

*Full Length Research Paper*

# Phenotypic and molecular characterization of inducible clindamycin resistance among *Staphylococcal* strains isolated from cancer patients with febrile neutropenia

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Received 14 September, 2018; Accepted 4 November, 2018

This work aimed at the phenotypic and molecular characterization of inducible clindamycin resistance among strains of *Staphylococcus aureus* isolated from cancer patients with febrile neutropenia. Out of 231 clinical specimens *Staphylococci* were isolated from 179 (77.48%) cases. Isolates were identified by conventional microbiological methods. Antimicrobial sensitivity to all isolates was done using disc diffusion methods. For strains that were erythromycin resistant, D-test was performed to screen the presence of inducible clindamycin resistance. Multiplex polymerase chain reaction (multiplex-PCR) was done for strains with constitutive or inducible resistance to detect the distribution of *erm* (e), and *erm* (C) genes. Out of 231 clinical specimens, staphylococci were isolated from 179 (77.48%). Staphylococcal isolates were tested for susceptibility to erythromycin; 100 (55.8%) of them were erythromycin resistant. of these 100, erythromycin resistant isolates (8, 4.46%) were resistant to both erythromycin and clindamycin indicating constitutive MLS<sub>B</sub> Phenotype; 92 isolates were erythromycin resistant, and clindamycin sensitive. Out of these, 45 (25.1%) isolates showed positive D test indicating inducible MLS<sub>B</sub> phenotype while 47(26.2%) gave negative D test indicating MS phenotype. Molecular study revealed that 8 strains (100%) of staphylococci with constitutive MLS<sub>B</sub> phenotype and 23 strains (51.1%) of staphylococci with inducible MLS<sub>B</sub> phenotype had both *erm*(e) and *erm*(c) gene, *erm* (e) gene was present in 33.3% and *erm* (c) gene present in 15.6% of staphylococcus isolates with inducible MLS<sub>B</sub> phenotype. Inducible resistance and MS phenotype were found to be higher in MRSA as compared to MSSA (27.6, 24.3 and 1.6 and 4% respectively). Rapid dissemination of inducible clindamycin-resistant *S. aureus* isolates is worrisome and calls for judicious use of antibiotics. Therefore, the D-test should be added as a routine procedure on each staphylococcal isolates to detect inducible clindamycin resistance to avoid failure of antibiotic therapy.

**Key words:** Clindamycin, *Staphylococcus aureus*, antimicrobial susceptibility.

## INTRODUCTION

Febrile neutropenia is defined as fever with other signs of infection, in a patient with neutropenia, which is

considered with abnormal low concentration of neutrophils granulocytes (<500 cells/mm<sup>3</sup>), the most

abundant circulating white blood cells that is considered the first line of the organism defence against infections (Viscoli et al., 2005). Percentage of patients with malignancy experience a decrease in the cells and other elements of the immune systems that make them more liable to different types of infections (Lustberg, 2012). Neutropenia could be considered as an oncology emergency and can lead to serious adverse consequences such as serious infection complications and death (Villafuerte et al., 2014). Bacteria, including Gram-positive and Gram-negative species, viruses, and fungi; all of these may be a causative agent of febrile neutropenia. The incidence and epidemiology of febrile neutropenia depends on different factors including type of cancer, the age, and sex of the patient, the type and cycle of treatment (Kristjanson, 2015).

*Staphylococcus aureus* (*S. aureus*) is considered as one of the most common organisms causing infections in patients with malignancy with increasing the risk of resistance to wide range of antibacterial drugs. Macrolide-Lincosamide-Streptogramin B (MLS<sub>B</sub>) is a group of antibiotics that are nowadays used in treatment of *S. aureus* infections. Clindamycin is the most preferred antimicrobial drug in this group due to its proper pharmacokinetic properties that led to increase development of clindamycin resistance among strains of *S. aureus* (Yilmaz et al., 2007).

Clindamycin resistance in *Staphylococcus* species can be either constitutive or inducible (Deotale et al., 2010). It is difficult to detect strains of *S. aureus* that have inducible clindamycin resistance by routine laboratory methods as they appears as erythromycin-resistant and clindamycin sensitive *in routine laboratory in vitro* disc diffusion tests when the two discs of erythromycin and clindamycin not placed adjacent to each other (Lim et al., 2006). When these cases are treated with clindamycin; the target will be constitutive *erm* genes that lead to failure of treatment (Drinkovic et al., 2001). Strains of *S. aureus* that have *mcrA* genes (efflux genes) have with another mechanism of resistance that presented clinically as erythromycin-resistant and clindamycin-sensitive both *in vivo* and *in vitro*, with no resistance to clindamycin resistance during therapy (Laclercq, 2002).

The aim of this study was to detect the incidence of *staphylococcus aureus* causing infection among cancer patients with febrile neutropenia with phenotypic and molecular characterization of inducible and constitutive clindamycin resistance among these strains.

## MATERIALS AND METHODS

A prospective study was carried out in the Department of Medical

Microbiology and Immunology, Faculty of Medicine, Tanta University over a period of 6 months from September 2017 to March 2018 on 273 cancer patients with febrile neutropenia admitted to Oncology Department in Tanta University Hospital with suspected clinical sepsis after the approval of ethical committee in Tanta Faculty of Medicine, and a written consent from the participated patients. Clinical sepsis was defined as per the criteria established by American College of Physicians and Society of Critical Care Medicine (ACOM, SCCM) which included temperature >38°C, heart rate >90/min and respiratory rate >20/min. Neutropenia was defined as an absolute neutrophilic count (ANC) of 500 mm<sup>3</sup> or less or a count that is expected to fall to that level in the next 1-2 days (Steven and John, 2008).

Out of 273 samples 100 staphylococci strains could be isolated that were eligible for the study.

Different patient samples (pus, throat swabs, blood, urine, sputum) were collected from the patients under complete asepsis and transferred immediately to microbiology laboratory. Samples were cultured on blood agar and mannitol salt agar. *Staphylococci* were identified using conventional microbiological methods and biochemical reactions; coagulase test and catalase test according to the standards of Clinical and Laboratory Standards Institute (2007).

### Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using the disk diffusion method. The antibiotics chosen were Erythromycin, Clindamycin, vancomycin, gentamicin, oxacillin, cefotaxime, ciprofloxacin, cefepime and meropenem, Interpretation of the results according to the standards of Clinical and Laboratory Standards Institute (2007).

### Phenotypic identification of clindamycin resistance (D-test)

All erythromycin-resistant isolates were further examined by double-disc test with erythromycin (15 µg) and clindamycin (2 µg) discs and the results were interpreted according to Clinical and Laboratory Standards Institute (2007) guidelines, to determine the resistance phenotype. According to the results of D-test; 3 phenotypes could be identified MS<sub>B</sub> phenotype, showed circular zone around clindamycin disc, iMLS<sub>B</sub> (inducible resistance) phenotype, demonstrated a flat zone around clindamycin disc, and cMLS phenotype were resistant to both discs of erythromycin and clindamycin (constitutive resistance).

### Multiplex PCR for Genotypic identification of staphylococcal strains with clindamycin resistance

#### DNA extraction

QIAamp DNA Mini Kit (Qiagen) was used for isolation of genomic presence of erythromycin resistance methylase *erm* genes *erm* (e), and *erm* (C) PCR using the primer pairs as described by Lim et al. (2006).

Multiplex PCR for *erm* (a) and *erm* (c) was performed in final volume of 20 µl by DFS Master Mix Kit (Cinagen) including Taq polymerase enzyme, MgCl<sub>2</sub>, dNTP, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, TrisHCl, Tween – 20. Reaction mixtures consisted of 12.5 µl Master Mix, 1 µl MgCl<sub>2</sub>,

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**Table 1.** Demographic and clinical characteristics studied cases.

Parameter		Number	%
Sex	Male	95	53.07
	Female	84	46.9
Sample	Pus	33	18.4
	Urine	33	18.4
	Blood	31	17.3
	Sputum	47	26.2
	Throat swab	35	19.4
Type of cancer	Colorectal	26	(3.3)
	Lymphoma	30	(11.1)
	Breast	29	(10.6)
	Lung	35	(19.5)
	HCC	21	(11.7)
	Prostate	19	(10.6)
	MUO	19	(10.6)

Patients (n = 179).

0.25 µl of each primer, 2 µl distilled water and 1 µl DNA template. Cycling conditions were primary denaturation at 94°C for 4 min, denaturation at 94°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 1 min, then 30 cycle, followed by a final extension at 72°C for 5 min. Primers used for *erm* (a) gene were: 5' TCA GGA AAA GGA CAT TTT ACC3', : 3'ATA TAGTGG TGG TAC TTT TTT GAG C5'detected at 118 bp, *erm* (c) gene 5'TAGCAAACCCGTATTCCACG3',3'CTTGTTGATCACGATAATTT CC5'detected at 495bp, (Matthew et al., 2006).

## RESULTS

Table 1 shows the demographic and clinical characteristics studied cases where among 179 cases from which *S. aureus* were isolated, 95 cases were male patients and 84 were female patients, with their age from 18 to 72 (33±24.66), samples were collected from different sites according to the site of infection; pus 33 (18.4%), urine 33 (18.4%), blood 31 (17.3%), sputum 47 (26.2), throat swab 35 (19.4%). Colorectal cancer represent 3.3%, lymphoma 11%, Breast 10.6%, lung 19.5%, Hepatocellular carcinoma (HCC) 11.7%, Prostate 10.6% and Metastasis of unknown origin (MUO) 10.6%.

Table 2 shows pattern of clindamycin resistance among studied cases where among 179 *S. aureus* that were eligible for the study, 79 (44.1%) strains were erythromycin sensitive, and 100 (55.8%) of them were erythromycin resistant. Of these 100 erythromycin resistant strains, 8 (4.46%) isolates were resistant to both erythromycin and clindamycin indicating constitutive MLS<sub>B</sub> phenotype; 92 isolates were erythromycin resistant, and clindamycin sensitive. Out of these, 45 (25.1%) isolates showed positive D test indicating inducible

**Table 2.** Pattern of clindamycin resistance among studied cases.

Pattern of clindamycin resistance	Number	%
Erythromycin Sensitive	79	44.1
CR (E resistant, CL resistant)	8	4.46
IR (E resistant, CL sensitive )+ve D test	45	45 (25.1)
MS (E resistant , CI sensitive ) -ve D test	47	47 (26.2)

clindamycin resistance (MLS<sub>B</sub> phenotype) while 47(26.2%) gave negative D test indicating MS phenotype.

Table 3 shows relation between pattern of clindamycin resistance and type of staphylococci as regard sensitivity to methicillin where constitutive MLS<sub>B</sub> Phenotype was found in higher percentage in MRSA [2 strains (25%)] of MSSA and 6 strains (70%) of MRSA; inducible MLS<sub>B</sub> phenotype found also in higher percentage in MRSA (10 strains (22.2%) of MSSA and 35 strains of MRSA (77.8%). As regard MS, phenotype was equally distributed in MSSA and MRSA [22 strains (46.8%) and 25 strains (53.2)] respectively.

Table 4 shows the distribution of *erm* (e) and *erm* (c) genes among different phenotypes of clindamycin resistance where it was found that 100% of the strains with constitutive resistance phenotype contain both *erm*(e) and *erm*(c) gene, and as regard strains with inducible resistance phenotype, 15 strains (33%) contain *erm*(e) gene, 7 strains (15.8%) contain *erm*(c) gene, 23 strains (51%) contain both *erm* (c) and *erm*(e) genes

Table 5 shows the antimicrobial sensitivity and resistance pattern of the *S. aureus* isolates with constitutive resistance phenotype where highest sensitivity was for vanomycin (98.7%), oxacillin (97.4%), cefpime and ciprofloxacin (83.5%) each, and all of the erythromycin sensitive strains were clindamycin sensitive.

Tables 6 and 7 shows the antimicrobial sensitivity and resistance pattern of *staphylococcal* strains with inducible resistance phenotype where 75% of the strains were resistant to meropenem, gentamycin, and ciprofloxacin, 62% of the strains were resistant to cefotaxim , 50% were resistant to cefepime, and 100% of the strains were vancomycin sensitive. Agarose gel electrophoresis showing positive amplification of 118 and 495 base fragments is shown in Figure 1.

## DISCUSSION

Cancer patients receiving cytotoxic antineoplastic therapy are at risk for invasive infection due to colonizing bacteria or fungi that translocate across intestinal mucosal surfaces. Since the magnitude of the neutrophil-mediated component of the inflammatory response may be muted in neutropenic patients. The first-line empirical treatment should cover the prevalent microorganism of the institute.

This study performed on 231 cases of febrile neutropenia from which *Staphylococcus aureus* could be

**Table 3.** Relation between pattern of clindamycin resistance and type of staphylococci as regard sensitivity to methicillin.

Type of staph		Pattern of clindamycin resistance			Total
		CR	IR	MS	
MSSA	N	2	10	22	34
	%	25.0%	22.2%	46.8%	34.0%
MRSA	N	6	35	25	66
	%	75.0%	77.8%	53.2%	66.0%
Total	N	8	45	47	100
	%	100.0%	100.0%	100.0%	100.0%
Chi-square	X <sup>2</sup>	6.507			
	P-value	0.039*			

**Table 4.** Distribution of *erm (e)* and *erm (c)* genes among different phenotypes of clindamycin resistance.

Gene		Pattern of clindamycin resistance			Total
		Constitutive MLS <sub>B</sub>	Inducible MLS <sub>B</sub>	MS	
<i>erm (e)</i>	N	0	15	0	15
	%	0%	33.3%	0%	15.0%
<i>erm (c)</i>	N	0	7	0	7
	%	0%	15.6%	0%	7.0%
<i>erm (e)+erm (c)</i>	N	8	23	0	31
	%	100.0%	51.1%	0%	31.0%
Total	N	8	45	47	100
	%	100.0%	100.0%	100.0%	100.0%
Chi-square	X <sup>2</sup>	112.616			
	P-value	0.001*			

**Table 5.** The antimicrobial sensitivity and resistance pattern of the *S. aureus* isolates with constitutive resistance phenotype.

Antibiotics	S		R	
	N	%	N	%
Erythromycin	0	00.00	00.00	00.00
Clindamycin	8	8	8	8
Gentamicin	7	7	1	1
Cefotaxime	2	2	6	6
Oxacillin	1	1	6	6
Vancomycin	7	7	1	1
Cefepime	5	5	3	3
Ciprofloxacin	4	4	4	4

S=sensitive; R=resistant.

isolated from 179 (77.4%). On reverse to this work, a study performed by Taj et al. (2015) found that Gram negative infections accounted for 68 (85%) and *Escherichia*

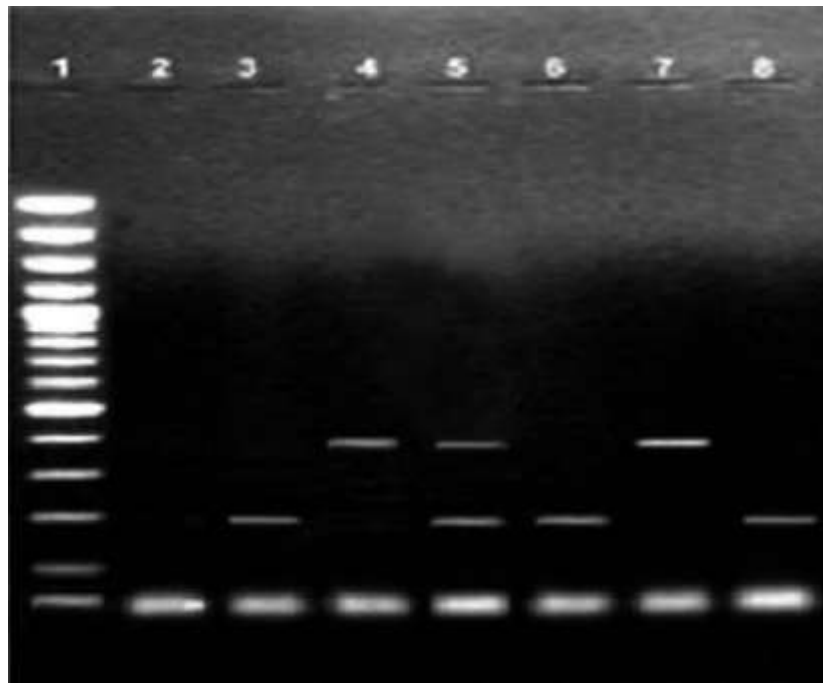
**Table 6.** Antimicrobial sensitivity and resistance pattern of the *S. aureus* isolates with inducible resistance phenotype.

Antibiotics	S		R	
	N	%	N	%
Erythromycin	00.00	00.00	45	45
Cefotaxime	23	23	22	2
Oxacillin	10	10	40	40
Vancomycin	33	33	12	12
Cefepime	31	31	14	14
Ciprofloxacin	22	22	23	3

*coli* was the commonest isolate. Gram positive microorganisms were isolated in 12 (15%) cases and most common was *S. aureus*. This study reported only one *staphylococcus* isolate (1%) with constitutive clindamycin resistance; resistant to vancomycin and 12 strains with inducible clindamycin resistance (12%) were

**Table 7.** Antimicrobial sensitivity and resistance of the *S. aureus* isolates with MS phenotype.

Antibiotics	S		R	
	N	%	N	%
Erythromycin	00.00	0.00	47	47
Cefotaxime	26	26	21	21
Oxacillin	13	13	34	34
Vancomycin	35	35	12	12
Cefepime	23	23	24	24
Ciprofloxacin	40	40	7	7

**Figure 1.** Agarose gel electrophoresis showing positive amplification of 118 and 495 base fragments specific for *erm* (e) and *erm* (c), respectively. Lane 1, 100-1500 bp ladder; lane 2, negative control; lane 3, control *erm* (e) (382 bp); lane 4, control *erm* (c) (188 bp); lanes 5-8, test strains.

resistant to vancomycin. Taj et al. (2015) also reported that the commonest Gram positive isolate is *S. aureus* and 4 cases of MRSA were isolated: all were sensitive to vancomycin; however, 2 cases of vancomycin resistant *enterococci* (VRE) were documented and were treated successfully with linezolid. In this study, sputum samples represents 26%, pus and urine 18.4 and 18.4% respectively, throat swab 19.4%, blood 17.3% of the clinical samples of cancer patients febrile neutropenia. Anderson et al. (2006) and Nasehi et al. (2010) reported that the most common infections that complicate cancer patients with febrile neutropenia include urinary tract infections, soft tissue infection, pneumonia, and septicaemia.

In this study, out of 179 *S. aureus* that were eligible for

the study, 79 (44.1%) strains were erythromycin sensitive, and 100 (55.8%) of them were erythromycin resistant. Of these 100 erythromycin resistant strains, 8 (4.46%) isolates were resistant to both erythromycin and clindamycin indicating constitutive MLS<sub>B</sub> Phenotype; 92 isolates were erythromycin resistant, and clindamycin sensitive. Out of these, 45 (25.1%) isolates showed positive D test indicating inducible MLS<sub>B</sub> phenotype while 47 (26.2%) gave negative D test indicating MS phenotype.

The study of Deotale et al. (2010) showed high percentage of erythromycin resistant isolates [80 (32.4%)]. Amongst them 36 (45%) isolates tested positive for inducible clindamycin resistance by D test while the rest of the isolates were negative for D test, out of which 9 (11.25%) were shown to have constitutive clindamycin

resistance and 35 (43.75%) showed true sensitivity to clindamycin (MS phenotype). They reported that these results indicate that D test must be done as a routine laboratory procedure otherwise most of the erythromycin resistant isolates will be misidentified as clindamycin sensitive resulting in failure of treatment. As regard the relation of pattern of clindamycin resistance to the type of sensitivity of *S. aureus* to mecithelline, the results of this study showed that constitutive  $MLS_B$  Phenotype was found in higher percentage in MRSA [2 strains (25%) of MSSA and 6 strains (70%) of MRSA], inducible  $MLS_B$  phenotype were found also in higher percentage in MRSA [10 strains (22.2%) of MSSA and 35 strains of MRSA (77.8%)]. As regard MS, phenotype was equally distributed in MSSA and MRSA [22 strains (46.8%) and 25 strains (53.2)] respectively. These results are in accordance with the results of Kavitha et al. (2011) who observed that percentages of inducible resistance and constitutive clindamycin resistance were higher amongst MRSA as compared to MSSA (20, 16.66, 6.15, and 6.15%, respectively).

As regard the antimicrobial sensitivity and resistance pattern of the *S. aureus*, isolates with constitutive resistance phenotype in this study showed that highest sensitivity was to vanomycin (98.7%), oxacillin (97.4%), cefpime and ciprofloxacin (83.5% each), and all of the erythromycin sensitive strains were clindamycin sensitive.

Moreover and as regard the antimicrobial sensitivity and resistance pattern of *staphylococcal* strains with inducible resistance phenotype, the results of this study showed that 75% of the strains were resistant to meropenem, gentamycin, and ciprofloxacin, 62% of the strains were resistant to cefotaxim, 50% were resistant to cefepime, and 100% of the strains were vancomycin sensitive.

## Conclusion

Rapid dissemination of inducible clindamycin-resistant among isolates of *S. aureus* is worrisome and calls for judicious use of antibiotics. Therefore, the D-test should be added as a routine procedure of antimicrobial susceptibility tests on *S. aureus* isolates to detect inducible clindamycin resistance to avoid failure of antibiotic therapy with improvement of the mortality rate among cancer patients with febrile neutropenia.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## ACKNOWLEDGEMENTS

The teamwork of this research greatly acknowledge

departments of Medical Microbiology and Immunology, Tropical Medicine, Internal Medicine, Clinical Oncology for their great help and support

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