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Virulence Potential and Characteristics of Multidrug-Resistant *Corynebacterium amycolatum* Strains Isolated from Nosocomial Infections

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ABSTRACT

The present study demonstrated *Corynebacterium amycolatum* as an emerging MDR nosocomial pathogen with emphasis on recognition of the class A β-lactamase encoding gene. Analysis of phenotypic and genotypic features of virulence mechanisms including *C. elegans* survival response to infection to *C. amycolatum* (imipenem - resistant and susceptible) strains were also investigated. Antimicrobial resistance genes were detected by polymerase chain reaction. We also evaluated the ability of *Corynebacterium amycolatum* in its ability to colonize and kill or host in a *C. elegans* infection model system and we investigated the adherence and invasion persistence of *C. amycolatum* within HEp-2 and U-937 cells. This study identified two *C. amycolatum* strains penicillin resistant and were positive for the *bla*gene, encoding a class A beta-lactamase. The ability to *C. amycolatum* to kill the nematode *C. elegans* was verified. All strains of *C. amycolatum* caused a decline in the survival curve of *C. elegans*. The interaction of nematode and *C. amycolatum* results in morphological changes: *Dar* formation and "bag of worms" in all tested strains. About U-937 macrophages assay, the data indicate that the samples of *Corynebacterium amycolatum* studied had greater ability to adhere to the surface in the first 3 hours of infection of the monolayers ($p < 0.05$). According to current knowledge, this is the first identification of *C. amycolatumbla* gene positive. So, we suggest that medical surveillance programs should include control strategies in order to decrease potential risk factors of nosocomial infections due to *C. amycolatum*.

INTRODUCTION

The genus *Corynebacterium* has more than 136 highly diversified species and 11 subspecies, with at least 50 species already recognized of medical, veterinary, and biotechnological relevance. Nowadays, multidrug-resistant (MDR) and multidrug-susceptible (MDS) *Corynebacterium* spp.strains have been reported with increased frequency as pathogens of severe nosocomial infections and outbreaks in different regions of the world. Nosocomial infections due to *Corynebacteriuma mycolatum* strains have been reported in both industrialized and developing countries, including India, Taiwan, Romania, Spain, and Brazil[1-7].

Antimicrobial resistance is one of the most currently important public health issues. Previous studies demonstrated *Corynebacterium* spp. isolates displaying MDR phenotypes originating either from hospitalized patients or from outpatients who declared no recent hospitalization. Their unusual MDR profiles were also correlated to the frequent antibiotic ambulatory therapy. Moreover, data also suggested the possibility of unrelated nosocomial transmission [1-3, 8,10-11].

Corynebacterium amycolatum have been isolated from bloodstream and invasive medical devices-related infections, endocarditis, septic arthritis, urinary tract infection, peritonitis, breast abscesses, including transplantation recipients, several different types of infection in patients with solid tumors, and fatal premature infant sepsis [2, 4,12-15].

Since *C. amycolatum* is a participant in the microbiota of mucous membrane and skin of humans, this pathogen remains recognized as a contaminant and systematically disregarded during clinical and laboratory diagnosis in many opportunities. Therefore, treatment of infections can be problematic as *C. amycolatum* clinical isolates were also found to express MDR profiles [5, 7, 13, 16-19].

According to current knowledge, further investigation related to resistance to antimicrobial agents and virulence mechanisms influencing the pathogenicity of *C. amycolatum* remains necessary. The present study demonstrated *C. amycolatum* as an emerging MDR nosocomial pathogen with emphasis on recognition of the class A β-lactamase encoding gene. Analysis of phenotypic and genotypic features of virulence mechanisms, including *C. elegans* survival response to infection to *C. amycolatum* (imipenem - resistant and susceptible) strains, were also investigated.

METHODS

Origin of strains

In the present investigation, irregular Gram-positive rods-IGPR clinical isolates were identified, including three *C. amycolatum* strains, during laboratorial culture procedures collected 214 patients with nosocomial infections, during a year period, attended at a hospital located in the mountainous region of Rio de Janeiro, Brazil.

C. amycolatum strains and microbiological features

Origin and microbiological features of partially investigated *C. amycolatum* strains (n= 03) analyzed in this study, were displayed in **Table 1**. Experiments were done with microorganisms grown on 5% sheep blood agar medium for 48 h at 37ºC. Phenotypic profiles were evaluated by colonial morphology, pigmentation, hemolysis and conventional biochemical assays, including catalase, DNAse and CAMP test. Microorganisms were also identified by matrix-assisted laser desorption/ionization-time of flight assays (MALDI-TOF MS; Bruker Daltonics, Wissembourg, France). All *C. amycolatum* strains were preserved in 10% skim milk (Difco Laboratories, USA) with 25% glycerol at -80ºC at the LDCIC/FCM-UERJ (Laboratório de Difteria e Corinebactérias de Importância Clínica / Faculdade de CiênciasMédicas - Universidade do Estado do Rio de Janeiro) [20,21].

The study was approved by the UFF (Universidade Federal Fluminense) Research Ethics Committee (Bioethics Committee number: 2.177.078/67843417.1.0000.5626, process 2.177.078).

Table 1. Origin and clinical data of *Corynebacterium amycolatum* strains used in the study

Strains	Origin	Gender/ Age	Hospital Sector
28	surgical wound	M/64	Post-operative unit
59	tracheal lavage fluid	F/74	UTI
134	vaginal discharge	F/22	Nursery

Legend: F, female; M, male.

MALDI-TOF mass spectrometry

All strains were sub-cultured on 5% horse blood agar (biorad, France), incubated aerobically for 24–48 h at 37^0C 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) and then covered with 1 ml of matrix (a-cyano-4-hydroxycinnamic acid HCCA, 50% acetronitrile, 2.5% trifluoroacetone) and 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, Wissembourg, 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.

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 (Clinical & Laboratory Standards Institute guidelines, 2021) except for cephalothin (*Enterobacteriaceae*), ampicillin, amoxicillin-clavulanic acid and imipenem (*Haemophilus* spp.). Intermediate results were considered resistant [21-24]. Antibiotic disks used (n=14) in this study were purchased from Oxoid (Hampshire, 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. amycolatum* strains presenting resistance to more than three different classes of antibiotics were classified as MDR [21,24].

Molecular detection of resistance genes

The presence of the *bla* gene, encoding a class A β-lactamase involved in resistance to penicillins and cephalosporins in *C.amycolatum*, and *amp*C gene, encoding a class C β-lactamase, were analysed based on previously described methods [8]. Briefly, PCR reaction

was performed in a final volume of 25µL containing 12µL de GoTaq® Master Mix Colorless 2X (Promega, EUA), 2µL of each primer (10µmol/µL), 2µL of DNA, and the remaining volume of water without DNAse. PCR amplification with primer sets *bla* e *amp*C was carried out under the following conditions: an initial cycle of 94ºC for 7 min, and then 35 cycles of 94ºC for 30 seconds, 58ºC for 45 seconds, and 72ºC for 45 seconds, followed by a cycle of 72ºC for 7 minutes. PCR products were resolved by electrophoresis on a 2% agarose gel (Invitrogen®) in 0.5X EDTA tris-borate (TBE) buffer (1X - 89 mM tris, 89 mM boric acid and 0.05 M EDTA [pH 8.2]) at 100V and, stained with ethidium bromide at 1µg/mL (Sigma®). The images of the gels were performed by using ImageQuant LAS 4000 (GE Healthcare Life Sciences®). PCR products were purified using the ExoSAP-IT (ThermoFisher Scientific, EUA), which had a final volume of 21µL containing 15 µL products and 6µL enzyme. The reactions were incubated at 37ºC for 15 minutes, and 80ºC for 15 minutes. Purified DNA was sequenced by Macrogen (Seul, Korea), using the Sanger methodology, using the automatic sequencer ABI3730 (Applied Biosystems). The sequences obtained were analyzed with the Geneious R10 software (Biomatters Ltd., New Zealand) and compared with sequences deposited in NCBI (National Center for Biotechnology Information, USA) using BLAST (https://blast.ncbi.nlm. nih.gov/Blast.cgi).

Table 2. Primers used to amplify *Corynebacterium amycolatum* resistance genes

PCR	Target	Primers	Oligonucleotide Sequence (5'→3')	Product size (bp)
Simplex bla-A	*bla*,	bla-F bla-R	CAGTCTAGCCACTTCGCCAAT TGACTGCACGGATGGAGATGG	808
Simplex AmpC	*ampC*	ampC-F ampC-R	CAATCGGATTCCTGGTCGCT TGGTTCGCGTGATGTTTTCG	965

Amplification and sequencing of 16S rRNA gene

Amplification of 16S rRNA gene was performed according to protocols previously described by Khamis, 2005[25]. Sequencing reactions were performed using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™, USA) following standard protocols. The 16S rRNA gene sequence was compared to type strains sequences available on the EzBioCloud (https://www.ezbiocloud.net) server using the 16S-basedID application, described by Yoon et al., 2017[26].

***Caenorhabditis elegans* assays**

In the present investigation, *C. elegans* N2 was maintained on agar plates inoculated with *Escherichia 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. amycolatum* 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 20ºC or 37ºC 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, aggregates of worms (Worm-star formation) and internal egg hatching (Worm bagging) [21,27].

Bacterial adherence patterns to HEp-2 epithelial cells related to virulence potential

Adherence patterns to HEp-2 lineage (human laryngeal epithelial carcinoma - ATCC CCL-23) of *C. amycolatum* strains were assayed by methods previously described. Adherence assays were performed with semi-confluent HEp-2 cells grown on circular coverslips in DMEM (13 mm diameter; Sigma, USA) supplemented with 5% fetal calf serum (Gibco-BRL, USA), 50 µg/mL gentamicin (Thermo fisher, USA), 2.5 µg/mL amphotericin B (Sigma-Aldrich, USA) and 0.5% L-glutamine (Sigma-Aldrich, USA) at 37°C in a 5% CO_2 atmosphere. Microorganisms were washed twice in 10 mM phosphate-buffered saline (PBS, pH 7.2) and resuspended in DMEM to a concentration of 10^7 colony-forming units per mL, and used in adherence assays (3h incubation). Giemsa-stained coverslips were examined by bright-field microscopy. Microorganisms were evaluated for their ability to display localised (LA; small clusters of bacteria resembling microcolonies), diffuse (DA; bacteria randomly distributed over HEp-2 cells surfaces) or aggregative (AA; clumps of bacteria with a "stacked-brick" appearance) phenotypes [39].

Bacterial adherence and intracellular viability within human macrophage U-937 cell line assays

Presently, aliquots (500 µl) of log-phase bacterial cultures with optical density of 0.2 at λ = 660 nm (MOI of 100) were used in experiments and macrophage cell line U-937 was maintained and differentiated based on methods previously described by Santos et al., 2010[41].

Bacterial adherence and intracellular viability experiments were carried out with U-937 monolayers grown to approximately 95% confluence in the presence of serum-free DMEM. At each incubation time (3 and 6h), supernatants and cell lysates were diluted and plated in Columbia Agar Base (Difco Laboratories, USA) to determine the number of viable bacteria (CFU/ml) and morphological profiles by Gram staining assays.

Statistical analysis

Data of experiment were analyzed by the Tukey test (one-way anova) with Graph Prism. Differences were considered significant when $P \leq 0.05$.

RESULTS

MALDI-TOF-MS Identification

MALDI-TOF-MS identified all *Corynebacterium* strains to the species level by direct colony testing (scores > 2.000) (Table 3).

Table 3. Strains identified by MALDI-TOF MS

Strain	Identification	Score
28	*Corynebacteriumamycolatum*	2.122
59B	*Corynebacteriumamycolatum*	2.152
134	*Corynebacteriumamycolatum*	2.226

C. amycolatum antimicrobial susceptibility profiles

C. amycolatum strains of varied clinical sites expressed MDR-profiles as displayed in **Table 4**. Phenotypic diversity was observed among antimicrobial susceptibility profiles of *C. amycolatum* isolated from nosocomial infections.

Table 4. Data of antimicrobial susceptibility assays and heterogenic antimicrobial resistance profiles of *Corynebacterium amycolatum* strain isolated from hospitalized patients.

Clinical Sample	PEN	AMP	TET	RIF	GEN	CFX	IMP	CIP	ERY	CLI	VAN	SUT	ASB	AMC	Antimicrobial Susceptibility Profiles
28 / surgical wound	R	R	S	S	S	R	R	S	R	S	S	S	R	R	MDR
59B / tracheal lavage fluid	R	R	S	S	S	R	S	R	R	S	S	S	S	S	MDR
134 / vaginal discharge	R	R	S	S	S	R	R	S	R	R	S	S	R	R	MDR

Legend: S, susceptible; R, resistance; CLI clindamycin, GEN gentamicin, 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-resistance;

PCR assay coding for resistance to antimicrobial agents

During experiments of PCR assay standardization primers used to amplify β-lactamase resistance *bla* gene (class A) and *amp*C gene (class C), were displayed in Figure 1.

PCR results revealed the presence of *bla*gene encoding class A beta-lactamase involved in resistance to penicillins and cephalosporins by the following MDR *C. amycolatum* strains isolated from nosocomial infections: 28/surgical wound and 134/vaginal secretion (**Figure 1**). Amplification profiles generated by PCR gave negative results for the *ampC* gene, encoding a class C beta-lactamase.

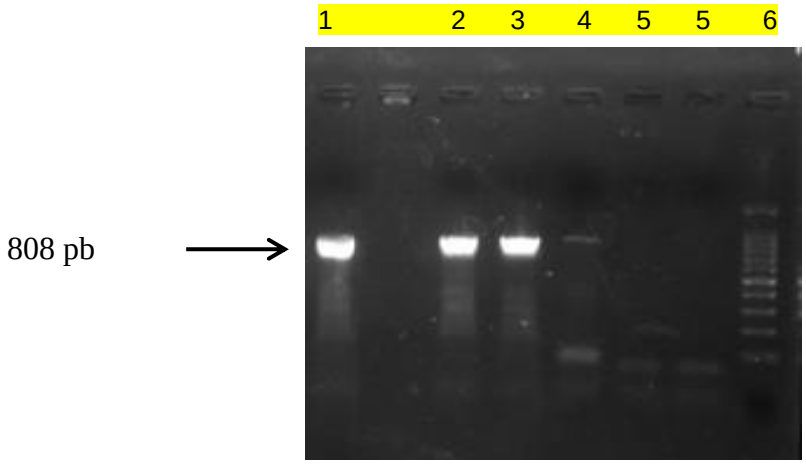

Figure 1 - Agarose gel electrophoresis (2%) of the PCR product of *bla* gene (808 pb) from *Corynebacterium amycolatum* clinical isolates.

Legend: 1: positive control, 2: *C. amycolatum* 28 strain, 3:*C. amycolatum* 134 strain, 4: *Enterococcus faecalis* - negative control, 5: negative control, 6: DNA ladder.

Accession numbers

The accession numbers of 16S rRNA gene sequences and *bla* gene sequences of isolates BR-LH-28 (strain 28) and BR-LH-134 (strain 134) were deposited at GenBank under the accession OP001419 / OP001418 and OP442521 / OP442522, respectively.

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

Data showed that all *C. amycolatum* strains expressed the ability to infect*C. elegans* and induce morphological changes as well as movement behavior alterations. As illustrated in Figure 3 and presented in Table 3, deformed anal region and abdominal distension were observed in *C. elegans* nematodes post-infection with all *C. amycolatum* strains. *C. amycolatum* strains were also capable of inducing internal egg hatching (worm bagging) in the nematodes, but at different levels. Similarly, reproductive development and behavior of infected nematodes were found affected, according to the strain tested (Table 5).

To study putative detrimental effects of *C. elegans* infection, survival of nematodes after infection with *C. amycolatum* strains was determined. As demonstrated in **Figure 4**, the clinical strains of *C. amycolatum* caused a decline in the survival of *C. elegans* when compared to *E. coli* OP50 ($p < 0.05$).

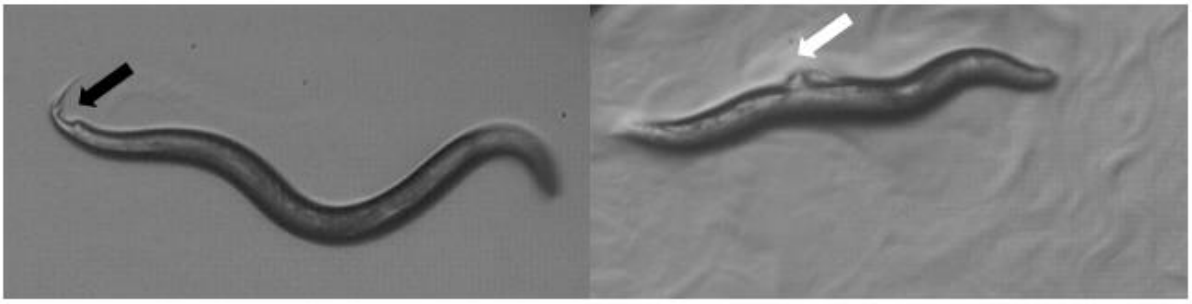

Figure 2. Morphological changes in *Caenorhabditis elegans* induced by infection with the clinical isolates of *Corynebacterium* amycolatum. Anal and abdominal regions of the worm are indicated by the black and white arrows, respectively. Microscopy at 4.5X magnification (bright field).

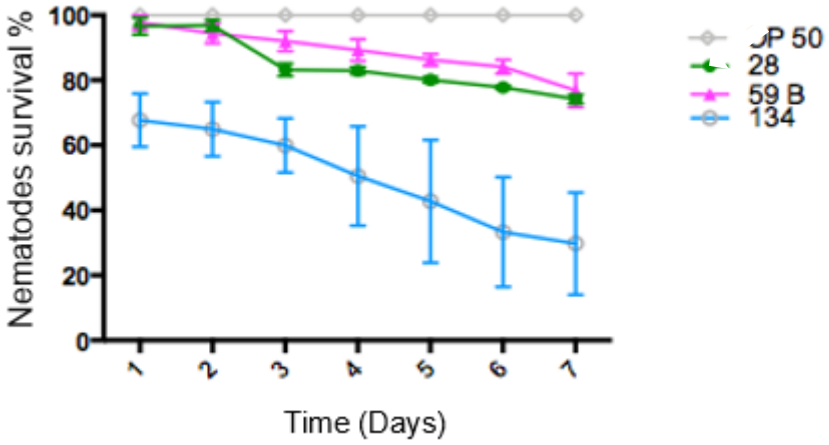

Figure 3.*Caenorhabditis elegans* survival post-infection with the clinical isolates of *Corynebacterium amycolatum*. *Escherichia coli* OP50 was used as a negative control. The Kaplan-Meier log rank analysis was used to compare survival curves. Survival curves were considered statistically different ($p<0.05$).

Table 5. Morphological changes, reproduction and survival ability of *Caenorhabditidis elegans* nematodes infected with multidrug-resistant *Corynebacterium amycolatum* strains.

Bacteria Samples / Origin	Bacteria and nematode interaction (surface)	Morphological changes		Slower movement behavior	Reduction of nematode reproduction	Nematode Death (%) in 7 days
		Abdominal distension	Deformed anal region			
C. amycolatum						
134 / vaginal discharge	+	++++	++++	+++	++	≥ 70%
28 / surgicalwound	+	++	++	+	+	≥ 30%
59B / tracheal lavage fluid	+	+	+	+	+	≥20%
E.coli						
(OP50) control	+	-	-	-	-	≤ 10%

Patterns of adherence to HEp-2 cells

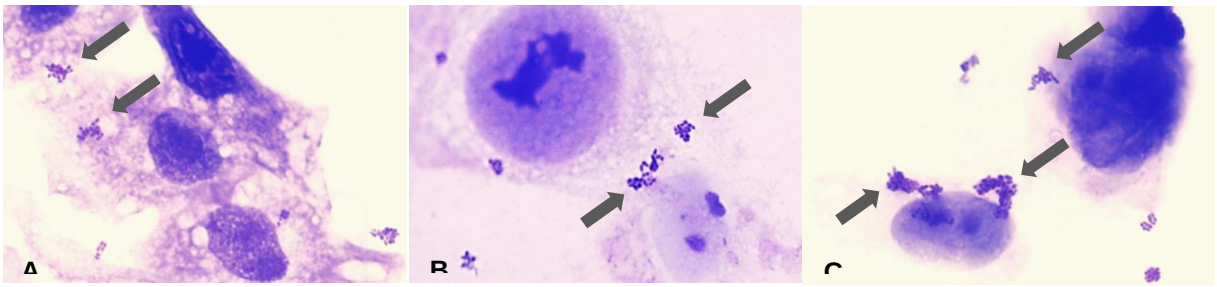

Figure 4. Adherence patterns on human epithelial HEp-2 cells line expressed by multidrug-resistance *Corynebacterium amycolatum* strains isolated from cases of nosocomial infection.

Aggregative pattern were characterize by clumps of bacteria with a “stacked-brick” appearance (narrows). A. Strain 28 B. Strain 59B. C. Strain 134. Magnification: 1,000XAs illustrated in Figure 4, all MDR *C. amycolatum* strains were found capable of interaction with

human epithelial cells (HEp-2 cells line), expressing an aggregative-like pattern 3h post-infection.

U-937 macrophages assay

Qualitative analysis

Corynebacterium amycolatum strains 28, 59B e 134 interacted with macrophages. Different cellular findings were also observed, at the interaction time of 3h (Table 6).

C. amycolatum was found to be capable of surviving in the presence of U-937 macrophages for a period of 3h. After the interaction of *C. amycolatum* strains with U-937 macrophages, a predominance of cells with pycnotic nuclei was observed after 3h infection interval assay.

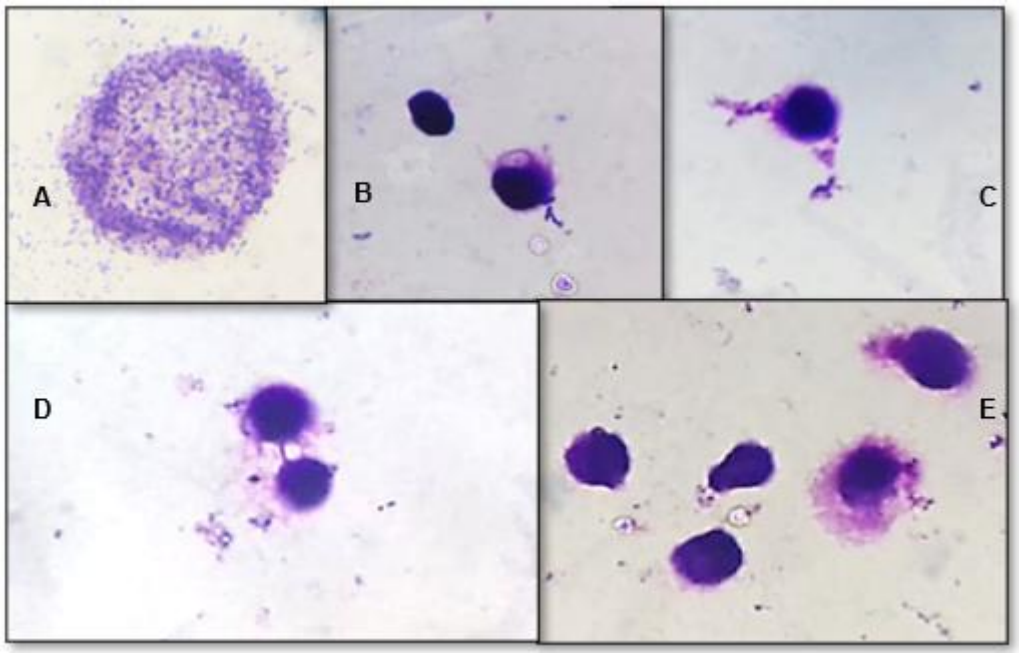

Figure 5. Light micrographs of U-937 macrophages infected with *Corynebacterium amycolatum* strains observed 3h and 6h post-infection. Images obtained by Giemsa staining.

Legend: A and B. *C. amycolatum*3hpost-infection C, D and E. *C. amycolatum* 6hpost-infection. Magnification: 1000X

Table 6 – Analysis of bacterial adherence and survival during interaction human macrophage U-937 cells and phenotypic and genotypic features of virulence mechanisms of multidrug-resistance *Corynebacterium amycolatum* clinical isolates presented as imipenem - resistant and susceptible strains

Strains/ Origin	Presence of a class A β-lactamase gene	*C. elegans* death/ Morphological changes	HEp-2 cells adherence patterns	Bacterial interaction with U-937 cells		
				Adherence 3h post-infection	Adherence levels 3h/6h post-infection (%)	Macrophages features
MDR 28/ Surgical wound	*bla* gene / IMP-R	≥ 30%	Aggregative	+	≥ 40 / ≥ 20	Predominance of cells with pyknotic nuclei
MDR 134/ Vaginal discharge	*bla* gene / IMP-R	≥ 70%	Aggregative	+	≥ 20 / ≥ 5	Predominance of cells with pyknotic nuclei
MDR 59B/ Tracheal lavage	ND / IMP-S	≥20%	Aggregative	+	≥ 10 / ≥ 5	Predominance of cells with pyknotic nuclei

Legend: ND – not determined; IMP-R – Imipenem resistant; IMP-S – Imipenem susceptible.

Quantitative assays

It was observed that all strains tested were able to adhere to U-937 macrophages. About the invasion capacity, a lower proportion was observed. The interaction cinetics profile of *Corynebacterium amycolatum* and U-937 macrophages, are shown in Graph 2.

The data indicate that the samples of *Corynebacterium amycolatum* studied had greater ability to adhere to the surface in the first 3 hours of infection of the monolayers ($p < 0.05$). During this period, the samples of *C. amycolatum* (28, 59B and 134) showed higher percentage values (Graph 2), revealing greater adherence capacity than the other samples tested.

The percentage of invasion of the samples was higher during the first 3 hours of the test, with the sample 59B (*C. amycolatum*) those with the highest percentage of invasion (Figure 6).

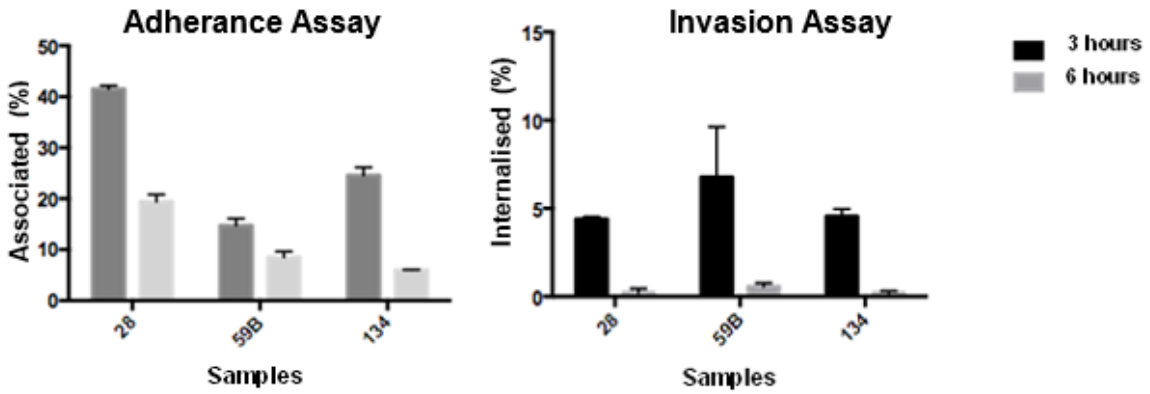

Figure 6– Adhesion cinetics profiles and intracellular viability of *Corynebacterium amycolatum* with U-937 macrophage monolayer. The percentages of viable bacteria associated and internalized in U-937 macrophage monolayers were obtained by counting CFU's. The values presented are averages resulting from tests performed in triplicate and repeated three times. The vertical lines projected above the bars represent standard deviations. Statistically different values, considering p <0.05.

DISCUSSION

Corynebacterium pathogenic species expressing MDR profiles have been reported as etiologic agents of nosocomial infections, especially in patients that have been experienced long hospitalization periods and/or those who have received several courses of antimicrobial agents, as well as immunocompromised patients, including those with acquired immune deficiency syndrome (AIDS), transplant and/or cancer. Antimicrobial resistance profiles have been increasingly verified among *Corynebacterium* spp. strains isolated from infected patients, such as *C. striatum e C. amycolatum* pathogens, and consequently, susceptibility tests are necessary for effective therapeutic procedures [2,16,28].

The current investigation emphasized the relevance of *C. amycolatum* strains, expressing resistance to antimicrobials agents commonly used in clinical practice, as etiologic agents of nosocomial infections regardless of the clinical sites. To the best of our knowledge, this is the first time that MDR *C. amycolatum* expressing class A β-lactamase encoding gene has been recognized as an emerging nosocomial pathogen. Moreover, phenotypic and genotypic features of virulence properties of *C. amycolatum* strains were also verified, especially penicillin-resistant clinical isolates related to the presence of the *bla*-gene encoding for class A beta-lactamase.

Presently, all MDR *C. amycolatum* strains were found resistant to penicillin, ampicillin, cefotaxime, and erythromycin. The dissemination of MDR pathogens in hospital environment has been of concern due to increase in rate of acquired resistance to β-lactam antimicrobials, erythromycin, clindamycin, ciprofloxacin, and gentamycin by *C. amycolatum* and other *Corynebacterium* species [1,2,16,29]. Moreover, increased antibacterial pressure could be the reason behind the emergence of *bla* gene positive from some strains. Selective pressure from naturally occurring beta-lactams and clinically overused beta-lactam-containing drugs has created an environment in which new beta-lactamases readily emerge, together with maintenance of some of the older enzymes [8,30].

Different studies reported an alarming rate of resistance from *Corynebacterium* species to beta-lactams [2,4,24]. Beta-lactams are the most broadly used class of antimicrobials. Hydrolysis of beta-lactam antibiotics by beta-lactamases is the most common mechanism of resistance for this class of antibacterial agents in clinically important bacteria [8]. Resistance to imipenem by *C. striatum* isolates has been reported in some countries, such as Spain [31], Brazil [24] and Italy [32]. However, to the best of our knowledge, this is the first time that resistance to imipenem has been reported for *C. amycolatum* strains. The indiscriminate use of carbapenems in hospitalized patients promotes selective pressure of the human microbiota and favors the survival and dissemination of subpopulations of bacterial cells expressing reduced sensitivity, or resistance to these antimicrobial agents [24,29].

The incidence of antimicrobial-resistant bacterial pathogens isolated from nosocomial infections is a global public health problem, including *Corynebacterium* spp.When *C. amycolatum* infections are linked to MDR strains, they are also difficult to treat. Glycopeptides (VAN), linezolid, and tigecycline are active against most strains, and macrolide (ERI) and fluoroquinolone (CIP) resistance is common [19]. Clinical samples of *C. amycolatum* showing variations in resistance patterns, were currently observed according to the isolation site. *C. amycolatum* (59B) strain, isolated from tracheal lavage fluid, was found to express resistance to fluoroquinolone – ciprofloxacin resistant. According to literature, the sequences encoding the A subunit of the gyrase enzyme in *C. amycolatum* have shown that resistance to fluoroquinolones is associated with mutations of a spontaneous nature in this gene and depends on the number of mutations and the type of amino acid that was exchanged [24,33]. Data are also of current value to health professionals, discouraging the use of these classes of antimicrobial agents for treatment of infections caused by *C. amycolatum* and *C. striatum* pathogens.

In this perspective, this study confirms that MALDI-TOF, 16S rRNA gene sequence and *bla* gene sequencing is an efficient mean for identification of MDR *C. amycolatum* in clinical practice. Reports show that 16S ribosomal RNA sequencing can diagnose infections caused by *Corynebacterium* spp. In infective endocarditis cases antibiotics commencement cannot be delayed before surgery, so surgical specimens culture results are often negative. Therefore, 16S ribosomal RNA sequencing is useful in diagnosing *Corynebacterium* spp infections. Furthermore, the usefulness of 16S ribosomal RNA sequencing has already been reported in the diagnosis of infective endocarditis caused by microorganisms that are difficult to culture like *Mycoplasma* and *Bartonella*. Previous reports have shown that 5-20% of infective endocarditis are culture-negative, and in such cases the causative organism may be identified with 16S ribosomal RNA sequencing [(34)].

Nowadays, *C. elegans* nematode is one of the major invertebrates used in infection model systems, including *Corynebacterium spp.* virulence and pathogenesis investigations [(21,23,27,35)]. *C. elegans* assays were used to distinguish between harmless *Corynebacterium durum and Corynebacterium glutamicum* and potentially pathogenic diphtheria toxin (DT)-producing *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* strains. *C. glutamicum* had minor influence on *C. elegans* mortality, while most of infected nematodes were killed by *C. diphtheriae* and *C. ulcerans*[(27,36)]. Moreover, morphological changes of infected *C. elegans* nematode, such as aggregates of worms, termed as worm-star, and rectal swelling (Dar formation) were also demonstrated by *C. diphtheriae* and *C. ulcerans* strains [(27,37)]. In a previous reported study, chemotaxis behavior, mortality, and morphological changes of *C. elegans* nematodes were also verified by *C. striatum* strains with different MDR profiles [(21)].

To the best of our knowledge, this is the first investigation concerning virulence potential of representative nosocomial clinical strains of *C. amycolatum* expressing MDR profiles using *C. elegans* asan animal infection model. Data showed heterogenic pathogenicity for MDR *C. amycolatum* strains isolated from vaginal discharge, surgical wound and tracheal lavage fluid of patients with nosocomial infections. *C. amycolatum* (134 strain / vaginal discharge) caused significant higher mortality level of nematodes since the first day of *C. elegans* infection, and approximately 70% mortality rates of 7 days post-infection.

Presently, morphological changes (*Dar* and bagging) and mortality at different levels of infected *C. elegans* nematodes were verified for MDR *C. amycolatum* strains isolated from nosocomial infections. Data indicated the virulence potential of *C. amycolatum* towards *C.*

elegans nematodes, similarly to previous reported studies with MDR *C. striatum* nosocomial pathogen [21]. Therefore, as already demonstrated for *C. amycolatum and C. striatum*, nematode survival assays may indicate that non-DT producing *Corynebacterium* spp. pathogens express the ability of host colonization and killing of *C. elegans*, as previously demonstrated for the DT-producing *C. diphtheriae* and *C. ulcerans*.

Investigation of virulence features is necessary, especially for prevention and dissemination of *C. amycolatum* and *C. striatum* strains among nosocomial patients worldwide, including cancer patients. The emergence of MDR *Corynebacterium* pathogenic strains and heterogeneity of MDR profiles in addition to the fact that different clones may be responsible for nosocomial infections and/or outbreaks have been problems of concern [2,21]. Interestingly, the pathogenicity of *C. amycolatum* for *C. elegans* was directly related to the MDR profile, in concordance to data previously described for other pathogens, including *Klebsiella pneumoniae* [38].

Adult *C. elegans* worms normally lay eggs that hatch outside the parental body, but internal egg hatching, an adaptative stress response called 'worm bagging' [27,36]. In this study, 'worm bagging' phenomenon was induced in *C. elegans* nematodes by all MDR *C. amycolatum* strains tested, which confirmed the virulence potential of non-diphtheria toxin producing *Corynebacterium* strains tested.

Moreover, morphological alteration Dar and abdominal distension were observed in different intensities for all *C. amycolatum* strains tested. In previous studies, internal egg hatching (bagging) of *C. elegans* nematodes was observed only during cultivation with *C. striatum* strains, and not with *C. diphtheriae* or *C. ulcerans*. Unlike *C. diphtheriae* and *C. ulcerans*, mortality rates in infected worms correlated with the extent of internal egg hatching (bagging) in *C. elegans* [21,27].

Present data regarding mortality and morphological changes in corynebacterial-infected nematodes warrant further investigation concerning a multiple factorial nature of virulence potential from *C. amycolatum* strains and influence of environmental conditions.

Host cells-pathogens interaction mechanisms may involve an array of virulence factors that facilitate their attachment to mucous surfaces and different types of cells, possibly disseminating to blood and/or tissues and causing invasive diseases. Bacterial adherence to respiratory mucosal and epithelial cell structures is important not only for microbial

persistence in the airways, but also for its systemic dissemination. Bacterial internalization behavior within epithelial cells provides some insight into the overall and strain-dependent pathogenic strategies indicates that such species have developed specialized mechanisms that co-opt normal host cell functions and stimulate their own uptake and adaptation to the intracellular environment. Invasive pathogens are frequently introduced into host cells by endocytosis. Cytoplasmic vacuolization and degradation suggest the death of infected cells, which could provide a route for the dissemination of microorganisms to distal sites, where foci of metastatic infection could be established. Previous molecular biology researches demonstrated the ability of bacterial pathogens to survive within host tissues and confer resistance to clearance by host immune mechanisms and antimicrobial agents, including *Corynebacterium* spp., such as *C. pseudo diphtheriticum* and *C. diphtheriae* strains [(39)].

Therefore, understanding the biology and recognition of virulence mechanisms may help to effectively prevent infections, especially nosocomial varied cases caused by MDR *C. amycolatum* strains. Bacterial adherence to human epithelial and macrophage cells structures is relevant not only for microbial infection ability, survival, persistence in airways and other tissues, but also for hematogenic dissemination.

Additional information is necessary to correlate the aggregative adhesive properties with the pathogenicity and characterize multiple virulence factors of *C. amycolatum* strains as observed for *C. diphtheriae, C. pseudo diphtheriticum, Aeromonas spp.* and enteroaggregative *Escherichia coli* [(39,40)]. It has been demonstrated that *E. coli* strains, which present aggregative adherence, are able to form biofilms. According to literature, biofilm formation enables several pathogens, including aggregative-adhering *C. diphtheriae, C. pseudodiphtheriticum* and *E. coli* strains, to produce more prolonged and/or invasive diseases, possibly leading to fatal outcome [(21,23,39,40)]. Therefore, virulence mechanisms of MDR *C. amycolatum* strains expressing AA-pattern and biofilm formation need further investigation.

It is well known that the interactions between bacteria and macrophages dictate the outcome of most infectious diseases. Macrophages can be heterogeneous and may bind, bind and internalize a bacterium, yet not be able to kill it. Assuming that the most bacteria are killed quite quickly by macrophages, we can conclude that bactericidal assays are the best measured within a few hours after co-incubation of cells and bacteria. Because, the receptors a macrophage uses to internalize bacterial cells may influence the intracellular fate of the

organism [41]. In this perspective, ours results showed that macrophages U-937 could be heterogeneous regarding their ability to kill *C. amycolatum*. Additional studies are necessary in order to investigate the type of membrane receptors involved in this process.

CONCLUSION

The study highlights the relevance of *Corynebacterium amycolatum* as a MDR nosocomial pathogen. Strains penicillin resistant were positive for *bla* gene, encoding a class A beta-lactamase. According to current knowledge, this is the first identification of *C. amycolatum* imipenem nonsusceptible. So, we suggest that medical surveillance programs should include control strategies in order to decrease potential risk factors of nosocomial infections due to *Corynebacterium* spp.

The ability to *C. amycolatum* to kill the nematode *C. elegans* was verified. The interaction of nematode and non-diphtheric *Corynebacterium* results in morphological changes observed might give new insights into aspects of bacterial pathogenicity and mechanisms underlying physiological processes in humans warrant further investigation and we will continue this work about *C. amycolatum* pathogenesis and host immune response.

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