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Multidrug-Resistant *Corynebacterium accolens* Strains Identified as Pathogens of Nosocomial Infections

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Lorraine Herdy Heggendornn[*1,2], Sara Wilis Cussuol Gomes[1,2], Luís Guilherme de Araújo Longo[3], Andrew Fernandes Farsura[2], Ana Luíza Mattos-Guaraldi[1], Louisy Sanches dos Santos[1], Helvécio Cardoso Correa Póvoa[2]

1 Laboratory of Diphtheria and Corynebacteria of Clinical Relevance, The Collaborating Centre for Reference and Research on Diphtheria/National Health Foundation/Ministry of Health - FNS/MS, Brazil-LDCIC/FCM/ UERJ, Faculty of Medical Sciences, State University of Rio de Janeiro – UERJ. Brazil

2 Laboratory of Experimental and Applied Microbiology, Health Institute of Nova Friburgo, Federal Fluminense University, Brazil

3 Paulo Góes Microbiology Institute (IMPG), Federal University of Rio de Janeiro (UFRJ), Brazil.

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ABSTRACT

We identified multidrug-resistant *C. accolens*, a rare human pathogen, isolated from nosocomial infections. Although nondiphtheroid *corynebacteria* have emerged as true pathogens in nosocomial infections and, the multidrug resistance pattern of these microorganisms has been emphasised, the virulence mechanisms of these pathogens have not been elucidated. We aimed to investigate the susceptibility of the *Caenorhabditis elegans* to *Corynebacterium accolens*. All isolates were identified by MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time of Flight, Bruker Daltonics™), submitted to antimicrobial susceptibility testing by disk diffusion and *Caenorhabditis elegans* assays were realized. MALDI-TOF-MS is a rapid and reliable method for the identification of *C. accolens*. All strains caused a decline in the survival curve of *C. elegans*. The interaction of nematode and *C. accolens* results in morphological changes, 100.0% of the strains caused deformed anal region (*Dar*) and abdominal distension in *C. elegans*. The “bag of worms” phenotype was observed in all tested strains. We suggest that medical surveillance programs should include control strategies in order to decrease potential risk factors of nosocomial infections due to *Corynebacterium accolens*. Furthermore, the results showed that interaction of nematode and non-diphtheric *Corynebacterium* might give new insights into aspects of bacterial pathogenicity and mechanisms underlying physiological processes in humans.

INTRODUCTION

Non-diphtherial *Corynebacterium* spp. may be part of the human microbiota. Varied species have been currently reported as the etiologic agent of human infections [1,2,3]. Cases of severe infections and nosocomial outbreaks due to multidrug-resistant (MDR) strains of *Corynebacterium* spp. have been reported in industrialized and developing countries, in immunocompetent and immunocompromised individuals. Moreover, different corynebacterial species have been also described as causal agents of infections with high morbidity and mortality rates. Data reflect that non-toxigenic strains have developed resistance to antibiotics, which makes it difficult for the treatment of infections. So, clinical relevance has been also recognized [4].

C. accolens is increasingly recognized as being medically relevant and has been isolated from a variety of human clinical specimens, including wound drainage, endocervix, blood, and valvular infections. Cases of abscess, granulomatous mastitis, pelvic osteomyelitis, and both aortic and mitral valve endocarditis have been reported [5]. Unfortunately, studies about virulence and prevalence of the infections caused by these microorganisms, either in hospitalized patients or out-patients, are scarce. However, many studies have reported difficulties in treating infections due to nontoxigenic strains of *Corynebacterium* spp. with resistance to antibiotics [6].

The nematode *Caenorhabditis elegans* has been used as a model host to study the pathogenic mechanisms of several human pathogens, including corynebacterial species. There is a growing body of work demonstrating the utility of using *C. elegans* as a model organism to study host–pathogen interactions for a variety of microbial infections. To the best of our knowledge, there are no data on the virulence potential of *C. accolens* towards *C. elegans* reported in the literature.

Therefore, the aim of this study was to report microbiological features and the virulence potential of *C. accolens* strains isolated from nosocomial infections in the mountainous region of Rio de Janeiro, Brazil.

METHODS

Bacterial strains and microbiological features

Origin and microbiological features were investigated *C. accolens* strains (n= 04) analyzed in this study, were displayed in **Table 1**. Experiments were done with microorganisms grown on 5% sheep blood agar medium or brain heart infusion enriched with 1% tween 80 for 48h at 37ºC. Phenotypic profiles were evaluated by colonial morphology, pigmentation, hemolysis, and conventional biochemical assays. Microorganisms were also identified by matrix-assisted laser desorption/ionization-time of flight assays (MALDI-TOF MS; Bruker Daltonics, France). All *C. accolens* strains were preserved in 10% skim milk (Difco Laboratories, USA) with 25% glycerol at -80ºC at the LDCIC/FCM-UERJ (Laboratory of Diphtheria and Corynebacteria of Clinical Relevance / Faculty of Medical Sciences / State University of Rio de Janeiro) [7,8].

Table No. 1: Origin and clinical data of *Corynebacterium accolens* strains used in this study.

Strains	Origin	Gender/ Age	Hospital Sector
29	tracheal lavage fluid	F/74	UTI
59T	tracheal lavage fluid	F/74	UTI
80	tracheal lavage fluid	M/29	UTI
173	tracheal lavage fluid	M/89	UTI

Legend: F, female; M, male.

Antimicrobial susceptibility testing

Antimicrobial susceptibility profiles were determined by the disk diffusion method on Mueller-Hinton agar (MHA) plate (Plast Labor®, Brazil) supplemented with 5% sheep blood using a bacterial inoculum in saline (0.9% NaCl) equivalent to a 0.5 McFarland standard, based on criteria used for *Staphylococcus aureus* according to CLSI except for cephalothin (*Enterobacteriaceae*), ampicillin, amoxicillin-clavulanic acid and imipenem (*Haemophilus* spp.). Intermediate results were considered resistant [9, 10, 11, 12, 13].

Antibiotic disks used (n=14) in this study were purchased from Oxoid (United Kingdom): amoxicillin-clavulanic acid (20 µg), ampicillin (10 µg), cefotaxime (30 µg), imipenem (10 µg), ampicillin-sulbactam (10 µg) and, trimethoprim-sulfamethoxazole (25 µg), clindamycin (2 µg), gentamicin (10 µg), rifampicin (5 µg), ciprofloxacin (5 µg), erythromycin (15 µg), benzylpenicillin (10 U) tetracycline (30 µg) and vancomycin (30 µg). *C. accolens* strains presenting resistance to more than three different classes of antibiotics were classified as MDR [8,13,14].

MALDI-TOF mass spectrometry

The four strains of *C. accolens* were sub-cultured on 5% sheep blood agar (Biorad, France), incubated aerobically for 24–48 h at 37 °C and analyzed by MALDI-TOF-MS using the following protocol: a small amount of a colony was transferred to a metallic MALDITOF MSP 96 plate (Bruker Daltonic GmbH), covered with 1 ml of matrix (a-cyano-4-hydroxycinnamic acid HCCA, 50% acetronitrile, 2.5% trifluoroacetone) and then allowed to visibly dry at room temperature. Each sample was spotted at least in duplicate to achieve proper identification and to verify reproducibility. Measurements were performed with a Microflex mass spectrometer (Bruker Daltonic, France) via the Flex Control software (version 3.0). The spectrum was imported into the BioTyper software (version 2.0; Bruker, Karlsruhe, Germany). Identification score criteria used were those recommended by the manufacturer: a score ≥ 2.000 indicated species-level identification, a score of 1.700–1.999 indicated identification to the genus level, and a score of < 1.700 was interpreted as no identification.

***Caenorhabditis elegans* assays**

Strain and culture conditions

In this study, the strain of *C. elegans* Bristol N2 (wild type WT) was used. It was obtained through the *Caenorhabditis Genetics Center* (Minnesota, USA), being maintained and propagated on *Escherichia coli* OP50 as previously described [15].

Nematode infection model

C. elegans nematodes was maintained on agar plates inoculated with *E. coli* strain OP50 for 6 to 7 days until the worms become starved, indicated by clumping behavior. Subsequently, the nematodes were infected with different *C. accolens* strains, as well as *E. coli* OP50. Infection

of L4 stage larval worms was carried out with 20 µl of each bacterial strain (from an overnight culture) on nematode growth media (NGM) plates. The plates were maintained at 20^{o}C and worms were assessed each day following infection and the dead nematodes were counted and removed every 24 h. For each strain, approximately 60 nematodes were used, and the assays were performed three times. The Kaplan–Meier survival analysis was used, and all statistical analyses were performed with GraphPad Prism 6.0 version, with P values of less than 0.05 considered significant. The following morphological changes in *C. elegans* after bacterial infection were inspected by light microscopy (Nikon C-DSD 230) and photographed: deformed anal region (*Dar*) morphology, abdominal distension, and internal egg hatching ("Worm bagging") [8,11,15].

RESULTS

C. accolens strains recovered from infected patients

During one-year period of this study, 04 clinical isolates were identified as *Corynebacterium accolens*. During laboratorial culture procedures of nosocomial infections samples collected from 214 hospital patients attended at a hospital located in the mountainous region of Rio de Janeiro, Brazil. All *C. accolens* (100%) strains isolated from tracheal lavage fluid samples of hospital patients with clinical signs of infection. Total strains were recovered from patients in numerically predominant colonies in association with *Klebsiella* sp. (n= 2, 50%), *Pseudomonas aeruginosa* (n=1, 25%) or *Candida albicans* (n=1, 25%). *C. accolens* were predominantly isolated from eldery (75%) – 50% from females and 50% from males**.**

MALDI-TOF-MS identification

MALDI-TOF-MS identified all *Corynebacterium* strains to the species level by direct colony testing (scores > 2.000) (**Table 2**).

Table No. 2: Strains identified by MALDI-TOF MS

Strains	Identification	Score
29 / MDR	*Corynebacteriumaccolens*	2.018
59T	*Corynebacteriumaccolens*	2.293
80 / MDR	*Corynebacteriumaccolens*	2.045
173	*Corynebacteriumaccolens*	2.195

C. accolens antimicrobial susceptibility profiles

Antimicrobial susceptibility testing results for 04 *C. accolens* tested strains are presented in **Table 3**. All *C. accolens* were sensitive to the following antibiotics: vancomycin, gentamicin, tetracycline, imipenem, and rifampicin. Two tracheal lavage fluidisolates (50%) were found resistant to penicillin, ampicillin and erythromycin. From a total of 04 *C. accolens* isolated from nosocomial patients with respiratory tract infections, 02 (50%) expressed MDR profiles (29 and 80 strains). MDR *C. accolens* strains were grown in numerically predominant colonies in association with *Klebsiella pneumoniae* (29 strain) and *C. albicans* (80 strains).

Table No. 3: **Antimicrobial resistance profiles of *Corynebacterium accolens* strains isolated from tracheal lavage fluid of hospitalized patients.**

Clinical Sample	PEN	AMP	TET	RIF	GEN	CFX	IMP	CIP	ERY	CLI	VAN	SUT	ASB	AMC
29 / MDR	R	R	S	S	S	S	S	S	R	S	S	S	S	S
59T / MDS	S	S	S	S	S	S	S	S	S	S	S	S	S	S
80 / MDR	R	R	S	S	S	R	S	R	R	R	S	R	R	R
173 / MDS	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Legend: R, resistant; S, susceptible; MDR, multidrug-resistant; MDS, multidrug-susceptible, CLI, clindamycin; GEN, gentamicina; RIF, rifampicin; CIP, ciprofloxacin; ERY, erythromycin; PEN, benzylpenicillin; TET, tetracycline; VAN, vancomycin; AMC, Amoxicillin-clavulanic acid; AMP, ampicillin; CFX, cefotaxime; IMP, imipenem; ASB, ampicillin-sulbactam; SUT, trimethoprim-sulfamethoxazole; MDR, multidrug-resistant; MDS, multidrug-susceptible.

Caenorhabditis elegans survival in response to *C. accolens* infection

Figure 1 displays the frequency (%) of nematode death induced by infection with multidrug-resistant (MDR) and multidrug-susceptible (MDS) *C. accolens* clinical isolates. Data showed that all *C. accolens* strains expressed the ability to infect *C. elegans* and induce morphological changes as well as movement behaviour alterations **(Table 4)**. As demonstrated in **Figure 1**, the clinical strains of *C. accolens* caused a decline in the survival of *C. elegans* when compared to *E. coli* OP50 ($p < 0.05$).

Five days post-infection, death of *C. elegans* nematodes were observed for *C. accolens* (173 strain). Deformed anal region (*Dar*) and abdominal distension were observed in *C. elegans* nematodes post-infection with all *C. accolens* strains (100%, n=4). The Dar phenotype was

observed from first day post-infection with the *C. accolens* strain 173 MDS isolated from tracheal lavage fluid. *Corynebacterium accolens* (strain 173) was the most pathogenic, followed by strain 80 MDR (*C. accolens*) **(Figure 1)**.

All *C. accolens* isolates were also capable of inducing internal egg hatching ("worm bagging") in the nematodes. Similarly, reproductive development and behavior of infected nematodes were found affected, according to the strain tested.

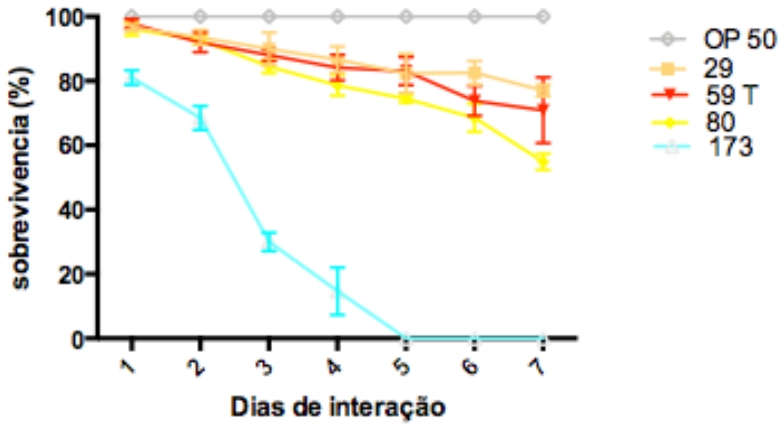

Figure No. 1: ***Caenorhabditis elegans* survival post-infection with the clinical isolates of *Corynebacterium accolens*. *Escherichia coli* OP50 was used as a negative control. The Kaplan-Meier log rank analysis was used to compare survival curves.**

Table No. 4: Morphological changes, reproduction, and survival hability of *Caenorhabditidis elegans* nematodes infected with multidrug-resistant *Corynebacterium accolens* strains.

Strain/ Antimicrobial profile	**Bacteria and nematode interaction (surface)**	**Morphological changes**	**Slower movement behavior**	**Reduction of nematode reproduction**	**Nematode Death (%) in 7 days or less**	
		Abdominal distension	**Deformed anal region**			
C. accolens						
29 / MDR	+	++	++	+	+	≥ 20%
59T / MDS	+	++	++	+	+	≥ 20%
80 / MDR	+	+++	+++	+++	++	≥ 40%
173 / MDS	+	++++++	++++++	-	+++++	100%
E.coli						
OP50 (control)	+	-	-	-	-	< 10%

DISCUSSION

Presently, four strains of *C. accolens* were isolated from tracheal lavage fluid of hospitalized patients. The colonies of this microorganism were small, gray, transparent and nonhemolytic. The growth was enhanced by lipids such as Tween 80. According to literature, *C. accolens* was first described by Neubauer *et al.* in 1991. It is a Gram-positive bacillus considered to be an inhabitant of the upper respiratory tract. It has been isolated from human clinical specimens from sites including wound drainage, endocervix, sputum, throat swab, breast abscess, and valvular vegetation. It has also been isolated from cases of sepsis, otitis media, keratoconjunctivitis, sinusitis maxillaris, and meningitis. *C. accolens* forms small colonies on sheep blood agar, growth is enhanced by lipids, and it also shows satellitism along a *Staphylococcus* strains. It ferments glucose and variably ferments sucrose and mannitol. It reduces nitrate and does not utilize esculin or produce urease. Rigorous efforts to confirm identification are important when these bacteria are isolated from previously unreported sites [16].

Antimicrobial susceptibility testing remains rarely performed on *Corynebacterium* spp. in many laboratories. The method of susceptibility by disk-diffusion is widely used by microbiology laboratories in Brazil and in other countries. Moreover, CLSI guidelines do not provide breakpoints for disk-diffusion while EUCAST document, provides breakpoints for corynebacteria susceptibility testing only for some antibiotics, excluding various importants antimicrobials classes. Thus, many researchers often use staphylococcal breakpoints [1,13,17,18]. The antibiogram might contribute to exposure to multiple-antibiotics and consequently extended hospital stays. The selective pressure exerted by prior antimicrobial treatments might favor the overgrowth of MDR *Corynebacterium* spp. as a secondary colonizer in immunocompromised hosts [21]. In this study, all samples analyzed were sensitive to vancomycin, gentamicin, tetracycline and rifampicin. Two samples (50%) presented a MDR profile.

C. accolens is a microorganism little mentioned in the literature. Ang & Brown (2007) [19] reported the resistant profile for penicillin G and sensitive for vancomycin for *C. accolens* isolated from breast abscess. Nhan *et al.* (2012) [20] reported *C. accolens* from clinical respiratory specimens susceptible to vancomycin, gentamicin, linezolid, amoxylin and imepenem. However, all samples were resistant to erythromycin and with a high percentage of resistance to third generation cephalosporins. Our findings emphasize that *C. accolens*

presented different resistance profiles. Strains 59T and 173 were sensitive to the 14 antimicrobials tested, while strain 29 had a profile resistant to penicillins and macrolides and strain 80 had a MDR profile. Many reports have mentioned that it remains a priority to identify *Corynebacterium* spp. at the species level in clinics [21]. So, its required correct identification of these microorganisms and the continuous surveillance of their antimicrobial resistance patterns.

Corynebacterium spp. have been increasingly recognized as causes of significant human infections [21]. Many infections caused by *Corynebacterim* spp. often remain undiagnosed. Generally, these species grow more slowly on usual culture media, or may be incorrectly classified. The possible involvement of these microorganisms in nosocomial infections should not be underestimated, giving their MDR pattern to frequently used antibiotics [6].

C. elegans has been used as an alternative host for the study of bacterial virulence factors required for pathogenesis in mammalian systems, despite the evolutionary distance and differences in growth temperatures between mammals (37ºC) and nematodes (20ºC) [8,22]. In this perspective, the virulence potential of *C. accolens*, human opportunistic pathogens that grow at 37ºC was verified at a nematode growth temperature (20ºC). According to the current literature, this is the first time that this assay has been performed for these species of corynebacteria.

In the present study, results showed that nematode survival assays indicated that the *C. accolens* strains expressed a high ability of host colonization and killing of *C. elegans*, as previously demonstrated for the toxigenic *Corynebacterium diphtheriae* CDC-E8392 strain [15,23,24]. Interestingly, the strain *C. accolens* 173 strain, susceptible to all tested antibiotics, was more harmful to *C. elegans* compared to the other strains tested, including MDR strains. So, the pathogenicity of *Corynebacterium* spp. for *C. elegans* was not directly related to the MDR profile.

Adult *C. elegans* worms normally lay eggs that hatch outside the parental body, but internal egg hatching, phenomenon called “worm bagging”, was reported to be induced at a high frequency by exposure to pathogenic bacteria. This phenomenon was observed at virulent *E. coli* strains and *Enterococcus faecalis* assays and can be regarded as a reliable population-wide stress reporter [15,25,26]. This finding suggested an adaptive response, as the parental body could provide enough food and physical protection for the small larvae under this condition

[27]. In this study, we can see this phenomenon in all *C. accolens* strains tested, which reaffirms the virulence potential of non-diphtheria *Corynebacteium* strains tested.

Cultures of *C. elegans* have been evaluated of a swollen tail or deformed anal region phenotype (*Dar*) presence, which is the morphological response of some rhabditid nematodes to rectal infection [28]. This response was discovered in laboratory stocks fortuitously infected by a coryneform bacterium, which has been repeatedly isolated in nematode laboratories, but never from nature [28,29]. The morphological alteration *Dar* and abdominal distension were observed in different intensities in all the *C. accolens* strains that we tested.

C. elegans was previously used to investigate harmless and harmful corynebacterial species. *Corynebacterium glutamicum* had significant but minor influence on *C. elegans* survival. On the other hand, *C. elegans* were killed by infection with *C. diphtheriae* and *C. ulcerans*. At a nematode growth temperature (20ºC), *C. elegans* mortality was observed at rates of approximately 20%, 70%, and 90% 5 days after infection with *C. glutamicum, C. diphtheriae,* and *C. ulcerans* strains, respectively [8,15]. In this study, we observed differences in pathogenicity levels in accordance for strain tested. *C. accolens* 173 strain demonstrated ability to kill all nematodes in 7 days of assay. So, results showed that *Corynebacterium* spp. possesses virulence potential towards *C. elegans*.

CONCLUSION

In conclusion, the study highlights the relevance of *C. accolens* as a nosocomial pathogen. MALDI-TOF-MS is a rapid, cost efficient and reliable approach for this specie. The study results showed ability to *C. accolens* to kill the nematode *C. elegans*. Factors associated with morphological changes in corynebacterial-infected nematodes warrant further investigation. Additional studies are necessary, and we will continue this work about *C. accolens* pathogenesis and host immune response.

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Image *Author -1*	***Lorraine Herdy Heggendornn – Corresponding Author*** *Laboratory of Diphtheria and Corynebacteria of Clinical Relevance, The Collaborating Centre for Reference and Research on Diphtheria/National Health Foundation/ Ministry of Health - FNS/MS, Brazil-LDCIC/FCM/ UERJ, Faculty of Medical Sciences, State University of Rio de Janeiro – UERJ.* *Av. 28 de Setembro, 87 - Fundos, 3º andar, Vila Isabel, Rio de Janeiro, RJ CEP 20551-030, Brazil.*
Image *Author -2*	***Sara Wilis Cussuol Gomes*** *Laboratory of Diphtheria and Corynebacteria of Clinical Relevance, The Collaborating Centre for Reference and Research on Diphtheria/National Health Foundation/ Ministry of Health - FNS/MS, Brazil-LDCIC/FCM/ UERJ, Faculty of Medical Sciences, State University of Rio de Janeiro – UERJ.* *Av. 28 de Setembro, 87 - Fundos, 3º andar, Vila Isabel, Rio de Janeiro, RJ CEP 20551-030, Brazil.*
Image *Author -3*	***Luís Guilherme de Araújo Longo*** *Paulo Góes Microbiology Institute (IMPG), Federal University of Rio de Janeiro (UFRJ), Brazil.*

Image Author -4	***Andrew Fernandes Farsura*** *Laboratory of Experimental and Applied Microbiology, Health Institute of Nova Friburgo, Federal Fluminense University, Brazil*
Image Author -5	***Ana Luíza Mattos-Guaraldi*** *Laboratory of Diphtheria and Corynebacteria of Clinical Relevance, The Collaborating Centre for Reference and Research on Diphtheria/National Health Foundation/ Ministry of Health - FNS/MS, Brazil-LDCIC/FCM/ UERJ, Faculty of Medical Sciences, State University of Rio de Janeiro – UERJ. Av. 28 de Setembro, 87 - Fundos, 3º andar, Vila Isabel, Rio de Janeiro, RJ CEP 20551-030, Brazil.*
Image Author -6	***Louisy Sanches dos Santos*** *Laboratory of Diphtheria and Corynebacteria of Clinical Relevance, The Collaborating Centre for Reference and Research on Diphtheria/National Health Foundation/ Ministry of Health - FNS/MS, Brazil-LDCIC/FCM/ UERJ, Faculty of Medical Sciences, State University of Rio de Janeiro – UERJ. Av. 28 de Setembro, 87 - Fundos, 3º andar, Vila Isabel, Rio de Janeiro, RJ CEP 20551-030, Brazil.*
Image Author -7	***Helvécio Cardoso Correa Póvoa*** *Laboratory of Experimental and Applied Microbiology, Health Institute of Nova Friburgo, Federal Fluminense University, Brazil*