Resistance profile from *staphylococcus aureus* and *pseudomonas aeruginosa* obtained from tracheostomized children in the four seasons of the year

Perfil de resistência de *staphylococcus aureus* e *pseudomonas aeruginosa* obtidos de crianças traqueostomizadas nas quatro estações do ano

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ABSTRACT
Objective: The objective of this study was to microbiologically characterize the tracheal secretion of tracheostomized children, evaluate biofilm formation and study the phenotypic and molecular profile of the antimicrobial resistance of *Sthapylococcus aureus* and *Pseudomonas aeruginosa* isolates. Methods: 88 samples of tracheal secretions were collected. The material was processed to perform phenotypic tests and bacterial identification. Tests were used to identify biofilms using Congo red agar test and microdilution in a 96-well plate, and the qPCR method was used to verify resistance. Results: Twelve *Staphylococcus aureus* samples and 30 *Pseudomonas aeruginosa* were isolated of pediactric tracheostomized patients. All the *S. aureus* samples were positive to biofilm formation in Congo red agar test. In the antibiogram test, *S. aureus* showed resistance to seven antimicrobials. Regarding the identification of resistance genes, *bla*Z was amplified in 57.1% and *mec*A in 28.6% of the isolated *S. aureus*. *Pseudomonas aeruginosa* showed *bla*OXA with 66,7% e *bla*KPC with 58,3%. In plasmid DNA, *bla*NDM stood out with 58,3% positive. Conclusions: The control of resistant bacteria involved in biofilms in the stoma of tracheostomized patients is a great challenge, since

the simple cannula change does not always allow the control of the microbiota, which increases the vulnerability of patients to future respiratory complications.

Keywords: anti-bacterial agents, resistance, beta-lactamases, pediatrics, biofilm.

RESUMO

Objetivo: O objetivo deste estudo foi caracterizar microbiologicamente a secreção traqueal de crianças traqueostomizadas, avaliar a formação do biofilme e estudar o perfil fenotípico e molecular da resistência antimicrobiana de Sthapylococcus aureus e isolados de Pseudomonas aeruginosa. Métodos: Foram coletadas 88 amostras de secreções traqueais. O material foi processado para realizar testes fenotípicos e identificação bacteriana. Testes foram usados para identificar biofilmes usando teste de ágar vermelho do Congo e microdiluição em uma placa de 96 poços, e o método qPCR foi usado para verificar a resistência. Resultados: Doze amostras de Staphylococcus aureus e 30 Pseudomonas aeruginosa foram isoladas de pacientes traqueostomizados pediátricos. Todas as amostras de S. aureus foram positivas para a formação de biofilme no teste de ágar vermelho do Congo. No teste antibiograma, o S. aureus mostrou resistência a sete antimicrobianos. Quanto à identificação dos genes de resistência, o blaZ foi amplificado em 57,1% e o mecA em 28,6% do S. aureus isolado. Pseudomonas aeruginosa mostrou blaOXA com 66,7% e blaKPC com 58,3%. No DNA plasmídeo, o blaNDM destacou-se com 58,3% positivo. Conclusões: O controle de bactérias resistentes envolvidas em biofilmes no estoma de pacientes traqueostomizados é um grande desafio, pois a simples troca de cânula nem sempre permite o controle da microbiota, o que aumenta a vulnerabilidade dos pacientes a futuras complicações respiratórias.

Palavras-chave: agentes antibacterianos, resistência, beta-lactamases, pediatria, biofilme.

1 INTRODUCTION

Tracheostomy consists of a surgical opening in the anterior tracheal wall, into which a tracheal cannula is inserted. Currently, the main indications are prolonged orotracheal intubation (OTI), upper airway obstruction due to craniofacial malformations (such as Robin Sequence, Treacher Collins Syndrome, Beckwith-Wiedemann Syndrome, Nager Syndrome and CHARGE association), stenosis laryngotracheal and hypoventilation associated with neurological diseases, as in cerebral palsy. As congenital and neurological disorders are on the rise, a greater number of tracheostomies are being performed in this population [1].

Due the lack of protection as filtration, humidification, and air heating performed by the upper respiratory tract, tracheostomized patients exhibit high risks of virus and bacterial infections, because the tracheal tube acts as a gateway to these microorganisms [2,3]. The contamination and the consequent lower respiratory tract colonization are facilitated, and the main bacteria found were *Pseudomonas aeruginosa* and

Staphylococcus aureu [4,5]. Both microorganisms exhibit high resistance rates to β-lactams, the most commonly group used in medical routine [6].

In addition, bacteria that are frequently found in tracheostomy tubes are biofilm-forming, such as *S. aureus*, which is often associated with lower respiratory tract infections [3,7,8].

The aim of this research was to study the physiology and antimicrobial resistant profile by phenotypic and molecular methods of *S. aureus* and *P. aeruginosa* obtained from the tracheal secretion of tracheostomized children in the four seasons of the year.

2 **METHODS**

Eighty-eight tracheal secretion samples were collected from 11 tracheostomized children at the Pediatric ENT Clinic, with age between zero months to eleven years old, which were using tracheostomy cannula. At the time of collection, the children were not infected and had no symptoms like fever, increased discharge, productive cough, or any other unusual symptoms. Samples were collected prior to cannula removal and were taken twice each season, during the time of cannula replacement, which was performed between 30 and 45 days, according to the protocol.

The samples were collected from May 2017 to October 2018, the first being performed in autumn and the others in the following order: winter, spring, and summer.

The secretion collection process aspirated the contents with a syringe containing saline solution, coupled to a probe, which was introduced into the cannula, and the saline solution was injected into it. The aspirated contents were deposited in a sterile vial and immediately sent for processing in the laboratory.

2.1 MICROBIOLOGICAL ANALYSIS

Samples were sown in salty mannitol, MacConkey, chocolate, and blood culture medium. Cultures were incubated for 24 hours at 37 °C under aerobic conditions. After this period, the morphocolonial and morphotintorial characterization was performed, as well as biochemical tests to identify isolated colonies, according to Procop and collaborators [9] and the ANVISA manual [10]. After microbiological identification, the presence of each bacterial species was computed once in each season of the year. Biofilm production was analysed according to Freeman et al. [11] and Tendolkar et al. [12], with adaptations.

Biofilm production for *S. aureus* was analysed according to Freeman et al. [11] in culture of samples on BHI agar with 0.08% (w/v) of Congo red dye, plus 5% (w/v) of sucrose. Congo red dye was used as a pH indicator, which shows a black color at intervals between 3.0 and 5.2. The plates were inoculated and incubated at 37°C, under aerobic conditions, for 24 hours. The sample was considered a biofilm producer when colonies showed black or brown color on the surface of the growing medium, and non-biofilm producers when they presented pink, red or intense red coloration.

Biofilm formation for *S. aureus* and *P. aeruginosa* by the plate microdilution method was analysed according to the method of Tendolkar et al.[12], with adaptations. The samples were inoculated in TSB, added with 1% glucose, and incubated for 18 hours. After growth, an aliquot of 50 µl of the sample was diluted in 450 µl of TSB without inoculum, in the proportion of 1:10. Then, 200 µl of the dilution was pipetted, in triplicate, into a 96-well flat-bottomed polystyrene plate, and then incubated at 37°C for 18 hours. Afterwards, the samples were individually aspirated and discarded with a pipette.

Then, each well plate was washed four times with phosphate buffered saline (PBS) buffer solution (pH 7.2). The plate was left to dry at room temperature for one hour. After that, each well received 200 µL of methanol for 15 minutes for fixation, methanol was removed using a pipette and the plate was left at room temperature for drying. Subsequently, 200 µL of 2% crystal violet was pipetted into each well and left for five minutes, then removed and washed three times with distilled water. The plate was left at room temperature until dry. Finally, 200 µL of methanol was pipetted into each well to perform the reading. As a positive control, the *P. aeruginosa* strain PA01 was used and, as a negative control, TSB with 1% glucose without bacterial inoculum.

The reading was performed in an ELISA plate reader (KHB ST-360), with a wavelength of 570 nm. The result of the optical densities of each isolate (ODi) was calculated through the mean of the triplicates, being compared with the mean of the optical density of the negative control (ODc). Biofilm formation intensity was defined as: non-adherent if DOi ≤ DOc; weakly adherent if DOc > DOi ≤ 2 x DOc; moderately adherent if 2 x DOc > DOi ≤ 4 x DOc; or strongly adherent if DOi > 4x DOc (35).

2.2 ANTIBIOGRAM TEST

The *S. aureus* and *P. aeruginosa* samples were subjected to the antibiogram test using the Kirby and Bauer method [13]. For *S. aureus*, Clindamycin resistance induction test, called D test, was performed to detect resistance to Macrolides, Lincosamines and

Streptogramins. For *P. aeruginosa* discs of Ceftazidime (CAZ) 30µg, Imipenem (IPM) 10 µg, Gentamicin (GEN) 10 µg, Ciprofloxacin (CIP) 5 µg, Piperacillin and Tazobactam associated (PPT) 100/10 µg, Aztreonam (ATM) 30 µg and Cefepime (CPM) 10 µg were used. For *S. aureus* Ciprofloxacin (CIP) 5 µg, Cefoxitin (CFO) 30 µg, Gentamicin (GEN) 10 µg, Penicillin (PEN) 10 µg, Clindamycin (CLI) 2 µg, Erythromycin (ERI) 15 µg, Sulfamethoxazole and Trimethoprim associated (SUT) 30 µg, Linezolid (LNZ) 30 µg, Rifampicin (RIF) 5 µg e Tetracycline (TET) 30 µg were used, as recommended by CLSI[14].

It was also evaluated the Oxacillin resistance *mec*A gene mediated. The penicillin halo measurement was considered to evaluate the activity of β-lactamase resistance mediated by *bla*Z gene in *S. aureus*. *Pseudomonas aeruginosa* tests were performed to evaluate the β-lactamases enzymes activity type AmpC, Extended Spectrum β-Lactamase (ESβL), metallo-β-lactamase and carbapenemase, using the disc diffusion method, according to ANVISA methodology [10].

2.3 RESISTANCE GENES AMPLIFICATION

The samples with positive resistance, phenotypic tests were used to plasmid and chromosomal DNA extraction, according to Pharmacia® Flexiprep extraction kit manual.

For *S. aureus*, specific primers *bla*Z and *mec*A genes were designed for one of the authors. The primer for *fem*A was also used gene to confirm the *S. aureus* phenotypic identification, according to store sequences in GenBank [15,16].

For *P. aeruginosa*, specific primers for *bla*VIM, *bla*KPC, *bla*SHV, *bla*OXA, *bla*CMY, *bla*IMP, *bla*NDM e *bla*TEM genes were designed by one of the authors, based on literature [17,18,19,20,21]. The qPCR technique conditions were performed according to the manufacturer's instructions.

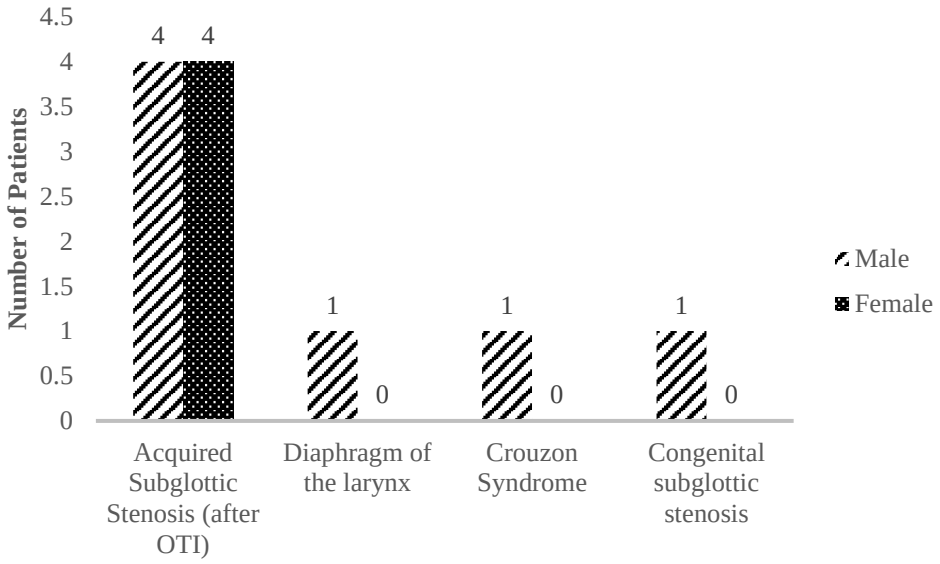
2.4 STATISTICAL ANALYSIS

For percentage analysis and graphs Microsoft Excel (Microsoft Corp., Redmond, WA, USA) was used. To associate the data obtained in the antibiogram test for phenotypic resistance and susceptibility, and the presence or absence of the resistance genes observed along the seasons of the year, the MATLAB script (version 8.1, Natwick, USA) was applied using the test Chi-square.

3 RESULTS

Children aged between one and 11 years old participated in the research, seven males and four females. Regarding the indications for tracheostomy, eight patients were tracheostomized for subglottic stenosis acquired after orotracheal intubation (OTI), of which three were premature. The others had diseases such as the laryngeal diaphragm, Crouzon syndrome and congenital subglottic stenosis, all of them being male (Figure 1).

Figure 1: Indications for tracheostomy according to the sex of 11 tracheostomized children.



OTI: Orotracheal Intubation

From the 88 samples of tracheal secretions, a total of 198 colonies of bacteria were isolated, 96 gram positive and 102 gram negative. For several patients, there was concomitant isolation of more than one type of bacteria.

Among the gram-positive bacteria, *Corynebacterium spp*. stood out, which was isolated in most patients, in all seasons of the year. Coagulase-negative Staphylococcus (CNS) and *S. aureus* were also isolated in all seasons, however in a smaller number of patients.

From the CNS isolates, the following species were identified: *S. epidermidis, S. haemolyticus, S. hominis and S. lugdunensis*. Some CNS had a lower occurrence, with *Planococcus* spp., *S. schileiferi and Stomatococcus* spp. found in one patient, in spring, autumn and summer, respectively (Table 1).

Table 1: Presence of gram-positive bacteria in the tracheal secretions of 11 tracheostomized children, according to the seasons of the year and number of patients.

Microorganisms	Number of Patients				Total of Samples
	Autumn	Winter	Spring	Summer	
Corynebacterium spp.	8	10	8	8	34
CNS	9	3	6	10	28
Staphylococcus aureus	4	3	2	3	12
Bacillus spp.	1	1		1	3
Microbacterium spp.			3		3
Staphylococcus hyicus		1	2	1	4
Staphylococcus intermedius		3	2	2	7
Micrococus spp.	1		1		2
Planococcus spp.			1		1
Staphylococcus schileiferi	1				1
Stomatococcus spp.				1	1
Total	**24**	**21**	**25**	**26**	**96**

As for the gram negatives, *P. aeruginosa* was the most prevalent, being present in most patients, in all seasons of the year. *Citrobacter* spp, *Enterobacter aerogenes* and *Klebsiella pneumoniae* also appear in the four seasons, but in a lower number of patients. *Acinetobacter Iwoffii, Enterobacter agglomerans, Klebsiella ascorbata, Plesiomonas* spp., *Serratia grimesii, Serratia marcescens, Serratia odorífera and Tatumella cítrea* were bacteria that appeared only once in different seasons (Table 2).

Table 2: Presence of gram-negative bacteria in the tracheal secretions of 11 tracheostomized children, according to the seasons of the year and number of patients.

Microorganisms	Number of Patients				Total of samples
	Autumn	Winter	Spring	Summer	
Pseudomonas aeruginosa	9	8	6	7	30
Moraxella spp.	4	1	5		10
Citrobacter spp.	2	2	1	1	6
Enterobacter aerogenes	2	1	2	1	6
Klebsiella pneumoniae	1	1	1	1	4
Morganella morganii			3	1	4
Yersinia aleksiciae		1	1	2	4
Enterobacter cloacae	1	2			3
Escherichia coli	1	1		1	3
Hafnia alvei			2	1	3
Klebsiella oxytoca	1		2		3
Proteus vulgaris			1	2	3
Providencia spp.		1	2		3
Serratia entomophila			2	1	3
Serratia liquefaciens	1		1	1	3

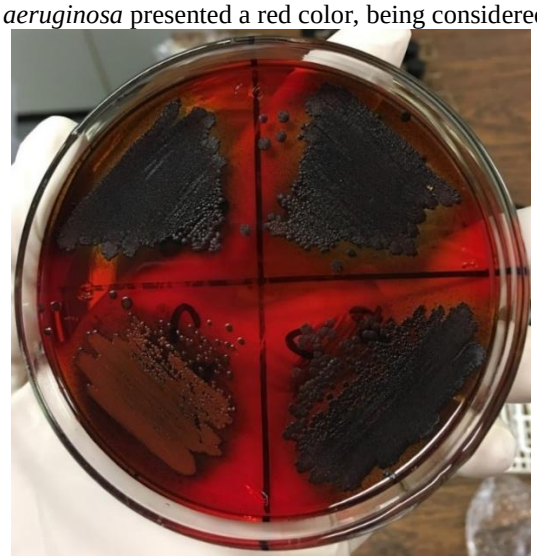
Serratia rubidae	1			1	2
Sphingomonas spp.	1	1			2
Stenotrophomonas maltophilia	1	1			2
Acinetobacter lwoffii	1				1
Enterobacter agglomerans			1		1
Klebsiella ascorbata				1	1
Plesiomonas spp.	1				1
Serratia grimesii				1	1
Serratia marcescens	1				1
Serratia odorifera	1				1
Tatumella citrea				1	1
Total	**29**	**20**	**30**	**23**	**102**

For the phenotypic tests for biofilm detection, the species of clinical importance that were more frequent in patients and that were present in all seasons of the year were chosen. Thus, biofilm detection was performed only in 12 samples of *S. aureus* and 30 of *P. aeruginosa*.

Despite *Corynebacterium* spp. being the most prevalent, the phenotypic test was not performed to detect the biofilm, as this bacterium is not commonly associated with respiratory infections. Regarding the SCN, although they were also prevalent, when evaluated separately, they were not isolated in all seasons of the year.

In tests using Congo red agar, only *S. aureus* isolates (12) were tested, being 100% positive, therefore, all biofilm formers (Figure 2).

Figure 2: *Staphylococcus aureus* cultured on Congo red agar for detection of biofilm formation. Colonies that presented black color and dry appearance were considered positive (A, B, C); colony of *Pseudomonas aeruginosa* presented a red color, being considered negative (D).

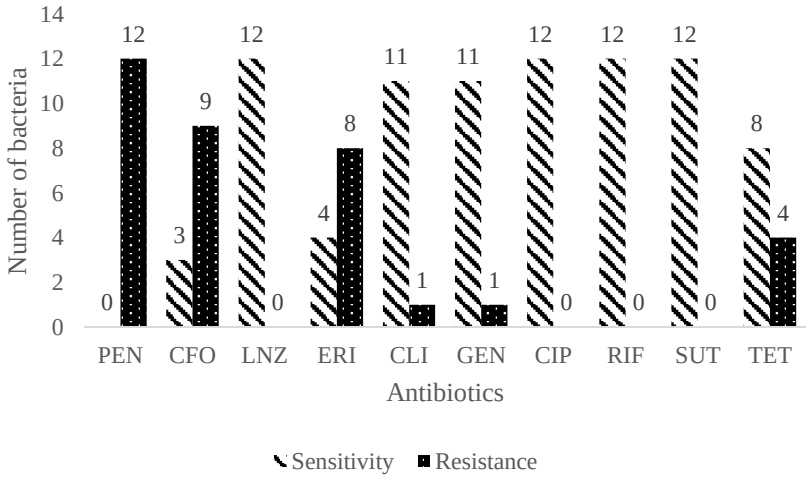


The microplate biofilm formation test was performed on 42 bacterial samples, of which 12 were *S. aureus* and 30 *P. aeruginosa*, and 100% of the samples were positive.

As for the biofilm adhesion intensity, *S. aureus* isolates were 8% (1/12) weakly, 42% (5/12) moderately and 50% (6/12) strongly adherent. As for the *P. aeruginosa* isolates, 50% (15/30) were weakly, 20% (6/30) moderately and 30% (9/30) strongly adherent.

The antibiogram tests performed on 12 *S. aureus* and 30 *P. aeruginosa* samples showed resistance to various antibacterial used in medical routine, especially *S. aureus* 100% (12/12) resistant to penicillin, 75% (9/12) to cefoxitin and 66,7% (8/12) to erythromycin (Figure 3). As for *P. aeruginosa*, resistance to aztreonam 56.7% (17/30) and imipenem 30% (9/30) stood out (Figure 4). In the phenotypic test to evaluate β-lactamase activity in *P. aeruginosa*, all were negative for ESβL, metallo-β-lactamase and carbapenemase, but 100% positive for AmpC.

Figure 3: Susceptibility profile of 12 samples of *Staphylococcus aureus* isolated from tracheal secretions of pediatric tracheostomized patients.



Subtitle: **PEN:** Penicillin; **CFO:** Cefoxitin; **LNZ:** Linezolide; **ERI:** Erythromycin; **CLI:** Clindamycin; **GEN:** Gentamicin; **CIP:** Ciprofloxacin; **RIF:** Rifampicin; **SUT:** Sulfamethoxazole + Trimetoprim; **TET:** Tetracycline.

It can be observed that in some cases it was possible to recover *S. aureus* and *P. aeruginosa* from the same patient in successive collections, which presented a similar susceptibility profile to the tested antimicrobials, as can be seen in tables 3 and 4.

Table 3: *Staphylococcus aureus* samples isolated from tracheal secretions of pediatric tracheostomized patients showing similar susceptibility profiles to the antimicrobials tested at different collection times.

Patient	Collect	Antibiotics										
		CIP[1]	CFO[2]	CLI[3]	ERI[4]	GEN[5]	LNZ[6]	PEN[7]	RIF[8]	SUT[9]	TET[10]	D-TEST
2	2ª Spring	S[11]	R[12]	S	R	S	S	R	S	S	S	+[13]
	1ª Summer	S	R	S	R	S	S	R	S	S	S	+
	2ª Summer	S	R	S	R	S	S	R	S	S	S	+
5	1ª Summer	S	R	S	R	S	S	R	S	S	S	+
	2ª Summer	S	R	S	R	S	S	R	S	S	S	+
11	2ª Autumn	S	S	S	S	S	S	R	S	S	R	-
	1ª Winter	S	S	S	S	S	S	R	S	S	R	-
	2ª Winter	S	S	S	S	S	S	R	S	S	R	-

[1]Ciprofloxacin, [2]Cefoxitin, [3]Cindamycin,[4] Erythromycin, [5]Gentamicin, [6]Linezolide, [7]Penicillin, [8]Rifampicin,[9] Sulfamethoxazole + Trimetoprim, [10]Tetracycline [11]Susceptibility [12]Resistance, [13]Positive test.

Table 4: *Pseudomonas aeruginosa* samples isolated from tracheal secretions of pediatric tracheostomized patients showing similar susceptibility profiles to the antimicrobials tested at different collection times.

Patient	Collect	Antibiotics							
		ATM[1]	CPM[2]	CAZ[3]	CIP[4]	GEN[5]	IPM[6]	PPT[7]	AmpC[8]
5	1ª Summer	R[9]	S[10]	S	S	S	S	S	+[11]
	2ª Summer	R	S	S	S	S	S	S	+
9	Autumn	R	S	S	S	S	S	S	+
	Winter	R	S	S	S	S	S	S	+
10	Summer	S	S	S	S	S	R	S	+
	Autumn	S	S	S	S	S	R	S	+
	Winter	S	S	S	S	S	R	S	+

[1]Aztreonam, [2]Cefepime, [3]Ceftazidime, [4]Ciprofloxacin, [5]Gentamicin, [6]Imipenem, [7]Piperacillin-tazobactam, [8]AmpC type β-lactamase, [9]Resistance, [10]Sensibility [11]Positive test.
[1]Aztreonam, [2]Cefepime, [3]Ceftazidime, [4]Ciprofloxacin, [5]Gentamicin, [6]Imipenem, [7]Piperacillin-tazobactam, [8]AmpC type β-lactamase, [9]Resistance, [10]Sensibility [11]Positive test.

Gene amplification tests were performed on seven isolates of *S. aureus* and 12 *P. aeruginosa*, which were selected based on a greater number of resistances detected in the phenotypic tests.

The *fem*A gene was detected in 100% (7/7) of the *S. aureus* samples. As for β-lactam resistance genes, in 57.1% (4/7) of *S. aureus* the *bla*Z gene was detected in both plasmid DNA and chromosomal DNA. The *mec*A gene was amplified in 28.6% (2/7) isolates (Table 5).

Table 5: Amplification of *fem*A, *bla*Z and *mec*A genes in *Staphylococcus aureus* samples isolated from tracheal secretions from tracheostomized children.

Isolates	**Gene Localization**	**Genes**		
		***fem*A**	***bla*Z**	***mec*A**
1	P		+	
	C	+	–	–
2	P		+	
	C	+	+	–
3	P		–	
	C	+	–	+
4	P		–	
	C	+	–	–
5	P		–	
	C	+	–	+
6	P		+	
	C	+	+	-
7	P		–	
	C	+	+	–

Subtitle: **P:** Plasmid; **C:** Chromosomal; **+:** Gene presence; **–:** Gene absences.

Based on the resistance found in *P. aeruginosa* antibiogram test, the literature search showed 12 genes responsible for β-lactams resistance. The genes description and their resistances are described in chart 1.

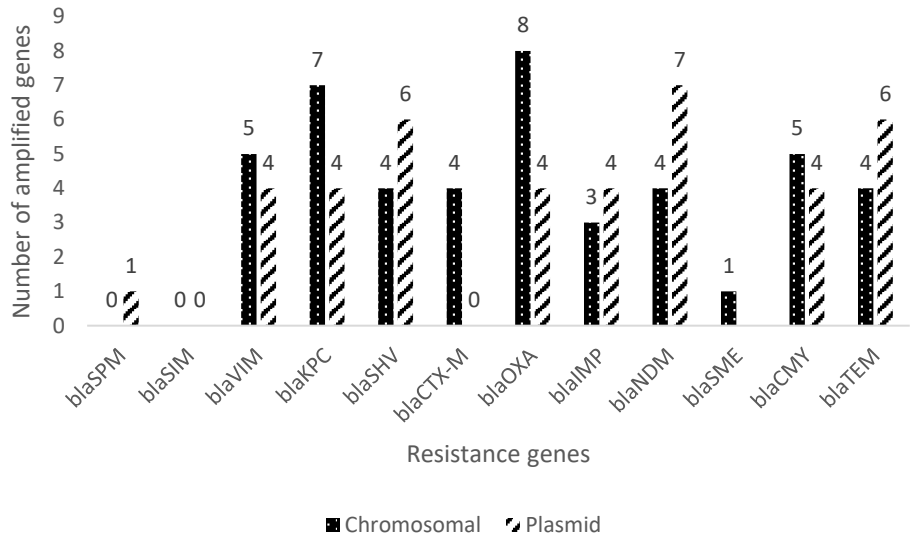
Chart 1: *Pseudomonas aeruginosa* resistance to β-lactams according to the genes described in the literature.

Antibiotics	**Gene Localization**	**Resistance Genes**											
		***bla*SPM**	***bla*SIM**	***bla*VIM**	***bla*KPC**	***bla*SHV**	***bla*CTX-M**	***bla*OXA**	***bla*IMP**	***bla*NDM**	***bla*SME**	***bla*CMY**	***bla*TEM**
ATM – AZTREONAM	P	-	-	+	+	+	-	+	-	+	-	+	+
	C	-	-	+	+	+	+	+	-	+	-	+	+
IPM – IMIPENEM	P	+	+	+	+	-	-	+	+	+	-	-	-
	C	-	-	+	+	-	-	+	+	+	+	-	-
CPM – CEFEPIME	P	-	-	+	+	-	-	+	+	-	-	-	+
	C	-	-	+	+	-	+	+	+	-	-	-	+
CAZ – CEFTAZIDIME	P	+	-	+	+	+	-	+	+	+	-	+	+
	C	-	-	+	+	+	+	+	+	+	-	+	+
PPT – PIPERACILLIN + TAZOBACTAN	P	-	-	+	+	+	-	-	-	-	-	+	+
	C	-	-	+	+	+	+	-	-	-	-	+	+

Subtitle: **P:** Plasmid; **C:** Chromosomal; **+:** Gene presence; **- :** Gene absence. Source: JACOBY et al. (2005), LEE et al. (2005), WALSH et al. (2005), PICOLI (2008), DOGONCHI et al. (2017).

Among the 12 *P. aeruginosa* samples studied, it was possible to amplify β-lactam resistance genes in all of them, 10 in chromosomal DNA and nine were found in plasmid DNA. In the samples tested, the most frequent chromosomal genes were *bla*OXA with 66.7% (8/12), *bla*KPC with 58.3% (7/12), *bla*VIM and *bla*CMY with 41.7% (5/12) positivity. For plasmid DNA, the most frequent were *bla*NDM with 58.3% (7/12), *bla*SHV and *bla*TEM with 50.0% (6/12) of positivity (Figure 5).

Figure 5: Amplification of resistance genes in samples of *Pseudomonas aeruginosa* isolated from pediatric tracheostomized patients.



When evaluating the types of genes amplified by each bacterium studied, the presence of the same types in both chromosomal and plasmid DNA stands out, as observed in samples D, G and J (Chart 2).

Chart 2: Amplification of Pseudomonas aeruginosa resistance genes isolated from tracheostomized pediatric patients, according to DNA origin.

Isolated	Gene Localization	Resistance Genes												Total of genes
		***bla*SPM**	***bla*SIM**	***bla*VIM**	***bla*KPC**	***bla*SHV**	***bla*CTX-M**	***bla*OXA**	***bla*IMP**	***bla*NDM**	***bla*SME**	***bla*CMY**	***bla*TEM**	
A	P			+	+	+						+	+	5
	C			-	-	-	-					-	-	0
B	P			-	-	+		-		-		-	-	1
	C			+	+	+	+	+		+		+	-	7
C	P	-	-	-	-			+	-	+				2
	C			+	+			+	+	-	-			4
D	P	-		-	-	+		-	+	+		+	+	5
	C			+	+	+	+	+	+	+		+	+	9
E	P	-	-	-	-			-	-	-				0
	C			-	+			+	-	-	+			3
F	P			-	-	-		-		+		-	-	1
	C			-	+	-	-	-		+		+	+	4
G	P	-		+	+	+		+	+	+		+	+	8
	C			+	+	+	+	+	-	-		+	+	7
H	P	+	-	+	-	+		+	-	+		+	+	7
	C			+	+	-	+	+	+	-	-	-	-	5
I	P			-	+	+		-		+		-	-	3
	C			-	-	-	-	-		-		+	-	1
J	P	-		+	-	-		+	+	+		-	+	5
	C			-	-	+	-	+	-	+		-	+	4
	P	-	-	-	+			-	+	-				2

K	C			-	-			-	-	-	-			0
L	P			-	-	-		-		-		-	+	1
	C			-	-	-	-	+		-		-	-	1

Subtitle: **C:** Chromosomal; **P:** Plasmid; **+:** Gene presence; **-:** Gene absence.
Source: BUSH; JACOBY (2010).

4 DISCUSSION

The patients of the presente study were newborn children up to eleven years old. Despite advances in pediatric tracheostomy indications, its incidence has not decreased in recent years. It is believed that this is related to the increased survival of patients admitted to pediatric and neonatal intensive care units, since congenital and neurological disorders are on the rise [1,22].

One of the main consequences of prolonged intubation is the occurrence of subglottic stenosis, since the presence of the tube in the region induces a healing process and collagen formation. After removal of the tube, the wall contracts, promoting stenosis, which can be partial or complete [23]. In the present study, in 72.2% (8/11) of the cases, the tracheostomy procedure was performed after OTI, which is consistent with the data reported in the literature.

Most of the patients were male, which is similar to those found in the literature, which demonstrate this predisposition. As for the occurrence of genetic or acquired diseases, which require tracheostomy, the literature also reports a higher occurrence in male children, similar to what was observed in the present study [8,21,24].

Studies report the importance of the technique to increase the survival of preterm infants, since the maturation of the fetal lung, as well as of several organs, are directly related to the gestational age [8,25]. Several physiological factors influence the form and response of ventilation, which makes preterm infants different in terms of oxygen requirements when compared to other age groups. As seen in this study, prematurity was present in 27.3% (3/11) of the patients, which allowed an improvement in pulmonary ventilation, as well as in quality of life.

The microbiological analysis of tracheal secretions detected the presence of a diversity of bacterial species, which can be explained by the presence of the device, especially when its use is prolonged. In these cases, the cannula can irritate the trachea and facilitate bacterial colonization, predisposing the development of secondary respiratory infections [7,21,24,26].

Among the microbiological findings, *S. aureus* and *P. aeruginosa* were the most prevalent bacteria of clinical importance, which can be seen as a risk for the worsening of the patients' condition, making them more vulnerable to IRT infections. Other studies with tracheal secretions also reported the two species as the most commonly isolated in this type of sample [6,21,24,26].

Pseudomonas aeruginosa is a bacterium that is easy to adapt to any type of environment, in addition to being an opportunistic pathogen that causes several serious infections such as pneumonia associated with mechanical ventilation [27]. Commonly, *S. aureus* is another bacterium also isolated in these conditions, as it is a skin colonizer, which can cause acute infections such as bacteremia and skin abscesses, as well as chronic infections [28].

It is known that there is an interaction between different microorganisms in the airways, and some studies report the concomitant infection by *P. aeruginosa* and *S. aureus*. In a study with patients diagnosed with cystic fibrosis, it was observed that the presence of co-infection by these two pathogens allowed a greater involvement of the lungs, as well as the need for the use of intravenous antibiotics, in addition to high mortality rates [29]. This demonstrates that the two bacteria in the same ecological niche have mechanisms that help in the progression of lung disease.

In these studies, the authors also observed that in some patients *P. aeruginosa* inhibited the growth of *S. aureus* during the growth and stationary phases, demonstrating that the former competed with the latter in the use of iron for its growth [29,30]. This data can be correlated with the fact that in four patients, the isolation of *S. aureus* was not observed, but only of *P. aeruginosa*, which was more prevalent. However, in the studies by Baldan et al. [29] and Filkins et al. [30] it was demonstrated that mutant strains of *S. aureus* can protect themselves from the attack of *P. aeruginosa*, also reinforcing the findings of the present study, since in five different patients it was possible to isolate the two species at the same time.

Regarding seasonality, no significant variation was observed in terms of microbiota diversity throughout the year. This finding was similar to that one described by Perez-Losada et al. [3], who also did not observe any difference in the microbiota of tracheal secretions during the seasons. It was expected to find changes in the microbiota mainly in winter, but the absence of this variation can be explained by the location of the tube, since it was in the trachea. Studies show that the colonization of the trachea and the TRI differs when compared to the TRS, where there is in fact a diversity of microbiota, which can be influenced by seasonality, due to greater contact with the external environment, as well as the presence of anatomical structures that contribute to microbiological control.[31] In addition, other factors influence the control of this microbiota, such as immunological mechanisms present in the upper airways, whose action is reduced in tracheostomized patients [32].. The cannula microbiota will be

influenced by different factors, such as time of exchange, care such as cleaning and sanitizing the cannula [31,33].

Regarding the detection of biofilm production, in the Congo red test, all *S. aureus* samples were biofilm producers. Despite being an easy test to perform, this method is not accurate, and its reading is subjective [34]. Also, the specific mechanism involved in the method is not yet known for sure. Lima et al. [35] suggest that the positivity on Congo red agar for *S. aureus* occurs due to the polysaccharide constitution of the extracellular matrix of the biofilm, since the dye binds to the polysaccharides present in the matrix of the biofilm, giving the colonies a blackish color [28].

The negativity of biofilm formation for *P. aeruginosa* by the same method may be associated with the deficiency of the *Pel* gene, which is responsible for the formation of the extracellular matrix abundant in polysaccharides, which would make it impossible for Congo red to bind to the matrix, with no the modification of the colonies' color [35]. Thus, the Congo red agar test is not as effective in detecting the production of biofilm, as the deficiency in the production of a matrix rich in polysaccharide in this type of agar, in some bacteria, can mask the result, which can be negative, even if the bacteria have the potential to form biofilms. Therefore, there is a need to also perform the test on a microplate.

In the microplate test, all the tested isolates were able to form biofilms, however at different intensities. In a study carried out by Lima et al. [35], 75% of *P. aeruginosa* isolates were positive for biofilm formation, being 40% weakly, 25% moderately and 10% strongly adherent. In another study, 67% of clinical isolates were biofilm formers, of which 92% were weakly adherent and 8% were moderately adherent [36]. The results described in the literature are similar to those of the present study, in which the weak adhesion of the biofilm was predominant.

The positivity in this test is explained by the various mechanisms involved in the formation of the *P. aeruginosa* biofilm, mainly by the constitution of polysaccharides in its matrix, such as *Psl*, *Pel* and alginate, which have primordial roles in the adhesion, maturation, protection and structuring of the biofilm. There is also the quorum sensing (QS) mechanism, which is of great importance for the formation of biofilms by *P. aeruginosa* [37].

In the present study, the number of strongly adherent isolates of *S. aureus* was higher than in other studies such as the one by Stepanovic et al. [37], in which the authors obtained 14 isolates, 14% being weakly adherent, 57% moderately and 28% strongly

adherent. In another study, Neopane et al. [38] found 69.8% of the biofilm-forming isolates, with 34.88% being weakly adherent, 27.90% moderately and 6.97% strongly adherent. The strong adherence of *S. aureus* isolates can be explained by the presence of the surface polysaccharide adhesin intracellular polysaccharide (PIA), which plays an important role in the interaction of the cell with abiotic surfaces, this adhesin being encoded by genes, such as the locus of intercellular adhesion (ica) [28,38].

The resistance detected in both *P. aeruginosa* and *S. aureus* samples is worrying, as these bacteria are inserted in an environment conducive to genetic exchange and perpetuation in the colonized site. The detection of these two species in successive collections from the same patient, with a similar susceptibility profile to antimicrobials, reinforces the hypothesis of microbial persistence, even with the cannula being changed once a month.

Studies have shown that biofilm formation by P. aerugiona and *S. aureus* on abiotic surfaces occurs within three days [39,40]. Once formed, its removal is a challenge, not just removing the device. Numerous strategies have been proposed to control biofilms on biotic surfaces, such as using phages and/or phage-derived products with other antimicrobial agents, such as antibiotics, nanoparticles, and antimicrobial peptides [41].

According to the literature, there is a consensus that among the care for tracheostomized patients is the daily or more frequent cleaning of the stoma, depending on the climatic conditions and general health of the child, as well as the excessive presence of secretions or local complications. This care aims at greater control of colonization by microorganisms in the stoma, as the site becomes a direct gateway to the lower respiratory tract. In addition to daily cleaning, it is necessary to change the cannula every 30 days or according to the manufacturer's recommendations [22].

In the present study, cannula replacement was recommended with an interval of 30 days. After removal, a new cannula was introduced through the stoma. The benefit of this exchange can be observed, for example in patient eight, because in all collections there was a change in the microbiota, and the isolation of *P. aeruginosa* and *S. aureus* was not constant throughout the collections. This fact may also be associated with better conditions of daily cleaning of the patient's stoma.

However, it was possible to observe that, in patients one and 11, *P. aeruginosa* and *S. aureus*, respectively, were present in all collections, which may indicate that the simple exchange of the cannula is not enough to remove all the bacteria that are colonizing the stoma, especially when surrounded by biofilm. Even if the daily cleaning of the stoma

is done correctly, it will hardly eliminate the biofilm that has already formed. For this reason, despite the benefits, the use of a tracheostomy cannula can result in complications, such as peristomal infectious processes.

Due to the occurrence of peristomal infectious processes, the culture of tracheal secretion is recommended before the surgery for reconstruction of the airways for the targeted choice of antibiotic therapy in the postoperative period. One of the most feared complications of surgery is exactly the infection and dehiscence of the sutures.

In Brazil, the factor that most impacts this frequent exchange is the lack of free availability of cannulas for patients by the Unified Health System, as the patient himself ends up having to bear this cost and does not always have the financial resources to do so [24].

The association of microorganisms in a self-produced extracellular matrix creates an environment that confers bacterial tolerance and antibiotic resistance by different mechanisms that depend on factors such as biofilm composition, architecture, biofilm development stage, and growth conditions [42,43].

The biofilm structure makes it difficult for antibiotics to penetrate and prevents the accumulation of bactericidal concentrations throughout the biofilm. Another important point is that nutrient and oxygen dispersion gradients within the biofilm generate different metabolic states of individual cells and support the development of antibiotic tolerance as well as bacterial persistence [42,43].

In addition, bacterial resistance can develop through the expression of efflux pumps, the occurrence of induced mutation, the presence of extracellular DNA, and close contact between bacterial cells of different species that favor horizontal gene transfer [42,43].

The presence of resistance genes, as shown in Table 4 and Chart 4, in samples of *S. aureus* and *P. aeruginosa*, respectively, points to the great challenge to control the biofilm formed in tracheostomized patients, who need to remain with the device for a long period.

For this reason, more research should be carried out to propose guidelines that will serve to guide health professionals regarding the care of this patient profile, in order to control the bacterial species involved in infectious processes and minimize possible complications arising from this type of procedure [33].

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Declaration of Competing Interest
The authors have no competing interests to declare that are relevant to the content of this article.

Ethical Approval
The research project was approved by the Ethics Committee of the Clinical Hospital of the Federal University of Goiás (CEP/HC/UFG), by number 32091014.6.1001.5078.

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