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DEVELOPMENT OF RAPID ANTIGEN DETECTION TEST FOR METHICILLIN RESISTANT *Staphylococcus aureus* INFECTION

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ABSTRACT:

Methicillin resistant *Staphylococcus aureus* (MRSA) detection is a challenge to any clinical microbiology laboratory and demands implementation of strict protocols for active screening. This study aims to determine the validity of rapid immunochromotographic (ICT) test for detection compared with the gold standard method. Duplicate swabs were collected from nasal or ear. First swab used for conventional culture and Cefoxitin disk sensitivity. From each culture positive, pure colony were obtained, DNA extracted and conventional polymerase chain reaction for mecA detection were done. The second swab was processed for lateral flow test that constructed in Department of Microbiology laboratory/College of Medicine/ Al-Nahrain University. Monoclonal capture anti-PBP2a (test line) and goat anti-mouse IgG antibodies (control line) were applied on each nitrocellulose membrane. Gold-in-a-Box^{TM} conjugation kit (40nm. 15OD) conjugated monoclonal anti- PBP2a were adsorbed in conjugate pad. Then assembled in the test cassette. Serially diluted Methicillin resistance *S. aureus* (1-10^8cfu/ml) and Methicillin sensitive *S. aureus* also used in concentration 10^8 (cfu/ml). Bacterial cells treated with lysozyme for 30 minutes and distilled water were loaded on a strip and visually evaluated after 30 min. The minimum detection limit of the immunochromatography test was 10^3cfu/ml. Furthermore, based on comparison of 82 culture positive of different clinical swabs, the specificity and sensitivity of this assay were 100% and 97.1% respectively. While, in comparison with mecA PCR detection the specificity and sensitivity of this assay were 100% and 93.03% respectively. However, the procedure used in this study was less complicated and gives the results within 1hour. This study recommends using of Rapid pbp2a as a new tool for the detection of MRSA directly from clinical samples.

Key words: Immunochromotography, Methicillin Resistance Staphylococci.

INTRODUCTION

Methicillin-resistant Staphylococcus aureus (M-R-SA) are bacteria, responsible for severe and hard-to-manage infections in human(Pantosti & Venditti, 2009). MRSA bacteria are pandemic and are often isolated in medical practice and nosocomial infections(Ahmad & Asrar, 2014; Davis, *et al.*, 2004). MRSA acquisition in the health care setting include invasive procedures, prior treatment with antibiotics, prolonged hospital stays, stay in an intensive care or burn unit, surgical wound infection and proximity to a colonized client/ patient/resident. Fomite transmission in any setting, particularly in special settings such as burn units or intensive care units(Amissah et al., 2017; Asghar, 2014). There is evidence that some individuals may act as '*super-shedders*' of MRSA when co-infected with a respiratory virus and that they can spread MRSA via respiratory droplets (the '*cloud*' phenomenon). In some settings, such as intensive care units, chlorhexidine gluconate (CHG) baths have resulted in lower acquisition rates of MRSA. In intensive care settings, daily bathing of all patients with 4% CHG has been shown to reduce new acquisition of MRSA by 32%,as well as reduce cases of bacteremia with MRSA (Altidotlessnbas, Shorbagi, Ascidotlessoglu, Zarakolu, & Cetinkaya-Sardan, 2013).

The MRSA detection is a challenge to any clinical microbiology laboratory and demands implementation of strict protocols for active screening. While more expensive molecular techniques have the potential of offering highly sensitive and rapid results, the cultural methods require longer time but can achieve a comparable sensitivity for lower price(Nihonyanagi *et al.*, 2012).

This study aims to determine the validity of rapid immunochromotographic test for pbp2a detection compared with the gold standard method.

MATERIALS AND METHODS

Samples collection: A convenient sample included a total of one hundred and twelve clinical samples collected from ear, nose and throat swabs from out-patients of both sexes who attended ENT clinic regardless of their chief complaints.

Staphylococcal strains identification: Staphylococcal type strains were identified in isolates used in this study collected from local clinical sample. Including methicillin-sensitive *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA) and *Streptococcus pneumoniae* as an irrelevant group genus.

All swab specimens (according to the routine work of the hospital) were immediately streaked onto Blood Agar (BA) (Mast diagnostic, UK) prepared in section and incubated at 37℃ for 24hours. The resulted growing colonies were Gram stained and streaked again onto Mannitol Salt Agar (MSA) Mast diagnostic, UK) prepared in section**.** This medium was used for the identification of pathogenic staphylococci; furthermore, and for more accurate identification of the bacterial isolates biochemical tests like API Staph test (Mast diagnostic, UK).

Cefoxitin (FOX 30 µg) disk diffusion test was performed on Mueller-Hinton agar (Mast diagnostic, UK) (agar thickness 4 mm) with an inoculum (s) equal to 0.5 McFarland turbidity according to Clinical and Laboratory Standards Institute (CLSI, 2017). The inhibition zone around the disks was measured and compared with standards of Clinical Laboratory Standards Institute (CLSI) and decided as susceptible (S) >22mm or resistant (R) ≤ 22 mm.

Lateral flow immunoassay for methicillin resistant staphylococci detection: The current lateral flow assay test strip was based on previously described system developed by Shin *et al*., 2010 with some modifications; however, the antigen target was selected as penicillin binding protein 2 A (PBP2A) to achieve greater sensitivity and specificity for detection MRSA. An affinity-purified mouse monoclonal anti–PBP2A antibody (ABIN-1110906; RayBiotech), raised specifically against *S. aureus* cell-wall peptidoglycan use as both the capture and detection antibody in the ICT test strip. The key principle of the test is the ability to gold nanoparticle-conjugated antibody through a sulfur bond has proven highly successful for visualization of antigen antibody aggregation at test band for this application.

Gold nanoparticles conjugation with anti-PBP-2A antibody: Gold-in-a-Box kit with 40 nm gold nanoparticles was purchased from BioAssay Works [Ijamsville, MD, USA] for preparing highly reactive antibody (purified) gold conjugates. 0.5 mL of naked gold solution added to ten Eppendorf tubes of 1.5 ml size labelled from 1 to 10 in order according to pH chart. For each tube rearrange. Vortex at low speed, 15 µg of antibody solution and thoroughly for 2 to 3 seconds. The reaction allowed to continue for a total of 30 minutes. Slight purple color tubes or no change in color are useful for immunological assays otherwise deep purple or black precipitate indicates insufficient conjugation. Then, the reaction stopped by adding 50µL of Bovine Serum Albumin (Blocking Solution).

Strip preparation and cassette assembly: The sample pad, nitrocellulose membrane, and conjugation pad were prepared, as previously described(Huang, 2006) with modifications: The sample pad (Cellulose fiber) was cut in 2x5 cm and treated with 50 mM borate buffer, pH 7.4, containing 1% BSA and 0.05% Tween-20, and then dried overnight at 37 °C. The nitrocellulose membrane was cut in 2x5cm and blocked with PBS buffer. Monoclonal capture anti-PBP2a (test line) and goat anti-mouse IgG antibodies (control line) were doted by Hamilton syringe with repeating dispenser consistently dispenses 0.5µl in each spot. The conjugation pad (glass fiber) was cut in 0.7x5cm and blocked with 50 mM borate buffer, pH 7.4, containing 10% sucrose, 2% BSA, and 0.05% Tween-20. The membrane and conjugation pad were dried at 37°C for 4 h. 0.5 µL of colloidal gold-Anti-pbp2A conjugate was applied to a conjugate pad and completely dried at 37 °C for 3 h. The sample pad, conjugate pad, nitrocellulose membrane, and absorption pad were assembled on thick adhesive tape then cut in 0.6 cm in width to form the lateral flow strip. This strip was inserted into a plastic cassette, and these were stored at room temperature until use.

***In-vitro* evaluation of sensitivity and specificity of ICT test strips:** One hundred microliters of normal saline (negative control), Methicillin Sensitive *S. aureus* (Test negative control) at concentration 10^8 CFU/ml and Methicillin Resistant *S. aureus* suspension in different concentrations (10^0, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 CFU/mL in normal saline as a positive control) were applied to the sample pad to evaluate the sensitivity and specificity of ICT test.

Antigen extraction was made enzymatically by adding 100µl of lysozyme to bacterial cell suspension, left for 20-30 minutes at room temperature, invert 2-3 times.

100µl of extracted antigen pipette and put on to the sample pad, the test signals developed within 30 min.

Interpretation of results: the results can be categorized in four possibilities: Valid positive result: The presence of a signal at both T- and C lines. Invalid positive result: The presence of a signal at T- line only. Valid negative result: The presence of a signal at C line only. Invalid negative result: no signal at both T and C lines. Test strip sensitiv ity was assessed by different concentrations of MRSA isolate concentrations of 10^0–10^9 CFU/mL to the sample pad. Specificity was assessed by applying MSSA and normal saline to the sample pad.

Molecular identification of MRSA by conventional PCR: The sequences of oligonucleotide primers that were used in conventional and multiplex PCR to detect the presence of mecA, gene was taken from ((Moussa & Shibl, 2009))(Lee *et al*, 2012) and synthesized in Bioneer® (South Korea). Foreword primer 5' GTG GAA TTG GCC AATACA GG 3', reverse primer 5' TGA GTT CTG CAG TAC CGG AT 3' with product size 1339bp.

DNA extraction made by Genomic DNA Mini (Genead ® South Korea) according to manufacturer instructions. The presence of bacterial DNA with the relevant concentration was determined by Quantus TM Fluorometer, optimized with preprogrammed setting for nucleic acid quantitation using Promega Quantifluor® Dye system. Users depend on the flexibility to create their own methods and quantitation settings for other dyes within the proper excitation and emission wavelengths.

One microliter of DNA was transferred to master mix tubes which contained 5µl of master mix and 1µl of each primer was added to the tubes. The volume was completed to 20 µl with Nuclease free distilled water. Tubes were then spun down with a mini centrifuge to ensure adequate mixing of the reaction components, non-template used as negative control. The tubes were placed on the PCR machine eppendroff- thermal cycler, Germany and the PCR program includes: Initial denaturation at 94°C for 5 min, Denaturation at 94°C for 60 sec, annealing at 60°C for 60 sec, Elongation at 72°C for 70 sec then repeated for 30 cycles. The final extension, at 72°C for 10min, then hold at 4°C. The electrophoresis done by adding 5 µl of 100bp DNA ladder to one well and mecA positive strains to others which showed positive band at 1399 bp.

Statistical Analysis: Data of the study sample were entered using EPI INFO7 Windows Version and analyzed by using statistical package for social sciences (SPSS) version 21 year. Descriptive statistics were presented as frequencies, proportion (%), means and standard deviation (SD). Chi square test was used to estimate the association between two categorical variables. Level of significance of ≤ 0.05 was considered as significant. Validity and predictability of immunochromotography screening test was assessed in relation to gold standard test (FOX 30 and mecA PCR) by calculating sensitivity, specificity, predictive value of positive and negative test results.

RESULTS

In-vitro estimation of sensitivity and specificity of the ICT test strip: The detection limit of the ICT test strip was determined using *S. aureus* isolates suspended in PBS or DW at concentrations ranging from 10^3 to 10^8CFU/mL. Visual analysis of the strips showed the appearance of positive T-line signal at 10^3 CFU/mL and greater (Figure: 1). All test strips showed strong positive signal at C-line, verifying that they were functioning correctly. The specificity of the ICT test strips was determined by positively detected MRSA isolates but didn't show development of signal with DW and MSSA species which was tested.

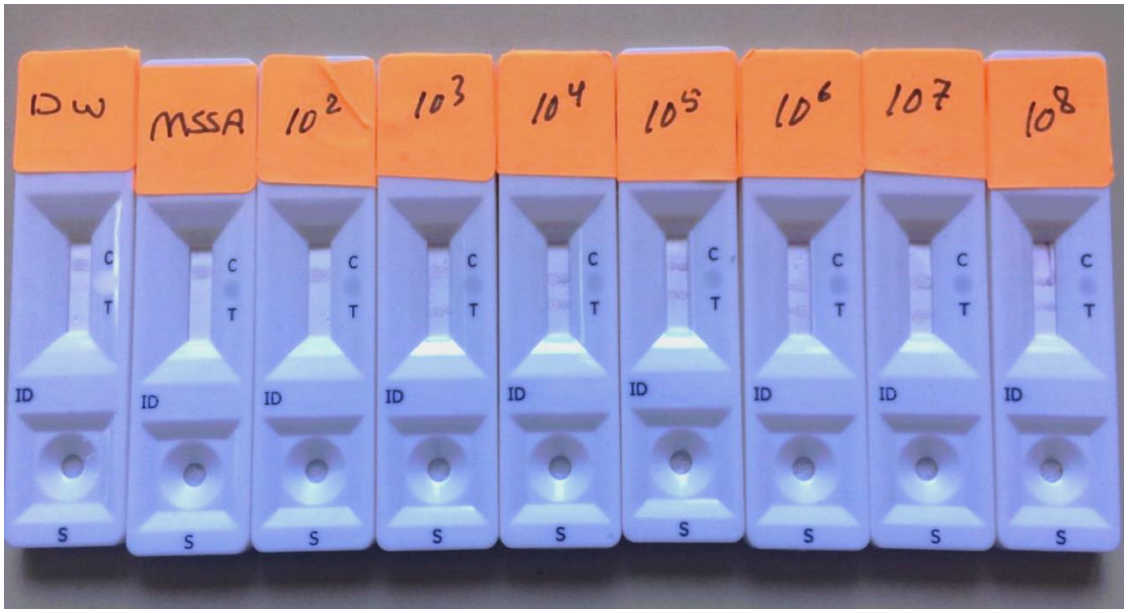

Figure 1: Immunochromotoghraphy test: Determination of the detection limit of ICT test strips.

The visual detection limit of the test was shown to be 10^3 CFU/mL. The results showed that this assay was specific for MRSA and does not react with MSSA or DW.

Methicillin testing methods of Staphylococci spp.: Depending on phenotypic characteristic of cefoxitin disk diffusion test, the immunochromotography assay for pbp2A detection and genotypic testing by polymerase chain reaction, the results showed that 69 (84.15%) were MRSA by cefoxitin (fox30) disk diffusion, 67 (81.71%) pbp-2A positive by lateral flow test and 72 (87.8%) mecA positive by PCR method (Figure 2).

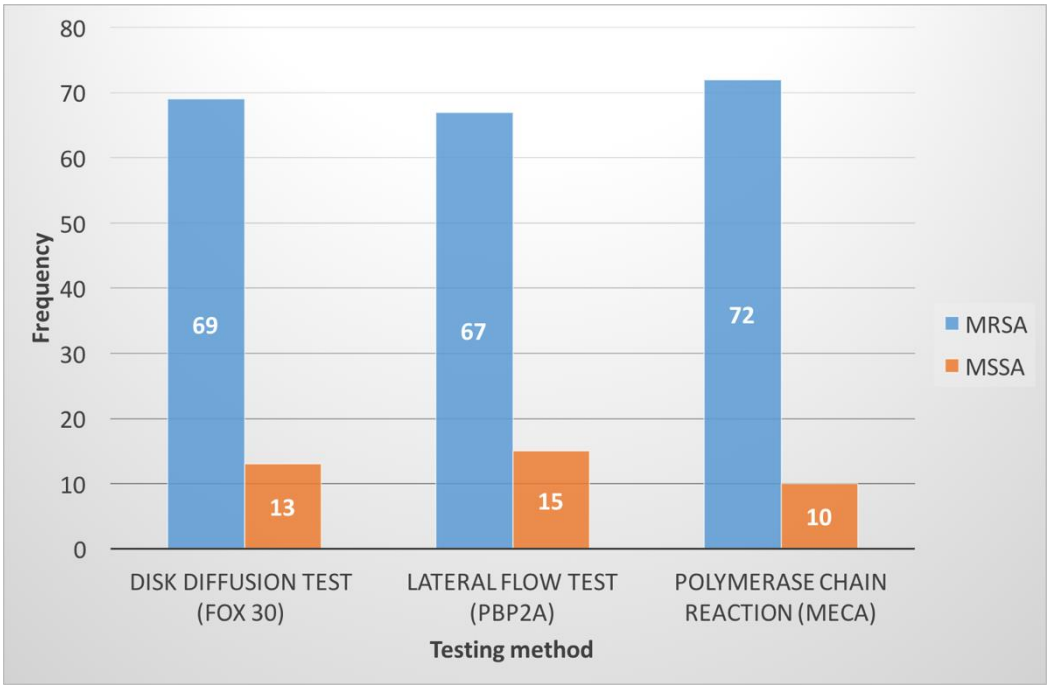

Figure 2: Distribution of MRSA according to different testing methods.

Sensitivity and specificity of ICT assay compared with disk diffusion test: Among coagulase negative staphylococci, the ICT assay showed 94.87 sensitivity and 100% specificity in compareson with FOX 30 disk diffusion test; that's because of only 2 of cases were detected as false negative result. While, coagulase positive staphylococci showed 100% of both sensitivity and specificity.

Overall, the results of 82 isolates were tested by ICT and FOX 30, when results of ICT were positive, detect 67 resistant isolates for cefoxitin, and when ICT was negative, detect 2 isolates were resistant and 13 isolates were sensitive for cefoxitin. Regarding sensitivity, the highest percentage was that of ICT (97.1%). On the other hand, ICT was highly specific (100%). ICT had high percentage in predicting positive results (100%) and showed lowest negative predictive value (86.67%) Table (1).

Table 1: Sensitivity and specificity of Immunochromotoghraphic test assay compared with FOX 30.

ICT		FOX 30			Sensitivity	Specificity	PPV	NPV
		+ve	-ve	Total				
Coagulase negative	+ve	37	0	37	94.87 (83.11-99.09)	100 (64.57-100)	100 (90.95-100)	77.78 (45.26-96.05)
	-ve	2	7	9				
	Total	41	5	46				
Coagulase positive	+ve	30	0	30	100 (88.65-100)	100 (60.97-100)	100 (88.65-100)	100 (60.97-100)
	-ve	0	6	6				
	Total	30	6	36				
Total (Staphylococci spp.)	+ve	67	0	67	97.1 (90.03-99.48)	100 (77.19 – 100)	100 (94.58 - 100)	86.67 (62.12 - 97.63)
	-ve	2	13	15				
	Total	71	11	82				

Data presented as count and percentage. PPV=Positive predictive value. NPV= Negative predictive value.

Sensitivity and specificity of Immunochromotoghraphy assay compared with mecA gene detection: In table 2, among coagulase negative staphylococci the ICT assay showed 90.24% sensitivity and 100 % specificity when compared with mecA gene detection by PCR. The coagulase positive staphylococci, the ICT assay showed 96.77% of sensitivity and 100% specificity when compared with mecA gene detection by PCR.

The results showed 82 isolates tested by PCR and ICT, when results of ICT were positive, detect 67 having mecA gene, and when ICT negative, detect 5 isolates had mecA gene and 10 isolates were negative for mecA gene. Regarding sensitivity, the highest percentage was that of ICT (93.07%). On the other hand, ICT was highly specific (100%). ICT had high percentage in predicting positive results (100%) and showed lowest negative predictive value (66.67%).

Table 2: Sensitivity and specificity of Immunochromotoghraphy assay compared with mecA gene.

ICT		mecA		Total	Sensitivity	Specificity	PPV	NPV
		+ve	-ve					
Coagulase negative	+ve	37	0	37	90.24 (77.45-96.14)	100 (56.55-100)	100 (90.59-100)	55.56 (26.67-81.12)
	-ve	4	5	9				
	Total	41	5	46				
Coagulase positive	+ve	30	0	30	96.77 (83.81-99.83)	100 (56.55-100)	100 (88.65-100)	83.33 (43.65-99.15)
	-ve	1	5	6				
	Total	31	5	36				
Total (Staphylococci spp.)	+ve	67	0	67	93.07 (84.75-97)	100 (72.25-100)	100 (94.58-100)	66.67 (41.71-84.82)
	-ve	5	10	15				
	Total	72	10	82				

Data presented as count and percentage. PPV=Positive predictive value. NPV= Negative predictive value.

DISCUSSION:

Early detection and isolation of Methicillin resistant *Staphylococcus aureus* (MRSA) are essential for successful prevention and control. The rate of methicillin resistance status was phenotypically and genetically tested. However, the higher rate was tested among both aureus and non-aureus Staphylococci, ranging from 84.15% by cefoxitin disk diffusion method (fox30), 81.71% by immunochromotography detection of pbp2A and 87.8% by mecA gene detection. Despite the clinical significance of *S. aureus* on human health worldwide (Tong *et al*, 2015), the current study reported a considerable number of Coagulase negative Staphylococci (46/112) isolated from Iraqi patients attending to the hospital. These isolates can possess a critical threat to human and non-human health (Von Eiff *et al*, 2002)ranging from skin, soft tissue infections and infective endocarditis to foreign-body-related infections(Becker *et al*, 2014).

Sensitivity and specificity of immunochromotographic essay: In order to identify the sensitivity at which range of bacterial cell density will be detected, diluted the stock bacteria in the range of 10^0-10^8 CFU/ml. While, MSSA and *S. pneumonia* were used in a concentration of 10^8cfu/ml. The results showed a highly specific and sensitive results because it gave valid positive signals from strip from 10^3 to 10^8cfu/ml and didn't gave for normal saline, MSSA, *S. pneumonia* and MRSA in the range of 10^0 to 10^2cfu/ml.

This study didn't show cross-reactivity of the purchased antibody between MSSA and MRSA strains and *S. pneumonae* bacteria tested, furthermore, this kit recognizes none aureus species of Staphylococci tested directly from clinical swabs, indicating that the antibody can specifically identify Methicillin resistant strains despite the species, although studies with a wider selection of strains would be required to confirm this.

This gives an advantage for using a primary antibody generated against peptidoglycan protein which is responsible for Methicillin resistance (pbp-2A). In addition to that, the current sensitivity seems to be adequate for detection nasal carrier state and true infection with higher number of MRSA.

So, it's much higher sensitive than those made by Yamada *et al*, (2013), who prepared an Anti-PBP-2a Chicken IgY Antibody in the test strip which was sensitive at the level of 10^8cfu/ml of MRSA whole cell grown on Tryptic soy agar. This might be due to using 1N NaOH as an antigen extraction buffer, makes their kit suitable for sample with higher bacterial load such as blood cultured cells. The developed kit is much sensitive than Wiriyachaiporn, *et al*., (2013), who used an affinity-purified mouse monoclonal antibody raised specifically against *S. aureus* cell-wall peptidoglycan (using UV- inactivated *S. aureus*, ATCC 29740; Biogenesis, Poole, UK), since it was gave a positive signal at 10^6cfu/ml of *S. aureus* (both MSSA and MRSA). This was related to using low sensitivity and specificity primary antibody was sensitive to 10^7-10^8cfu/ml of *S. aureus*, MSSA, and MRSA clinical isolates measured by dot blot and ELISA analysis before use as both the capture and detection antibody in the ICT test strip (Wiriyachaiporn *et al*, 2013). Together with that this kit is much better than the original one made by Huang, *et al*., 2006.,who used a primary antibody against protein A of *Staphylococcus aureus* without mention on its sensitivity for cfu/ml because they mentioned the detection limit was 25ng/ml of protein

A in phosphate buffered saline (PBS)(Huang, 2006).

The enzymatic extraction method using lysozyme is preferable; since this enzyme is capable of detaching or disintegrating the cell wall of *Staphylococcus aureus* at pH 5 in vitro(Slifkin & Interval, 1980) acting hydrolyzes the bond between N-acetyl glucosamine and N-acetyl muramic acid (muramidase activity) leading to degradation of peptidoglycan in the cell wall of Gram-positive bacteria (Huff & Silverman, 1968; Wecke *et al*, 1982; Salazar & Asenjo, 2007). This method gives stable results on repeated testing or same isolates.

The detection of presence of pbp2A is not restricted by one species of Staphylococci allowing detection of all true human pathogens and none human pathogens lacking the ability to discriminate between species or identifying new one (Mantion *et al*, 2015). ICT were highly sensitive and specific for both coagulase negative and coagulase positive Staphylococci when compared with either FOX30 disk diffusion method or mecA gene detection by polymerase chain reaction.

This developed kit provides a significant advantage by enabling a direct qualitative determination of the actual Methicillin Resistance Staphylococci. (aureus and non-aureus species) present within the clinical sample.

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