, Chongqing 400016, China

Received 19 June 2017; accepted 2 July 2017

Available online 8 July 2017

KEYWORDS

Clinical technology;
Disinfectants
resistance;
Inhibitory efficacy;
Oxford plate method;
Staphylococcus aureus

Abstract In clinical practice, the important hygienic prevention of bacterial pathogen spread is disinfection of potentially contaminated area. Benzalkonium bromide and chlorhexidine acetate are commonly used disinfectants with a broad spectrum of antimicrobial effect. It is vital to inhibit the spread of pathogen in hospital. However, a large number of pathogens with the decreased antiseptic susceptibility have been isolated from clinical samples which showed an increased minimal inhibitory concentration (MIC) against those antiseptics. These resistant pathogens are the major causes for nosocomial cross-infections in hospital. The present study demonstrated the utility of Oxford plate assay system in determining the potential disinfectant resistance of bacteria. The microbiological assay is based on the inhibitory effect of tested disinfectants upon the strains of *Staphylococcus aureus* and *Escherichia coli*. Statistical analysis of the bioassay results indicated the linear correlation ($r = 0.87-0.99$, $P < 0.01$) between the diameter of growth inhibition zone and the log dosage of the tested disinfectants. Moreover, comparison of inhibitory efficacy of benzalkonium bromide upon 29 *S. aureus* strains isolated from clinical samples by both Oxford plate method and broth dilution method showed that the diameter of growth inhibition zone has significantly negative correlation with the minimal inhibitory concentration (MIC) ($r = -0.574$, $P < 0.001$). These results suggest that the Oxford plate is a simple

* Corresponding author.

E-mail address: yinwen@fmmu.edu.cn (W. Yin).

Peer review under responsibility of Chongqing Medical University.

¹ The authors contribute equally to this work.

and time-saving method in detecting potential clinical disinfectant resistance and its usefulness for routine surveillance of pathogenic resistance to disinfectants warrants further investigation.

Copyright © 2017, Chongqing Medical University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

In clinical practice, the important hygienic prevention of bacterial pathogen spread is disinfection of potentially contaminated area, such as rooms, utensils and hands. Some antiseptics and disinfectants, sharing common characteristics,¹ such as irritation, contact dermatitis, and urticaria, have to be carefully used at minimal bactericidal concentrations in controlling pathogen contamination. For example, povidone-iodine and chlorhexidine are important antiseptics for decreasing skin contaminations of Gram-positive and Gram-negative bacteria, especially those antibiotic-resistant strains, such as MRSA and CA-MRSA. Aldehydes and quaternary ammonium compounds, such as benzalkonium bromide and benzalkonium chloride, are widely used as disinfectants for sterilization of non-living objects or surfaces.^{2,3} The usage of those chemical antiseptics and disinfectants is the vital way to inhibit the spread of pathogen in hospital. However, a large number of pathogens with the decreased antiseptic susceptibility have been isolated from clinical samples which showed an increased minimal inhibitory concentration (MIC) against those antiseptics.^{4–8} These resistant pathogens are the major causes for nosocomial cross-infections in hospital. Sometimes, the infection is fatal. Since 1980s, *Pseudomonas* and *Pseudomonas cepacia* have been isolated in iodophor and polyvinylpyrrolidone-iodine complex (PVP-I) respectively. And the last one has resulted in pseudo-bacteremia.¹ Similarly, Gram-positive bacteria resistance has also been reported in the past decades. The activity of three *Enterococcus* spp strains can last for 5 min in 100 ppm available chlorine, while only 2 min for non-resistant *Enterococcus* in 0.5 ppm chlorine.⁹ Based on these clinical observations it's important and crucial to monitor pathogen resistance against system antiseptics and disinfectants.

To closely monitor the bacterial adaptation and resistance to antiseptics and disinfectants, some microbiological assays used in evaluation of antibacterial activity of antibiotics, such as agar diffusion assay,¹⁰ cylinder plate assay,¹¹ could be applied. Although less accurate and less precise than HPLC assays, the microbiological assays are still widely used for clinical practice because of their advantages of short turn-around time, simplicity, and low cost. One of the agar diffusion bioassay, Oxford plate assay, is commonly used for the measurements in antibiotics for antibacterial activities and cytotoxicity based on the correlation of the size of bacteriostatic ring and the dosage of antibiotics.^{12–16} However, the usefulness of the Oxford plate method in detecting the resistance of pathogen against chemical disinfectant is not well studied. To explore such potential Oxford plate method was used in the

present study to determine the potential disinfectant resistance of *Staphylococcus aureus* and *Escherichia coli* against benzalkonium bromide and chlorhexidine acetate in dosage forms of MIC.

Materials and methods

Bacteria strains

The *S. aureus* (ATCC6538) and *E. coli* (8099) were routine strains in chemical disinfectant and antibiotic-resistance detection. They were provided by Chinese PLA Center for Disease Control & Prevention, Academy of Military Medical Sciences. The 29 *S. aureus* clinical strains were automatically separated by VITEK 2 Compact (Biomérieux, France) from the Department of Infection Control, Air Force General Hospital.

Chemicals

The stainless steel Oxford plates with inner diameter (6.0 ± 0.1) mm, outer diameter (7.8 ± 0.1) mm, height (10.0 ± 0.1) mm were purchased from Shanghai Huake Labware Co., LTD. The gradient dilutions of benzalkonium bromide from 50 mg/mL stock solution (Nanchang Baiyun Pharmaceutical) were 32 000, 16 000, 8 000, 4 000, 2 000, 1 000, 500, 250, 125, 62.5, 31.25, 15.625, 7.812 5 $\mu\text{g/mL}$ with ddH₂O. And chlorhexidine acetate (Jiutai Pharmaceutical) was dissolved with ddH₂O to a final concentration of 2 000, 1 000, 500, 250, 125, 62.5, 31.25, 15.625, 7.812 5, 3.906 $\mu\text{g/mL}$. The MUELLER-HINTON (MH) agar plates (90 mm) and MH broth powder were purchased from BIOMERIEUX and OXOID.

Microbiological assay

Oxford plate assay

3–4 bacteria colonies were picked up and resuspended in sterile PBS. The suspension was adjusted to 0.5 McF (equal to 1.5×10^8 cfu/mL) by turbidimeter and then diluted by 10 folds in which the concentration was around 1.5×10^7 cfu/mL. The suspension was homogeneously smeared on the MH plate using sterile cotton swab. 2 cylinders were placed on the surface of inoculated medium. The cylinders were filled with 250 μL containing the titrations of benzalkonium bromide or chlorhexidine acetate. After incubation for 24 h at 37 °C, the zone diameters of the growth inhibition were measured and compared. Same experiments were performed with the 29 clinically isolated *S. aureus* with 60 $\mu\text{g/mL}$ benzalkonium bromide. Each concentration listed in 2.2 chemicals was tested for quadruplicates.

Suspension quantitative germicidal test

2.5 mL of each concentrations (as above) of benzalkonium bromide was added to MH broth tube to make a final concentration of 8, 6, 4, 3, 2, 1.5, 1, 0.5 $\mu\text{g/mL}$ respectively. The suspension of *S. aureus* strains (named as sau + numbers) was adjusted to 0.5 McF. Adding 0.1 mL of those into the benzalkonium bromide MH broth medium, the bacteria were cultured at 37 °C for 48 h. The minimal inhibitory concentration (MIC) was determined as the highest dilution of medium in which none bacteria could grow. Each experiment was conducted in quadruplicates.

Statistical analysis

The data were analyzed by SPSS20.0. The correlation analysis of the diameter of growth inhibition zone and the minimal inhibitory concentration was performed by Spearman ($\alpha = 0.01$). The linear correlation between the diameter of growth inhibition zone and the log concentration ($\mu\text{g/mL}$) of tested disinfectants was analyzed by Origin 7.5 software.

Results

Comparison of the disinfectant susceptibility of *S. aureus* and *E. coli*

Generally, the Oxford plate assay is an agar diffusion bioassay which is used to detect the antibacterial activity of antibiotics. In order to explore if it's useful for evaluating the antibacterial effects of disinfectants, it was used in this study to detect the bactericidal activities of benzalkonium bromide and chlorhexidine acetate against *S. aureus* (ATCC6538) and *E. coli* (8099) respectively. Using the Oxford plate method, well-defined inhibition growth zones were formed allowing accurate measurements (Fig. 1A). The corresponding mean diameter of bacteriostatic zone was positively correlated with the logarithm of

the applied doses of benzalkonium bromide (Table 1). Similar results were found with the chlorhexidine acetate (Table 2). We also found the bacteriostasis of tested disinfectants on *S. aureus* is similar. However, *E. coli* is more sensitive to chlorhexidine acetate than to benzalkonium bromide (Tables 1 and 2).

Further calculation assumed a linear relationship between the observed diameter of bacteriostatic zone and the logarithm of the applied dose. The calibration curves for disinfectants were constructed by the log concentration ($\mu\text{g/mL}$) versus the zone diameter (mm). The representative linear equation for benzalkonium bromide against *S. aureus* and *E. coli* was $y = 9.05 + 6.28x$ ($r = 0.92$, $P < 0.0001$) (Fig. 1B) and $y = 2.47 + 4.54x$ ($r = 0.87$, $P = 0.002$) respectively (Fig. 2). For the activity of chlorhexidine acetate, the representative linear equation for *S. aureus* was $y = 11.74 + 5.17x$ ($r = 0.97$, $P < 0.0001$) (Fig. 3) and *E. coli* was $y = 9.8 + 5.26x$ ($r = 0.99$, $P < 0.0001$) (Fig. 4). All the correlation coefficient was statistically significant for the method.

Antibacterial effect of benzalkonium bromide on clinically isolated *S. aureus*

To determine the clinical application in screening disinfectant resistant bacteria strain, the Oxford plate assay was used to detect the potency of disinfectants by comparing the growth inhibition zone among 29 clinically isolated *S. aureus* and reference standard (ATCC6538) induced by 60 $\mu\text{g/mL}$ benzalkonium bromide. After incubating at 37 °C for 24 h, the diameter of inhibition growth zone is ranging from 11.5 mm to 19.5 mm for the clinical strains compared with 17.0–19.5 mm for standard strain. The minimal inhibitory concentration (MIC) was evaluated using broth dilution method. The results showed that the MIC of the reference strain (ATCC6538) was around 1.5 $\mu\text{g/mL}$ (Table 3). The results of the statistical analysis indicated that there is significant negative correlation between the

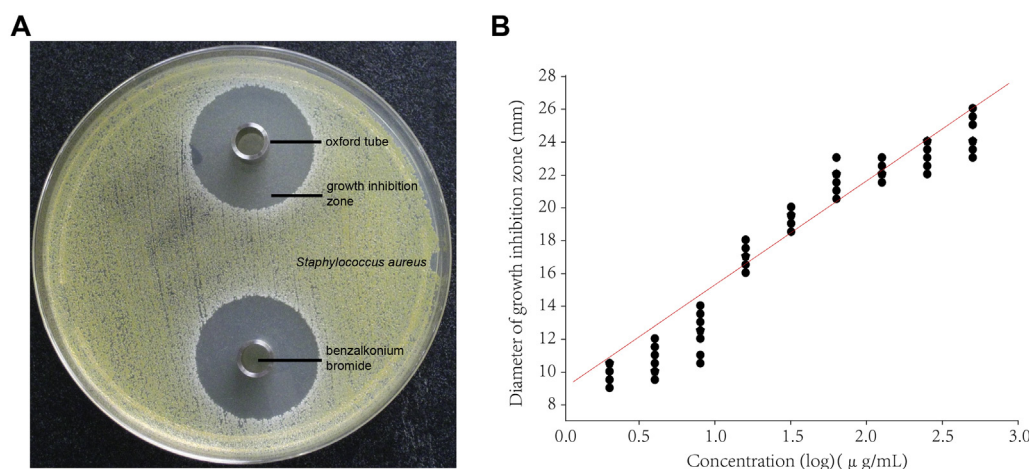


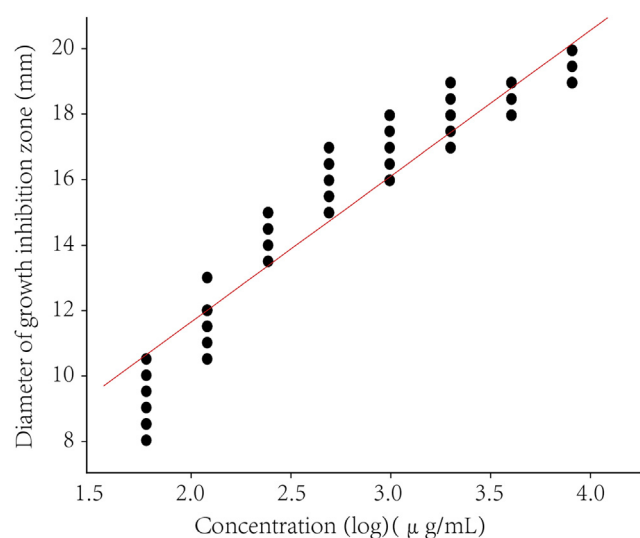
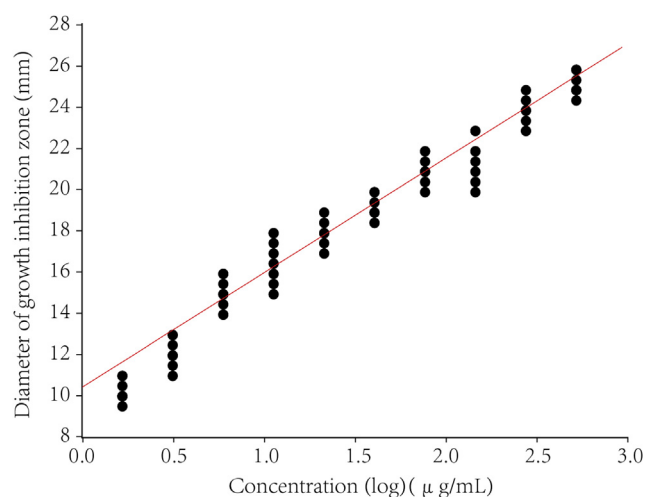
Fig. 1 The correlation of benzalkonium bromide against *S. aureus* tested by Oxford plate assay. (A) An example photo of the Oxford plate assay. (B) Calibration curves of the log concentration ($\mu\text{g/mL}$) versus the zone diameter (mm) for the inhibition of benzalkonium bromide on *S. aureus* (ATCC6538).

Table 1 Antibacterial susceptibility of benzalkonium bromide against *S. aureus* and *E. coli*.

Concentration ($\mu\text{g/mL}$)	<i>S. aureus</i> (ATCC6538)		<i>E. coli</i> (8099)	
	Mean diameter of inhibition zone (SEM $\pm$ S) (mm)	Variation coefficients (%)	Mean diameter of inhibition zone (SEM $\pm$ S) (mm)	Variation coefficients (%)
7.81	9.88 $\pm$ 0.52	5.24	Ns	Ns
15.63	10.75 $\pm$ 0.89	8.25	Ns	Ns
31.25	12.31 $\pm$ 1.19	9.69	Ns	Ns
62.5	17.17 $\pm$ 0.82	4.76	Ns	Ns
125	19.38 $\pm$ 0.52	2.67	Ns	Ns
250	21.88 $\pm$ 0.88	4.01	9.06 $\pm$ 0.86	9.53
500	22.38 $\pm$ 0.58	2.6	11.69 $\pm$ 0.75	6.44
1000	23.17 $\pm$ 0.82	3.52	14.42 $\pm$ 0.58	4.05
2000	24.5 $\pm$ 1.04	4.22	15.88 $\pm$ 0.74	4.69
4000	Ns	Ns	17.13 $\pm$ 0.88	5.12
8000	Ns	Ns	18.00 $\pm$ 0.80	4.45
16,000	Ns	Ns	18.63 $\pm$ 0.44	2.38
32,000	Ns	Ns	19.44 $\pm$ 0.50	2.55

Table 2 Antibacterial susceptibility of chlorhexidine acetate against *S. aureus* and *E. coli*.

Concentration ($\mu\text{g/mL}$)	<i>S. aureus</i> (ATCC6538)		<i>E. coli</i> (8099)	
	Mean diameter of inhibition zone (SEM $\pm$ S) (mm)	Variation coefficients (%)	Mean diameter of inhibition zone (SEM $\pm$ S) (mm)	Variation coefficients (%)
3.91	10.25 $\pm$ 0.53	5.21	Ns	Ns
7.8125	12.13 $\pm$ 0.69	5.73	10.81 $\pm$ 0.80	7.39
15.625	15.00 $\pm$ 0.80	5.35	12.44 $\pm$ 0.56	4.53
31.25	16.69 $\pm$ 1.13	6.78	14.69 $\pm$ 0.84	5.74
62.5	18.00 $\pm$ 0.71	3.93	16.25 $\pm$ 0.76	4.65
125	19.19 $\pm$ 0.59	3.1	17.63 $\pm$ 0.58	3.3
250	21.13 $\pm$ 0.69	3.29	19.00 $\pm$ 0.80	4.22
500	21.63 $\pm$ 1.09	5.06	20.94 $\pm$ 0.68	3.24
1000	23.88 $\pm$ 0.69	2.91	21.83 $\pm$ 0.52	2.37
2000	25.38 $\pm$ 0.58	2.3	23.06 $\pm$ 0.62	2.7

**Fig. 2** Calibration curves of the log concentration ($\mu\text{g/mL}$) versus the zone diameter (mm) for the inhibition of benzalkonium bromide on *E. coli* (8099).**Fig. 3** Calibration curves of the log concentration ($\mu\text{g/mL}$) versus the zone diameter (mm) for the inhibition of chlorhexidine acetate on *S. aureus* (ATCC6538).

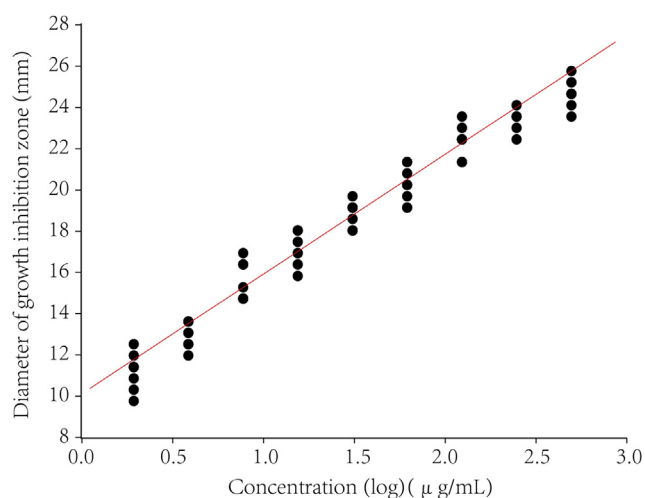


Fig. 4 Calibration curves of the log concentration ($\mu\text{g/mL}$) versus the zone diameter (mm) for the inhibition of chlorhexidine acetate on *E. coli* (8099).

diameter of growth inhibition zone from Oxford plate assay and the MIC value ($r = -0.574$, $P_{\text{two-tailed}} = 0.000$).

Antibacterial coefficient of variation of disinfectants on *S. aureus* and *E. coli*

Generally, the coefficient variation of disinfectant vs. bacteria was determined by Oxford plate method in the current study. The data was summarized and calculated by SPSS20.0 in Table 4. And the index of disinfectant against 29 clinical isolated *S. aureus* was $(3.61 \pm 0.71)\%$, indicating a good reproducibility of Oxford plate assay.

Discussion

In healthcare facilities, antiseptics and disinfectants are used to reduce the cross-transmission of pathogenic microbes. Numerous studies demonstrated the resistance of the pathogenic microbes to antiseptics and disinfectants.^{4,7,8}

Table 4 Antibacterial coefficient of variation of disinfectant against *S. aureus* and *E. coli* (SEM $\pm$ S, %).

Antiseptic	<i>S. aureus</i>	<i>E. coli</i>
Benzalkonium bromide	5.00 ± 2.44	4.90 ± 2.29
Chlorhexidine acetate	4.36 ± 1.46	4.24 ± 1.59

Table 3 Antibacterial activities of benzalkonium bromide against clinically isolated *S. aureus* strains.

Clinically isolated strain	Oxford plate assay		Broth dilution method
	Mean diameter of inhibition zone (SEM $\pm$ S) (mm)	Variation coefficients (%)	MIC _{median} ($\mu\text{g/mL}$)
ATCC6538 (control)	18.19 ± 0.90	4.94	1.5
sau33	17.94 ± 0.58	3.25	1.5
sau34	12.38 ± 0.60	4.84	3
Sau41	14.56 ± 0.63	4.36	3
Sau46	18.19 ± 0.50	2.73	1.5
Sau52	16.56 ± 0.39	2.36	2
Sau53	13.25 ± 0.43	3.27	2
Sau54	17.94 ± 0.58	3.25	1.5
Sau55	17.81 ± 0.50	2.79	1.5
Sau56	14.81 ± 0.66	4.45	2
Sau70	17.44 ± 0.53	3.02	1.5
Sau76	15.31 ± 0.56	3.63	1.5
Sau77	16.94 ± 0.53	3.11	2
Sau78	16.56 ± 0.46	2.8	1.5
Sau80	16.83 ± 0.47	2.8	1.5
Sau81	14.75 ± 0.56	3.79	1.5
Sau82	14.94 ± 0.58	3.9	2
Sau83	15.00 ± 0.61	4.08	2
Sau133	14.56 ± 0.63	4.36	2
Sau135	14.75 ± 0.43	2.94	2
Sau142	15.19 ± 0.66	4.34	2
Sau143	17.50 ± 0.71	4.04	1.5
Sau149	18.06 ± 0.77	4.25	1.5
Sau155	13.50 ± 0.50	3.7	3
Sau157	17.00 ± 0.50	2.94	1.5
Sau159	16.56 ± 0.46	2.8	1.5
Sau161	16.06 ± 0.63	3.95	1.5
Sau162	18.31 ± 0.61	3.33	1.5
Sau165	14.94 ± 0.68	4.56	1.5
Sau167	16.00 ± 0.61	3.83	1.5

Standardized methods of susceptibility testing and minimum inhibitory concentrations (MICs) were routinely applied in defining bacterial resistance to antibiotics. However, fewer reports demonstrated the utility of those methods in susceptibility testing for antiseptics and disinfectants. The use-dilution and agar-dilution methods have been used to evaluate the disinfectant activity and minimum inhibitory concentration (MIC).¹⁷ Alternatively it could be approximately determined by calculating the viable colonies in agar plates before and after exposure to a disinfectant.¹⁸ But these methods are less cost-effective and time-consuming. The emerging challenges of bacterial resistance to antiseptics and disinfectants in healthcare facilities call for more frequent susceptibility testing.^{8,19–21} For routine quality control in hospitals, validation of time-saving and less expensive methods to monitor resistance to antiseptics and disinfectants is highly warranted.

In this study, the Oxford plate assay, a standard microbiological method for evaluating the susceptibility and resistance to antibiotics,²² was used to evaluate the disinfectant susceptibility of *S. aureus* and *E. coli*. The results obtained in this study showed a significant correlation between the applied dosages of disinfectants, benzalkonium bromide and chlorhexidine acetate, and the diameter of growth inhibition zone for *S. aureus* (ATCC6538) and *E. coli* (8099). These results confirmed that the Oxford plate assay is suitable for its application in detecting bactericidal activities of disinfectants. However, we also noticed the limitations of the Oxford plate assay in the cases of o-phthalaldehyde and chlorine. Specifically we were not able to get a well-defined growth inhibition zone when using o-phthalaldehyde and chlorine against *S. aureus* (ATCC6538) and *E. coli* (8099). It might be due to the chemical properties of tested disinfectants as alcohols or aldehyde.

While the pathogen resistance to antibiotics has been known for decades, pathogen strains resistant to disinfectants are relatively recent findings. The spread of disinfectant-resistant pathogens has resulted in serious secondary cross-infections and deaths in hospitals. In the present study, both Oxford plate assay and broth dilution method were used to determine disinfectant susceptibility of 29 clinically isolated *S. aureus* strains to benzalkonium bromide. The results showed increased MICs using broth dilution method, indicating an acquired resistance in *S. aureus*, which corresponded to shrunk sizes of growth inhibition zones in Oxford plates. Our results also showed that MICs from broth dilution method have a negative correlation with the size of growth zone in Oxford plate. These comparisons gave further validation on the merit of Oxford plate assay in detecting disinfectant resistance, raising a promising prospect for this method to be used in the surveillance of bacterial resistance in hospitals.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

All authors would like to thank Prof. Fei Li for helpful comments and revision on this manuscript.

References

- Craven DE, Moody B, Connolly MG, Kollisch NR, Stottmeier KD, McCabe WR. Pseudobacteremia caused by povidone-iodine solution contaminated with *Pseudomonas cepacia*. *N Engl J Med*. 1981;305(11):621–623.
- Boyce JM, Pittet D. Guideline for hand hygiene in health-care settings: recommendations of the healthcare infection control practices advisory committee and the HICPAC/SHEA/APIC/IDSA hand hygiene task Force. *Infect Control Hosp Epidemiol*. 2002;23(12 suppl):S3–S40.
- McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. *Clin Microbiol Rev*. 1999;12(1):147–179.
- Akinkunmi EO, Lamikanra A. Susceptibility of community associated methicillin resistant *Staphylococcus aureus* isolated from faeces to antiseptics. *J Infect Dev Ctries*. 2012;6(4):317–323.
- Horner C, Mawer D, Wilcox M. Reduced susceptibility to chlorhexidine in staphylococci: is it increasing and does it matter? *J Antimicrob Chemother*. 2012;67(11):2547–2559.
- Ivanov IB, Gritsenko VA, Kuzmin MD. The effect of brief exposure to sub-therapeutic concentrations of chlorhexidine digluconate on the susceptibility of staphylococci to platelet microbicidal protein. *Surg Infect (Larchmt)*. 2015;16(3):263–266.
- Russell AD. Bacterial adaptation and resistance to antiseptics, disinfectants and preservatives is not a new phenomenon. *J Hosp Infect*. 2004;57(2):97–104.
- Kawamura-Sato K, Wachino J, Kondo T, Ito H, Arakawa Y. Reduction of disinfectant bactericidal activities in clinically isolated *Acinetobacter* species in the presence of organic material. *J Antimicrob Chemother*. 2008;61(3):568–576.
- Kearns AM, Freeman R, Lightfoot NF. Nosocomial enterococci: resistance to heat and sodium hypochlorite. *J Hosp Infect*. 1995;30(3):193–199.
- Schmidt CA, Carazzo M, Laporta LV, Bittencourt CF, Santos MR, Friedrich M. Development and validation of an agar diffusion assay for determination of ceftazidime in pharmaceutical preparations. *J AOAC Int*. 2008;91(1):59–66.
- Souza MJ, Kulmann RR, Silva LM, Nogueira DR, Zimmermann ES, Schmidt CA. Development and in-house validation of a microbiological assay for determination of cefepime in injectable preparations. *J AOAC Int*. 2006;89(5):1367–1372.
- Costa MC, Barden AT, Andrade JM, Oppe TP, Schapoval EE. Quantitative evaluation of besifloxacin ophthalmic suspension by HPLC, application to bioassay method and cytotoxicity studies. *Talanta*. 2014;119:367–374.
- Paim CS, Fuhr F, Barth AB, et al. Gemifloxacin mesylate (GFM) stability evaluation applying a validated bioassay method and in vitro cytotoxic study. *Talanta*. 2011;83(5):1774–1779.
- Mahmoudi A, Fourar RE, Boukhechem MS, Zarkout S. Microbiological assay for analysis of certain macrolides in pharmaceutical dosage forms. *Int J Pharm*. 2015;491(1-2):285–291.
- Lopes CC, Salgado HR. Development and validation of a stability-indicative agar diffusion assay to determine the potency of linezolid in tablets in the presence of photo-degradation products. *Talanta*. 2010;82(3):918–922.
- Wagner RD, Johnson SJ, Cerniglia CE, Erickson BD. Bovine intestinal bacteria inactivate and degrade ceftiofur and ceftriaxone with multiple beta-lactamases. *Antimicrob Agents Chemother*. 2011;55(11):4990–4998.
- Fekadu S, Merid Y, Beyene H, Teshome W, Gebre-Selassie S. Assessment of antibiotic- and disinfectant-resistant bacteria in hospital wastewater, south Ethiopia: a cross-sectional study. *J Infect Dev Ctries*. 2015;9(2):149–156.

18. Reichel M, Schlicht A, Ostermeyer C, Kampf G. Efficacy of surface disinfectant cleaners against emerging highly resistant gram-negative bacteria. *BMC Infect Dis.* 2014;14:292.
19. Harbarth S, Tuan Soh S, Horner C, Wilcox MH. Is reduced susceptibility to disinfectants and antiseptics a risk in healthcare settings? A point/counterpoint review. *J Hosp Infect.* 2014; 87(4):194–202.
20. Ledder RG, Gilbert P, Willis C, McBain AJ. Effects of chronic triclosan exposure upon the antimicrobial susceptibility of 40 ex-situ environmental and human isolates. *J Appl Microbiol.* 2006;100:1132–1140.
21. Sasatsu M, Shimizu K, Noguchi N, Kono M. Triclosan-resistant *Staphylococcus aureus*. *Lancet.* 1993;341(8847):756.
22. Commission CP. Pharmacopoeia of the People's Republic of China. In: *China Medical Science Press*. vol. II. Beijing: China Medical Science Press; 2010. Appendix 93–130 [in Chinese version].