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Effect of Incubation Time on the Biofilm Formation by Methicillin Resistant *Staphylococcus aureus*

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ABSTRACT

Background: Methicillin resistant *Staphylococcus aureus* (MRSA) refers to a group of gram-positive bacteria that are genetically distinct from other strains of *Staphylococcus aureus* that has developed, through horizontal gene transfer, natural selection and multiple drug resistance to beta lactam antibiotics and thus causing many severe diseases which are very problematic to treat as they are resistant to different antibiotics. The above situation is further aggravated by the formation of biofilms which are structured aggregation of surface attached microbes encased in an extracellular matrix. Thus, the time required to develop biofilms becomes an important point to study.

Methods: In the present study, detection of the extent of biofilm formation by MRSA isolates, was performed using the Microtitre Plate Assay. The test was performed in triplicates and was continued for a time period of 7 days to detect the extent of biofilm formation with increase in incubation interval.

Result: On comparing the biofilm forming ability of different isolates from Day 1 to Day 7, it could be clearly observed that the biofilm forming capacity of isolates gradually increased with increase in incubation time and most of the isolates became strong biofilm producers from Day 4.

Key words: Biofilm, Incubation time, Microtitre plate assay, MRSA.

INTRODUCTION

Biofilms are communities formed by unicellular individuals with spatial and functional heterogeneity and are ubiquitous in nature. These biofilms are covered with an exopolysaccharide matrix (EPS). In biofilm communities, the multi cellular structure of the biofilm makes it possible for the bacteria to undergo dormancy and hibernation, enabling them to survive and to disseminate their genomes (Bordi and Bentzmann, 2011). The staphylococcal biofilm life cycle is believed to occur in following stages, *i.e*., the initial attachment of cells to a surface, formation of micro colonies into an established biofilm and dispersal of the bacteria from the biofilm (Chung and Toh, 2014). Thus, the present study was performed to detect the extent of biofilm formation by MRSA isolates for a time period of 7 days by Microtitre Plate Assay. The outcome of the research may help to form timely strategies for successful prevention of biofilm formation in various clinical diseases in animals especially mastitis.

MATERIALS AND METHODS

The present work was conducted in the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University, Jabalpur. The study was conducted for a period of 24 months from January 2020 to December 2021. In the present investigation, 20 MRSA isolates from cases of mastitis were studied for the extent of biofilm production.

This assay was performed in 96 well microtiter plates for a period of Day 01 to Day 07. This was performed to see the effect of incubation time on growth of biofilms. All the isolates of MRSA were sub cultured into Tryptone Soy broth individually and incubated aerobically at 37°C for 24 hrs. Biofilm formation was investigated at 37°C. From each individual culture, 20 µl samples of exponential phase and 180 µl of fresh sterile broth were dispensed in the wells of sterile 96 well flat-bottomed microtiter plate and kept for incubation at 37°C. Each isolate was inoculated into at least

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03 wells. The negative control well contained only broth without inoculation. *Staphylococcus aureus* ATCC 6538 and *Staphylococcus aureus* ATCC 25923 were used as positive control. After incubation, unbound cells were removed by inversion of microtiter plate, followed by vigorous tapping on absorbent paper. Subsequently, adhered cells were fixed with methanol. Adhered cells were stained by addition of 220 µl of crystal violet (0.5%) for 01 min. The stain was removed by exhaustive washing with distilled water. The plates were then allowed to dry. In order to quantify adhered bacteria, 220 µl of decolouring solution (ethanol / acetone, 80:20%) was added to each well for 15 min. The absorption of the eluted stain was measured at 570 nm. The strains were classified into the three categories: weak, moderate and strong biofilm producers as per Stepanovic *et al*. (2007). The following calculations were used to categorize the results:

$OD \leq OD_C$ (No Biofilm Production)

$OD_C < OD \leq 2 \times OD_C$ (Weak biofilm production)

$2 \times OD_C < OD \leq 4 \times OD_C$ (Moderate biofilm production)

$4 \times OD_C < OD$ (Strong biofilm production)

The readings of OD are presented as mean values and their standard error of the mean. Data analysis was performed using One-way ANOVA to determine the significance of differences. Results were considered significant at a P value of < 0.01 (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

To detect the effect of incubation time on extent of biofilm formation the microtitre plate assay was done for a time period of 7 days. The analysis was done on the basis of OD at 570 nm which are present in the adjoining table (Table 1 to 3 and Fig 1).

On comparing the biofilm forming ability of different isolates from Day 1 to Day 7, it could be clearly observed that the biofilm forming capacity of isolates gradually increased with increase in incubation time and most of the isolates became strong biofilm producers from Day 4. However, on Day 6, 90% of the isolates formed strong biofilms. It was also interesting to note that none of the isolates were weak biofilm formers from Day 4. Hence, Day 4 could be considered as the most crucial day for biofilm formation which transformed majority of the samples as strong biofilm formers and none of the samples as weak biofilm formers. The present study also revealed that Day 3 was also an important day, as from Day 3 all the isolates were biofilm producers.

Not many studies are present to validate our findings. However, Al-kafaween *et al.* (2019) performed a study to determine the optimum incubation time for formation of *Pseudomonas aeruginosa* and *Streptococcus pyogenes* biofilms in microtiter plates. The cultures were incubated for 7 days at 37°C to justify the formation of biofilm. It was found that the organisms strongly adhered to the plates on days 3, 4, 5 and 6. This finding is in affirmation with our study where we found that 100% isolates were biofilm forming from Day 3. Al-kafaween *et al.* (2019) also observed that biofilm formation by *P. aeruginosa* and *S. pyogenes* were strong on days 4, 5 and 6. This is also similar to our

Table 1: Comparison of biofilm formation assay of MRSA isolates from Day 1 to Day 7.

Isolate	OD_{570}						
no.	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
2	$0.035^{c}\pm0.0097$	$0.043^{c}\pm0.0174$	$0.087^{c}\pm0.0082$	$0.147^{c}\pm0.0203$	$0.496^{b}\pm0.0329$	$0.235^{bc}\pm0.0292$	$0.867^{a}\pm 0.2430$
18	$0.040^{c}\pm0.0109$	$0.080^{c}\pm0.0085$	$0.184^{c}\pm0.0067$	$0.226^{bc}\pm0.0300$	$0.460^{ab}\pm0.0389$	$0.549^{a}\pm0.1583$	$0.639^{a}\pm0.1307$
20	$0.043^{d}\pm0.0069$	$0.084^{d}\pm0.0113$	$0.065^{d}\pm0.0015$	$0.190^{c}\pm0.0217$	$0.319^{b}\pm0.0292$	$0.255^{bc}\pm0.0354$	$0.746^{a}\pm0.0484$
21	$0.056^{c}\pm0.0103$	$0.200^{c}\pm0.0311$	$0.148^{c}\pm0.0114$	$0.145^{c}\pm0.0514$	$0.427^{b}\pm0.0180$	$0.498^{b}\pm0.0748$	$1.200^{a}\pm0.1277$
22	$0.019^{d}\pm0.0017$	$0.044^{d}\pm0.0026$	$0.114^{cd}\pm0.0120$	$0.176^{c}\pm0.0708$	$0.309^{b}\pm0.0415$	$0.226^{bc}\pm0.0384$	$0.469^{a}\pm0.0389$
23	$0.029^{d}\pm0.0062$	$0.043^{d}\pm0.0031$	$0.104^{cd}\pm0.0065$	$0.275^{cd}\pm 0.0620$	$0.348^{bc}\pm 0.0093$	$0.529^{b}\pm0.0810$	$0.955^{a}\pm0.1995$
24	$0.100^{cd}\pm 0.0159$	$0.045^{d}\pm0.0035$	$0.111^{cd}\pm0.0067$	$0.257^{bc}\pm0.0951$	$0.372^{b}\pm0.0160$	$0.329^{b}\pm0.0067$	$1.310^{a}\pm0.1149$
38	$0.018^{d}\pm0.0035$	$0.053^{cd}\pm 0.0006$	$0.135^{c}\pm0.0045$	$0.080^{cd}\pm0.0238$	$0.375^{b}\pm0.0284$	$0.287^{b}\pm0.0430$	$0.508^{a}\pm0.0587$
39	$0.010^{d}\pm0.0047$	$0.061^{d}\pm0.0067$	$0.180^{cd}\pm0.0230$	$0.278^{bc}\pm0.0035$	$0.472^{b}\pm0.0298$	$0.365^{bc}\pm0.1192$	$1.356^{a}\pm0.1161$
40	$0.078^{d}\pm0.0022$	$0.107^{cd}\pm 0.0319$	$0.163^{cd}\pm0.0070$	$0.142^{cd}\pm0.0317$	$0.370^{b}\pm0.0238$	$0.195^{c}\pm0.0054$	$1.173^{a}\pm0.0810$
41	$0.056^{b}\pm0.0086$	$0.084^{b}\pm0.0018$	$0.116^{b}\pm0.0067$	$0.231^{b}\pm0.1023$	$0.379^{ab}\pm 0.0125$	$0.405^{ab}\pm0.0822$	$0.698^{a}\pm0.2790$
42	$0.062^{b}\pm0.0043$	$0.060^{b}\pm0.0052$	$0.124^{b}\pm0.0117$	$0.187^{b}\pm0.0300$	$0.521^{a}\pm0.0967$	$0.208^{b}\pm0.0331$	$0.692^{a}\pm0.1204$
46	$0.065^{c}\pm0.0085$	$0.061^{c}\pm0.0015$	$0.132^{c}\pm0.0092$	$0.200^{bc}\pm0.0598$	$0.450^{b}\pm0.0100$	$0.716^{a}\pm0.2064$	$0.838^{a}\pm0.0797$
47	$0.101^{c}\pm0.0072$	$0.071^{c}\pm0.0105$	$0.170^{c}\pm0.0193$	$0.237^{bc}\pm0.0233$	$0.396^{b}\pm0.0437$	$0.344^{b}\pm0.0382$	$1.063^{a}\pm0.1335$
48	$0.054^{c}\pm0.0125$	$0.113^{c}\pm0.0542$	$0.137^{c}\pm0.0246$	$0.304^{b}\pm0.0502$	$0.422^{b}\pm0.0330$	$0.343^{b}\pm0.0740$	$1.493^{a}\pm0.0746$
49	$0.081^{d}\pm0.0188$	$0.142^{d}\pm0.0228$	$0.218^{cd}\pm0.0116$	$0.503^{b}\pm0.1101$	$0.570^{b}\pm0.0946$	$0.446^{bc}\pm0.0800$	$1.440^{a}\pm0.1458$
171	$0.081^{e}\pm0.0172$	$0.175^{de}\pm 0.0181$	$0.202^{de}\pm0.0135$	$0.279^{cd}\pm0.0267$	$0.645^{b}\pm0.0713$	$0.392^{c}\pm0.0392$	$1.401^{a}\pm0.0745$
346	$0.077^{d}\pm0.0196$	$0.132^{d}\pm0.0313$	$0.134^{d}\pm0.0049$	$0.336^{c}\pm0.0648$	$0.511^{b}\pm0.0304$	$0.493^{b}\pm0.0482$	$0.855^{a}\pm0.0143$
214	$0.077^{c}\pm0.0040$	$0.162^{bc}\pm 0.0283$	$0.150^{bc}\pm0.0026$	$0.365^{bc}\pm0.0771$	$0.447^{b}\pm0.0203$	$0.379^{bc}\pm0.0386$	$1.057^{a}\pm0.2739$
235	$0.082^{d}\pm0.0033$	$0.113^{d}\pm0.0112$	$0.133^{d}\pm0.0054$	$0.283^{c}\pm0.0056$	$0.531^{b}\pm0.0381$	$0.315^{c}\pm0.0432$	$0.674^{a}\pm0.0537$
Control	$0.046^{g}\pm0.0003$	$0.074^{c}\pm0.0003$	$0.072^{d}\pm0.0003$	$0.066^{f}\pm0.0003$	$0.082^{b}\pm0.0003$	$0.069^{e}\pm0.0003$	$0.169^{a}\pm0.0003$

Means with different superscripts differed significantly ($p \leq 0.01$) at different time intervals.

Table 2: Comparison of Biofilm formation assay of MRSA isolates from Day 1 to Day 7.

Isolate No.	Type of biofilm formed						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
02	Non-biofilm producer	Weak	Weak	Moderate	Strong	Strong	Moderate
18	Non-biofilm producer	Weak	Moderate	Moderate	Moderate	Moderate	Strong
20	Non-biofilm producer	Weak	Weak	Moderate	Strong	Strong	Strong
21	Weak	Weak	Moderate	Moderate	Moderate	Moderate	Moderate
22	Non-biofilm producer	Non-Biofilm Producer	Weak	Strong	Strong	Strong	Strong
23	Non-biofilm producer	Non-Biofilm Producer	Weak	Moderate	Strong	Strong	Strong
24	Non-biofilm producer	Weak	Moderate	Moderate	Strong	Strong	Moderate
38	Non-biofilm producer	Non-Biofilm Producer	Weak	Strong	Strong	Strong	Strong
39	Non-biofilm producer	Non-Biofilm Producer	Moderate	Moderate	Strong	Strong	Moderate
40	Weak	Weak	Moderate	Moderate	Strong	Strong	Strong
41	Weak	Weak	Weak	Moderate	Moderate	Strong	Strong
42	Non-biofilm producer	Weak	Weak	Moderate	Strong	Strong	Strong
46	Non-biofilm producer	Weak	Weak	Moderate	Strong	Strong	Strong
47	Non-biofilm producer	Moderate	Moderate	Strong	Strong	Strong	Strong
48	Weak	Weak	Weak	Strong	Strong	Strong	Strong
49	Weak	Moderate	Moderate	Strong	Strong	Strong	Strong
171	Weak	Moderate	Moderate	Strong	Strong	Strong	Strong
346	Weak	Weak	Weak	Strong	Strong	Strong	Strong
214	Weak	Moderate	Moderate	Strong	Strong	Strong	Strong
235	Weak	Weak	Weak	Moderate	Strong	Strong	Strong

Table 3: Total biofilm producers from Day 1 to Day 7.

Total no. of isolates (20)	Total biofilm producers						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Strong	0 (0%)	0 (0%)	0 (0%)	08 (40%)	17 (85%)	18 (90%)	16 (80%)
Moderate	0(0%)	03 (15%)	09 (45%)	12(60%)	03 (15%)	02 (10%)	04 (20%)
Weak	09 (45%)	13 (65%)	11 (55%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total biofilm producers	09 (45%)	16 (80%)	20 (100%)	20 (100%)	20 (100%)	20 (100%)	20 (100%)

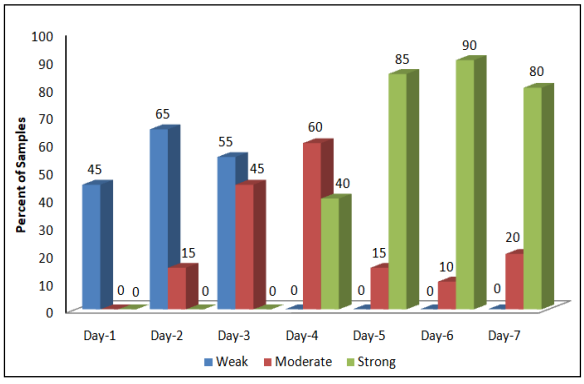

Fig 1: Biofilm producers from Day 1 to Day 7.

findings, where 80-90% of the isolates are strong biofilm formers on days 04, 05 and 06 (Jama *et al*., 2017 and Rossi *et al*., 2016). A slight decrease in biofilm formation is observed on day 07. Many factors such as integration of diverse signals from the environment might play a role in biofilm formation, concurrent with other events such as phenotypic and genetic switching during biofilm production and also EPS production (Ismael, 2013 and Bakar *et al*., 2018). This decrease in biofilm formation could be attributed to the loss of exopolymers from the biofilm and in particular of exopolysaccharides, which may suggest that an active process of detachment may have started, probably mediated by enzymatic degradation (Allison *et al*., 1998).

CONCLUSION

The comparative analysis of biofilm formation assay from Day 1 to Day 7 suggests that the biofilm forming capacity of isolates gradually increased with increase in incubation time and most of the isolates became strong biofilm producers from Day 4. It was also interesting to note that none of the isolates were weak biofilm formers from Day 4. Hence, Day 4 could be considered as the most crucial day for biofilm formation which transformed majority of the isolates as strong biofilm formers.

Conflict of interest

All authors declare that they have no conflict of interest.

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