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Phenotypic detection and comparison of biofilm production in methicillin resistant *Staphylococcus aureus*

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Abstract
Biofilms are structured aggregation of surface attached microbes encased in an extracellular matrix. Biofilm formation by resistant organisms like Methicillin Resistant *Staphylococcus aureus* makes the problem more difficult to treat. Hence, methods to detect the biofilm production by these resistant organisms is the need of the hour so that timely preventive measures can be taken. The study was conducted with the aim to detect the capability to form biofilm by 20 MRSA organisms isolated form cases of bovine mastitis which is the leading cause of decline in milk production in the dairy industry. Biofilm formation was detected by Congo Red agar method, Tube method and Microtitre plate assay. By Congo Red agar method, 05/20 (25%) isolates produced characteristic black coloured biofilm forming colonies and rest 15/20 (75%) isolates produced pink coloured non biofilm forming colonies. By Tube method, 07/20 (35%) isolates were weak biofilm producers, 05/20 (25%) isolates were moderate biofilm producers, 05/20 (25%) isolates were strong biofilm producers, while rest of the 03/20 (15%) isolates were designated as Non biofilm producers. Quantitative detection of biofilm was done by Microtitre plate method. The result was analysed by taking OD in ELISA reader at 570 nm. After 24 hours of incubation, results indicated that only 09/20 (45%) isolates were biofilm producers, which only produced weak biofilms. Rest 11/20 (55%) isolates were non biofilm forming. However, we can conclude from our study that the Tissue Culture Plate method is a more quantitative and reliable method for the detection of biofilm forming microorganisms as compared to Tube Method and Congo Red Agar methods and it can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories.

Keywords: Biofilm, MRSA, Congo red, tube method, microtitre plate assay

Introduction
The ability to form biofilm is one of the many factors affecting pathogenicity of MRSA. The ability to form biofilm helps microorganisms to survive hostile environments within the host and is considered responsible for chronic or persistent infections (Costerton *et al.*, 1999) [1]. Biofilm formation is considered to be a two-step process in which the bacteria first adhere to a surface mediated by a capsular antigen, namely capsular polysaccharide / adhesin (PS/A), followed by multiplication to form a multilayered biofilm, which is associated with production of polysaccharide intercellular adhesin (PIA). The PIA and PS/A are structurally similar with a common backbone of beta-1-6- linked polyglucosamine, but differ in the primary substitutions in their amino groups (Mack *et al.*, 1996) [2]. Biofilm production can be detected by phenotypic (qualitative and quantitative) and genotypic methods. The present study aims to detect biofilm production qualitatively by Congo red agar method and Tube method and quantitatively by Microtitre plate assay which is also known as Tissue culture Plate method.

Materials and Methods
A total of 20 MRSA samples isolated from cases of bovine mastitis were used for detection of biofilm forming ability. For qualitative detection of biofilm production, Congo red agar method and Tube method were used. Quantitative detection of biofilm forming ability was done by Tissue culture plate method. *Staphylococcus aureus* ATCC 6538 and ATCC 25923 were used as a standard positive control for biofilm production.

Congo red agar method

Qualitative detection of Biofilm formation in MRSA isolates to differentiate between biofilm producers and non-biofilm producers was determined by cultivation on Congo red agar plates (Mariana *et al.*, 2009) [3]. Inoculated CRA plates were incubated at 37°C for 24hr. The production of rough black colonies by biofilm forming strains was used to differentiate them from pink-coloured colonies produced by non-biofilm forming isolates.

Tube Method

The isolated organisms were inoculated in 5ml Trypticase Soy Broth in test tubes and incubated overnight at 37°C. After incubation, the tubes were decanted, dried and stained with 0.1% Crystal violet. Subsequently, the tubes were washed gently and placed upside down for drying. Visible lining of the wall and bottom of the tube by a film was considered as positive. The results were scored visually as non-producers, or weak, moderate or strong biofilm producers (Neopane *et al.*, 2018) [4].

Microtitre Plate Assay

This assay was performed in 96 well microtiter plates for 24 hours of incubation. All the isolates of MRSA were sub cultured into Tryptic 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 plates and kept for incubation at 37°C. Each isolate was inoculated into at least 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 (Stepanovic *et al.*, 2007) [5].

The following calculations were used to categorize the results:

$OD \leq OD_C$ (No Biofilm Production)

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

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

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

Results and Discussion

To differentiate the biofilm producing isolates from non-biofilm formers, all the 20 MRSA isolates were subjected to Congo Red agar method. It is a basic screening tool which differentiates biofilm formers from non-biofilm producers with the presence of different colony colours. Isolates which were biofilm producers gave black colour colonies, whereas non biofilm producers gave pink-coloured colonies. Typical black coloured colonies were given by 05/20 (25%) isolates and pink-coloured colonies were seen in 15/20 (75%) isolates (Table 01, Plate 01 and Fig. 01).

Table 1: Detection of Biofilm by Congo Red Agar Method

S. No.	Isolate No.	Colony Colour in Congo Red Agar	Type of Biofilm Producer
1.	02	Pink	NonBiofilm Producer
2.	18	Pink	NonBiofilm Producer
3.	20	Black	Biofilm Producer
4.	21	Pink	NonBiofilm Producer
5.	22	Pink	NonBiofilm Producer
6.	23	Pink	NonBiofilm Producer
7.	24	Pink	NonBiofilm Producer
8.	38	Pink	NonBiofilm Producer
9.	39	Pink	NonBiofilm Producer
10.	40	Black	Biofilm Producer
11.	41	Pink	NonBiofilm Producer
12.	42	Black	Biofilm Producer
13.	46	Pink	NonBiofilm Producer
14.	47	Black	Biofilm Producer
15.	48	Black	Biofilm Producer
16.	49	Pink	NonBiofilm Producer
17.	171	Pink	NonBiofilm Producer
18.	346	Pink	NonBiofilm Producer
19.	214	Pink	NonBiofilm Producer
20.	235	Pink	NonBiofilm Producer
Total			Biofilm Producer- 05/20(25%) NonBiofilm Producer-15/20(75%)

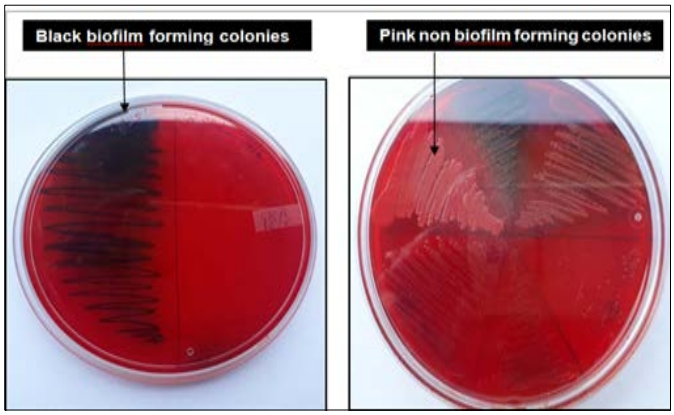

Plate 1: Biofilm forming and non-biofilm forming colonies on Congo Red agar

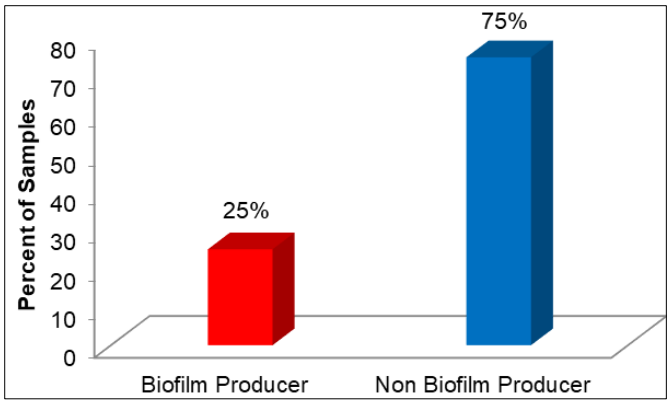

Fig 1: Biofilm Produced by Congo Red Agar Method

Many researchers have studied the biofilm forming capacity of *Staphylococcus aureus* and MRSA by various methods. Khan *et al.* (2011) [6] detected biofilm formation in *Staphylococcus aureus*. They screened 262 clinical strains of *S. aureus* by tissue culture plate method, tube method and Congo red agar method. The study finally concluded that the Congo red agar method had a low sensitivity and specificity of 67.65% and 89.13%. Similarly, Sharlee and Sumangala (2020) [7] detected biofilm production among *Staphylococcus aureus* by Congo red method and Tube method. Congo red agar method showed 63/150 (42%) black colonies with dry crystalline consistency indicating biofilm production. Out of 63 isolates 62% of isolates were MRSA and 38% of isolates were MSSA. The study finally concluded that Congo red method was less accurate when compared to tube method as screening test for the detection of biofilm.

Senobar *et al.* (2021) [8] depicted by means of Congo red agar plates, 41% of MSSA and 44% of MRSA were positive for biofilm production. Ebineshan *et al.* (2020) [9] demonstrated that out of the 67 bacterial isolates screened for biofilms by the CRA method, only 29.8% (n = 20) were biofilm producers and rest were biofilm non-producers (NBP). Most of the studies have concluded that Congo red agar method shows a low sensitivity to biofilm production and could not be relied on for differentiation of biofilm producers and non biofilm producers. However, we detected lower biofilm producers; it may be due to the difference in type and size of samples screened. Further studies on larger samples across various settings are recommended, in order to further validate the utility of Congo Red Agar method.

All the 20 MRSA isolates were also subjected to Tube method for qualitative detection of biofilm and to categorize them as weak, moderate and strong biofilm formers. The present study depicts that 03/20 (15%) isolates were non biofilm producers, while 17/20 (85%) isolates were categorized as Biofilm producers. On the basis of visual examination of tubes, these biofilm forming isolates were also categorized as weak, moderate and strong biofilm producers. It was found that 07/20 (35%) isolates were weak biofilm producers, 05/20 (25%) isolates were Moderate biofilm producers and only 05/20 (25%) isolates were confirmed as Strong biofilm producers (Table 02, Plate 02, 03 and Fig.02).

Table 2: Qualitative Detection of Biofilm by Tube Method

S. No.	Isolate No.	Type of Biofilm Producer
1.	02	Weak
2.	18	Moderate
3.	20	NonBiofilm Producer
4.	21	Weak
5.	22	Moderate
6.	23	NonBiofilm Producer
7.	24	Strong
8.	38	Weak
9.	39	Weak
10.	40	Moderate
11.	41	Strong
12.	42	Weak
13.	46	Strong
14.	47	Strong
15.	48	Strong
16.	49	NonBiofilm Producer
17.	171	Moderate
18.	346	Moderate
19.	214	Weak
20.	235	Weak
Total (20)		Weak- 07 (35%) Moderate-05 (25%) Strong-05 (25%) NonBiofilm Producer-03 (15%) Total Biofilm Producer-17 (85%)

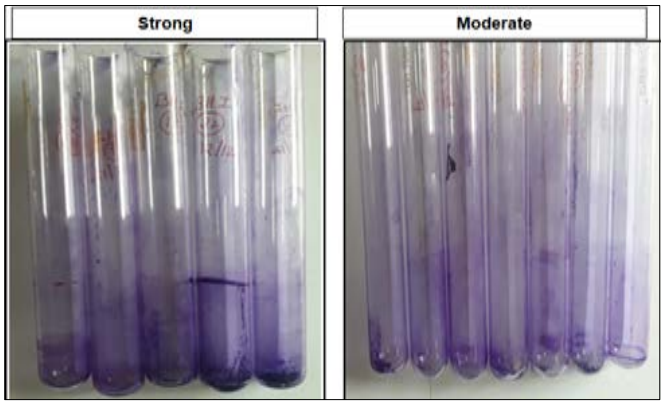

Plate 2: Strong and moderate biofilm formers by Tube method

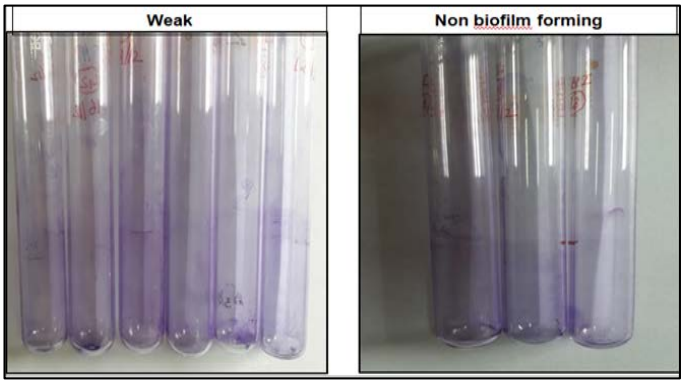

Plate 3: Weak and non-biofilm formers by Tube Method

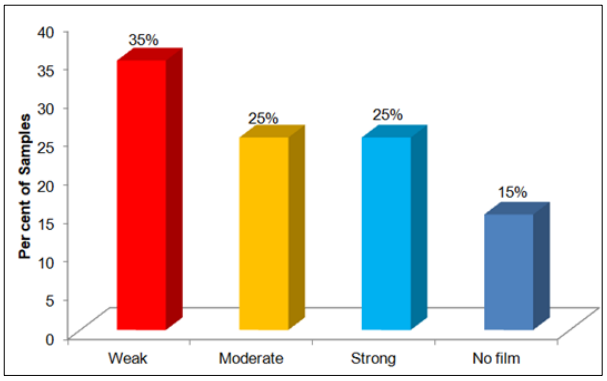

Fig 2: Biofilm produced by Tube method

When the results of Congo Red agar method were correlated with results of Tube method, it was found that all isolates except one (Isolate no. 20) which were concluded as biofilm forming by Congo red agar method were also categorized as biofilm forming by Tube method. This shows that 04/05 (80%) of the isolates positive by Congo red agar method were also positive with Tube method. However, only 02/05 (40%) isolates which were positive by Congo red method were designated as strong biofilm formers by Tube method.

Various studies of biofilm formation by tube method suggest a variety of inferences. This may be due to the fact that the interpretation of Tube method is solely dependent on the interpretation of the observer. Our findings were contrary to the findings of Khan *et al.* (2011) [6] who detected biofilm formation in *Staphylococcus aureus* from clinical strains. The study depicted 13.36% isolates as strongly positive, 50.38% as moderate and 36.26% of the samples as non-biofilm producers. Similarly, our findings were also not in agreement with Sharlee and Sumangala (2020) [7] who detected biofilm production among MRSA isolates by Tube method. The study depicted 06/65 (9.2%) as strong biofilm producers, 11/65 (17.1%) as moderate biofilm producers, 36/65 (55.3%) as weak producers and 12/65 (18.4%) were designated as non biofilm formers. However, our study agrees with the percentage of non-biofilm formers, as our study depicts 15% of samples as non-biofilm producers. The findings of Tube method in the literature review do not correlate much, as the tube method may be easy to perform and is much cheaper, which is good for diagnosis in developing countries like India, but the interpretation of result is observer dependent and there are chances of subjective errors.

Microtitre Plate Assay has always been a gold standard test to detect the persistence and invasiveness of organisms to form biofilms (Mathur *et al.*, 2006) [10]. Thus, to detect the extent of biofilm formation by MRSA isolates, the Microtitre Plate Assay, in 96 well polystyrene plates were performed. The test was performed in triplicates. After 24 hrs. of incubation at 37°C, the extent of biofilm formation was read by ELISA reader at 570nm (Table 03 & 04, Plate 04 and Fig. 03).

Table 3: Biofilm formation assay of MRSA by Microtitre Plate Method

S. No.	Isolate No.	OD_{570}	Type of Biofilm Producer
1.	02	0.035 ± 0.0097	NonBiofilm Producer
2.	18	0.040 ± 0.0109	NonBiofilm Producer
3.	20	0.043 ± 0.0069	NonBiofilm Producer
4.	21	0.056 ± 0.0103	Weak
5.	22	0.019 ± 0.0017	NonBiofilm Producer
6.	23	0.029 ± 0.0062	NonBiofilm Producer
7.	24	0.100 ± 0.0159	NonBiofilm Producer
8.	38	0.018 ± 0.0035	NonBiofilm Producer
9.	39	0.010 ± 0.0047	NonBiofilm Producer
10.	40	0.078 ± 0.0022	Weak
11.	41	0.056 ± 0.0086	Weak
12.	42	0.062 ± 0.0043	NonBiofilm Producer
13.	46	0.065 ± 0.0085	NonBiofilm Producer
14.	47	0.101 ± 0.0072	NonBiofilm Producer
15.	48	0.054 ± 0.0125	Weak
16.	49	0.081 ± 0.0188	Weak
17.	171	0.081 ± 0.0172	Weak
18.	346	0.077 ± 0.0196	Weak
19.	214	0.077 ± 0.0040	Weak
20.	235	0.082 ± 0.0033	Weak
21.	Control	0.046 ± 0.0003	NonBiofilm Producer

Table 4: Total Biofilm formation assay

Total no. of isolates	Biofilm Producer			NonBiofilm producer	Total Biofilm producer
	Weak	Moderate	Strong		
20	09 (45%)	0 (0%)	0 (0%)	11 (55%)	09 (45%)

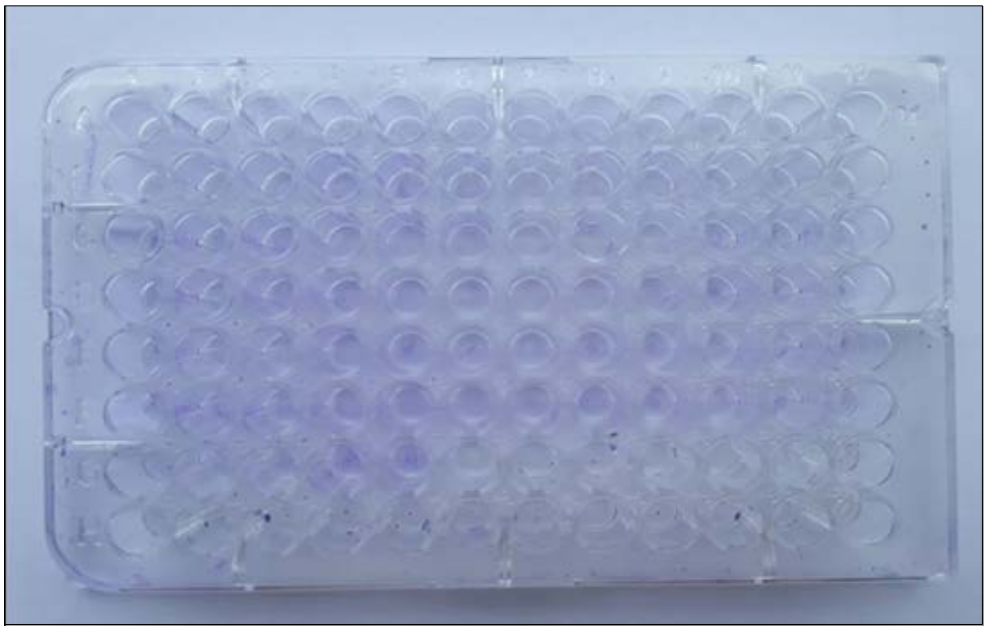

Plate 4: Microtitre plate showing biofilm formation

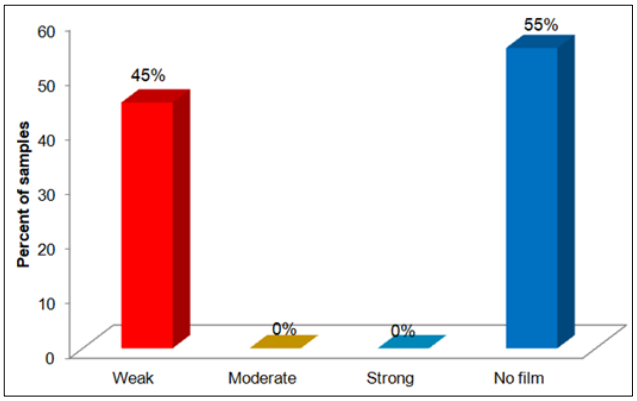

Fig 3: Biofilm formation Assay

The results indicate that only 09/20 (45%) samples were able to form biofilm. Rest 11/20 (55%) samples did not form any biofilm. It was also noted that all the samples 09/20 (45%) which were Biofilm forming after 24 hours of incubation were only weak biofilm formers. None of the samples could form moderate or strong biofilms.

Congo red agar detected biofilm formation in 05/20 (20%) isolates, Tube method in 17/20 (85%) and Tissue Culture Plate method in 09/20 (45%).Only 02 isolates, i.e., isolate no.40 and 48 were positive by all the three methods of phenotypic detection of biofilm. Similarly, only 01 isolate, i.e., isolate no. 23 was negative by all the methods of detection. Only 01 isolate (isolate no. 20) was found to be positive by Congo Red Agar method and negative by other methods. Similarly, only 01 isolate (isolate no.49) was positive by Tissue culture Plate method and was found to be negative by all other methods.

Many workers have reported that various factors (growth medium, incubation period, fixation of adhered cells and staining) affect development of biofilm on microtiter plate (Stepanovic *et al.*, 2004) [11]. Oliveira *et al.* (2007) [12] showed that the 34.6% *S. aureus* isolates were able to produce biofilm at 24 hours incubation. Our findings suggest a little higher percentage of biofilm forming ability which may be due to the involvement of Methicillin Resistant *Staphylococcus aureus* as the samples for study. Lade (2021) [13] suggests no biofilm formation at 24 hours interval which is contradictory to our findings. The difference in results may be due to the difference in source and type of samples involved. Previously in a similar study, Lee *et al.* (2014) [14] studied ability of the 31 *S. aureus* pulsotypes to produce biofilms in the polystyrene microplate assays. Seventeen pulsotypes (54.8%) did not produce biofilms, whereas 14 (45.2%) pulsotypes were classified as weak (n = 9), moderate (n = 2), or strong (n = 3) biofilm producers, respectively.

Biofilm production is recognized as an important virulence factor for bacteria of the genus *Staphylococcus* (Cucarella *et al.*, 2002 [15], Vasudevan *et al.*, 2003 [16] and Fox *et al.*, 2005) [17]. Khan *et al.* (2011) [6] detected 14.51% *S. aureus* isolates as strong biofilm producers, 50.38% as moderate biofilm producers and 35.11% as non-producers of biofilm. Piechota *et al.* (2018) [18] studied the biofilm formation ability of MRSA isolates from hospitalized patients in Poland. The study revealed that 47.9% of strains were moderate biofilm formers, 39.7% were strong biofilm producers and 11% were weak producers of biofilm. Hosseini *et al.* (2020) [19] also revealed that 52.9% MRSA isolates were strong, 45.3% were moderate and 22.5% were weak biofilm formers. Hence, formation of biofilms by *S. aureus* is a major concern for the dairy industry and is frequently associated with a lack of monitoring of operational standards established for processing milk (Zadoks *et al.*, 2002) [20]. The high frequency of *S. aureus* biofilm-producing isolates highlights the need for constant improvement of quality assurance systems in the dairy farms evaluated.

Conclusion

Our study did not find any correlation between different methods of biofilm assay, i.e., Congo red agar method, Tube method and Microtiter Plate method. However, we can conclude from our study that the Tissue Culture Plate method is a more quantitative and reliable method for the detection of biofilm forming microorganisms as compared to Tube Method and Congo Red Agar methods and it can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories.

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