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Original Article

Desiccation and ethanol resistances of multidrug resistant *Acinetobacter baumannii* embedded in biofilm: The favorable antiseptic efficacy of combination chlorhexidine gluconate and ethanol

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Antiseptic efficacy

Abstract *Background/purpose:* Globally, multidrug-resistant *Acinetobacter baumannii* (MDRAB) has emerged as an important pathogen in nosocomial outbreaks. This study aimed to investigate the correlation between the biofilm formation and survival of MDRABs, and to investigate the antiseptic efficacy of hand sanitizers for the MDRABs, embedded with biofilm (MDRAB-Bs).
Methods: The MDRABs were selected randomly after pulsed-field gel electrophoresis (PFGE), and their biofilm formation was analyzed. Desiccation and ethanol tolerances were assayed to test the bacterial survival. The antiseptic efficacy of combined chlorhexidine gluconate

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(CHG) and 70% ethanol agents against MDRAB-Bs were compared with the 70% ethanol cleanser.
Results: Eleven MDRABs, which varied in biofilm formation (MRDAB-B) and planktonic type (MDRAB-P), were tested. In desiccation survival, the mean survival time for the MDRAB-Bs was 49.0 days which was significantly higher than that of their planktonic type (17.3 days) ($P < 0.005$). The MDRAB-Ps could be eliminated after a 10 min contact with a 30% ethanol agent, however, it took 10 min of 70% ethanol to eliminate the MDRAB-Bs. On the other hand, a 2% CHG in 70% ethanol solution completely eliminated all MDRAB-Bs after 1 min contacted time. The 2% CHG in 70% ethanol agent provided a significantly superior efficacy than the 70% ethanol solution at eliminating the MDRAB-Bs ($P < 0.005$).
Conclusion: MDRAB with biofilm-formation presented significantly higher desiccation and ethanol resistances than their planktonic type. Moreover, the 2% CHG in 70% ethanol agent provided a superior antiseptic efficacy for MDRAB-Bs than that of the 70% ethanol agent.

Introduction

Acinetobacter baumannii (AB) is a non-fermenting Gram-negative bacterium that is widely distributed in the natural environment.[1] Globally, multi-drug-resistant AB (MDRAB) has emerged as an important pathogen in nosocomial outbreaks, associated with environmental and health care worker (HCW) contamination.[2,3] MDRAB is a successful pathogen in a variety of nosocomial infections due to its environmental resilience and widespread antibiotic resistance.[4,5] Infection control strategies, such as contact precautions, hand washing, and alcohol hand decontamination, are crucial for preventing MDRAB outbreaks.[6] A previous report suggested that 70% alcohol antiseptic agents might be the most effective hand-cleansing agents for removing AB strains from heavily contaminated hands.[7] Currently, an alcohol-based hand rub, recommended by the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO), is available for hygienic hand disinfection of HCWs to decrease the spread of AB infections.[8,9]

High capacity of biofilm formation for clinically isolated ABs prolongs their survival and persistence in hospital environments, which results in the higher probability of nosocomial infections.[10–13] ABs displays high variability of biofilm production,[10] however, the correlation between biofilm production and environmental resilience is largely unknown. Moreover, the scientific reports on the efficacy of alcohol-based antiseptic agents for eliminating the MDRABs with biofilm (MDRAB-Bs) are limited. Chlorhexidine gluconate (CHG) has been proven to reduce the rate of nosocomial infections more effectively than the alcohol-based agents.[14] While microorganisms in biofilms are resistant to CHG, Bonez et al. reported that 2% CHG showed an unfavorable efficacy to eliminate AB with biofilm.[15] Of note, there were limited studies investigating the antiseptic efficacy of CHG in combination with 70% alcohol for eliminating MDRAB-B for hand hygiene.

The purposes of this study were to investigate the desiccation and ethanol resistances of MDRABs, and to compare the antiseptic efficacy between the CHG combined 70% ethanol agents and the traditional 70% alcohol cleanser for MDRAB-Bs.

Methods and results

Microorganisms

Sixty-four MDRABs, isolated from sporadic clinical cases at the Chi-Mei Medical Center, were used as test organisms. The organisms were identified by the Phoenix 100 system (BD Biosciences, Franklin Lakes, NJ), and were used throughout the experiment. Aliquots of these stains were prepared and frozen at −80 °C until use. Antibiotics susceptibility testing of amikacin, ceftazidime, ciprofloxacin, carbapenems (meropenem, imipenem, and doripenem), gentamicin, minocycline, trimethoprim-sulfamethoxazole, piperacillin-tazobactam were determined by the routine disk-diffusion method to assay the susceptibility of bacteria to antimicrobial agents, followed by the Clinical and Laboratory Standards Institute.[16] In present study, MDRAB was defined as an *A. baumannii* isolate resistant to the antibiotics including carbapenem antibiotics and five of the others antibiotics.[17]

Bacterial strain selection

Molecular typing by pulsed-field gel electrophoresis (PFGE) was performed as previously described for molecular typing and bacterial selection.[18] Briefly, the preparation of genomic deoxyribonucleic acid (DNA) of the AB isolates was performed. Clusters of possibly related isolates were identified using the Dice coefficient of similarity and the unweighted group method with arithmetic averages of 80%, which indicates four to six fragment differences in gels with an average of 20 bands for analysis.[19]

Compounds

Three antiseptic agents (ethanol alcohol, CHG, and isopropyl alcohol) were prepared; 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, or 80% ethanol alcohol were prepared by diluting from 100% ethanol (Sigma–Aldrich, St. Louis, MO, USA) with sterile distilled water. 0.5% (w/v) CHG in 70% (v/v) isopropyl alcohol (Steridal Solution®, Panion & BF biotech,

Taoyuan, Taiwan) and 2% (w/v) CHG in 70% (v/v) ethanol (Easy Antiseptic Liquid®, Panion & BF biotech, Taoyuan, Taiwan) were purchased from market. These agents were used according to the manufacturer's recommendations.

Determining biofilm formation

Biofilm formation was detected as previously reported.[20] Briefly, the bacteria were enriched for 1 day at 37 °C in 5 mL of tryptic soy broth (TSB). Cultures were diluted 1:1000 in TSB with 1% D-glucose (TSBG), and 1 mL was added to each well of a 24-well tissue culture-treated polystyrene plate. For biofilm formation, the incubation times were 1, 3 and 5 days at 37 °C. Plates were then washed vigorously three times with phosphate-buffered saline (PBS) to remove unattached bacteria and were stained with a 0.5 mL 1% crystal violet solution at room temperature for 30 min. The stain was then removed, and the wells were washed 3 times with 1 mL sterile deionized water before the addition of 0.5 mL of dimethyl sulfoxide (DMSO) to solubilize the crystal violet. The plate was then read spectrophotometrically at an absorbance wavelength of 570 nm. For each strain, the assay was run in triplicate wells, the results were averaged, and the standard deviation (S.D.) was calculated. Wells containing medium only were used as the control. The definition of biofilm-positive phenotype (D1, D3, and D5) was modified as an optical density (OD) 570 nm value of $\geq$0.2.[21]

Desiccation survival assay for MDRAB-Bs and MDRAB-Ps

The desiccation survival assay for MDRAB-Bs was performed as previously described.[22] After 5 days of biofilm formation, the plates were washed vigorously three times with PBS to remove unattached bacteria. The plates were kept dry in a laminar-flow hood. All samples were stored in a dark, dust-protected climate chamber at 22 $\pm$ 2 °C with 50% $\pm$ 5% relative humidity. At various time intervals (after 1, 3, 5, 7, and 10 days and after 2, 3, 4, 5, 6, 7, 8, and 9 weeks), 1 mL PBS was added in per well, viable cells were recovered from the contaminated samples. Bacteria were removed by sonication, and 100 μL aliquots were inoculated onto nutrient agar plates after appropriate dilutions were made. The number of bacteria per sample was calculated from the results. For planktonic-type MDRAB-Bs (MDRAB-Ps), 1-mL aliquots of overnight TSB cultures were centrifuged at 11,600 $\times$ g for 5 min in a microcentrifuge. The cell pellet was washed twice with PBS and suspended in distilled water to an OD 600 of 2.0. One hundred microliters of each suspension was deposited onto a 24-well culture plate to produce an inoculum of $\sim$1 $\times$ 10^8 colony-forming units (CFUs) per well. 1 ml PBS was added in per well, then the viable cells were recovered. Under the same desiccated conditions and checking time intervals as those of MDRAB-Bs, the number of bacteria was assayed.

Antiseptic efficacy assay of ethanol on MDRAB-Bs and MDRAB-Ps

The antiseptic effects of ethanol against MDRAB-Bs and MDRAB-Ps were tested as previously reported.[23] Briefly, for MDRAB-Bs, after 5 days of biofilm formation, the plates were washed vigorously three times with PBS to remove unattached bacteria. Two hundred microliters of 20, 30, 40, 50, 60, 70, or 80% ethanol alcohol was added to each 24-well plate. After 10 min, the wells were washed once with sterile PBS. Bacterial numbers were counted by plating as described above. For MDRAB-Ps, 1 mL aliquots of overnight TSB cultures were centrifuged at 11,600 $\times$ g for 5 min in a microcentrifuge tube. The cell pellet was washed twice with PBS and suspended in distilled water to an OD600 of 0.2. One milliliter of each suspension was deposited onto a 15 mL centrifuge tube to produce an inoculum of $\sim$1 $\times$ 10^8 CFUs. The tubes were centrifuged at 11,600 $\times$ g for 5 min to remove supernatant. Two hundred microliters of 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, or 80% ethanol alcohol was added to each tube. After 10 min, 10 mL of sterile PBS was added to the tubes, and the supernatant was removed completely by centrifugation. The bacterial number was counted by plating as described above.

Comparison of disinfectant efficacies against MDRAB-Bs

After 5 days of biofilm formation, the plates were washed vigorously three times with PBS to remove unattached bacteria. Two hundred microliters of the three disinfectants was added to each 24-well plate. After 1, 3, 5, or 10 min, the supernatants were gently removed and added to 1 mL of neutralizing agent [2% (v/v) Tween 80, 1.17% (w/v) lecithin, 0.1% (v/v) Triton X-100 and 0.5% (w/v) sodium thiosulphate, all purchased from Sigma; St Louis, USA], mixed, and incubated for 5 min. The bacteria were removed from plates by sonication, harvested in 100 μL aliquots and inoculated onto nutrient agar plates, then, the CFUs were measured by serial dilutions. The evaluations were performed in triplicate. Three tested hand antiseptic agents (70% ethanol, 0.5% CHG combined 70% isopropyl alcohol, and 2% CHG combined 70% ethanol) were compared.

Statistical analysis

The data are presented as means $\pm$ standard deviations. The assumptions of normality and homoscedasticity (Levene-test) were satisfied for each group. In the desiccation survival assays and comparative antiseptic effectiveness experiments, the differences between groups were tested using repeated measures analysis of variance (rANOVA). The assessment of the bactericidal activities of the diverse ethanol agents was derived from three-way ANOVA with Scheffe's post hoc test. The data were analyzed using Sigma Plot Software 10.0 and, in all cases, statistical significance was set at $P < 0.05$.

Results

Bacterial selection

According the results of PFGE study, MDRABs were randomly selected from each pulsotype. PFGE analysis yielded eleven

different pulsotypes (including three PFGE subtypes), showing a great genetic heterogeneity. These strains were selected for further study (Fig. 1). No relevant differences in growth yields were observed among the strains.

Biofilm formation of the tested MDRABs

Three cohorts of biofilm producers from the tested MDRAB-Bs were identified after 5 days inoculation, including three strains of high biofilm producers (Group H; $OD_{570} \geq 3.0$), five strains of medium biofilm producers (Group M; $2 \leq OD_{570} < 3.0$), and three strains of low biofilm producers (Group L; $0.2 \leq OD_{570} \leq 2$) (Fig. 2A). The biofilm formation capacities of the three cohorts were showed in Fig. 2B. All were enrolled for further study.

Desiccation survival assay of MDRAB-Bs and MDRAB-Ps

The desiccation survival profiles of the Group H, M, and L in MDRAB-Ps and MDRAB-Bs were analyzed (Fig. 3A and B). Both MDRAB-Ps and MDRAB-Bs exhibited capacities of desiccation resistance overall, the survival times ranged from 14 to 56 days depending on their capacity of biofilm formation. The mean survival time for the Group H, M, and L of MDRAB-Bs was 49.0 days (range, 35—56 days, Fig. 3A), which was significantly longer than that of their planktonic types (MDRAB-Ps) (mean: 17.3 days, range of 14—21 days, Fig. 3B) ($P < 0.005$). In particular, Group H and Group M of MDRAB-Bs were extremely highly resistant to desiccation, with significantly longer survival times (56 days) than that of Group L (35 days, $P < 0.005$) (Fig. 3A). MDRAB-Ps were lesser resistant to desiccation with the survival time less than 21 days (Fig. 3B). The lowest desiccation survival day of the Group L MDRAB-P (14 days) was found. Biofilm formation increased the survival time of MDRAB-Bs in the desiccated condition. Therefore, high capacity of biofilm formation of MDRAB-Bs may be inherently more resistant in desiccated environments.

Bactericidal activity assay of ethanol against MDRABs

Survival assays were performed to test the ethanol effectiveness of ethanol on the MDRAB-Bs and MDRAB-Ps (Fig. 4A and B). Ethanol resistance developed after their biofilm formation in the MDRABs. It took 10 min of dwelling time of 70% ethanol agent to completely eliminate all the Group H, Group M, and Group L cohorts of MDRAB-Bs (Fig. 4A). By contrast, the Group H, M, and L cohorts of MDRAB-Ps could be completely eliminated after 10 min dwelling time in 30% ethanol agent only (Fig. 4B). Thus, a significantly higher bactericidal concentration of ethanol was found for MDRAB-Bs than that of the MDRAB-Ps (70% vs. 30%, respectively, $P < 0.005$).

Comparative antiseptic efficacies of the tested agents for MDRAB-Bs

Three tested agents (70% ethanol, 0.5% CHG combined 70% isopropyl alcohol, and 2% CHG combined 70% ethanol) were investigated to compare their antiseptic efficacies against the MDRAB-Bs (Fig. 5). MDRAB-B CFUs were obtained before and after dwelling with the three agents for 10 min. The mean (±SD) bacterial counts of the MDRAB-Bs before disinfection was 2.1×10^8 ($\pm 1.4 \times 10^8$) CFUs/well. Application of the 2% CHG in 70% alcohol agent for 1 min yielded a significantly higher efficacious reduction of the tested MDRAB-Bs; it eliminated the bacteria at 1 min time point completely. This agent showed a superior antiseptic efficacy to reduce the bacterial titer, compared with those titers of the 0.5% CHG in 70% alcohol group ($1.0 \times 10^5 \pm 2.1 \times 10^5$ CFUs/well, $P < 0.005$) and the 70% ethanol agent group ($6.3 \times 10^6 \pm 8.2 \times 10^6$ CFUs/well, $P < 0.005$) at 1 min time point. By contrast, a much longer time required for the 0.5% CHG in 70% isopropyl alcohol agent (3 min) and the 70% ethanol agent (10 min) to completely eliminate the MDRAB-Bs. Of note, a number of MDRAB-Bs ($2.7 \times 10^5 \pm 5.6 \times 10^5$ CFUs/well at 3 min and

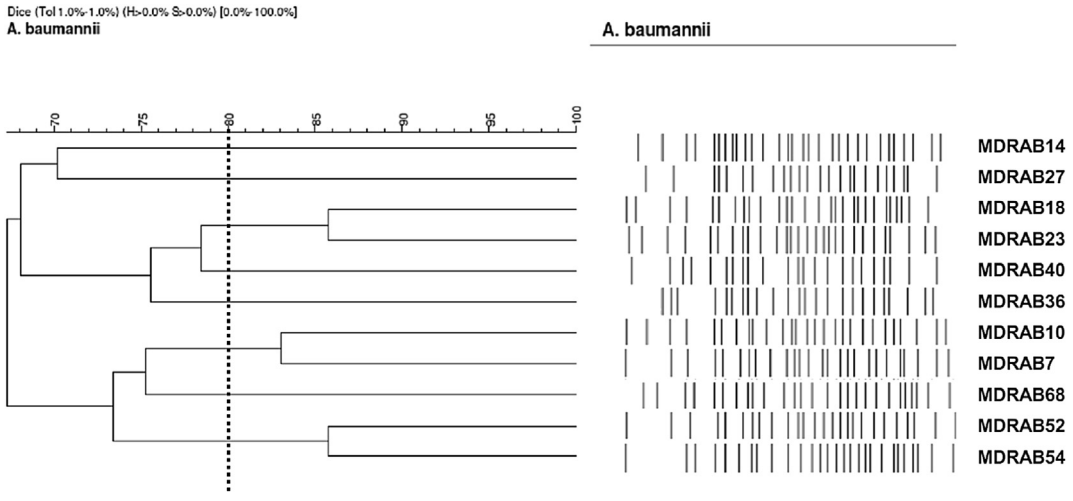

Figure 1. The pulsotypes of the MDRAB strains in the pulsed-field gel electrophoresis analysis. A total of eight pulsotypes of the MDRABs in PFGE analysis were found. There was a subtype MDRAB with Dice coefficient values (between 80 and 90) in three pulsotypes. The three subtype (possibly related clusters) MDRABs were enrolled also for analysis.

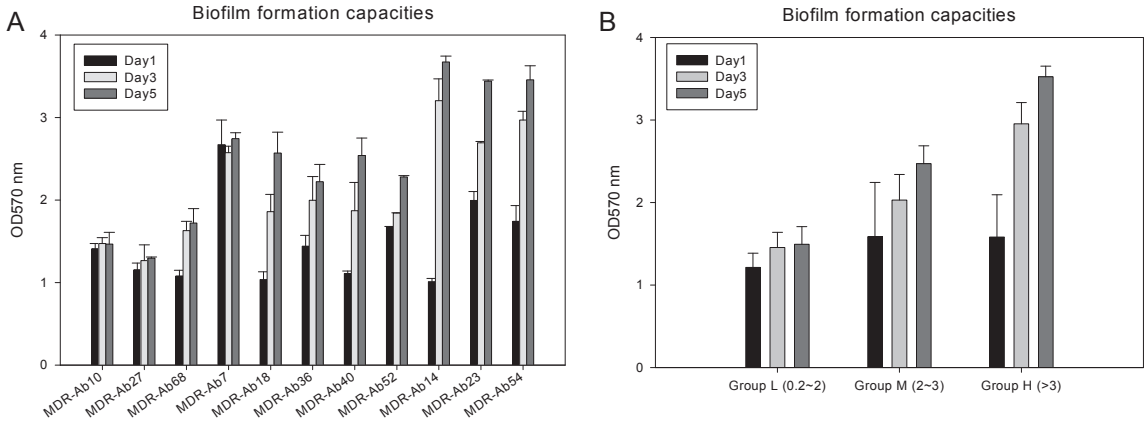

Figure 2. (A and B) Biofilm-formation levels (expressed as OD $\pm$ SD) of the tested MDRABs. Group H: the high-producer group ($OD_{570} \geq 3.0$). Group M: the medium-producer group ($2 \leq OD_{570} < 3.0$), and Group L: the low-producer group ($0.2 \leq OD_{570} \leq 2$) (Fig. 2A). The mean levels of biofilm formation of the Group H, Group M, and Group L of the MDRABs were presented from 1st to 5th day (Fig. 2B).

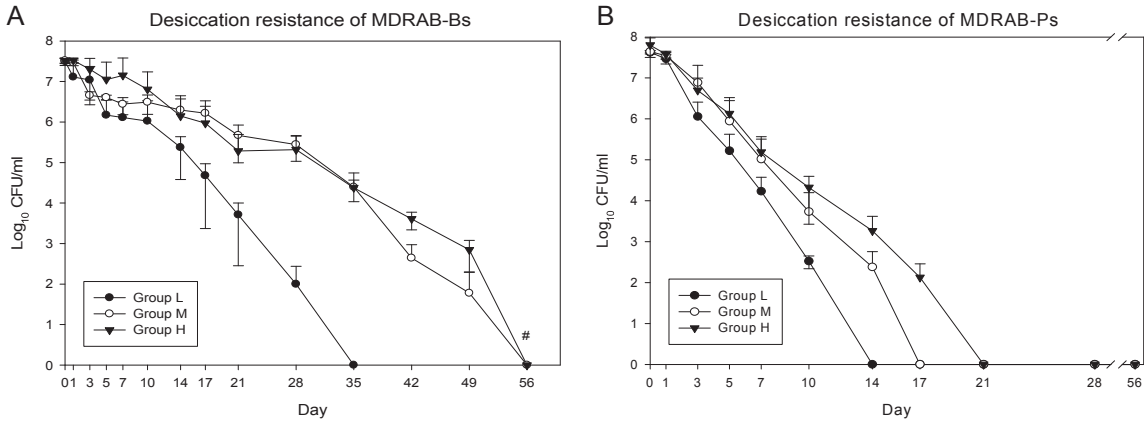

Figure 3. Desiccation survival assay for the MDRAB-Bs and the MDRAB-Ps. Groups H and M of MDRAB-Bs exhibited extremely high resistance to desiccation with significantly longer survival times (both 56 days) than that of Group L (35 days) (Fig. 3A). The Group H, M and L of MDRAB-Ps were lesser resistant to desiccation with 21 survived days, the lowest survival time was 14 days for Group L (Fig. 3B), but there were no significant difference of the survival times among the three subgroups of MDRAB-Ps. #. Indicates significant longer survival times (both 56 days) than that of Group L (35 days). (Three-way ANOVA with Scheffe's post hoc test, $P < 0.005$).

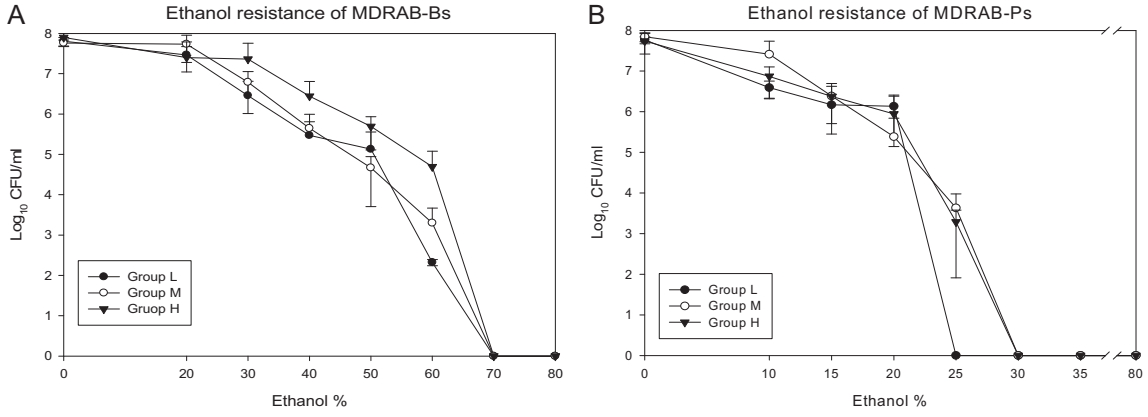

Figure 4. Antiseptic efficacies of ethanol for MDRAB-Bs and MDRAB-Ps. Ethanol resistance developed for the MDRAB-Bs; exposure to 70% ethanol for 10 min was required to eliminate the MDRAB-Bs completely (Fig. 4A). Exposure to only 30% ethanol for 10 min was required to eliminate the MDRAB-Ps completely (Fig. 4B).

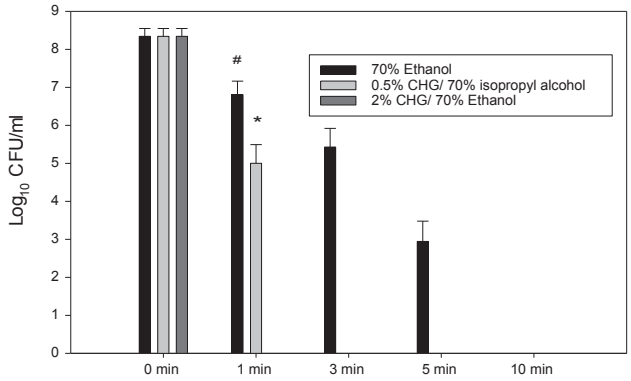

Figure 5. Antiseptic efficacies of the three tested agents. The 2% CHG in 70% ethanol eliminated the MDRAB-Bs completely at 1 min time point. The 0.5% CHG in 70% isopropyl alcohol eliminated the MDRAB-Bs completely at 3 min time point. However, the 70% ethanol eliminated the MDRAB-B completely at 10 min time point. * Indicates significant lower MDRAB CFUs treated with 2% CHG in 70% ethanol agent than 0.5 CHG in 70% isopropyl alcohol. (Three-way ANOVA with Scheffe's post hoc test, $P < 0.005$). #. Indicates significant lower MDRAB CFUs treated with 2% CHG in 70% ethanol agent than 70% ethanol agent. (Three-way ANOVA with Scheffe's post hoc test, $P < 0.005$).

$8.8 \times 10^2 \pm 2.1 \times 10^3$ CFUs/well at 5 min time point) still could be detected after contacting with the 70% ethanol agent.

Discussion

In present results, the MDRABs, embedded in biofilms, had higher resistance to desiccation and ethanol than the MDRAB-Ps. The agent, 2% CHG and 70% alcohol, provided a significantly superior antimicrobial activity than the 70% ethanol agent to eliminate the MDRABs, embedded in biofilms. In viewing the aspect of hand hygiene, the superior antiseptic efficacy of this combined agent may be more convenient and feasible for clinicians in clinical practices.

Biofilm formation is an important feature of most clinical isolates of *Acinetobacter* spp., facilitating the spread of antibiotic-resistant microorganisms.[24,25] In our results, all of the clinically isolated MDRABs, without pulsotypic variations, exhibited variabilities in biofilm formation and could be classified into time-dependent high, medium, and low producers, consistent with a previous report.[10] In previous reports, 50–92% of clinically isolated ABs were biofilm producers,[11,26–28] although the relation of biofilm formation capacities and the antibiotics resistances are largely unknown, there are individual differences in biofilm formation among the isolates, existence of correlation between antibiotic resistance and biofilm-specific resistance in ABs were reported.[28]

Several investigators have studied the survival of AB in clinical environments. Previous investigators showed that AB strains could be isolated from a hospital bed rail 9 days after an infected patient was discharged from the hospital.[29] Jawad et al. have shown that there is no statistically significant difference between the survival times of sporadic and outbreak strains (27.2 versus 26.5 days, respectively).[22] However, the survival times for the biofilm-forming AB strains were significantly longer (36 days) compared to the non-biofilm forming strains (15 days).[12]

Our results demonstrated that the desiccation tolerance of MDRAB-Bs significantly correlated with its capacity of biofilm formation (Group H: 56 days, Group M: 56 days, and Group L: 35 days, respectively). No significant difference of the survival times in their planktonic types, however, the planktonic type of Group H isolates had longer survival times than the other groups still (Group H: 21 days, Group M: 17 days, and Group L: 14 days, respectively). The constitutional difference in biofilm production may explain why certain strains are able to establish themselves in the hospital environment while others are isolated only sporadically.

The hospital environments may become contaminated with microorganisms responsible for healthcare associated infections. Attachment and adherence to medical equipment and environmental surfaces appear to be important for *A. baumannii* pathogenesis[13]; especially because ABs is one of the most common bacteria to induce the biofilm-related contamination of medical devices.[30] Indeed, contact transmission, by hands of healthcare staffs, is the most common mechanism of transmission attributed to MDRAB infections.[31] Pittet et al. reported that 17%–30% of nurses carried gram negative bacilli on their hands (median counts: 3400–38,000 CFUs) in a general hospital.[32] Also, Markogiannakis et al. recovered that imipenem-resistant A. baumannii isolates were isolated from 12 (28.6%) of 42 cultures of samples from HCWs' hands in a ICU.[33] Moreover, about 4.5% HCWs were contaminated with MDRABs in their unwashed hands after glove removal.[34] Indeed, transmission of clonal strains by HCWs' hands episodes of the MDRAB outbreaks have also been reported.[33,35] Rising threat of MDRABs, hand hygiene and contact precaution are the two most important elements of infection control for the infections.[36] Even, hand hygiene was considered in a priority for the infection control,[37] as the ease contamination of MDRABs.[34,37,38]

For the hand sanitizers, alcohol-based hand rub is a very effective and widespread measure in hospitals for preventing the transmission of AB currently.[39,40] However, in present study, a 30% ethanol concentration was able to eliminate all the MDRAB-Ps, however, the 10 min was needed for a 70% ethanol concentration to eliminate for the MDRAB-Bs completely. By contrast, 2% CHG combined with 70% ethanol disinfectant could eliminate the MDRAB-Bs completely at 1 min time point.

Our findings have raised several concerns for the recommendation of alcohol-based hand sanitizers in hospitals for MDRAB infections. First, the WHO has recommended 60%–80% concentrations alcohol solutions as the most effective and safe agents for post-contamination hand cleansing,[9] however, most of the formulations used in US hospitals contain quite low ethanol concentrations (60–70%).[41] Our results revealed that alcohol solutions below a concentration of 70% cannot achieve a favorable result to eliminate the MDRAB-Bs. Second, Hand hygiene is the primary measure, proven to be effective in preventing health care-associated infections,[42] especially, it is a priority for the infection control for MDRABs infections.[37]

However, the compliance with hand hygiene practices is known to be low (compliance rates of 5—81%, overall average: 40%) for HCWs.[8,42] The duration of hand cleansing episodes ranged on average from as short as 6.6 s to 30 s.[42] In 2009, WHO published the guidance, estimating 20—30 s with alcohol-based hand rubs and 40—60 s for hand washing with soap and water were required for hand disinfection.[43] Nevertheless, from our results, these required times by WHO may be insufficient for eradication the MDRABs. In our results, a 10 min contacted time for the 70% alcohol agent was required to eliminate the MDRAB-Bs completely. This time period is not feasible for a HCW with a high work loading; insufficient time to perform the hand-sanitizing procedure may develop poor compliance of hand hygiene to facilitate MDRABs spreading and infections. Third, lower efficacy of alcoholic hand rubs do not provide improved infection control.[44] The widespread use of antiseptic agents with 70% alcohol products may provoke the development of microbial resistance in AB embedded with biofilm, particularly cross-resistance to antibiotics.[45,46] Addition of 2% of CHG to alcohol-based preparations results in significantly greater antiseptic activity than alcohol alone.[47] Thus, the antiseptic efficiency of 2% CHG in 70% ethanol merits further evaluation as a hand hygiene method for MDRAB-Bs infection control critically.

Conclusion

All of the clinically isolated MDRABs were able to produce biofilms, giving them greater desiccation and ethanol resistances than those without biofilm formation capacity. Moreover, the 2% CHG in 70% alcohol agent had a more efficacious to eliminate MDRAB-Bs completely than the 70% ethanol agent. Our findings may display important roles in the MDRABs infection control.

Conflict of interest

The authors have reported to the JMII that no potential conflicts of interest exist with any companies or organizations.

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