ORIGINAL ARTICLE

Clinical relevance, Speciation, and Antibiogram of Non -Diphtherial *Corynebacteria* isolated from various clinical samples in a tertiary care hospital in Zagazig, Egypt

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Key words:
Corynebacterium, Diphtheroids, speciation, Antibiogram

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ABSTRACT

Background: *The non-diphtherial Corynebacteria, also called "Coryneforms" bacteria are a diversified group of gram positive non sporing bacilli belonging to the genus Corynebacteria. Such bacteria are considered members of human microbiota (skin, respiratory and genital mucus membranes). Coryneform bacteria's pathogenic capacity has been undervalued until recently. Despite of frequently deemed as contaminants, these bacteria have been correlated to diverse clinical infections recently.* ***Objectives:*** *To isolate, speciate, and determine antimicrobial susceptibility pattern of clinically relevant non-diphtherial Corynebacteria from various clinical samples.* ***Methodology:*** *Different clinical samples (blood, urine, sputum, wound swabs, pus) collected from hospitalized patients attending at Zagazig University Hospital. The samples were processed and cultured as per conventional bacteriological methods. A total of 75 clinically relevant corynebacterial isolates exhibited speciation utilizing matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis and their antibiogram was done by disc diffusion method by means of combined guidelines of Clinical and Laboratory Standards Institute (CLSI) as well as British Society for Antimicrobial Chemotherapy (BSAC) because of lack of definite CLSI guidelines.* ***Results:*** *The mean age of the studied patients was 64.6 ± 14.9 years, 60% were male and 40% female. A total 75 clinically relevant Corynebacteria species were obtained from different clinical samples, including wound swabs and pus (53%), sputum (20%), and blood (17%). Forty two percent were isolated from ICUs. The most prevalent isolated species was C.amycolatum (27%), C. striatum (20%), and C.jeikieum (16%). Beta lactam antibiotics showed least activity against Corynebacteria species with resistance rate against penicillin 76% and ceftriaxone 72%, while all isolates exhibited uniform sensitivity (100%) against vancomycin as well as linezolid.* ***Conclusion:*** *This study showed isolation of different clinically relevant non-diaphterial Corynebacteria from different clinical samples with pus and wound swabs as the most common samples from which Corynebacteria were isolated. In particular, C.amycolatum was the most common isolated species. Beta lactam antibiotics (penicillin, ceftriaxone) showed the least activity while vancomycin and linezolid were the most active agents against non-diapdhterial Corynebacteria isolates. Herein, we confirm diphtheroids' clinical importance among different infections that necessitate evaluating their susceptibility patterns to some common antibacterial agents for guide the best antibiotic to treat infections caused by these species.*

INTRODUCTION

Corynebacteria are club-shaped Gram-positive, catalase-positive, non-motile, aerobic or facultatively anaerobic, non-spore forming rods. The genus includes two species such as *Corynebacterium diphtheriae* and non-diphtherial *Corynebacteria* that are generally called "diphtheroids"[1]. Such species are widespread in environment (water and soil), and some species exist as a part of human skin in addition to mucous membranes microbiota. More than 100 species are recognized, and 54 of them exhibit a relation to human infections [2]

Non-diphtherial *Corynebacteria's* clinical relevance was long discussed. Their presence is generally found on skin as well as mucosa, rendering them in clinical microbiology a harmless status [1]. The majority of such isolates exhibit no speciation or identification because these were considered as contaminants, particularly in the case of samples derived from non-sterile locations. Nevertheless, their extensive isolation from clinical

DOI: https://doi.org/10.51429/EJMM30207

samples as opportunistic pathogens exists in patients with immunosuppression[2]. Nevertheless, clinical importance of being isolated from immunocompetent patients is often ambiguous [3].

The past two decades showed a greater association of non-diphtherial *Corynebacteria* in various clinical infections, such as catheter-associated blood stream infections, peritonitis, neurosurgical shunt infection, osteomyelitis, meningitis brain abscess, urinary tract infections, septic arthritis, empyema in addition to pneumonia [4].

The most widely recognized species isolated from human clinical samples include lipophilic and non-fermentative species such as *C. jeikeium* as well as *C. urealyticum* in addition to non-lipophilic and fermentative species such as *C. striatum* as well as *C. amycolatum* [3].

The exact prevalence of these group of organisms are unknown, however there are increasing reports from both India and the western world [5]. The rising role of diphtheroids as medically relevant pathogens is due to both host factors and microbial determinant of pathogenicity. Though poor knowledge about virulence factors and pathogenesis of infections originated from non-diphtheriae *Corynebacterium*, there are many aspects linked to their increasing pathogenic potential. The leading aspect of this potential is a frequent multidrug antibiotic resistance of coryne bacteria. The ability to adhere to biotic and abiotic surfaces and/ or to form biofilms in which bacteria are protected both against antimicrobial agents and the host immune responses are also considered an important strategy promoting the involvement of bacteria in both medical devices- and tissue-associated chronic infections [6,7]

Although the growing frequency of infections due to diphtheroids, their relevance has been disregarded because of profound lack of awareness about their clinical significance and pathogenic potential. This study was carried out to isolate, speciate the clinically relevant *Corynebacteria* from various clinical samples by MALDI-TOF MS and to determine their antimicrobial susceptibility patterns utilizing disc diffusion method.

METHODOLOGY

Study location and design:

The current cross-sectional research was carried out from June 2016 to May 2018 at Medical Microbiology Department and Clinical Pathology Department, Zagazig University Hospitals, Egypt. All patients admitted at different Hospital Departments were enrolled in this study. This study got approval from Research and Ethic Committee of Faculty of Medicine, Zagazig University.

Samples criteria and collection:

Different clinical samples were collected from various wards (Surgery, Gynaecological, Orthopaedic, ICU, Oncology unit) from patients with different infections (UTI, Ventilator associated pneumonia, wound infections, bacteraemia). The obtained samples included sputum, blood, pus, wound swabs, urine. All samples were processed in Microbiology Unit within 2 hours of collection.

Samples showing growth of diphtheroids which fulfilled one or more of the following criteria were taken as clinically relevant and was included in the study.

Inclusion criteria:

- Direct gram staining showing pus cells with or without gram positive bacilli.
- Pure and predominant growth of diphtheroids.

Exclusion criteria:

- Diphtheroid growth is scanty with predominance of other bacteria in culture.
- Pus cells are absent by direct examination.

Microscopic examination and processing:

Except for blood samples, all samples were subjected to direct gram stain examination (Figure 1). The samples were cultured on sheep blood agar (5%) in addition to MacConkey agar (Oxoid) and incubated for 24-48 hours at 37ºC.

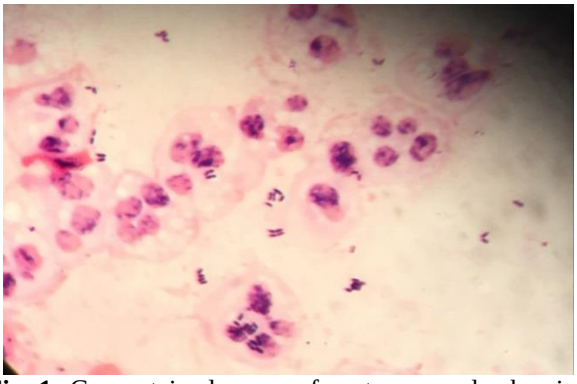

Fig. 1: Gram-stained smear of sputum sample showing pus cells, gram positive bacilli with pallisading pattern and "V forms shaped arrangement, 1000X oil immersion.

Identification of *Corynebacteria*:

The genus *Corynebacteria* was presumptively identified based on the presence of the below features: Mostly aerobic, non-motile, non sporing, non-capsulated, catalase positive, oxidase negative, club shaped gram-positive rods arranged in pallisading pattern or "V forms" on gram staining. Diphtheroids were differentiated from *Corynebacterium diphtherium* by characteristics like their capability of growing on ordinary media like MacConkey and Nutrient Agar, urease hydrolysis and clinical correlation [8]. Further confirmation and speciation were done by MALDI-TOF.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis:

Samples Preparation:

A part of a well identified single colony was directly applied to a disposable target slide (product no. 11111149BM; bioMérieux, Marcy l'Etoile, France) comprised a polypropylene carrier having a stainless-steel layer. This part exhibited lysis directly by applying a 1-l loop. One ùl of matrix solution cyano-4-hydroxycinnamic acid, product no. 1002317170; bioMérieux) was added, followed by room-temperature drying before analysis by mass spectrometry. When it comes to Vitek MS, isolates were subjected to preparation to be analyzed utilizing mass spectrometry. After that, analysis of samples was carried out by means of Vitek MS MALDI-TOF mass spectrometer[9]

Antimicrobial Susceptibility Testing*:*

The modified Kirby Bauer disc diffusion method on 5% sheep blood agar was used for antibiogram determination of *Corynebacteria* species. One percent Tween 80 (Oxoid) was added to 5% Sheep blood Agar when testing lipophilic Corynebacterial species (*C. jeikieum* and *C.urealyticum*) [2] .The following antibiotic susceptibility discs obtained from ((Bioanalyse , Turkey) were used: Penicillin (10 U), ceftriaxone (30 µg), clindamycin (2 µg), erythromycin (15 µg), Ciprofloxacin (5 µg), gentamicin (10 µg), linezolid (30 µg), , tetracycline (30 µg), vancomycin (30 µg).

For disc diffusion method of *Corynebacteria*, because of having no developed CLSI guidelines, another approach was undertaken:(a) penicillin, ciprofloxacin, in addition to vancomycin tests followed the guidelines of British Society for Antimicrobial Chemotherapy (BSAC)[10]. (b) Regarding other antibiotics, applied CLSI guidelines can be for *Staphylococcus aureus*, having an *S.aureus* ATCC 25923 as control strain [11]

Statistical analysis:

Data were collected, tabulated, followed by analysis by SPSS version 16.0.

RESULTS

The demographic and clinical properties of patients involved in this study are presented in (Table 1).

The mean age of studied patients was 64.6 ± 14.9 years (range, 35–85 years) and 40% of patients age exhibits a range of 45-65 years old. Our 75 patients under study include 45 (60%) as males, and 30 (40%) as females.

Of the 75 *Corynebacteria* isolates 32(43%) were isolated from ICUs, followed by General Surgery Ward 18(24%), Medical Oncology Ward 15(20%).

All patients suffered from at least one underlying disease, and such diseases includes diabetes mellitus (27%), ischemic heart disease (15%), and chronic liver failure (13%).

Table-1: Demographics and clinical characteristics distribution between patients group n=75

Data	N (%)
Age	
▪ 35-45 years	▪ 10 (13%)
▪ 46-65 years	▪ 40 (53%)
▪ 66-80 years	▪ 15(20%)
▪ >81	▪ 10(13%)
Sex	
▪ Male	▪ 45(60%)
▪ Female	▪ 30(40%)
Hospital units	
▪ ICUs	▪ 32(43%)
▪ General surgery Ward	▪ 18(24%)
▪ Oncology Ward	▪ 15(20%)
▪ Orthopedic Ward	▪ 6(8%)
▪ Gynecological ward	▪ 4(5%)
Underlying co-morbid diseases	
▪ Diabetes mellitus	▪ 20(27%)
▪ Ischemic heart disease	▪ 11(15%)
▪ Chronic Liver disease	▪ 10(13%)
▪ Chronic kidney disease	▪ 8(11%)
▪ Malignancy	▪ 10(13%)
▪ COPD*	▪ 8(11%)
▪ Neutropenia	▪ 5(7%)
▪ Recent surgery	▪ 3(4%)

*****COPD: Chronic obstructive pulmonary disease

The *Corynebacterium* isolates obtained were 40 (53%) from wound swabs and pus, 15 (20%) from sputum, blood 13 (17%) and 7 (9%) from urine (Table-2).

Table 2: Distribution of isolate according to sample

Sample	No (%)	Percentage (%)
▪ Pus, wound swabs	▪ 40	▪ 53%
▪ Sputum	▪ 15	▪ 20%
▪ Blood	▪ 13	▪ 17%
▪ Urine	▪ 7	▪ 9%

Out of 75 *Corynebacteri*a isolates, most prevalent species isolated were *C.amycolatum* (27%), *C. striatum* (20%) , *C.jeikieum* (16%), *C.ulcerans* (15%) (Table -3).

Table 3: Distribution of the isolates according to species n =75.

Species	No of isolate	Percentage of isolate
C.amycolatum	20	(27%)
C. striatum	15	(20%)
C.jeikieum	12	(16%)
C.ulcerans	11	(15%)
C.urealyticum	8	(11%)
C.pseudodiphtheriticum	7	(9%)
C.minutissmum	2	(3%)

The isolates' resistance pattern **(**Table-4**)** revealed resistance with high frequency to penicillin (76%), ceftriaxone (72%), erythromycin (70%), clindamycin (64%), and ciprofloxacin (50%). Perfect activities were shown by linezolid, vancomycin which all the isolates were sensitive.

Table 4: Resistant pattern of the isolated *Corynebacterium* species n=75

	Penicillin (10 U)	Ceftriaxone (30 µg)	Erythromycin (15 µg)	Clindamycin (2 µg)	Ciprofloxacin (5 µg)	Gentamycin (10 µg)	Tetracycline (30 µg)	Vancomycin (30 µg)	Linezolid (30 µg)
C.amycolatum (20)	15(75%)	15(75 %)	16 (80%)	17(85 %)	10(50 %)	14(70 %)	8(40 %)	0(0%)	0(0%)
C. striatum (15)	11(73%)	11 (73%)	10 (67 %)	11(73%)	9(60%)	12(80 %)	9(60 %)	0(0%)	0(0%)
C.jeikieum (12)	9(75%)	9(75%)	10(83%)	9 (75 %)	5(42%)	9(75%)	5(42%)	0(0%)	00(0%)
C.ulcerans (11)	8 (73%)	7(64 %)	8(73 %)	7(64%)	7(64%)	8(73%)	4(36%)	0(0%)	00(0%)
C.urealyticum (8)	7(88 %)	6(75 %)	7(88 %)	4(80%)	5(63%)	6(88%)	4(506%)	0(0%)	0(0%)
C.pseudodiphtheriticum (7)	5(72 %)	6(86 %)	2(29%)	0(0 %)	2(29%)	0(0%)	0(0%)	0(0%)	0(0%)
C.minutissmum (2)	2(100 %)	0 (0%)	0(0%)	0(0%)	0(0%)	1(50 %)	0(0%)	0(0%)	0(0%)
Total (75)	57(76%)	54(72%)	53(70%)	48 (64%)	38(50%)	50(67%)	30(40%)	0(0%)	0(0%)

DISCUSSION

Non-diphtherial *Corynebacterium* are commonly considered as contaminants from the skin in the routine diagnostic practice. They are usually not recognized to species level and antimicrobial susceptibility testing is not performed [12]

Non-diphtherial *Corynebacterium*, were found to have rising incidence as possible pathogens, particularly as nosocomial pathogens. By the time, patients exposed to immunosuppression in terms of duration and intensity in addition to excessive deployment of indwelling intravenous devices, pathogens function exhibits higher significance than before. Evolving antimicrobial resistance in different species generated extra requirements towards exact identification for clinically relevant coryneform organisms to species level in addition to continuous surveillance regarding their patterns of resistance [13]

Identifying *Coryneform* bacteria still poses challenging task to be identified by routine diagnostic laboratories due to widespread spectrum of species related to such group, causing scarce isolation for such organisms. As all of them build up part of a commensal flora at one or other sites in body, ultimate decision is required for investigating its clinical relevance[13]

By implementing advanced MALDI-TOF MS in laboratory of clinical microbiology, most unknown *diphtheroids* are easily recognized to species level [9,14]. Patel and coworkers [15]stated that identification of isolates of diphtheroid to species level can influence treatment decisions.

In this study a total 75 isolates of clinically relevant *Corynebacteria* were isolated, subjected to speciation and antibiotic susceptibility testing. Majority of the patients were males (60%) when compared to females (40%) and predominantly belonged to the age group 46-65 years (50%). Comparable results were obtained by a study done by Archana[16] in which, he reported his study patients' group were males (62%) when compared to females (38%) and predominantly belonged to the age group 41-50 years (42%) .

In our study, all patients had associated co-morbid conditions, including diabetes mellitus (27%) followed by ischaemic heart diseases (15%). Similarly, various other previous case reports of infections due to diphtheroids in patients with diabetes mellitus have been reported [17,18,19]

In this research, most isolates were obtained from the pus and wound swab (50%) and sputum (20%) followed by blood (17%) and urine (9%). Reddy *et al*[20] in his study on 114 clinically relevant diphtheroids isolated from different samples reported that most isolates were attained from urine (37.7%) and pus (32.4%) samples.

The commonly isolated non-diphtheritic *Corynebacteria* in our study were *C. amycolatum* followed by *C. straitum*, C. *jeikieum*, *C. ulcerans*, *C. urealyticum*, *C. pseudodiaphteriticum*, *and C. minutissimum*. Samuel and coworkers stated that the commonly isolated non-diphtheritic *Corynebacteria* were *C. pseudodiphtheriticum, C. ulcerans, C. straitum, C. minutissimum* and *C. xerosis* [21]

In the present study, *C. amycolatum* exhibited mostly prevalent isolated species representing 27% of total isolates and was mostly isolated from all specimens, such as pus, sputum and wound swabs. Langrou and coworkers[22] stated an analogous result, having isolation rate of 53% for such organism, representing main organism of their series. Reddy *et al*[20] and Archana[16] respectively showed the same results, with 35.9% and 33% isolation rate of this organism in their series.

C. striatum was regarded as the second most predominant isolated species, representing 20% of total isolates, and mainly originated from blood. Such findings exhibit coherency with *C. striatum* as an evolving pathogen in numerous sites in addition to being a colonizer of indwelling medical devices [23]. In addition to multidrug resistance, nosocomial outbreaks were also reported [24,25]

C. urealyticum is a recognized reason for chronic UTIs, particularly in patients having advanced age, genitourinary disorders, in addition to immunosuppression [26]. In our study we reported 5 *C. urealyticum* isolates of all *Cornebacterium* species isolated from urine samples. All such patients had moderate to severe UTI symptoms, and half of them were above 80 years old.

Among Corynebacterium species, many studies showed a worrisome rate regarding antibiotic resistance. Resisting β-lactams, erythromycin clindamycin, gentamicin, in addition to ciprofloxacin is relatively common, demonstrating that vancomycin and linezolid are the only drugs to be effective [20,27,28]

In this study beta-lactam antibiotics showed least activity against the *Corynebacteria* species with resistance rate against penicillin 76% and ceftriaxone 72%. These finding were similar to other studies which also reported *Corynebacteria* as highly resistant to beta-lactam antibiotics with resistance rate of 65% [20-29-30]

Regarding sensitivity rate of ciprofloxacin and tetracycline our result showed moderate activity against *Corynebacterium* with a sensitivity rate 49% and 60%, respectively. This finding is quite near to a research conducted by Mathavi and Coworkers[31] in which they stated that sensitivity rates 56% and 57% to ciprofloxacin and tetracycline respectively.

The overall susceptibility pattern of our study revealed high susceptibility pattern against vancomycin (100%), linezolid (100%). Other studies reported the same results [27,29, 31]

The majority of isolated *Corynebacteria* species in this study exhibited multidrug resistant pattern, which is identified as resistance to ≥3 classes of antibiotics. *C. amycolatum*, the main species in our research, demonstrated resistance with high frequency against penicillin and ceftriaxone (75%), erythromycin (80%), in addition to clindamycin (85%). Such isolates exhibited fairly less resistance to ciprofloxacin (50%) as well as tetracycline (40%). Such findings exhibited consistency with other researches [22, 32,33.]

C. striatum is considered an important multidrug resistant organism which is transmitted between hospitalized patients and medical personnel. Its engagement in human infections is frequently linked to catheterization, intubation and immunosuppression [34,35.]

Herein, *C. striatum* was the second common isolated species. This pathogen showed multidrug resistance pattern with high resistance to gentamycin (80%), penicillin and ceftriaxone (73%), erythromycin (67%) and clindamycin (73%). The strains were moderate resistance to ciprofloxacin and tetracycline (60%) while, all strains were (100%) sensitive to vancomycin in addition to linezolid. These results were comparable to a conducted research by Suh and Coworkers[36] in which they stated resistance pattern of 67 *C.striatum* clinical isolates with moderate resistance to high levels of resistance to penicillin (97%), ampicilllin (94%), cefotaxime (95.5%), and levofloxacin (91%), while all strains exhibited susceptibility to erythromycin, vancomycin, in addition to linezolid.

Another study[37] was done on 81 *C.striatum* isolated form different clinical samples ,they reported that all strains (100%) exhibited resistance to penicillin, cefotaxime, ciprofloxacin, and tetracycline with susceptibility to vancomycin as well as linezolid. While resistance rates exhibited trending behavior against gentamicin (34.6%), erythromycin (79%), and clindamycin (87.7%).

CONCLUSION

We need to keep Non- diaphterial *Corynebacterium* in mind before precluding them as commensals. This study showed isolation of different clinically relevant *Corynebacteria* from different clinical samples with pus and wound swabs were the most common samples from which *Corynebacteria* isolated .*C.amycolatum* was the most common isolated species. Beta lactam antibiotics (penicillin, ceftriaxone) showed the least activity while vancomycin and linezolid were the most active agents against non-diapdhterial *Corynebacteria* isolates. This study also highlighted existence of multidrug resistance *Corynebacterium spp.* that necessitate evaluating their susceptibility patterns to some common antibacterial agents for guide the best antibiotic to treat infections caused by these species.

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.

- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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