



Rapid detection of methicillin resistance and biofilm formation in *Staphylococcal* species

Snehali Majumder^{1*}, P. F. G. Wolffs², Christian J. P. A. Hoebe² and Mohammed Rahmatullah³

¹Department of Microbiology and Serology, NH Health City, Bangalore India

²Faculty of Health, Medicine and Life Sciences Department of Medical Microbiology, Maastricht Infection Center (MINC), School of Public Health and Primary Care (CAPHRI), Maastricht University Medical Center (MUMC+), 6202 AZ Maastricht, The Netherlands

³Department of Pharmacy, University of Development Alternative, Dhaka, Bangladesh

ABSTRACT

Staphylococcus aureus (including Methicillin-resistant *S. aureus*) and coagulase negative *Staphylococcus* spp (including methicillin-resistant coagulase negative) permanently or transiently colonize humans and act as sources of complicated skin and soft tissue infections, bacteraemia and infective endocarditis. They also cause chronic infection of indwelling medical devices. Treatment of these infections has become problematic due to development of methicillin resistance, ability to cause hospital outbreaks and 2-3 days required for their proper identification. Rapid, sensitive detection of methicillin resistant *Staphylococcus* spp from clinical samples is a necessity. 242 bacterial isolates (162 methicillin-resistant *S. aureus* and 80 methicillin-resistant coagulase negative *S. aureus*) from clinical samples was included in the study; detection of *mecA*, *femA* and *icaD* gene was done by Multiplex-Colony Polymerase Chain Reaction technique. Out of the 242 *Staphylococcal* strains included in the study, in 192 (79.3%) isolates the presence of *mecA* gene was detected. Interestingly the presence of *femA* gene was detected in all 162 methicillin-resistant *S. aureus* isolates. However none of the 80 methicillin-resistant coagulase negative *S. aureus* isolates harbored the *femA* gene. The *icaD* gene Polymerase Chain Reaction revealed that 59.25% of methicillin-resistant *S. aureus* and 10% of methicillin-resistant coagulase negative *S. aureus* harbored this gene. Multiplex colony Polymerase Chain Reaction is a comprehensive alternative for rapid sensitive and accurate detection of methicillin resistance and biofilm production in *Staphylococcus* spp from clinical samples.

Keywords: MRSA/MRCoNS, *mecA*, *femA*, *icaD*, Biofilm

INTRODUCTION

Timely detection of methicillin-resistant *Staphylococcus aureus* (MRSA) is crucial for an effective management of infection and isolation policy in any hospital setup [1]. MRSA has become endemic in many hospitals worldwide where it is causing excess nosocomial infection, particularly in the intensive care setting. There is mounting public concern about this situation, as evidence shows that invasive MRSA infection is associated with a significant increase in mortality and prolonged hospital care [2, 3]. *Staphylococcus* spp is causing both nosocomial and community acquired infections ranging from minor skin infections to endocarditis and sepsis and septic shock [4]. Conventional methods are slow for early identification of MRSA carriers. Culture-based screening methods usually require 48–96 hours for MRSA identification. New-generation selective agar media with chromogenic enzyme substrates perform better but still require 24–48 hours for presumptive MRSA detection [5]. This delay in detection increases the chance of cross-infection. To address this diagnostic delay, a cautious alternative is to place Intensive Care Unit (ICU)-admitted patients in preemptive isolation until proven MRSA-negative. The arrangement results in an increased cost burden on both the hospital and the patient. In cases of sepsis turnaround time (TAT) for positive cultures are important so that appropriate antimicrobial can be selected immediately, unnecessary treatment of likely contaminants and antibiotic exposure can be avoided and expenditure on

antimicrobials decreased. Timely detection of, distinction of *S.aureus* from coagulase negative *S. aureus* (CoNS), methicillin-susceptibility and screening for pathogenic traits results have great therapeutic, prognostic and economic significance. [6-9].

The detection of *mec A* encoding a PBP (Penicillin Binding Protein) with low affinity with beta-lactam antibiotics (PBP2a) is considered a “Gold Standard” technique for oxacillin resistance detection [8]. Phenotypic tests for oxacillin resistance provide unsatisfactory results since the microorganisms may carry a gene for resistance factor, though expression of this gene may be influenced by environmental conditions and culture factors. Resistance to methicillin may be extrinsic, non-*mecA* mediated, in *S. aureus* strains with low-level resistance to oxacillin, known as borderline oxacillin-resistant *Staphylococcus aureus* (BORSA) [9-12]. Typically, this borderline phenotype results from excess production of β -lactamase. It was described initially by McDougal and Thornsberry in 1986 [11]. According to these authors, these strains were neither Heteroresistant nor multi-resistant, and they produced large amounts of normal *Staphylococcal* beta-lactamase which partially hydrolyze oxacillin and became fully susceptible to oxacillin in the presence of beta-lactamase inhibitors [12]. However, the borderline phenotype has been attributed to other mechanisms, i.e., the production of an inducible, plasmid-mediated methicillinase or different modifications in the PBP genes due to spontaneous amino acid substitutions in the trans-peptidase domain [10, 13]. Molecular assays can provide a rapid, sensitive, specific alternative to the conventional method of MRSA detection. Thus from an epidemiological and infection management point of view the detection of only *mec A* gene is not enough to control and treat MRSA and MR-CoNS. It is known from the literatures that *Staphylococci* are the most frequent causative agent of medical device-related and surgical site infections and surgical-site infections (13, 14). It is well known that slime-producing *Staphylococci* on medical devices are the most important cause of chronic implant-related infections. According to reports from the Centers for Disease Control and Prevention, more than 65% of nosocomial infections involve slime.

The present study is a two-phased study. Phase one study validates the performance of a 3 gene comprehensive multiplex colony PCR. The aim of the study was to develop a PCR assay that could differentiate between *Staphylococcus aureus* and other coagulase negative *Staphylococcal* spp (CoNS), detect methicillin resistance and biofilm producing ability of a *Staphylococcal* isolates within hours from clinical samples. Using this approach, named Multiplex-Colony PCR it is possible to identify, and assess micro-organisms resistance and virulence in the same reaction [15, 16]. The second phase of the study used the same protocol for detection of *Staphylococcus* in positive blood cultures by GCT method. The guanidinium isothiocyanate (GCT) method for DNA isolation was originally developed by Pitcher et.al [17] in 1989. Since its publication the method has been modified many times by researchers [18-20]. Species differentiation can be achieved by targeting *fem A* gene (factor essential for methicillin resistance) belongs to the *fem AB operon*. It is a housekeeping gene and is found in all *S.aureus* strain. *fem A* gene is responsible for the addition of the second and third glycine residues to the intra-peptide bridge [21]

The *ica D* gene, of *icaADBC* operon has been reported to play a significant role in biofilm formation in *S. aureus* and *S. epidermidis*. This gene enhances the maximal expression of N-acetylglucosaminyltransferase, leading to the phenotypic expression of the capsular polysaccharide [22, 23]. Hence *ica D* gene in clinical isolates can be a potential candidate marker for detection of biofilm synthesis by *Staphylococcus* spp. In the present study the Multiplex-Colony PCR technique was employed to detect genes *fem A* (Species specific), *mec A* (methicillin/oxacillin resistant) and *icaD* (biofilm producing ability). The aim was to develop a more efficient technique for the rapid detection of *Staphylococcal* infection.

EXPERIMENTAL SECTION

Bacterial Isolates

242 bacterial isolates were collected from cases of suspected clinical infections. The isolates collected comprised of phenotypically identified Methicillin resistant *Staphylococcus aureus* (MRSA) and Methicillin-resistant coagulase-negative *Staphylococci* (MR-CoNS) only. The isolates were recovered from clinically significant specimens (blood, pus, other body fluid, etc.). All isolates were non-duplicate, consecutive clinical strains collected from patients from Narayana Hrudayalaya Health City, Bangalore, India

Identification and Antimicrobial Susceptibility Testing

Cefoxitin disc diffusion was conducted as per CLSI guidelines (*S.aureus* Cefoxitin sensitive cut off ≥ 22 mm and resistant ≤ 21 mm and CoNS Cefoxitin sensitive cut off ≥ 25 mm and resistant ≤ 24 mm). Identification and Oxacillin MIC was determined using Vitek 2 compact System (*S.aureus* Oxacillin MIC, sensitive breakpoint $\leq 2\mu\text{g/ml}$ and Resistant breakpoint $\geq 4\mu\text{g/ml}$ and CoNS Oxacillin MIC, sensitive breakpoint $\leq 0.25\mu\text{g/ml}$ and Resistant breakpoint $\geq 0.5\mu\text{g/ml}$) [24].

Conventional identification of bio-film formation, by Tissue Culture Plate (TCP) method

In this study, 242 isolates were tested for their ability to synthesize biofilm by TCP method as described by Christensen et al with a modification in duration of incubation which was extended from 16 to 24 hours [25]. Isolates from fresh blood agar plates (Delta Biologicals, Bangalore) were inoculated in brain heart infusion (BHI, Difco) with 2% sucrose (BHISuc) and incubated for 18 hours at 37°C in stationary condition. Inoculated media was diluted 1 in 100 with fresh medium. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates (Tarson, Kolkata, India) were filled with 0.2 ml aliquots of the diluted cultures. Sterile broth served as control to check sterility and non-specific binding of media. The tissue culture plates were incubated for 18 hours - 24 hours at 37°C. After incubation content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free-floating 'planktonic' bacteria. Biofilm formed by adherent 'sessile' organisms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v) (Hi-media Ltd). Excess stain was rinsed off by thorough washing with deionized water and the plates were kept for air drying. Adherent *Staphylococcal* cells usually forming Biofilm on all side wells were uniformly stained with crystal violet. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader (model 680, Bio-rad) at wavelength of 570 nm (OD570 nm). As a control, un-inoculated medium was used. The mean OD570 value from control wells subtracted from the mean OD570 value of tested wells. These OD values were considered as an index of bacteria adhering to surface and forming bio-film. The known MRSA strain (ATCC BAA-1026) and MSSA (ATCC 25923) were used as controls in all the experiments. The interpretation of biofilm production was done according to the criteria of Stepanovic et al [26].

Interpretation of biofilm production

Average OD value \leq ODc / ODc $< \sim \leq$ 2x ODc
 2x ODc $< \sim \leq$ 4x ODc
 > 4x ODc

Biofilm production

Non/weak
 Moderate
 Strong

Optical density cut-off value (ODc) = average OD of negative control + 3x standard deviation (SD) of negative control

Conventional identification of Blood cultures

A total of 50 blood culture. (BACTEC FX BD) was collected over a period of one month from Jan to Feb 2104 from the microbiology department of NH Health City, Bangalore, India. All the bottles were positive for *Staphylococci*. All positive cultures were aliquoted into 1.5ml micro-centrifuge tubes as soon as positive signal and Gram's Stain showed the presence of bacteria. The micro-centrifuge tubes were stored at -70°C until testing. *Staphylococci* isolates were identified by standard methods including Gram stain, catalase, and tube coagulase. Screening for cefoxitin and MIC was determined as for other clinical samples described earlier [27].

Multiplex Colony PCR

Overnight culture of *Staphylococcus* spp grown on Blood Agar was used for DNA extraction. A single colony of *Staphylococcus* was picked up by the tip of a sterile tip and was re-suspended into 25µl of distilled autoclaved water. This inoculum was subjected to heat shock at 95°C for 5 minutes in a thermo-cycler. The primers used for multiples PCR is listed in Table 1. Multiples PCR was performed in a 25µl volume that comprised of 10x Buffer 5µl, 200 mM dNTP mix, 10 pmoles of each primer, Taq polymerase 1U, and 1µl of DNA suspension (Colony lysis). Cycling parameters were as follows: 95°C for 5 min, 40 cycles of 94°C for 1 min, 55.5°C for 1 minute and extension at 72°C for 1 min with a final extension at 72°C for 5 min. The amplified PCR fragments were analyzed by 2% agarose gel electrophoresis with 100bp DNA ladder. The amplicon size was 320 for *mecA*, 132 for *femA* and 238 for *icaD*. The resulting bands were visualized by UV trans-illumination (Bio-rad, USA).

Table 1: List of primers used

Gene	Primers used	Base Pair Size
<i>mec A</i>	5'-AAA-ATC-GAT-GGT-AAA-GGT-TGG-C-3' 5'-AGT-TCT-GCA-GTA-CCG-GAT-TTG-C-3'	310
<i>fem A</i>	5'-AAA-AAA-GCA-CAT-AAC-AAG-CG-3' 5'-GAT-AAA-GAA-GAA-ACG-AGC-AG-3'	132
<i>ica D</i>	5'-CTTCGATGTCGAAAATAAACTC-3' 5'-GCTTCTGGAATGAGTTTGCT-3'	238

DNA extraction from positive blood culture bottles by Guanidine Thiocyanide Method (GTC)

GTC (8 molar) 390µl was added to 200 µl of whole blood and mixed vigorously for 1 minute and was then kept on ice for 5 minutes. 60µl Sodium acetate, 300µl chloroform and 600µl Phenol were added and again kept on ice for 10 minutes. The mixture was centrifuged at 1200 rpm for 10 minutes. The aqueous phase was transferred to another tube and 30 µl of chloroform and isopropyl alcohol was added in 24:1 ratio. The tubes were kept on ice for

10 minutes and then centrifuge at 1200 rpm for 10 minutes. The aqueous phase was transferred to another tube and 60 μ l of ice cold isopropanol was added and kept at -20°C for 1 hour and centrifuge at 15000 rpm for 10 minutes. The pellet formed was washed with 70% Ethanol (0.5ml) and excess liquid was drained on a tissue paper. The Dry the pellet was then suspended in water (20 μ l). [28, 29] The sample can be stored at -20°C for future use. The amount of protein contamination in isolated DNA was assessed by calculating the ratio of absorbance at 260nm to that at 280nm. Absorption ratios between 1.92 and 2.20 indicates effective removal of proteins. The DNA was quantified by measuring the UV-induced emission fluorescence from intercalated ethidium bromide. [30]

$$\text{dsDNA concentration} = 50\mu\text{g/ml} \times \text{OD}_{260} \times \text{dilution factor}$$

This method is useful if there is not enough DNA to quantify with a spectrophotometer. The DNA sample was used for multiplex PCR for *mec A*, *fem A* and *icaD* gene.

RESULTS

Multiplex PCR results as compared to conventional technique

When conventionally identified MRSA isolates were subjected to Multiplex-colony PCR in strains 128 (79%) isolates *mecA* and in all 162 (100%) isolates *femA* gene were detected. They were confirmed to be MRSA with no biofilm producing trait. Among 64(80%) isolates *mecA* gene was detected and identified to be methicillin resistant coagulase negative *Staphylococcus* spp (MR-CoNS) with no biofilm forming ability. In 96 (40%) strains all the three genes were detected which indicates the strains to be MRSA with biofilm producing ability conventionally identified MR-CoNS isolates were subjected to Multiplex-colony PCR. None of the isolates harbored the *femA* gene. 8 (3.3%) isolates were positive for both *mecA* and *icaD* gene. These isolates were confirmed to be MR-CoNS which produce biofilms. 66 isolates were positive only for *mecA* gene which identified them to be MR-CoNS with no biofilm producing ability. The results are summarized in Table 2–3 and Fig-1.

Multiplex PCR direct testing from blood cultures

The GCT method of DNA extraction directly from blood samples yielded a DNA sample that had a purity of [A1 (260nm) to A2 (280nm) ratio] 2.05 and DNA concentration (50X OD 260) of 21.89ng/ μ l. Direct multiplex PCR testing with DNA from positive blood cultures did not misidentify any isolate compared to the PCR test results with actual bacterial growth. The total time required to perform the multiplex PCR assay directly from blood culture was 4hrs (Fig 2).

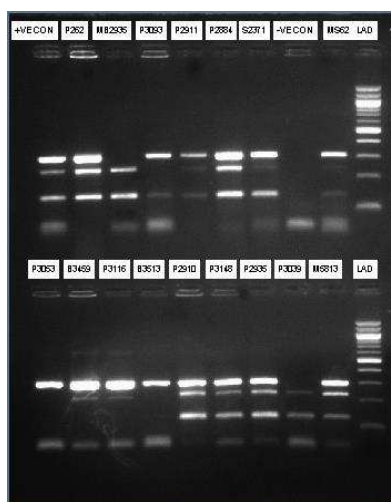
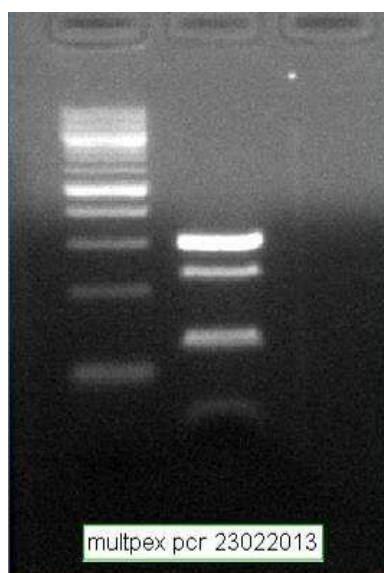
Table 2: Comparison between phenotypic and genotypic identification of MRSA/MR-CoNS

SPECIMENS	MRSA					MR-CoNS				
	Culture Method (Cefoxitin resistant)	<i>mec</i>	<i>fem A</i>	TCP	<i>icaD</i>	Culture Method (Cefoxitin resistant)	<i>mec A</i>	<i>fem A</i>	TCP	<i>icaD</i>
Pus & Wound Swab	100	91	100	68	68	32	23	0	3	3
Respiratory Samples	13	9	13	9	9	2	1	0	0	0
Blood	24	10	24	7	7	29	25	0	4	4
Fluids	3	2	3	0	0	1	1	0	0	0
Urine	2	1	2	1	1	6	4	0	0	0
Miscellaneous	20	15	20	11	11	10	10	0	1	1

TCP: Tissue Culture Plate Method; MRSA: Methicillin resistant *Staphylococcus aureus*; MR-CoNS: Methicillin Resistant Coagulase Negative *Staphylococcus* spp

Table 3: Tissue Culture Plate Method (TCP) and Distribution of bio-film formation.

SPECIMENS	MR-CoNS			MRSA		
	Strong Adherence	Weak Adherence	No Adherence	Strong Adherence	Weak Adherence	No Adherence
Pus	2	1	42	67	1	76
Blood	1	3	22	7	0	27
Miscellaneous Samples	0	1	6	11	0	12
Respiratory Sample	0	0	2	9	0	1
Fluids	0	0	1	0	0	1
Urine	0	0	6	1	0	6

Fig 1: Multiplex Colony PCR for *mec A*, *fem A* and *icaD* gene**Fig 2: Multiplex PCR from direct blood sample by GTC method**

DISCUSSION

We describe a direct DNA isolation technique from blood followed by a multiplex colony PCR. This technique can be used for survey of clinically relevant antibiotic resistance genes frequently encountered in *Staphylococci* spp. The method is simple, easy to perform and rapid. We have compared multiplex PCR assays for the identification and detection of methicillin resistance genes with classical methods [10]. Overall we found 79.33% co-relation between these two methods. Several studies have used PCR for the detection of the *mec A* gene only or combined in multiplex with *S.aureus* specific amplification assays [1-3, 10]. Although culture methods are generally reliable for detect MRSA, the detection of *mecA* is now considered the gold standard method mainly because

- (i) Phenotypic methods may be difficult to interpret [10, 31-33];
- (ii) Some isolates do not express their *mec A* gene unless selective pressure via antibiotic treatment is applied [34].

To deal with applicability and accuracy we applied the PCR assay to test a total of 162 MRSA and 80 MR-CoNS from routine clinical specimens. In the study we found 44 strains of *S.aureus* and 66 strains of CoNS strains harbored the *mec A* Gene. In 50 (21%) isolates *mec A* gene was not detected. The differentiation of MRSA/MR-CoNS from BORSA (Borderline Resistant *Staphylococcus aureus*) strain is crucial for therapeutic advice. MRSA strains produce PBPs with low intensity to beta-lactum antibiotics, except for methicillin and oxacillin [32]. BORSA strains are beta-lactamase hyper producers and do not produce a modified PBP (PBP2a) that

is they have *mec*-independent resistance [33, 35]. Hence the combination of a β -lactum and a β -lactum inhibitor could be useful in treating infections caused by those strains [34] implying the less frequent use PCR does not differentiate the BORSA strains since they do not have the *mec A* gene Another gene *fem A*, essential for the expression of methicillin resistance in *S.aureus* and is universally present only in *S.aureus* isolates. This gene has been implicated in cell metabolism and is present in large amounts in actively growing cultures. [36] All 162 isolates of *S.aureus* cultures examined regardless of the presence or absence of *mec A* gene produced a positive result in PCR for *fem A* gene. The *fem A* gene encodes a protein (Fem), essential for the expression of resistance to methicillin. In spite of this involvement in resistance, the gene *fem A* is also present in MSSA strains. When 80 CoNS strains were examined for the presence of *fem A* gene by multiplex colony PCR all were negative. The results are in agreement with the fact that inter-species variants of *fem A* gene exists that may be used as species specific primers. [37-39]. *icaD* gene was detected in 96 MRSA isolates and 8 of MR-CoNS isolates. It was confirmed that all biofilm producing strains were positive for *icaD* gene the results are in agreement with previous reports (40-42). It is important to diagnose to give prophylactic antibiotics just before and during the surgical procedure to eliminate planktonic bacteria before they can form biofilm [43].

CONCLUSION

The multiplex-colony PCR will provide a simple, rapid, reliable diagnosis of MRSA/MR-CoNS and their bio-film producing ability.

Acknowledgment(s)

The authors would like to thank the staff and management of NH Narayana Health city for their support.

REFERENCES

- [1] MJ Struelens; D Olivier, *Critical Care*, **2006**, 10, 128.
- [2] SE Cosgrove; Y Qi; KS Kaye; S Harbarth; AW Karchmer; Y Carmeli, *Infect. Control Hosp. Epidemiol.*, **2005**, 26, 166-174.
- [3] CA Muto; JA Jernigan; BE Ostrowsky; HM Richet; WR Jarvis; JM Boyce; BM Faror, *Infect. Control Hosp. Epidemiol.*, **2003**, 24, 362-386.
- [4] AJM Loonen; AR Jansz, H Kreeftenberg; CA Bruggeman; PFG Wolfs; AJC van den Brule, *Eur. J. Clin. Microbiol. Infect. Dis.*, **2011**, 30, 337-342.
- [5] DF Brown; DI Edwards; PM Hawkey; D Morrison; GL Ridgway; KJ Towner; MWD Wren, *J. Antimicrob. Chemother.*, **2005**, 56, 1000-1018.
- [6] D Annane; E Bellissant; JM Cavaillon, *Lancet*, **2005**, 365, 63-78.
- [7] SE Beekmann; DJ Diekema; KC Chapin; GV Doern, *J. Clin. Microbiol.*, **2003**, 41, 3119-3125.
- [8] KB Anand; P Agrawal; S Kumar; K Kapila, *Indian J. Med. Microbiol.*, **2009**, 27, 27-29.
- [9] O Massidda; MP Montanari; M Mingoia; PE Varaldo, *Antimicrob. Agents Chemother.*, **1996**, 40, 2769-2774.
- [10] LK McDougal; C Thornsberry, *J. Clin. Microbiol.*, **1986**, 23, 832-839.
- [11] MP Montanari; E Tonin; F Biavasco; PE Varaldo, *Antimicrob. Agents Chemother.*, **1990**, 34, 911- 913.
- [12] MP Montanari; O Massidda; M Mingoia; PE Varaldo, *Microb. Drug Resist.*, **1996**, 2, 257-269.
- [13] J Nadarajah; MJ Lee; L Louie; L Jacob; AE Simor; M Louie; MJ McGavin, *J. Med. Microbiol.*, **2006**, 55, 1675-1683.
- [14] J Huebner; DA Goldmann, *Ann. Rev. Med.*, **1999**, 50, 223-236.
- [15] LA Pereira; DA Fisher, *Ann. Acad. Med. Singapore*, **2008**, 37, 891-896.
- [16] P Markoulatos; N Siafakas; M Moncany, *J. Clin. Lab. Anal.*, **2002**, 216, 47-51.
- [17] DG Pitcher; A Saunders; RJ Owe, *Lett. Appl. Microbiol.*, **1989**, 8, 151-156.
- [18] MG La Montagne; FC Michel; PA Holden; CA Reddy, *J. Microbiol. Meth.*, **2002**, 49, 255-264.
- [19] T Wilson; J Carson, *J. Appl. Microbiol.*, **2001**, 32, 326-330.
- [20] AL McOrist; M Jackson; AR Bird, *J. Microbiol. Meth.*, **2002**, 50, 131-139.
- [21] BR Heinrich Maidhof; P Blumel; L Brigitte Berger-Bachi; H Labischinsk, *J. Bacteriol.*, **1991**, 173, 3507-3513.
- [22] M Cralbl, *J. Clin. Microbiol.*, **2001**, 39, 2151-2156.
- [23] GF Gad; MA El-Feky; MS El-Rehewy; MA Hassan; H Abolella; RM El-Baky, *J. Infect. Dev. Ctries.*, **2009**, 3, 342-351.
- [24] Clinical and Laboratory Standards Institute. 2010. Performance standards for antimicrobial susceptibility testing; 20th informational supplement. Document M100-S20. CLSI, Wayne, PA.
- [25] GD Christensen; WA Simpson; JJ Younger; LM Baddour; FF Barrett, DM Melton et al, *J. Clin. Microbiol.*, **1985**, 22, 996-1006.
- [26] S Stepanovic; I Dakic; N Opavski; P Jezek; L Ranin, *Ann. Microbiol.*, **2003**, 53, 63-74.

- [27] District Laboratory Practice in Tropical Countries, Part 2. Front Cover · *Monica Cheesbrough*. Cambridge University Press, Mar 2, 2006 - Medical - 440 pages.
- [28] SC Tan; BC Yiap, *J. Biomed. Biotechnol.*, **2009**, 2009, Article ID 574398, 10 pages.
- [29] R Boom; CJ Sol; MM Salimans; CL Jansen; PM Wertheim-van Dillen; J van der Noordaa, *J. Clin. Microbiol.*, **1990**, 28, 495-503.
- [30] Barbas, C.F. 3rd., Burton, D.R., Scott, J.K., and Silverman, G.J. 2007. Quantitation of RNA and DNA. "General Procedures," Appendix 3, in *Phage Display*, by Carlos F. Barbas III, Dennis R. Burton, Jamie K. Scott, and Gregg J. Silverman. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2001.
- [31] T Siripornmongkolchai, *Southeast Asian J. Trop. Med. Public Health*, **2002**, 33, 758-763.
- [32] FD Lowy, *J. Clin. Invest.*, **2003**, 111, 1265-1273.
- [33] F Nicola; C Bantar; LF Canigia; S Relloso; H Bianchini; J Smayevsky, *Diagn. Microbiol. Infect. Dis.*, **2000**, 36, 91-93.
- [34] F Martineau; FJ Picard; L Grenier; M Ouellette; MG Bergeron, *J. Antimicrob. Chemother.*, **2000**, 46, 527-534.
- [35] AC Fluit; MR Visser; FJ Schmitz, *Clin. Microbiol. Rev.*, **2001**, 14, 836-871.
- [36] P Vannuffel; J Gigi; H Ezzedine; B Vandercam; M Delmee; G Wauters; JL Gala, *J. Clin. Microbiol.*, **1995**, 33, 2864-2867.
- [37] P Vannuffel; M Heusterspreute; M Bouyer; B Vandercam; M Philippe; JL Gala, *Res. Microbiol.*, **1999**, 150, 129-141.
- [38] P Francois; D Pittet; M Bento; B Pepey; P Vaudaux; D Lew; J Schrenzel, *J. Clin. Microbiol.*, **2003**, 41, 254-260.
- [39] H Al-Talib; CY Yean; A Al-Khateeb; H Hassan; KKB Singh; K Al-Jashamy; M Ravichandran, *BMC Microbiol.*, **2009**, 9, 113-120.
- [40] De Silva, G.D., Kanatazanou, M., Massey, R.C., Wikinson, A.R., Day, N.P., and Peacock, S.J. 2002. The *ica* operon and biofilm production in coagulase negative *Staphylococci* associated with carriage and disease in neonatal intensive care unit. *J. Clin. Microbiol.* 40, 382-388.
- [41] D Mack; H Rohde; S Dobinsky; J Riedewald; M Nedelmann; JK Knobloch; HA Elsner; HH Feucht, *Infect. Immun.*, **2000**, 68, 3799-3807.
- [42] CR Arciola; D Campoccia; S Gamerini; ME Donati; L Baldassarri; L Montanaro, *Acta Orthop. Scand.*, **2003**, 74, 617-621.
- [43] GFM Gad; MA El-Feky; MS El-Rehewy; MA Hassan; H Abolella; RMA El-Baky, *J Infect. Dev. tries.*, **2009**, 3, 342-351.