academicJournals

Vol. 8(11), pp. 1112-1117, 12 March, 2014
DOI: 10.5897/AJMR2014.6604
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Assessment of five phenotypic tests for detection of methicillin-resistant staphylococci in Cotonou, Benin

Dissou Affolabi[1,2]*, Mathieu Odoun[2], Lucrece Ahovegbe[2], Ablo Prudence Wachinou[2], Aderemi Kehinde[3], Frederic Sogbo[1], Frank Faïhun[2], Wilfried Bekou[2], Achille Massougbodji[1] and Severin Anagonou[1,2]

[1]Centre National Hospitalier et Universitaire Hubert Koutoukou Maga, Cotonou, Benin.
[2]Centre National Hospitalier de Pneumo-Phtisiologie, Cotonou, Benin.
[3]Department of Medical Microbiology and Parasitology, College of Medicine, University of Ibadan, Ibadan, Oyo State, Nigeria.

Received 3 January, 2014; Accepted 17 February, 2014

The study aimed at assessing performance and cost of phenotypic tests for detecting methicillin resistance in *Staphylococcus* spp. isolates in Cotonou, Benin. Isolates consecutively collected from various specimens from four medical laboratories in Cotonou from December 2012 to April 2013 were included in the study. The isolates were subjected to five phenotypic tests: disk diffusion tests with cefoxitin (Cefox) and moxalactam (Moxa) on Mueller Hinton agar incubated at 37°C, oxacillin on Mueller Hinton agar incubated at 30°C (Oxa30), oxacillin on salt Mueller Hinton agar incubated at 37°C (Oxa37) and agglutination test for PBP2a detection (TPBP 2a). Results were compared with polymerase chain reaction (PCR) of *mec*A gene which was used as the gold standard. In addition, cost per reagent of each phenotypic test was assessed. Considering the general agreement with PCR, Cefox and Moxa were the best tests in *S. aureus* while in non-*aureus Staphylococcus* isolates, TPBP 2a was the best test but its cost was 20 times higher than that of disk diffusion tests.

Key words: *Staphylococcus* spp., methicillin resistance, diffusion disk tests, PBP 2a, *mec*A.

INTRODUCTION

Staphylococcus spp. has been recognized as one of the most frequent bacteria isolated in routine laboratory practice. Even though, *Staphylococcus aureus* is the most pathogen among the staphylococci, non-*aureus* species commonly called “coagulase negative staphylococci” have become increasingly important in human pathology due to various reasons among which is the rising prevalence of immunocompromised patients, particularly in sub-Saharan Africa (Adeyemi et al., 2010; Reddy et al., 2010).

Staphylococcus spp. can show resistance to several antibiotics. Of particular importance in clinical practice is the case of methicillin resistance in which almost all the β-lactams are inactive against this pathogen (Gould et al., 2012). Due to their affordability, low toxicity and high efficacy in treating common diseases, β-lactams are

*Corresponding author. E-mail: affolabi_dissou@yahoo.fr. Tel: +229-66-61-48-62.

among the most prescribed groups of antibiotics in medical practice. Therefore, rapid and accurate detection of methicillin resistance in infections caused by *Staphylococcus* species is of paramount importance.

Methicillin resistance is mediated by the production of an altered penicillin-binding protein (PBP), 2a coded by the *mec*Agene complex (Dumitrescu et al., 2010). Detection of *mec*A gene by polymerase chain reaction (PCR) is the most accurate method for detecting methicillin resistant *Staphylococcus* spp. isolates (Akpaka et al., 2008; Majouri et al., 2007; Mohanasoundaram et al., 2008). However, the use of this molecular assay is largely restricted to reference centres in developed countries, and this test is not currently available in most routine diagnostic laboratories, particularly in resource constraints settings. Indeed, PCR for *mec*A gene is expensive and in its basic form includes many steps such as DNA extraction, amplification and electrophoresis. When it is used in the form of Real Time PCR, it is shorter but also expensive to use in resource-limited settings.

In low-income countries like Benin, only phenotypic methods are used for detecting methicillin resistant isolates (Seydi et al., 2004; Affolabi et al., 2012). These include disk diffusion tests with either cefoxitin (Cefox) or moxalactam (Moxa) on Mueller Hinton (MH) agar incubated at 37°C, oxacillin on MH agar incubated at 30°C (Oxa30) and oxacillin on salt MH agar incubated at 37°C (Oxa37). It is also possible to detect PBP 2a in isolates using a commercial agglutination test.

Several studies have evaluated phenotypic tests, however these studies were either performed in developed countries or did not use comparison, an appropriate reference method such as the detection of *mec*A gene. In addition, most of these studies were restricted only to *S. aureus* while non-*aureus Staphylococcus* species were not included. Furthermore, cost of tests which is an important parameter in developing countries, was not also assessed (Mohanasoundaram et al., 2008; Olowe et al., 2013).

The present study was carried out in Cotonou, Benin to evaluate five phenotypic tests for detecting methicillin resistant *Staphylococcus* spp. using PCR-based *mec*A gene as a gold standard.

MATERIALS AND METHODS

Bacterial isolates

Isolates were collected from Medical Microbiology Laboratories of the University Teaching Hospital, Hubert Koutoukou Maga (the reference hospital for the country), Saint Luc Hospital, Menontin Hospital and that of the Ministry of Health, Cotonou. The first three laboratories receive specimens from outpatients as well as inpatients while the latter receives specimens mainly from non-hospitalized patients. All isolates were Gram-positive cocci occurring in pairs or clusters, have grown on mannitol salt agar and were catalase-positive. Differentiation between *S. aureus* from non-*aureus Staphylococcus* species was done using PCR-amplification of the *nuc* gene (Brakstad et al., 1992).

Phenotypic tests

Disk diffusion tests

After preparation of 0.5 McFarland standard suspension of each isolate, a 1:10 dilution of the suspension was inoculated on MH agar plate (Biorad, France) supplemented with or without 4% NaCl as recommended by the Antibiogram Committee of the French Society of Microbiology (AC-FSM, 2012). Antibiotic disks (Biorad, France) were applied on the plate and incubation was done at 37°C aerobically for 24 h. The inhibition zone diameter for each isolate was measured and compared with interpretative standards (AC-FSM, 2012).

Detection of PBP 2a (TPBP 2a)

PBP 2a was detected using a commercial agglutination kit (Oxoid, United Kingdom) according to the manufacturer's instructions. Briefly, protein extraction was carried out by heating a heavy bacterial suspension at 100°C for 3 min. After addition of the extraction reagent, the mixture was centrifuged at 4,500 rpm for 5 min and the supernatant (the extract) collected. Then, the test and the control reagents were mixed with the extract for 3 min and agglutination was then observed. As recommended by the manufacturer, for non-*aureus* species, only colonies around the oxacillin disk on MH agar plate were used since a PBP 2a induction is needed for these species prior detection.

mecA gene amplification by PCR

DNA extraction

DNA extraction from each isolate was carried out as previously described (Mayoral et al., 2005). Briefly, colonies were emulsified in 500 µl of sterile distilled DNA-free water. The mixture was boiled at 100°C for 15 min, cooled on ice and then centrifuged at 13,000 rpm for 5 min. The supernatant containing the DNA was stored at 4°C before use.

DNA amplification

It was carried out as previously described by Majouri et al. (2007) with minor modifications on amplification program. Primers used were *mecA1*: *5'-GTAGAAATGACTGAACGTCCGATAA-3'* and *mecA2*: *5'-CCAATTCCACATTGTTTCGGTCTAA-3'* (Eurogentec, Belgium). The 50 µl mix reaction contained 200 µM for each dNTP (Sigma, USA); 1X enzyme buffer (Sigma, USA); 0.4 µM of each primer, 1.25 U of Jump Start Taq polymerase (Sigma, USA) and 5 µl of DNA extract. PCR amplification program was as follows: initial denaturation at 94°C for 5 min, 37 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min and 30 s, extension at 72°C for 1 min and final extension at 72°C for 10 min. The size of the final amplification product was 310 bp.

Quality control

Methicillin resistant *S. aureus* strains ATCC 43300 and a well-characterized methicillin susceptible *S. aureus* were used as positive and negative controls, respectively for phenotypic as well as molecular tests. In addition, for molecular tests, standard microbiological procedures were strictly followed in order to

Table 1. Characteristics of disk diffusion tests studied.

Disk	Incubation temperature	Medium	Critical diameter (mm)		
			R	I	S
Cefoxitin (30 µg)	37°C	MHA	< 25	25-27	≥ 27
Moxalactam (30 µg)	37°C	MHA	< 23	23-24	≥ 24
Oxacillin (5 µg)	37°C	Salt MHA	< 20		≥ 20
Oxacillin (5 µg)	30°C	MHA	< 20		≥ 20

MHA: Mueller Hinton agar; R: resistant; I: intermediate; S: susceptible; salt MHA: MHA supplemented with 4% NaCl.

Table 2. Comparison between phenotypic tests and PCR of *mec*A gene.

Disk		PCR *mec*A					
		S. aureus			Non-*aureus Staphylococcus*		
		Positive	Negative	Total	Positive	Negative	Total
Cefoxitin	R	27	01	28	43	03	46
	S	01	86	87	06	49	55
Moxalactam	R	27	01	28	43	05	48
	S	01	86	87	06	47	53
Oxacillin 30°C	R	22	00	22	19	03	22
	S	06	87	93	30	49	79
Oxacillin37°C	R	23	00	23	38	02	40
	S	05	87	92	11	50	61
PBP2a	R	27	03	30	48	02	50
	S	01	84	85	01	50	51

R: Resistant; S: susceptible.

minimize cross contamination. DNA extraction and PCR-amplification were done in molecular laboratories that were separated from the routine clinical microbiology laboratory. The PCR laboratory has designated sections for pre-amplification, DNA extraction and amplification/post-amplification with a unidirectional movement of staff.

Cost assessment of reagents per test

While one technician was performing a test, a second technician recorded the quantity of reagents used. Prices of reagents collected from a local supplier were presented in US dollars (US$) (change rate on 15th December, 2013). Only reagents were taken into account for cost calculation. Some reagents were prepared in batches and the whole cost for the batch was first recorded. In the final assessment, the cost for the test was calculated by taking into account the portion of the batch used for the test.

Data analysis

Data were entered and analysed using Excel software. The sensitivity, specificity and the agreement of each phenotypic test were calculated using PCR of the *mec*A gene as gold standard with the formulas:

Sensitivity = [True Positive/(True Positive + False Negative)] × 100
Specificity = [True Negative/(True Negative + False Positive)] × 100
Agreement = [(True Positive + True Negative)/Total strains tested] x 100

RESULTS

A total of 216 *Staphylococcus* spp. isolates (*S. aureus*, N=115; non-*aureus Staphylococcus*, N=101) were recovered from various clinical specimens. Of these, 127 (58.80%) were urine samples, 53 (24.54%) were wound swabs, 30 (13.89%) were genital fluid, while 6 (2.78%) were blood cultures.

Table 1 summarizes characteristics of each test performed. Results of the comparison between phenoltypic tests and PCR of *mec*A gene are shown in Table 2, while Table 3 shows performance of each phenotypic test. Of all the phenotypic tests performed, the best agreement with the gold standard (98.26%) was

Table 3. Performances of phenotypic tests.

Test	*S. aureus*			Non-*aureus Staphylococcus*		
	Sensitivity (%)	Specificity (%)	Agreement (%)	Sensitivity (%)	Specificity (%)	Agreement (%)
Céfoxitin	96.43	98.85	98.26	87.76	94.23	91.09
Moxalactam	96.43	98.85	98.26	87.76	90.38	89.11
Oxacillin 30°C	78.57	100	94.78	38.78	94.23	67.33
Oxacillin 37°C	82.14	100	95.65	77.55	96.15	87.13
PBP 2a	96.43	96.55	96.52	97.96	96.15	97.03

Table 4. Performances of tests combinations

Combination	*S. aureus*			Non *aureus Staphylococcus*		
	Sensitivity	Specificity	Agreement	Sensitivity	Specificity	Agreement
A	82.14	100	95.65	77.55	94.23	86.14
B	96.43	98.85	98.26	87.76	92.31	90.1
C	96.85	98.85	98.26	87.76	90.38	89.11
D	96.43	96.55	96.52	97.96	92.31	95.05
E	96.43	98.85	98.26	87.76	94.23	91.09
F	96.43	98.85	98.26	87.76	90.38	89.11
G	96.43	96.55	96.52	97.96	94.23	96.04
H	96.43	98.85	98.26	89.80	88.46	89.11
I	96.43	96.55	96.52	97.96	92.31	95.05
J	96.43	96.55	96.52	97.96	88.46	93.07

A : Oxacillin 30°C + oxacillin 37°C; B: oxacillin 30°C + cefoxitin; C: oxacillin 30°C + moxalactam; D: oxacillin 30°C + PBP2a; E: oxacillin 37°C + cefoxitin; F: oxacillin 37°C + moxalactam; G: oxacillin 37°C + PBP2a; H: cefoxitin + moxalactam; I: cefoxitin + PBP2a; J : moxalactam + PBP2. The final result of a combination was considered as resistant if at least one the test showed a resistant result, otherwise it was considered as susceptible.

obtained with Cefox and Moxa for *S. aureus* isolates while for non-*aureus* strains, TPBP 2a had the best agreement, 97.03% (Table 3). Although, Oxa30 and Oxa37 had the lowest agreement rates of 78.57 and 82.14% respectively when compared with gold standard, they had the highest specificities (100%) (Table 3).

The results of performance of combining phenotypic tests are presented in Table 4. The final result of a combination was considered as resistant if at least one of the tests showed a resistant result; otherwise, it was considered as susceptible. For *S. aureus* isolates, the best agreement of 98.26% was obtained with the following tests combinations: Oxa30 + Cefox; Oxa30+Moxa; Oxa37+Cefox; Oxa37+Moxa and Cefox+Moxa while for non-*aureus* isolates, Oxa37 + TPBP 2a showed the best agreement (96.04%) with PCR-based *mec*A gene (Table 4).

In addition, two non-*aureus Staphylococcus* isolates showed methicillin resistance in all diffusion disk tests done while PCR for *mec*A gene and TPBP 2a were negative. Concerning the cost of reagents per phenotypic test, the cost of all diffusion disk tests was close and varied from US$ 0.57 to 0.64, while TPBP 2a was 20 times more expensive than diffusion disk tests (Table 5).

DISCUSSION

Medical bacteriology laboratories in most French speaking countries in sub-Saharan Africa usually follow recommendations from the French Society of Microbiology without performing their own studies to determine the suitability of use of such recommendations in their settings (Seydi et al., 2004; Affolabi et al., 2012; AC-FSM, 2012). In order to test these recommendations in Cotonou (Benin), we compared five phenotypic tests for detecting methicillin resistant *Staphylococcus* spp. isolates (*S. aureus* as well as non-*aureus Staphylococcus*) using PCR-based *mec*A gene as gold standard.

We observed that oxacillin disk diffusion tests had the lowest performances. This finding is in agreement with other studies (Boutiba-Ben Boubaker et al., 2004; Majouri et al., 2007; Datta et al., 2011; Olowe et al., 2013). Despite these similar findings, these tests are still in the 2012 recommendations of the French Society of

Table 5. Reagents cost per test.

	Test				
	Cefoxitin	**Moxalactam**	**Oxacillin, 37°C**	**Oxacillin, 30°C**	**PBP 2a**
Reagents cost/ test (US $)	0.57	0.57	0.64	0.57	11.20

Microbiology and are being used in several laboratories in sub-Saharan African countries like Benin (Seydi et al., 2004; Affolabi et al., 2012). One of the advantages of oxacillin disk diffusion tests is their high specificity but their sensitivity is low for recommendation in routine work. Other disadvantages of these tests are the need to use a salt MH agar (for Oxa37) or an incubation temperature of 30°C (for OXA 30). This incubation temperature is not readily accessible in several medical laboratories in low-income countries.

From this study, Cefox and Moxa were found to be the best phenotypic tests for detecting methicillin resistance in *S. aureus* as each of them (Cefox and Moxa) had the highest performance agreement of 98.26% when compared with the gold standard (Table 3). This is in agreement with submissions of other workers within the region and elsewhere (Boutiba-Ben Boubaker et al., 2004; Majouri et al., 2007; Datta et al., 2011; Olowe et al., 2013) where Cefox and Moxa have been recommended for use in detection of methicillin resistance in *S. aureus*-based infections. These tests even performed better than TPBP 2a in *S. aureus* (Table 3), reinforcing the need of using one of these tests in our routine diagnostic laboratories.

For non-*aureus Staphylococcus* isolates, Cefox and Moxa still performed well but the best test was TPBP 2a (Table 3). Only few studies have evaluated methicillin resistance detection tests in non-*aureus Staphylococcus* isolates (Majouri et al., 2007; Souza Antunes et al., 2007). In fact, these species are often considered as contaminants when isolated from clinical samples. However, it is now well known that *Staphylococcus saprophyticus* is a common cause of urinary tract infection ("honey-moon cystitis") in immunocompetent sexually active women (Raz et al., 2005). Furthermore, in immunocompromised patients, non-*aureus Staphylococcus* species can cause severe infections such as blood stream infections and endocarditis (Adeyemi et al., 2010; Reddy et al., 2010). As these species often yield resistance to several groups of antibiotics, thus an accurate identification of methicillin resistance is of utmost importance for correct management of such infections. In this study, we observed that the best test was TPBP 2a. The test is rapid; time to get result is less than 30 min while antibiotics disk diffusion tests require 24 h to give results. However, TPBP2a is too expensive (about 20 times the cost of diffusion disks tests) (Table 5) to be routinely used in laboratories in low resource countries. In spite of this limitation, TPBP 2a could be proposed for severe infections due to non-*aureus Staphylococcus* species in reference laboratories even in resource-limited settings. If TPBP2a is not available, Cefox and Moxa can be used as their performances are quite good even in non-*aureus Staphylococcus* species, as seen in this study (Table 5).

A surprising observation in this study was a result from two non-*aureus Staphylococcus* isolates that showed methicillin resistance in all diffusion disk tests performed while PCR for *mec*A gene and TPBP 2a were repeatedly negative. Even though, primers used in the present study were from a highly conserved region of *mec*A gene, false negative PCR results cannot be excluded (Geha et al., 1994). Similar observations were made for *S. aureus* in Switzerland, United Kingdom and Denmark (García Álvarez et al. 2011; Monecke et al., 2013). This may be due to a new variant of *mec*A, recently named *mec*C, which codes for a new protein (PBP 2c). Further investigations are needed to confirm this finding in non-*aureus Staphylococcus* isolates.

In conclusion, Cefox and Moxa are were found to be the best phenotypic tests to detect methicillin resistance in *S. aureus* isolates but in non-*aureus Staphylococcus* isolates, TPBP 2a is the best although 20 times more expensive than diffusion disks tests.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

We would like to thank technical staff of all participating laboratories in Cotonou, namely medical microbiology laboratories of the University Teaching Hospital, Hubert Koutoukou Maga, Saint Luc Hospital, Menontin Hospital and that of the Ministry of Health. The assistance of Professor Honore Bankole is also appreciated with thanks.

REFERENCES

AC-FSM, Antibiogram Committee of the French Society of Microbiology (2012). Recommendations for 2012. http://www.sfm-microbiologie.org.

Adeyemi AI, Sulaiman AA, Solomon BB, Chinedu OA, Victor IA (2010). Bacterial blood stream infections in HIV-infected adults attending a Lagos teaching hospital. J. Health Popul. Nutr. 28:318-326.

Affolabi D, Ade G, Bankolé H, Hounkpe C, Satchi A, Soclo Dansi E, Anagonou S (2012). Trends on antibiotic resistance in common bacterial infections in Cotonou. J. de la Société de Biologie Clinique. 16:79-83.

Akpaka PE, Kissoon S, Rutherford C, Swantson WH, Jayaratne P (2008). Evaluation of methods and costs for detecting methicillin-resistant *Staphylococcus aureus* isolates from clinical specimens at regional hospitals in Trinidad and Tobago. West Indian Med. J. 57:24-27.

Boutiba-Ben Boubaker I, Ben Abbes R, Ben Abdallah H, Mamlouk K, Mahjoubi F, Kammoun A, Hammami A, Ben Redjeb S (2004). Evaluation of a cefoxitin disk diffusion test for the routine detection of methicillin-resistant *Staphylococcus aureus*. Clin. Microbiol. Infect. 10:762-765.

Brakstad OG, Aasbakk K, Maeland JA (1992). Detection of *Staphylococcus aureus* by Polymerase Chain Reaction Amplification of the *nuc* Gene. J. Clin. Microbiol. 30:1654-1660.

Datta P, Gulati N, Singla N, Rani Vasdeva H, Bala K, Chander J, Gupta V (2011). Evaluation of various methods for the detection of meticillin-resistant *Staphylococcus aureus* strains and susceptibility patterns. J. Med. Microbiol. 60:1613-1616.

Dumitrescu O, Dauwalder O, Boisset S, Reverdy MÉ, Tristan A, Vandenesch F (2010). *Staphylococcus aureus* resistance to antibiotics: key points in 2010. Med. Sci. 26:943-949.

GarcíaÁlvarez L, Holden MT, Lindsay H, Webb CR, Brown DF, Curran MD (2011). Meticillin-resistant *Staphylococcus aureus* with a novel *mec*A homologue in human and bovine populations in the UK and Denmark: a descriptive study. Lancet Infect. Dis. 11:595-603.

Geha DJ, Uhl JR, Gustaferro CA, Persing DH (1994).Multiplex PCR for identification of methicillin-resistant staphylococci in the clinical laboratory. J. Clin. Microbiol. 32:1768-1772.

Gould IM, David MZ, Esposito S, Garau J, Lina G, Mazzei T, Peters G (2012). New insights into meticillin-resistant *Staphylococcus aureus* (MRSA) pathogenesis, treatment and resistance. Int. J. Antimicrob. Agents 39:96-104.

Majouri D, Touati A, Achour W, Bouchami O, Ben Hassen A (2007). Comparison of phenotypic methods with PCR to screening methicillin resistance in coagulase negative staphylococci. Pathol. Biol. 55:361-365.

Mayoral C, Noroña M, Baroni MR, Giani R, Zalazar F (2005). Evaluation of a nested-PCR assay for *Streptococcus pneumoniae* detection in pediatric patients with community-acquired pneumonia. Rev. Argent. Microbiol. 37:184-188.

Mohanasoundaram KM, Lalitha MK (2008). Comparison of phenotypic versus genotypic methods in the detection of methicillin resistance in *Staphylococcus aureus*. Indian J. Med. Res. 127:78-84.

Monecke S, Gavier-Widen D, Mattsson R, Rangstrup-Christensen L, Lazaris A, Coleman DC, Shore AC, Ehricht R (2013).Detection of *mec*C-Positive *Staphylococcus aureus* (CC130-MRSA-XI) in Diseased European Hedgehogs (Erinaceuseuropaeus) in Sweden. PLoS One 8: e66166.

Olowe OA, Kukoyi OO, Taiwo SS, Ojurongbe O, Opaleye OO, Bolaji OS, Adegoke AA, Makanjuola OB, Ogbolu DO, Alli OT (2013). Phenotypic and molecular characteristics of methicillin-resistant *Staphylococcus aureus* isolates from Ekiti State, Nigeria. Infect. Drug Resist. 6:87-92.

Raz R, Colodner R, Kunin CM (2005). Who are you, *Staphylococcus saprophyticus*?Clin. Infect. Dis. 40:896-898.

Reddy EA, Shaw AV, Crump JA (2010). Community-acquired blood stream infections in Africa: a systematic review and meta-analysis. Lancet Infect. Dis. 10:417-432.

Seydi M, Sow AI, Soumaré M, Diallo HM, Hatim B, Tine R, Diop BM, Sow PS (2004). *Staphylococcus aureus* bacteremia in the Dakar Fann University Hospital. Med. Mal. Infect. 34:210-215.

Souza Antunes AL, Secchi C, Reiter KC, Rodrigues Perez LR, Peixoto de Freitas AL, Alves d'Azevedo P (2007). Evaluation of oxacillin and cefoxitindisks for detection of resistance in coagulase negative staphylococci. Mem. Inst. Oswaldo Cruz. 102:719-723.