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Full Length Research Paper

Evaluation of modified Congo red agar for detection of biofilm produced by clinical isolates of methicillin–resistance *Staphylococcus aureus*

N. S. Mariana[1, 2], S. A. Salman[1]*, V. Neela[1] and S. Zamberi[1]

[1]Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Science,
[2]Laboratory of Marine Science and Aquaculture, Institute of Bioscience, Universiti Putra Malaysa, 43400Serdang, Selangor

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Methicillin resistance *Staphylococcus aureus* (MRSA) notoriety is not limited to nosocomial or community acquired infection but is so much the cause of biofilm related infection. Use of highly selective or differential medium, published Congo Red Agar (PCRA) has important shortcomings such as variations in black pigment formation while the intracellular adhesion locus (ica) gene required for biofilm production received equivocal outcomes since contradictory results. The evaluation of modified Congo Red Agar (MCRA) was conducted based on the characteristics of 100 MRSA isolated from different clinical samples and controls. All MRSA isolates showed presence of icaA and icaD genes by the PCR method and then formed intense black pigmented colonies on the Modified Congo Red Agar with increased times in contrast growth of 78% MRSA strains were exhibited black pigmentation on the published CRA but pigmentation decreased with time. The phenotypic coloration on agar improved upon modification of agar ingredients. The reduction in the concentration of several agar constituents resulted in permanent formation of intense black pigment in isolates with ica A and D genes, without any decreased in pigmentation over time. The agar constituent modification allowed stability of black pigment formation and also reduced agar preparation cost.

Key words: MRSA, biofilm, icaA and icaD genes, polymerase chain reaction (PCR), modified CRA, unmodified CRA.

INTRODUCTION

Emergence of multi-antibiotic-resistant strains including *Staphylococcus aureus* infection are becoming more critical, since chronic infection due to biofilm growth, renders antimicrobial agents and the host immune response ineffective in clearing these biofilms (Kloos and anner-man ,1994; Raad ,2000; Donlan , 2001). The sessile community of cells, attached to a substratum embedded in a matrix of extracellular polymeric substance exhibit altered growth, gene expression, and protein production phenotypes can cause major medical and economic sequel (OGara and Humphreys, 2001) The differentiation of staphylococci with respect to its biofilm phenotype might help to elucidate the impact of staphylococci in diagnosis of infections related to biomedical devices and and these observations may have utility in the prevention of device related infections (Raad et al., 1995).

Assays and methods without severe analytical limitations to detect multi-antibiotic-resistant strains including *S. aureus* biofilm accurately would be a valuable approach in diagnosis of resistant biofilm infection. The first used routine methods (tryptic soy broth tube) on isolates of coagulase negative staphylococci induced biofilm from carriage sites, blood cultures, and infected peritoneal dialysis fluids was not always successful for detecting weak slime production and variations in media may affect the result (Christensen et al., 1982). Congo Red Agar used as new alternative method for detecting slime production by coagulase negative staphylococci and more reliable than Christensen method (Freeman et al., 1989) but this media has also been shortcoming in variations in black pigment formation. However, modification on the agar constituent is hypothesized to improve

*Corresponding author. E-mail: salmanatshan@yahoo.com

Table 1. The composition of both Congo red agar and modified Congo red agar used in this study. BHIA, Brain Heart Infusion Agar, BAB-2, Blood Agar Base-2.

Composition/Litter	Congo red agar	Modified Congo red agar
Congo red dye	0.8 g	0.4 g
Sucrose	36 g	---
Glucose	---	10 g
BHIA	52 g	---
BAB-2	---	40 g
Water	1000 ml	1000 ml

the outcome on biofilm identity determination. Therefore the aim of this study were to: (i) evaluate a Modified Congo Red Agar (MCRA) for its utility and reliability as alternative media for biofilm production phenomenon (ii) analyze the prevalence of the intracellular adhesion locus (ica), icaA and icaD genes required for biofilm in our collection of Methicillin Resistant Staphylococcus aureus (MRSA) strains and the correlation between the presence of the ica genes and the ability to produce biofilm *in vitro.*

MATERIALS AND METHODS

Bacterial strains

Three reference strains, they are ATCC35556 for positive biofilm producing *S. aureus,* ATCC700698 for positive methicillin resistance *S. aureus* and ATCC12228 for negative biofilm producing *Staphylococcus epidermidis* under study were assessed by cultured onto CRA, and test samples include 100 MRSA isolates from different clinical sign. All of bacterial strains were obtained and maintained in trypticase soy broth (TSB), in which 20% glycerol was added, at -80℃. from the collection of Research Laboratory of Medical Microbiology , Department of Medical Microbiology and Parasitology , Faculty of Medicines and Health Sciences, University Putra Malaysia , Malaysia. All isolates were confirmed as MRSA by the conventional microbiological methods.

Phenotypic characterization of slime-producing ability

Production of slime was studied by cultivation all of (100) MRSA clinical isolates strains on published Congo Red Agar (CRA) comprising 0.8 g of Congo red [Sigma] and 36 g of saccharose [Sigma] to one litter of brain heart infusion agar [Bacton, Dickinson, rance] (Freeman et al.,1989).

Comparative growth on Modified CRA (MCRA) was determined. The modifications include changing the concentration of Congo red dye and saccharose, omission of glucose [Baker.UK] and replacement of BHIA by an alternative agar, Blood Base Agar-2 (BAB-2) [Oxoid, Basingstoke, Hampshire, England]. Inoculated agar was incubated for 48 h at 37℃ and subsequently 2 - 4 days at room temperature (Table 1).

PCR method for amplification of icaA and icaD genes

The GF-1 Bacterial DNA Extraction Kit was used for bacterial DNA extraction according to manufacturer's protocol. Briefly, DNA was extracted from bacteria cells in broth culture and DNA trapped in column following several steps in the extraction procedure was eluted out using a specified buffer. Pure DNA was stored at -80℃.

The sequences of icaA and icaD were taken from the Gen Bank sequence database (http://www.ncbi.nlm.nih.gov/).The primers were derived from the icaA and ica D sequence data of S. aureus ATCC 35556. Primers were designed using the Primer-3 program [http://www.genome.wi.mit.edu/genome_software/other/primer3.html].The sequences of primers and PCR parameters used are as previously described (Arciola et al., 2001a, b; Rohde et al., 2001) The primers were commercially synthesized by eurogenetic (Singapore) and sequences are as shown in (Table 2) The PCR reaction mixture of 25-µl was used and amplification parameters with minor modification and conditions are as presented in (Table 3) Amplified products were analyzed by agarose gel electrophoresis (1.2% agarose was used in which 0.72 g of the agarose powder (Seakem) is added to 60ml of 1xTBE buffer). The gel was viewed using Alphalmager R Imaging System.

RESULTS

Detection of slime-producing phenotype of MRSA strains By MCRA plate test

On unmodified CRA, slime producing strains formed black (complete) colonies to slightly black, whereas non producing strains develop strong red colonies (Table 4) and (Figure 1. A1, A2) both reference strains ATCC35556, ATCC700698 were found to be slime producers and ATCC12228 to be non-slime producers. Among the 100 MRSA clinical strains investigated, the classification was as follows: 78% black (complete) to slightly black and 22% red color on published Congo red agar and all of strains harbored icaA and icaD genes. In the Modified Congo Red Agar (MCRA), all of MRSA strains formed strong black pigmentation and harbored icaA and icaD genes (Table 5 and Figure 1B).

PCR detection of icaA and icaD genes

The PCR technique was applied to the 100 methicillin resistance *S. aureus* (MRSA) clinical isolates and three reference strains, they are, positive biofilm producing *S. aureus* ATCC35556, MRSA ATCC 700698 and negative biofilm producing *S. epidermidis,* As shown in (Table 4, 5) and (Figure 2) shows icaA and icaD bands on agarose gel electrophoresis of representative MRSA strains and reference strains. The amplification of slime-producing reference strain ATCC 35556 was found to be positive for icaA and icaD genes giving a 188 and 198 bp band respectively. The presence of icaA and icaD genes are seen in all black pigment forming colonies of MRSA isolates grown on MCRA and unmodified CRA also in those colonies that remained red on unmodified CRA . Ica A and D genes are consistently present in all strains regardless they form black pigment.(Table 5) Therefore genotypic based biofilm detection strongly associated to black pigment forming colonies but couldn't still be a re-

Table 2. The sequence of primers used in this study with the respective products sizes.

Gen	Sequence	Product size
Ica A	Forward 5-ACACTTGCTGGCGCAGTCAA-3 Reverse 5-TCTGGAACCAACATCCAACA-3	188 bp
Ica D	Forward 5-ATGGTCAAGCCCAGACAGAG-3 Reverse 5-AGTATTTTCAATGTTTAAAGCAA-3	198 bp

Table 3. PCR amplification parameters with minor modification and conditions

Process	ica A	ica D
Initial denaturation	94℃ 2 min	94℃ 2 min
Denaturation	94℃ 1 min	94℃ 1 min
Annealing	52℃ 1 min	52℃ 1 min
Elongation	72℃ 2 min	72℃ 2 min
Extension elongation	94℃ 4 min	94℃ 4 min
Cycle	25	25

Table 4. Phenotypic characterisation of biofilm formation production on published Congo red agar and the presence of the icaA and icaD genes in the MRSA strains investigated in this study. ATCC35556 for positive biofilm producing *S. aureus*, ATCC700698 for Positive Methicillin Resistance *S. aureus* and ATCC12228 for negative biofilm producing *Staphylococcus epidermidis*

No.	Strain no.	Biofilm formation on BAB-2 after 48 h at 37℃	Biofilm formation on BAB-2 after 2 to 4 days at room temp.	icaA	icaD
0	ATCC35556	Strong black	Strong black	+	+
0	ATCC700698	Strong black	Strong black	+	+
0	ATCC12228	Red	Red	-	-
1	13	Strong black	Strong black	+	+
2	15	Strong black	Strong black	+	+
3	23	Strong black	Strong black	+	+
4	44	Strong black	Strong black	+	+
5	46	Strong black	Strong black	+	+
6	110	Strong black	Strong black	+	+
7	116	Strong black	Strong black	+	+
8	132	Strong black	Strong black	+	+
9	197	Strong black	Strong black	+	+
10	200	Strong black	Strong black	+	+
11	215	Strong black	Strong black	+	+
12	216	Strong black	Strong black	+	+
13	227	Strong black	Strong black	+	+
14	232	Strong black	Strong black	+	+
15	233	Strong black	Strong black	+	+
16	234	Strong black	Strong black	+	+
17	235	Strong black	Strong black	+	+
18	240	Strong black	Strong black	+	+
19	273	Strong black	Strong black	+	+
20	278	Strong black	Strong black	+	+
21	323	Strong black	Strong black	+	+
22	347	Strong black	Strong black	+	+
23	359	Strong black	Strong black	+	+
24	214	Strong black	Strong black	+	+
25	219	Strong black	Strong black	+	+
26	238	Strong black	Strong black	+	+
27	249	Strong black	Strong black	+	+
28	267	Strong black	Strong black	+	+
29	322	Strong black	Strong black	+	+
30	325	Strong black	Strong black	+	+
31	376	Strong black	Strong black	+	+

Table 4. Contd

32	380	Strong black	Strong black	+	+
33	338	Strong black	Strong black	+	+
34	366	Strong black	Strong black	+	+
35	378	Strong black	Strong black	+	+
36	336	Strong black	Strong black	+	+
37	319	Strong black	Strong black	+	+
38	324	Strong black	Strong black	+	+
39	326	Strong black	Strong black	+	+
40	327	Strong black	Strong black	+	+
41	335	Strong black	Strong black	+	+
42	340	Strong black	Strong black	+	+
43	342	Strong black	Strong black	+	+
44	345	Strong black	Strong black	+	+
45	348	Strong black	Strong black	+	+
46	350	Strong black	Strong black	+	+
47	353	Strong black	Strong black	+	+
48	354	Strong black	Strong black	+	+
49	363	Strong black	Strong black	+	+
50	367	Strong black	Strong black	+	+
51	368	Strong black	Strong black	+	+
52	369	Strong black	Strong black	+	+
53	370	Strong black	Strong black	+	+
54	372	Strong black	Strong black	+	+
55	374	Strong black	Strong black	+	+
56	375	Strong black	Strong black	+	+
57	377	Strong black	Strong black	+	+
58	379	Strong black	Strong black	+	+
59	383	Strong black	Strong black	+	+
60	358	Strong black	Strong black	+	+
61	332	Strong black	Strong black	+	+
62	230	Strong black	Strong black	+	+
63	337	Strong black	Strong black	+	+
64	30	Strong black	Strong black	+	+
65	57	Strong black	Strong black	+	+
66	351	Strong black	Strong black	+	+
67	112	Strong black	Strong black	+	+
68	160	Strong black	Strong black	+	+
69	185	Strong black	Strong black	+	+
70	1	Strong black	Strong black	+	+
71	2	Strong black	Strong black	+	+
72	3	Strong black	Strong black	+	+
73	4	Strong black	Strong black	+	+
74	5	Strong black	Strong black	+	+
75	8	Strong black	Strong black	+	+
76	10	Strong black	Strong black	+	+
77	11	Strong black	Strong black	+	+
78	14	Strong black	Strong black	+	+
79	19	Strong black	Strong black	+	+
80	20	Strong black	Strong black	+	+
81	28	Strong black	Strong black	+	+
82	29	Strong black	Strong black	+	+
83	32	Strong black	Strong black	+	+
84	35	Strong black	Strong black	+	+
85	37	Strong black	Strong black	+	+
86	38	Strong black	Strong black	+	+
87	39	Strong black	Strong black	+	+
88	40	Strong black	Strong black	+	+
89	41	Strong black	Strong black	+	+
90	45	Strong black	Strong black	+	+
91	47	Strong black	Strong black	+	+
92	49	Strong black	Strong black	+	+
93	50	Strong black	Strong black	+	+

Table 4. Contd

94	52	Strong black	Strong black	+	+
95	52	Strong black	Strong black	+	+
96	53	Strong black	Strong black	+	+
97	56	Strong black	Strong black	+	+
98	63	Strong black	Strong black	+	+
99	66	Strong black	Strong black	+	+
100	320	Strong black	Strong black	+	+

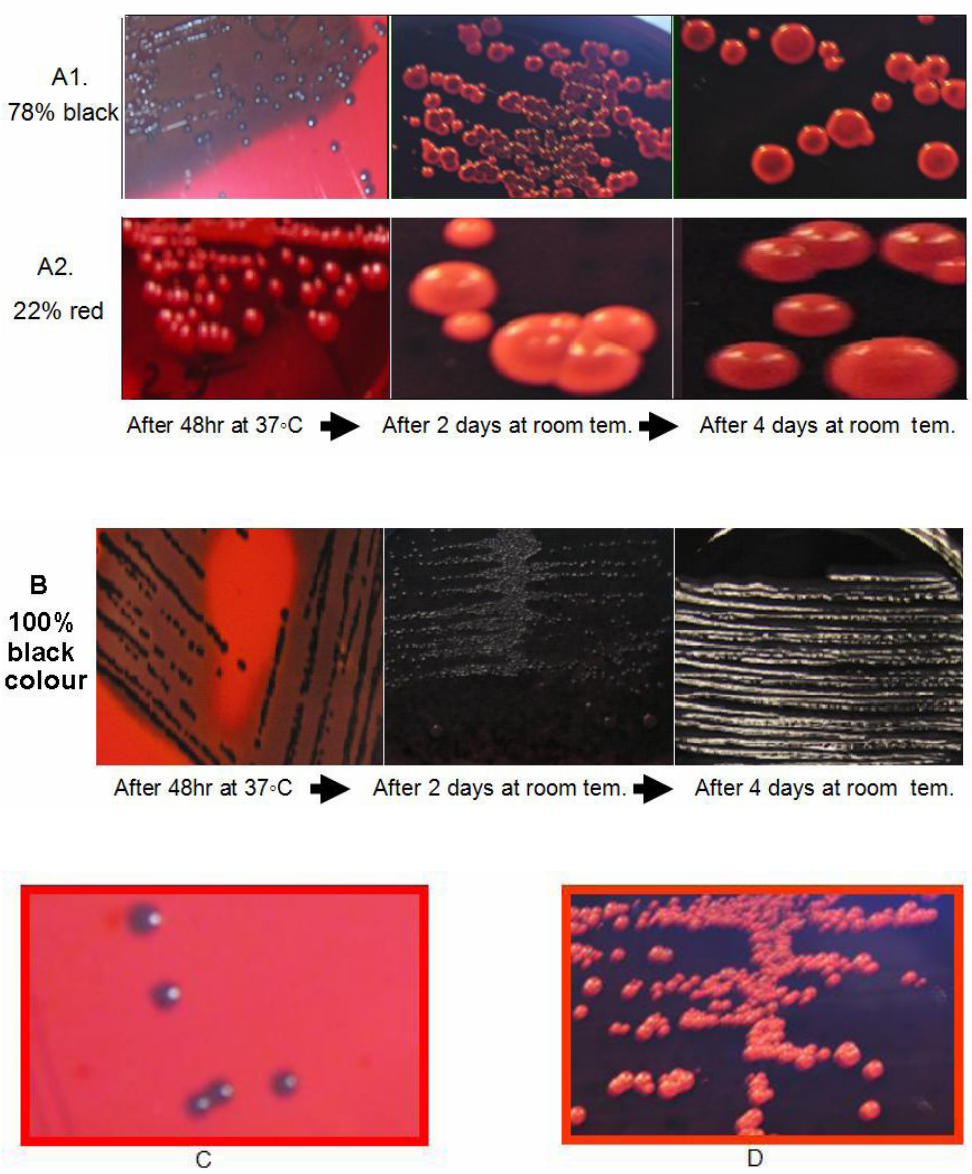

Figure 1. Congo red agar has showed pigmentation decreased with the time from black color to red color as shown in colonies of 78 MRSA strains, picture A1, red color exhibited was represented a falls negative results of 22 MRSA strains on CRA at the first 48 h at 37°C and then at room temperature for more than 4 days, picture A2. Picture B, Modified Congo Red Agar was performed a strong black pigmentation with all colonies of 100 MRSA clinical isolates after 48 h at 37°C and then at room temperature for more than 4 days. Picture C, represented black color of ATCC35556 for positive biofilm producing S. aureus on Unmodified and Modified Congo Red Agar, picture D. represented red color of ATCC12228 for negative biofilm producing *Staphylococcus epidermidis*.

Table 5. Phenotypic characterisation of biofilm formation production on Modified Congo red agar and the presence of the icaA and icaD genes in the MRSA strains investigated in this study. ATCC35556 for positive biofilm producing *S. aureus*, ATCC700698 for Positive Methicillin Resistance *S.aureus* and ATCC12228 for negative biofilm producing *Staphylococcus epidermidis*

No.	Strain no.	Biofilm formation on BAB-2 after 48 h at 37°C	Biofilm formation on BAB-2 after 2 to 4 days at room temp.	icaA	icaD
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0	ATCC700698	Strong black	Strong black	+	+
0	ATCC12228	Red	Red	-	-
1	13	Strong black	Strong black	+	+
2	15	Strong black	Strong black	+	+
3	23	Strong black	Strong black	+	+
4	44	Strong black	Strong black	+	+
5	46	Strong black	Strong black	+	+
6	110	Strong black	Strong black	+	+
7	116	Strong black	Strong black	+	+
8	132	Strong black	Strong black	+	+
9	197	Strong black	Strong black	+	+
10	200	Strong black	Strong black	+	+
11	215	Strong black	Strong black	+	+
12	216	Strong black	Strong black	+	+
13	227	Strong black	Strong black	+	+
14	232	Strong black	Strong black	+	+
15	233	Strong black	Strong black	+	+
16	234	Strong black	Strong black	+	+
17	235	Strong black	Strong black	+	+
18	240	Strong black	Strong black	+	+
19	273	Strong black	Strong black	+	+
20	278	Strong black	Strong black	+	+
21	323	Strong black	Strong black	+	+
22	347	Strong black	Strong black	+	+
23	359	Strong black	Strong black	+	+
24	214	Strong black	Strong black	+	+
25	219	Strong black	Strong black	+	+
26	238	Strong black	Strong black	+	+
27	249	Strong black	Strong black	+	+
28	267	Strong black	Strong black	+	+
29	322	Strong black	Strong black	+	+
30	325	Strong black	Strong black	+	+
31	376	Strong black	Strong black	+	+
32	380	Strong black	Strong black	+	+
33	338	Strong black	Strong black	+	+
34	366	Strong black	Strong black	+	+
35	378	Strong black	Strong black	+	+
36	336	Strong black	Strong black	+	+
37	319	Strong black	Strong black	+	+
38	324	Strong black	Strong black	+	+
39	326	Strong black	Strong black	+	+
40	327	Strong black	Strong black	+	+
41	335	Strong black	Strong black	+	+
42	340	Strong black	Strong black	+	+
43	342	Strong black	Strong black	+	+
44	345	Strong black	Strong black	+	+

Table 5. Contd

45	348	Strong black	Strong black	+	+
46	350	Strong black	Strong black	+	+
47	353	Strong black	Strong black	+	+
48	354	Strong black	Strong black	+	+
49	363	Strong black	Strong black	+	+
50	367	Strong black	Strong black	+	+
51	368	Strong black	Strong black	+	+
52	369	Strong black	Strong black	+	+
53	370	Strong black	Strong black	+	+
54	372	Strong black	Strong black	+	+
55	374	Strong black	Strong black	+	+
56	375	Strong black	Strong black	+	+
57	377	Strong black	Strong black	+	+
58	379	Strong black	Strong black	+	+
59	383	Strong black	Strong black	+	+
60	358	Strong black	Strong black	+	+
61	332	Strong black	Strong black	+	+
62	230	Strong black	Strong black	+	+
63	337	Strong black	Strong black	+	+
64	30	Strong black	Strong black	+	+
65	57	Strong black	Strong black	+	+
66	351	Strong black	Strong black	+	+
67	112	Strong black	Strong black	+	+
68	160	Strong black	Strong black	+	+
69	185	Strong black	Strong black	+	+
70	1	Strong black	Strong black	+	+
71	2	Strong black	Strong black	+	+
72	3	Strong black	Strong black	+	+
73	4	Strong black	Strong black	+	+
74	5	Strong black	Strong black	+	+
75	8	Strong black	Strong black	+	+
76	10	Strong black	Strong black	+	+
77	11	Strong black	Strong black	+	+
78	14	Strong black	Strong black	+	+
79	19	Strong black	Strong black	+	+
80	20	Strong black	Strong black	+	+
81	28	Strong black	Strong black	+	+
82	29	Strong black	Strong black	+	+
83	32	Strong black	Strong black	+	+
84	35	Strong black	Strong black	+	+
85	37	Strong black	Strong black	+	+
86	38	Strong black	Strong black	+	+
87	39	Strong black	Strong black	+	+
88	40	Strong black	Strong black	+	+
89	41	Strong black	Strong black	+	+
90	45	Strong black	Strong black	+	+
91	47	Strong black	Strong black	+	+
92	49	Strong black	Strong black	+	+
93	50	Strong black	Strong black	+	+
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95	52	Strong black	Strong black	+	+
96	53	Strong black	Strong black	+	+

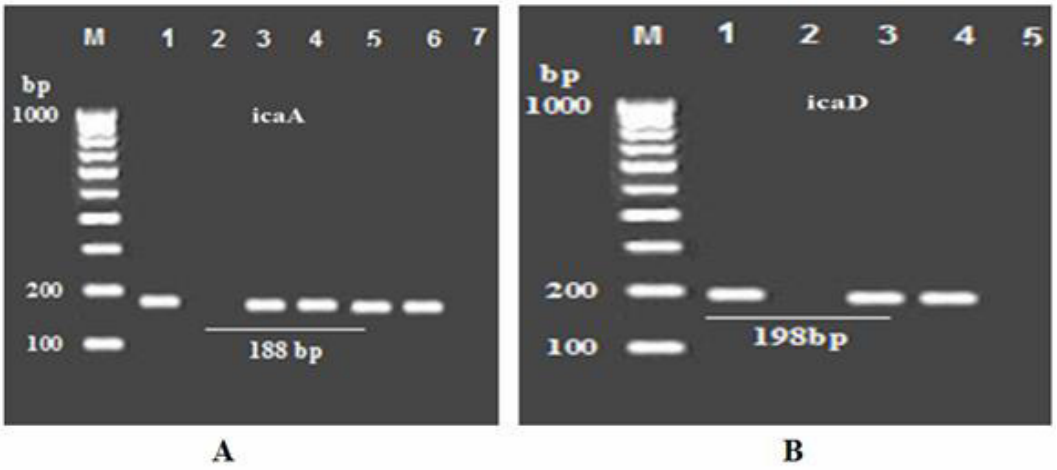

Figure 2. Amplification of icaA and icaD genes. M: Molecular size marker 100 bp A and B. lanes 1.A, 1.B, positive biofilm producing Reference strain ATCC35556. Lanes 2A, 2B, negative biofilm producing reference strain ATCC12228. Lanes 7A and 5.B negative control. Lanes 3A, 4A, 5A, 6A, represented of icaA gene at 188bp band from DNA all of MRSA clinical isolates strains exhibited a black color on MCRA. Lanes 3B and Lane 4B, 198bp band obtained with icaD gene from DNA all of MRSA clinical isolates strains forming black pigmentation in this study.

relevant approach to rapidly diagnose biofilm strains if further investigations involving other genes or other bioassay media are required. The phenotypic assay of black pigment formation is however associated with the presence of glucose in the CRA medium

DISCUSSION

The evaluation of modified Congo Red Agar based on the phenotypic characteristics of 100 Methicillin Resistance *S. aureus* (MRSA) isolated from different clinical samples and controls. All MRSA isolates showed presence of icaA and icaD genes by the PCR method. The *in vitro* slime production ability on the published Congo red Agar by Freeman et al. (1989) is diffusion of black pigment in the agar with growth of black pigmented colonies but pigmentation decreased with time. In the present study the Modified Congo Red Agar (MCRA) was optimized to get strong black pigmented in all colonies of MRSA isolates with constant pigmentation at 48 h incubation and then for 2 - 4 days at room temperature. A black color interpreted as positive biofilm producing strains in contrast with red colonies which interpreted as negative biofilm producing.

The phenotypic coloration on agar improved upon modification of agar ingredients. The reduction in the concentration of agar constituents resulted in permanent formation of intense black pigment in isolates with ica A and D genes, without any decreased in pigmentation over time. The agar constituent modification allowed stability of black pigment formation and also reduced agar preparation cost. Stable black pigment is an added value to the identification accuracy of biofilm agents. Accurate identity of biofilm agents will improve drugs or antibiotics prescription, rendering effective and rapid killing of pathogen. Further studies will be conducted to determine the true economic return from the development of a modified Congo Red Agar.

Conclusion

The exists a correlation between a constant black colony-forming phenotype on modified Congo red agar and presence of ica A and icaD global genes in this findings, suggest that phenotypic and genotypic approaches are complementary for accurate and rapid identification of bacterial types, namely MRSA in a biofilm matrix in contrast with limited efficacy of identification between genotypic and phenotypic on published Congo Red Agar.

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REFERENCES

Arciola CR, Baldassarri L, Montanaro L (2001a). Presence of icaA and icaD genes and slime production in a collection of *staphylococcal* strains from catheter-associated infections. J. Clin. Microbiol.39: 2151–2156.

Arciola CR, Campoccia D, Borelli AM, Montanaro ME (2001b).Congo red agar plate method: improved accuracy and new extended application to *Staphylococcus aureus*. New Microbiol.24: 355–363.

Christensen GD, Simpson WA, Bisno AL, Beachey EH (1982). Adherence of slime-producing strains of *Staphylococcus* epidermidis to smooth surfaces. Infect. Immun.37: 318-326.

Donlan RM (2001). Biofilms and device associated infections. Emerg. Infect. Dis. 7: 277-281.
Freeman DJ, Falkiner FR, Keane CT (1989). New method for detecting slime production by coagulase negative *staphylococci*. J. Clin. Pathol. 42: 872-874.
Kloos WE, annerman TL (1994). Update on clinical significance of coagulase-negative *Staphylococci*. Clin. Microbiol. Rev. 7: 117-405.
OGara JP, Humphreys H (2001). *Stphylococcus epidermidis* biofilms: Importance and implications. J. Med. Microbiol. 50:582-587.
Raad I (2000). Management of intravascular catheter-related infection. J. Antimicrob. Chemother. 45: 267-270.
Raad I, Darouiche R, Hachem R, Sacilowski M, Bodey GP(1995).Antibiotics and prevention of microbial colonization of catheter. Antimicrob. Agents Chemother. 39: 2397-2400.
Rohde H, Knobloch JKM, Horstkotte MA, Mack D (2001). Correlation of *Staphylococcus aureus* icaADBC genotype and biofilm expression phenotype. J. Clin. Microbiol. 39: 4595–4596.