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Towards the development of rapid biofilm antibiotic sensitivity testing (BAST)

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This study presents a modification of the antibiotic susceptibility testing (AST), which is a rapid means of determining the response of planktonic bacteria to different antimicrobial agents, for application to biofilms. Colony biofilm was first developed on a cellulose filter/membrane disc, over which an antibiotic disc was imposed. Zone of inhibition was measured after incubation on nutrient agar. Biofilms were not as susceptible to the test antibiotics as compared to the planktonic cultures. The results point to the possibility of this method as a rapid means for antibiotics for treating biofilm infections. Limitations and potential application for biofilm AST are discussed.

Key words: Biofilm, antibiotic susceptibility testing.

INTRODUCTION

The antibiotic susceptibility testing (AST) is a rapid means of determining the response of bacteria to different antimicrobial agents. AST utilizes the disc diffusion method, which is simple and widely used for sensitivity testing (Bauer et al., 1966; Clinical and Laboratory Standards Institute, 2009). Disc diffusion is very flexible, with a wide variety of antibiotic discs of varying concentrations available. This test effectively identifies organisms that are susceptible/resistant to certain antibiotics through visual means. Identification of effective antibiotics will lead to the successful treatment of bacterial infections. However, the test is conducted on planktonic cultures, which differ from biofilms in their response to antimicrobials *in vivo* (Jorgensen and Ferraro, 2009).

Studies show that 80% of infectious diseases are due to biofilm bacteria that are more difficult to treat than planktonic bacteria. (Lewis, 2005; Walters et al., 2003). Most biofilm antibiotic susceptibility tests utilized polystyrene wells for growing biofilms in vitro (Cerca et al., 2005; Ceri et al., 1999; Olson et al., 2002; Amorena et al., 1999). Results are not easily visualized compared to the disc assay. Therefore, it is necessary to develop a rapid test that will identify antibiotics for effectively treating biofilm infections.

This study presents a modification of the antibiotic susceptibility testing performed on planktonic cultures for application to biofilm cultures.

MATERIALS AND METHODS

Preparation of bacterial culture

Overnight cultures of *Pseudomonas aeruginosa* PA01 and *Staphylococcus aureus* SH1000 were prepared by dispersing a single colony in 5-ml test tubes in Mueller-Hinton broth (MHB). After incubation at 37°C in air with shaking, 500 µl of the overnight cultures were transferred to a 50 ml flask with 10 ml MHB, and

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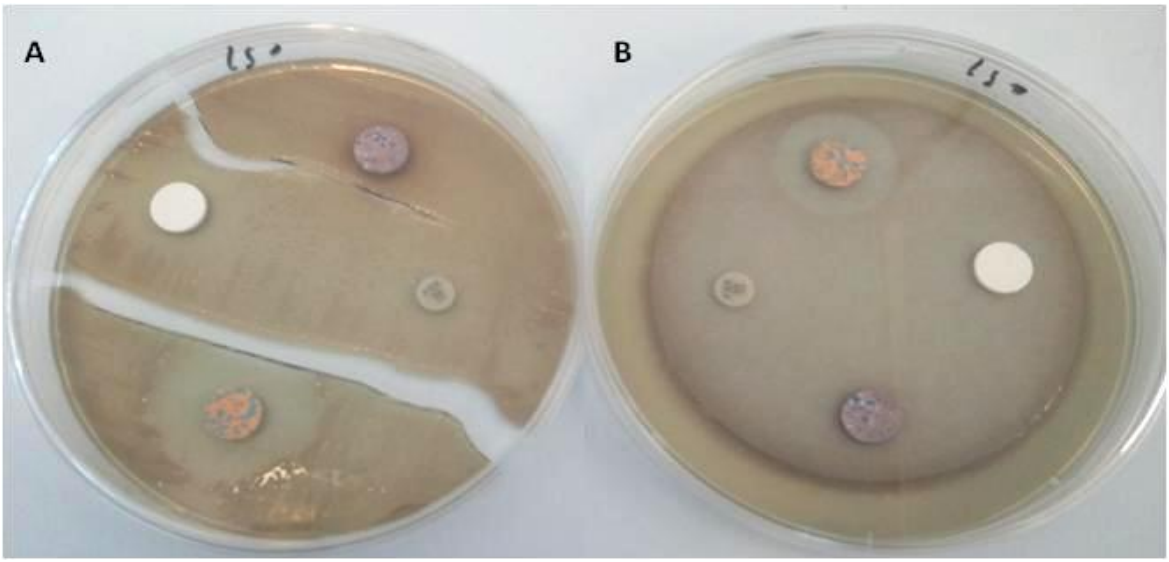

Figure 1. Comparison of the results of the antibiotic sensitivity assay for (A) planktonic culture, and (B) biofilm of *Pseudomonas aeruginosa* PA01. Antibiotic discs: white, chloramphenicol; orange, imipenem; small greyish, cefoxitin; and blue, vancomycin.

incubated with shaking at 120 rpm at 37°C. After 4 h, the bacterial cultures were diluted to an optical density (OD) of 0.05 at 600 nm.

Initial antibiotic sensitivity tests

For the AST, 1 ml of the culture was added to 10 ml of lukewarm broth, and poured over brain heart infusion (BHI)agar plates for *S. aureus* SH1000 and Iso-Sensitest™ Agar plates for *P. aeruginosa* PA0. Four antibiotic discs, with the following concentration were arranged over the agar: 30 µg chloramphenicol, 30 µg cefoxitin, 30 µg vancomycin, and 10 µg imipenem. The plates were incubated at 37°C in air for 48 h, after which the zone of inhibition was measured.

To perform the biofilm AST (BAST), cellulose ester discs or filters with 0.22 µm pore size and 7 mm diameter were sterilized by autoclaving for 15 min at 121°C. After cooling, the discs were soaked overnight in phosphate buffered saline. These were then dipped into overnight bacterial cultures that have been diluted to an OD 0.05 at 600 nm. The filters were placed carefully on the surface of BHI agar for *S. aureus* and Iso-Sensitest agar for *P. aeruginosa*, followed by incubation at 37°C for 48 h. Discs were then carefully removed and dipped in 4% human plasma in order to promote the adherence of bacteria to the membranes. After dipping, the membranes were placed onto new plates with BHI and Iso-Sensitest media. The same antibiotic discs used in the AST were then superimposed over the membrane filters and the zone of inhibition of bacterial growth was measured after 48 h.

Verification tests with strain-specific antibiotics

Using the same protocol as mentioned earlier, a second antibiotic sensitivity test was conducted; this time the antibiotics used were specific for the bacterial strains. The following antibiotics were tested on *S. aureus*: erythromycin (15 µg), ampicillin (10 µg), vancomycin (30 µg), gentamicin (10 µg) and cefotaxime (30 µg). For *P. aeruginosa*, the following antibiotic discs and concentrations were utilized: amikacin (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), and chloramphenicol (30 µg).

RESULTS AND DISCUSSION

The preliminary tests show the zones of inhibition of bacterial growth produced by the antibiotics (Figures 1 and 2). It is clear that the planktonic cultures were all sensitive to the 4 test antibiotics as compared to the biofilms. Figure 1 shows that it is only imipenem that was able to inhibit growth of the biofilm. *S. aureus* planktonic cultures exhibited overlapping zones while the growth zones in the biofilm were distinctly separated.

Table 1 summarizes the inhibitory action of the different antibiotics on the bacterial cultures of the two strains tested. In the preliminary trial, planktonic cultures of *S. aureus* were most sensitive to the four test antibiotics. *S. aureus* biofilm was sensitive to the action of three of the four antibiotics tested. However, the zones of inhibition were smaller than those observed for the planktonic cultures, which implies that the biofilms were able to grow despite the antibiotics. Disc zones were also observed around the antibiotics imipenem and chloramphenicol in planktonic cultures of *P. aeruginosa.* Imipenem was the only antibiotic that produced a zone of inhibition on the *P. aeruginosa* biofilm.

After the initial tests, strain-specific antibiotics were used. Planktonic *S. aureus* cultures differed in their response to the five test antibiotics. Its growth was highly inhibited by cefotaxime and ampicillin, while least inhibited by vancomycin. Compared to planktonic cultures, the biofilms were generally less affected by the antibiotics. Cefotaxime, which was highly inhibitory to planktonic growth, did not produce growth inhibition against the biofilm. The same was observed for vancomycin-treated biofilm. Erythromycin, ampicillin, and gentamicin all inhibited biofilm growth, but not as effectively as compared to

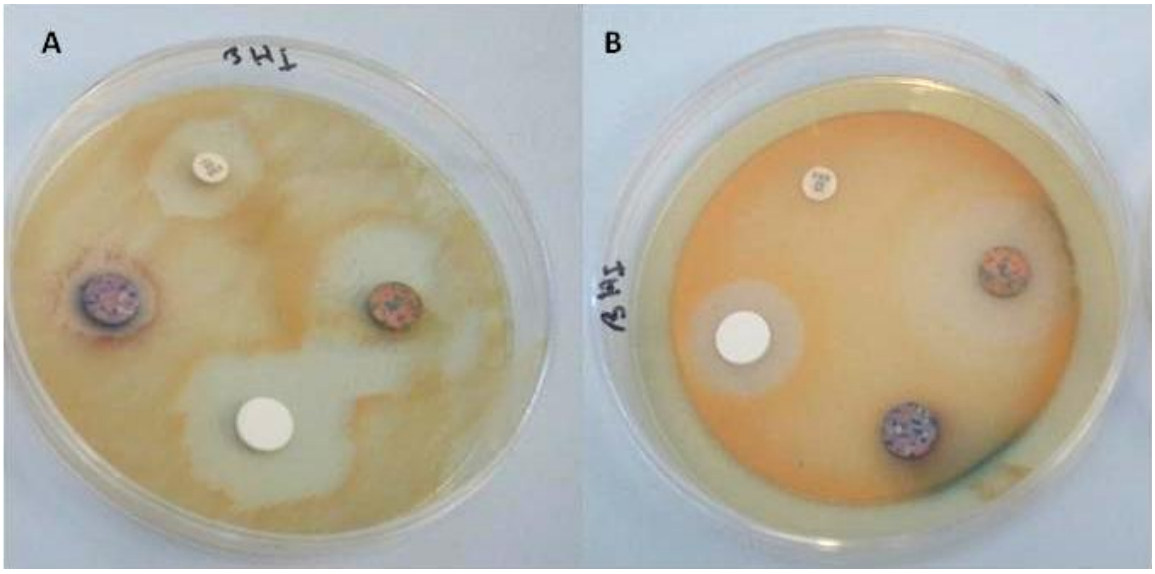

Figure 2. Comparison of the results of the antibiotic sensitivity assay for (A) planktonic culture, and (B) biofilm of *Staphylococcus aureus* SH1000. Antibiotic discs: white, chloramphenicol; orange, imipenem; small greyish, cefoxitin; and blue, vancomycin.

Table 1. The zone of inhibition of specific antibiotics on the growth of planktonic and biofilm cultures of *S. aureus* and *P. aeruginosa*.

Antibiotic		Zone of inhibition (mm ± SD)			
		***S. aureus* SH1000**		***P. aeruginosa* PA01**	
		Planktonic	**Biofilm**	**Planktonic**	**Biofilm**
Initial test	Cefoxitin	15 ± 0.5	0	0	0
	Imipenem	25 ± 1.0	20 ± 2.5	13 ± 1.5	9 ± 0.6
	Vancomycin	10 ± 2.0	5 ± 1.2	0	0
	Chloramphenicol	25 ± 1.0	15 ± 1.2	12 ± 1.15	0
Verification test	Erythromycin	15 ± 1.0	8 ± 1.0	Not tested	Not tested
	Ampicillin	25 ± 1.5	8 ± 2.0	Not tested	Not tested
	Vancomycin	8 ± 0.6	0 ± 0	Not tested	Not tested
	Gentamicin	15 ± 1.5	10 ± 2.0	Not tested	Not tested
	Cefotaxime	23 ± 1.0	0 ± 0	Not tested	Not tested
	Amikacin	Not tested	Not tested	20 ± 1.7	6 ± 0.6
	Ceftazidime	Not tested	Not tested	20 ± 0.6	6 ± 1.5
	Ciprofloxacin	Not tested	Not tested	30 ± 2.5	10 ± 2.0
	Imipenem	Not tested	Not tested	25 ± 1.2	0 ± 0
	Chloramphenicol	Not tested	Not tested	0 ± 0	0 ± 0.6

*Values are means of 3 replicates. SD: Standard deviation.

their inhibition of planktonic cultures. Except for gentamicin, growth of biofilms was more than 50% higher in the presence of erythromycin and ampicillin; gentamicin proved to be most inhibitory of the antibiotics to the *S. aureus* biofilm.

In the planktonic culture of *P. aeruginosa*, four of the five test antibiotics were able to produce zones of growth inhibition ranging from 20 to 30 mm (Table 1). Based on the zone of inhibitions produced, the growth of biofilms was 70% more than planktonic growth despite the addition of amikacin, ceftazidime, and ciprofloxacin. In the case of imipenem, it was highly effective against planktonic cells, but not against the biofilm. This last result was not in agreement with results of the initial tests

where imipenem inhibited biofilm growth of *P. aeruginosa*. Chloramphenicol was ineffective against *P. aeruginosa* because there was no zone of inhibition produced in both planktonic cells and biofilms.

The presence of some conflicting results indicates that more precision is necessary in carrying out the antibiotic tests. As in the case of the antibiotic sensitivity test for planktonic cultures, strict quality standards have to be met before BAST is performed. Extreme care is necessary during the preparation of the materials (that is, culture media, pH, and antibiotic discs) for the test. In addition, the preparation of biofilms on membranes has to be perfected to ensure that growth is homogeneous and there are no contaminating strains. Although such a condition is ideal; since natural biofilms are heterogeneous (Boles et al., 2004), a single strain biofilm could prove sufficient for antibiotic sensitivity testing. The interpretation of the antibiotic disc inhibition zones has to follow standards in order to attain reliable results and to give proper recommendation. Therefore, the standards for the performance and the interpretation of BAST results also have to be developed.

Conclusion

The proposed BAST method appears to be effective in identifying antibiotics for treating biofilm infection. These preliminary results show that there is a potential of developing BAST as a sensitive assay for biofilms. More studies should be conducted for producing biofilms *in vitro*, standardizing methods, reagents, and conditions for BAST.

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