Diclofenac-Cefoxitin Double-Disk Method is a Novel Tool for Phenotypic Detection of Methicillin Resistant *Staphylococcus Aureus*: a Preliminary Report

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Abstract

Background: the detection of methicillin resisatance is essential for both the institution of appropriate antimicrobial therapy and infection control measures. The development of largely divergent *mecA* genes by the new methicillin resistant *Staphylococcus aureus* (MRSA) made the present 'gold-standard' tests unable to determine whether they are MRSA or not. Therefore, there is an essential need to develop a new MRSA testing method.
Aim of Study: to demonstrate diclofenac (Dc) to be a very strong inducer of low expression methicillin resistance in MRSA.
Materials and Methods: standard disk diffusion method was performed for 26 *Staphylococcus aureus* isolates obtained from blood culture and wound swabs from patients admitted to Ramadi Teaching Hospital (from October, 2010 to March, 2011), against cefoxitin (FOX) 30µg disk, and MRSA screen test (Denka Seiken, Japan), as indicators to detect the presence of MRSA, and against selected β-lactam and non-β-lactam antimicrobial agents. Double disk tests (D-test) were performed to determine MLS_B-inducible resistance mechanism and to assess the activity of Dc 50µg disk in approximation with selected antimicrobial agents.
Results: Both in complete agreement, FOX disk diffusion test and MRSA screen test revealed that 19/26 (73.1%) of the isolates appeared as MRSA, and 7/26 (26.9%) as methicillin susceptible *Staphylococcus aureus* (MSSA). In MRSA isolates only, Dc produced a paradoxical antagonistic effect upon combination with β-lactam drugs only, and this effect appeared in all the 19 MRSA isolates; while with non-β-lactam drugs, it potentiated their antistaphylococcal activity including inducible-MLS_B isolates.
Conclusion: Dc is suggested to be a strong inducer of the expression of methicillin resistance. The use of Dc in detecting MRSA could be considered as a backup test with FOX to improve the accuracy of phenotypic detection of MRSA.
Key words: Diclofenac, MRSA, Methicillin resistance induction

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Introduction

Staphylococcus aureus is an aggressive human pathogen and a leading cause of disease in the community and health care settings[1]. In Staphylococcal isolates, the most clinically concerning resistance mechanism to β-lactam antimicrobials is the acquisition of a *mecA* gene encoding a modified penicillin-binding protein (PBP), known as PBP2a, found in methicillin resistant *Staphylococcus aureus* (MRSA). This PBP2a is intrinsically resistant to inhibition by β-lactams [2]. The phenotypic expression of methicillin resistance is variable among MRSA strains. In strains exhibiting heterotypic methicillin resistance, only a sub-population of bacteria expresses high level resistance, whereas homotypic resistance is characterized by the expression of high level resistance by the entire population [3]. Thus, detection of methicillin resistance is essential for both the institution of appropriate antimicrobial therapy and infection control measures [4].

The accurate detection of methicillin resistance poses a great challenge. For the past three decades, several phenotypic detection methods have been adopted worldwide with continuous modification by well recognized expert bodies in an attempt to improve the accuracy and thus avoiding false results. Recent investigations suggest that the cefoxitin (FOX) disk diffusion is a superior test and a surrogate for the oxacillin disk diffusion test due to its ease of reading and higher sensitivity [5, 6, 7]. Further, adopting the molecular technique PCR has been considered as a gold-standard one[8, 9, 10].

Nevertheless, in the past five years, several reports have questioned the sensitivity of the PCR approach in detecting *mecA* gene. This alarm has been raised as several findings documented the presence of phenotypic methicillin resistance and yet negative results were found by the PCR technique on the isolates. These authors suggested the development and spread of non-conventional *mecA* genes like $mecA_{LGA251}$ homologue[11, 12].Thus, these novel genetic elements are not detectable by the present 'gold-standard' methods and hence cannot determine whether they are MRSA or not. It follows that the problems in detecting methicillin resistance are not settled yet. In this paper, we demonstrate Diclofenac (Dc) to be a very strong inducer of low expression *mecA* gene in MRSA.

Materials and Methods

Twenty six isolates of *S. aureus* isolated from blood culture and wound swabs from patients admitted to Ramadi Teaching Hospital (from October, 2010 to March, 2011). Identification of *S. aureus* depends on the morphology and cultural characteristics on the mannitol salt agar, coagulase and catalase test, Mastastaph kit (Mast Diagnostic, France) and API Staph kit (biomerieux, France) as described by [13].

Antimicrobial susceptibility test of the isolates was done by disk diffusion method on Mueller-Hinton agar (MHA) with 24 h incubation at 37°C.[14]; the antibiotic disks were from Bioanalyse Company, Ankara-Turkey. The phenotypic detection of MRSA was performed by MRSA screen test (Denka Seiken, Japan), and FOX 30µg disk diffusion method. The double disk diffusion technique, disk approximation methods described [15], was used to determine the action and interaction (synergism or antagonism) between Dc (50µg) which was placed 6mm to 20mm (edge to edge) from selected antimicrobial agents oxacillin (OX 1µg), cloxacillin (CX 1µg), cefoxitin (FOX 30µg), cefotaxime (CTX 30µg), meropenem (MEM 10µg), erythromycin (E 15µg), clindamycin (DA 2µg), ciprofloxacin (CIP 5µg), rifampicin (RA 5µg), co-trimoxazole (COT 25µg), vancomycin (VA 30µg), gentamicin (GEN 10µg), amikacin (AK 30µg), and tetracycline (T 30µg).

Plates are then incubated overnight at 37°C. The identification of MLS_B

resistance was performed by the D-test demonstrating erythromycin (15µg) antagonism to clindamycin (2µg) by placing the two disks at a distance of 15-26mm apart from edge to edge, a flattening or blunting of the clindamycin zone of inhibition adjacent to the erythromycin disk, giving a D shape to the zone as recommended by[7]. *S. aureus* ATCC 25923 (MSSA β-lactamase-negative strain) used as a control strain[16].

Diclofenac (Dc 50µg) disks were prepared as described by[17], diclofenac sodium (Voltarin(R), 75mg/3ml ampoule, Novartis pharma AG, Switzerland).

Results

Both in complete agreement, FOX disk diffusion test and MRSA screen test revealed that 19/26 (73.1%) of the isolates appeared as MRSA, and 7/26 (26.9%) as MSSA. Further, the antibiogram results with E and DA, and the D-test are described in table 1. In MSSA isolates, Dc produced a substantial antistaphylococcal activity on its own and it also potentiated the activity of all β-lactam and non-β-lactam drugs; these results were also observed with *S. aureus* ATCC 25923. In MRSA isolates only, Dc produced a **paradoxical** antagonistic effect upon combination with β-lactam drugs **only**, and this effect appeared in all the 19 MRSA isolates; while with non-β-lactam drugs, it potentiated their antistaphylococcal activity including inducible-MLS_B isolates (figure 1, 2,3).

Table 1. Susceptibility to erythromycin (E) and clindamycin (DA) and detection of MLS_B resistance by D-test among all the *Staphylococcus aureus* isolates.

Phenotype	MRSA	MSSA	Phenotypic interpretation
E-S, DA-S	5 (19.2%)	6 (23.1%)	Classical 11/26 (42.3%)
	11		
E-R,DA-S, D^-	0	1 (3.9%)	may have macrolide efflux-mechanism encoded by the *msrA* gene 1/26 (3.9%)
	1		
E-R, DA-S, D^+	14 (53.8%)	0	MLS_B inducible encoded by the *erm* gene 14/26 (53.8%)
	14		
Total	26		26

D= D-test, S= sensitive, R= resistance, *erm*= erythromycin ribosome methylase, *msrA*= macrolide streptogramin resistance

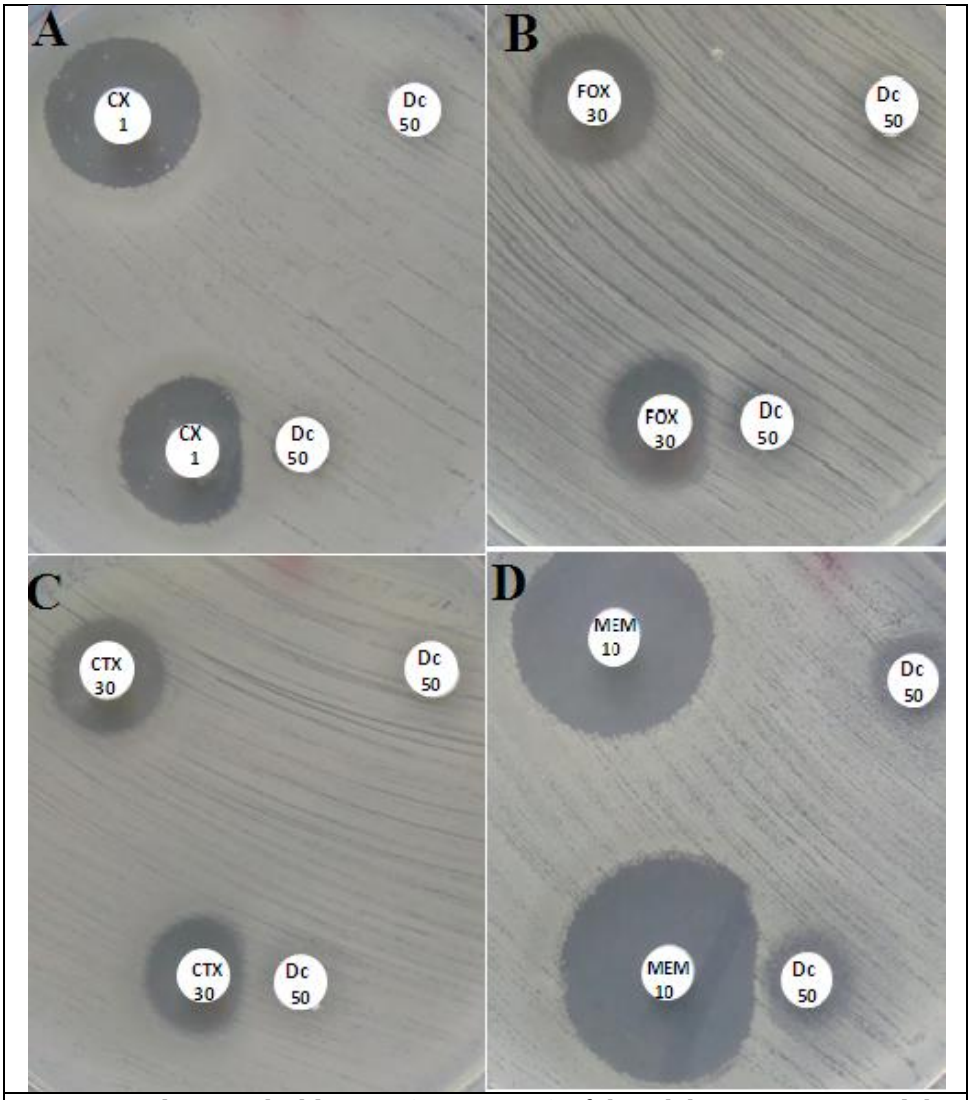

Figure. 1. Showing the blunting (antagonism) of the inhibition zone around the β-lactam drugs (A) cloxacillin (CX 1µg), (B) cefoxitin (FOX 30µg), (C) cefotaxime (CTX 30µg), (D) meropenem (MEM 10µg) in all MRSA isolates towards the diclofenac (Dc 50µg) disk.

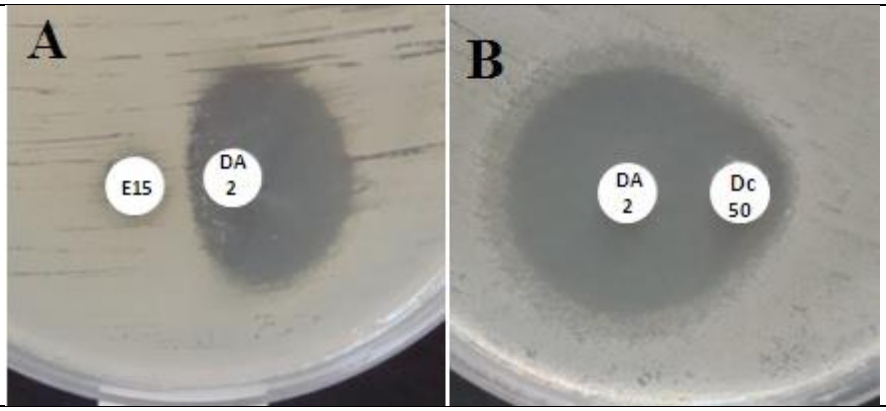

Figure. 2. Showing A. blunting (antagonism) of the inhibition zone around clindamycin (DA 2 µg) towards the erythromycin (E 15µg) disk, i.e. representative of inducible MLS_B resistance. B. synergism around clindamycin (DA 2 µg) towards the diclofenac (Dc 50µg) disk in the same MRSA isolates.

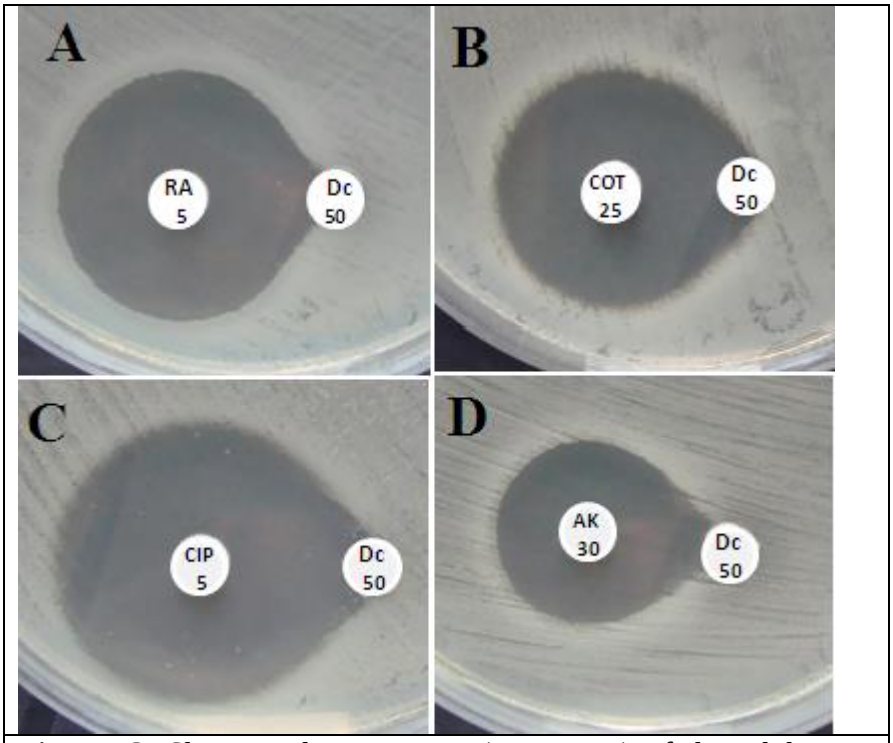

Figure. 3. Showing the extension (synergism) of the inhibition zone around the non-β-lactam drugs represented by (A) rifampicin (RA 5µg), (B) co-trimoxazole (COT 25µg), (C) ciprofloxacin (CIP 5µg) , and (D) amikacin (AK 30µg) in all **MRSA** isolates towards the diclofenac (Dc 50µg) disk.

Discussion

Detection of MRSA adopting laboratory phenotypic and molecular methods is a complex balance between rapidity of result, sensitivity, specificity and cost. Up to now, no standard laboratory test for MRSA detection has been worldwide approved. Therefore, there is a need for backup tests to enhance the above desired parameters.

Dc produced antistaphylococcal activity and synergistic effects with the tested β-lactam and non-β-lactam antimicrobial agents, including DA in MLS_B phenotypes; the antibacterial action of Dc has been described previously[18]. They explained these effects of Dc by its ability to inhibit bacterial DNA synthesis, which was demonstrated using 2u Ci (3H) deoxy thymidine uptake.

However, these observations were made consistently only in MSSA isolates while all results obtained with Dc and the β-lactam agents only in MRSA isolates were consistently of a paradoxical blunting (antagonistic) effect as in figure. 1. Up to our knowledge, no such observation has been reported previously. This blunting effect failed to appear when testing Dc against DA in MLS_B isolates whereby E gave positive blunting tests in these isolates. This differential blunting effect of Dc on MRSA is suggestive of a good specificity and hence has the potential to be utilized to detect MRSA.

It is tempting to propose a working hypothesis to explain the mechanism(s) of the antagonistic (blunting) action of Dc which appears to be specific to β-lactam antibiotics class in isolates which were classified as MRSA. It is suggested that Dc mimics the action of β-lactam antibiotics in inducing *mecA* gene and producing PBP2a because this action did not appear when using double disk method with non β-lactam antibiotics. Also, the inducibility of β-lactam antibiotics for *mecA* gene varies depending

on which member of the β-lactams. As a result, the CLSI guidelines recommend the usage of FOX instead of oxacillin when using the disk diffusion method to determine resistance against methicillin for *S. aureus* by [7]. FOX results are easier to interpret and are thus more sensitive for the detection of *mecA* gene mediated resistance than oxacillin results [19, 20].

Further, FOX has been suggested to be superior to oxacillin in detecting methicillin resistance because of the rare occurrence of resistance mechanisms other than *mecA*, if susceptibility testing is performed by dilution in addition to disk diffusion methods, isolates for which oxacillin MICs are ≥ 4 μg/mL and are *mecA* negative or PBP2a negative should be reported as oxacillin resistant. These isolates may test as susceptible to FOX by disk diffusion[7]. Furthermore, unlike β-lactamase synthesis, expression of PBP2a is not strongly inducible in isolates carrying the normal regulatory genes (*mecA* and *mecR1-mecI*) and induction is much slower (15 minutes for β-lactamase expression compared to up to 48 hours for PBP2a synthesis). This is because *mecI* is a tight regulator of *mecA* transcription [21, 22, 23, 24], hence, Dc appears to act as a very strong inducer and more selective for *mecR1* and can initiate *mecA* gene expression in a short period. So that evidently, phenotypically, the induction can be detected in an overnight growth procedure and this has not apparently been demonstrated previously for this β-lactam class resistance.

It appears that Dc is more efficacious on *mecR1* sensor than on PBP2 while β-lactam antibiotics appear to be more efficacious on PBP2 than *mecR1* sensor, a receptor located on the outer side of the bacterial cell membrane[24] in MRSA isolates. As a result, the expression of PBP2a takes up to two days [23] in case of β-lactam agents while Dc apparently works within minutes in a similar rapidity to that described with the expression of β-lactam inducible β-lactamase synthesis[25].

Despite carrying the *mecA* gene, such strains may appear susceptible to FOX in laboratory susceptibility tests; similar suggestions were made elsewhere [26]. Therefore, the double disk method of Dc-FOX could be a better tool for the phenotypic detection of MRSA isolates.

Although the accuracy of the double disk method of Dc-FOX, and FOX disk diffusion method is similar in the present study, it is tempting to introduce the former method to be used as a backup test for the phenotypic detection of *mecA* gene carrying *S. aureus* isolates. While, the FOX disk diffusion method is already recognized as screen test by CLSI [7] and EUCAST[27] and depends on reading the diameter of the inhibition zone with a certain breakpoint which can be influenced by several experimental factors [14] and thus carries the possibility of false result and interpretation, the double disk method of Dc-FOX has the advantage of revealing a growth of bacteria distorting the inhibition zone in an overnight incubation period.

This Dc-FOX test appears to show a good specificity as judged by the failure of the appearance of the blunting effect with the non-β-lactam drugs or with the inducible MLS_B isolates, and a good sensitivity as none of the 19 MRSA isolates was missed by this test. However, further studies are suggested to evaluate more comprehensively the sensitivity and specificity of this suggested test.

This blunting phenomenon-based principle of phenotypic detection of resistance mechanisms has been used for detecting AmpC β-lactamases and MLS_B resistance [7]. Further, up to our knowledge, the recognition of this Dc-FOX blunting phenomenon in MRSA and its introduction as a method for detecting MRSA has not been made previously. The use of Dc in detecting MRSA could be considered as a backup test with FOX to improve the accuracy of phenotypic detection of MRSA.

Conflicts of interest: All authors have nothing to disclose.

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