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Keywords: *enterobacteriaceae, oral cavity, saliva, adolescents.*

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I. Introduction

The microflora of the mouth is different from that present in other sites of the body because it owns exclusive biologic and physical properties(Marsh & Percival 2006) Despite the diversity that the oral flora has, the majority of the microorganisms from elsewhere in the body are not able to colonise the oral cavity (Poeta et al. 2009).The homeostasis of the mouth depends on the harmonic relationship existent among the extensive range of microorganisms present in the oral environment. This balanced interaction can be affected by several factors such as alimentation habits, systemic diseases, debilitated conditions, immunological suppression, or the prolonged use of drugs (Reddy et al. 2013).

When an imbalance occurs in the oral environment, some opportunistic microorganisms can colonise the oral cavity. *Enterobacteriaceae* is an example of a family that includes some pathogenic bacteria that may colonise the oral cavity under uncommon physiological conditions. Escherichia, Klebisiela and Serratia are some of the representative microorganisms of this group, and they can cause diseases such as meningitis, dysentery, and food poisoning (Hejazi & Falkiner 1997),(Paju et al. 2003), (Bremer et al. 2005)

These pathogens are able to invade either the oral cavity of those patients systemically compromised and of those undergoing cytotoxic drugs or a broad-spectrum antibiotic therapy (Hejazi & Falkiner 1997). Nonetheless, investigations have been demonstrating that species of *Enterobacteriaceae* can be isolated from the oral cavity of subjects systemically healthy(Slots et al. 1990), (Ali et al. 1994), (Barbosa et al. 2001). Whenentericbacilli are isolated from the oral cavityorfluids, thisoccurrence may also be correlated to deficient sanitary conditions, to the consumption of contaminated water or food, or to an inadequate personal hygiene (Ali et al. 1996)

Since the mentioned microorganisms are involved in severe infections, it is relevant to highlight their presence in the oral cavity as concern when considering the potential that these bacteria have to cause disease (Hejazi & Falkiner 1997), (Paju et al. 2003), (Bremer et al. 2005). Moreover, infections caused by these microorganisms can be difficult to treat due to the resistance that they show to a variety of antibiotics, including β-lactams, amoxicillin with clavulanic acid, cephalosporin, aminoglycosides, carbapenems, chloramphenicol, aztreonam, trimethoprim/ sulfamethoxazole and tetracycline(Karlowsky et al. 2002), (Lee et al. 2005), (Okimoto et al. 2005), (Park et al. 2005).

The characterization of the prevalence of Enterobacteriaceae in the oral cavity is extremely important, since the mouth can serve as a reservoir for opportunistic pathogens that may cause severe systemic infections. Therefore, the aim of this study is to investigate the prevalence and antimicrobial

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susceptibility of Enterobacteriaceae isolated from saliva of high-school students from Sobral-CE, Brazil.

II. Materials and Method

This study was a cross-sectional microbiology investigation, conducted on saliva samples of 30 adolescents aged 15 to 19 years old. Public schools from Sobral, a city in the Northeast of Brazil, were randomly selected to participate of the research. Those subjects presenting any medical illness, undergoing antibiotic therapy, or who were pregnant were excluded from the investigation. In regards of the ethical aspects, the Ethics Committee of the Universidade Estadual Vale do Acaraú approved this investigation under the protocol number FR-186159. Additionally, the volunteers were informed about the aims of the research, and signed a consentient term for the collection of the material to be analysed.

To collect the saliva, it was given to each volunteer a sterile bottle containing 10 ml of a phosphate-buffered sterile saline (PBS; 0.1 M, pH 7.2). The subjects were instructed to perform a mouth rinsing for 60 seconds with the saline solution, and then they were asked to expectorate the rinsing into a labelled sterile bottle. The samples were transported to the laboratory within no more than 24 hours. To isolate the microorganism, an aliquot of 0.1 ml of the sample was inoculated on MacConkey agar medium culture using a sterile Drigalski handle. Then, the plates were incubated at 37 °C for 24 hours.

After 24 hours, the samples were removed from the incubator, and the bacterial growth was examined. The lactose fermenting colonies were identified as those that appeared pink in colour, and the non-lactose fermenting as those in a pale red colour. The colonies were submitted to the Gram's stain, examined under microscope for gram-negative bacilli, and tested to the activity of the cytochrome oxidase (Newprov, Pinhais, Brazil). The samples that were oxidase negative and gran-negative were classified as Enterobacateriaceae. The final sample identification of the bacteria was performed using the NEWPROV (Brazil) kit for Enterobacteriaceae, as well as with the BBL CRYSTAL ENTERIC/NONFERMENTER system (Becton Dickinson Micronbiology Sistems, Cockeysville MD, USA).

The susceptibility to antimicrobial agents was tested through the disk diffusion method(Bauer et al. 1966)and according to the recommendations of the Clinical and Laboratorial Standards Institute (CLSI 2015). The concentrations of bacterial cultures grown overnight in BHI broth were adjusted to a standard density of 0.5 McFarland (10^8CFU/mL) and seeded with a swab on petri plates containing Mueller-Hinton agar (Acumedia Manufacturers, Inc., Baltimore, MD, USA). Antibiotic discs (Sensifar-Cefar, Sao Paulo, Brazil) were distributed over the surface of the inoculated agar, and the diameters of the inhibition zones were measured after 18 hours of aerobic incubation at 37 ° C. The antimicrobials used for the susceptibility test included: amoxicillin (10mg), amoxicillin/ clavulanic acid (20 / 10mg), doxycycline (30µg), tetracycline (30µg), tobramycin (10mg), imipenem (10mg), cefotaxime (30µg) and ciprofloxacin (5µg). The isolated samples were classified as sensitive, intermediate or resistant according to the rules of CLSI. *Serratia marcescens* CDC 4112 was standardized as control.

III. Results

In this research, out of 33.3% of the subjects were men, while women accounted for 66.6% and were more numerous than men in all age groups, except among the volunteers aged 18, who were all male (Table 1). Seven of the 30 samples analysed were infected (23.3%) with Enterobacteriaceae. Enteric bacilli were cultured from 3 (30%) out of 10 men, while 4 (20%) out of 20 women were infected. (Table 2).

Thus, Entereobacteriaceae were prevalent in 6 (85%) out the 7 contaminated samples. The same volunteer could be contaminated with more than one type of bacterium. Serratia liquefaciens and *Enterobactercloacae* were the most commonly isolated microorganisms, corresponding to 31.8 and 18.1%, respectively (Table 3).

The antimicrobial susceptibility tests showed that al the isolates of Serratia liquefaciens, the most prevalent microorganism, were susceptible to tobramycin and ciprofloxacin, while 4 out of the 7 (57%) isolates of this bacterium demonstrated an intermediate susceptibility to imipenem and ciprofloxacin. *Enterobactercloacea* exhibited resistance to cefotaxime, tetracycline, and amoxicillin associated with clavulanic acid. Overall, the most effective antibiotic against the isolated microorganisms was tobramycin, since 80% of the samples demonstrated susceptibility to this drug. Moreover, 90% of the *Enterobacteriaceae* isolated in this investigation demonstrated an intermediary level of resistance to ciprofloxacin, and 65% to imipenem. More than half of the microorganisms evaluated were resistant to amoxicillin associated with clavulanic acid. The least effective antibiotic was ciprofloxacin, since only 5% of the samples demonstrated susceptibility to this drug. (Chart 01).

IV. Discussion

Although studies of the prevalence of Enterobacteriaceae in the oral cavity are not usual, investigations of this type are of extreme importance because of the multi-resistant character that these bacteria show to a wide range of antibiotics. Furthermore, the mouth can be a reservoir for these pathogens, and as soon as the organism of the carrier faces an imbalance, these bacteria can act as

opportunists and intensify existent illness. Hosting enterobacteria in the oral cavity is a predisposing and aggravating factor for many oral and systemic diseases, as well as it can be a way from which these pathogens can be spread to the environment.

In this study, the prevalence of isolation of Enterobacteriaceae from saliva of healthy subjects was 23.3%, which was higher than the 18.7% found in similar a study with workers of a hospital in Sao Paulo-Brazil (Leão-Vasconcelos et al. 2015).The prevalence obtained in this study was three times higher than that found by Barbosa et al. (2001) in the biofilm of individuals with periodontitis and more than twice of that found by Sedgley et al. (1996), who analysed samples of mouth rinses of monks in Hong Kong. Our findings were almost two times higher than the results presented by Sedgley et al. (1997), who analysed the prevalence of enteric bacilli in the oral cavity of Chinese children. As long as *Enterobacteriaceae* is not an indigenous microorganism of the oral flora, its isolation from saliva can be related to aging or poor hygiene habits. Moreover, most of the studies present in the current literature associate the occurrence and prevalence of this type of microorganism with immunocompromised patients, and our investigations highlights the fact that these pathogenic and multi-resistant bacteria might be present in the saliva even before the diagnose of some serious illness, which is a risk factor for complications during the treatment.

Among the isolated bacteria, Serratia liquefaciens represented 31,8% of the microorganisms isolated, while *Enterobater cloacae* was 18,1%. Some studies have been relating the oral carriage of these tow microorganisms to systemic diseases. Back-Brito *et al.* (2011) observed a significantly higher number such pathogens in the oral cavities of HIV positive patients compared to HIV negative patients(Back-Brito et al. 2011).

In regards of the antimicrobial susceptibility, among the microorganisms studied 90% of them were susceptible to tobramycin, which was different from the results obtained by Barbosa et al. (2001) who observed that ciprofloxacin, the antibiotic with the least effectiveness in this investigation, was the most effective in inhibiting the growth of such organisms In our study. This study and other published studies showed that the initial choice of treatment of antibiotic resistant Gram-negative infections is carbapenems (English & Gaur 2010). However, there has been the emergence of resistance to carbapenems (Gupta et al. 2006), which was also observed in the present study.

V. Conclusion

The occurrence of *Enterobacteriaceae* in the saliva of healthy individuals must be seen as a sign of alert, since the mouth can serve as reservoir, and those who carry these pathogenic and multidrug resistant microorganisms in the mouth might spread them in the community.

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Tables

Table 1 : Subject Distribution per Age and Sex

Age (years)	Men N*	%	Women N	%	Total N	%
5	3	10	7	23,3	10	33,3
16	2	6,6	3	10	5	16,6
17	3	10	9	30	12	40
18	2	0	0	0	2	6,6
19	0	0	1	3,3	1	3,3
Total	**10**	**33.3**	**20**	**66.6**	**30**	**100**

*N = Number of subjects % = Percentage of the total of subjects

Table 2 : Distribution of Bacterium Species Isolated from 10 Men and 20 Women

Microorganism	Men N *	%	Women N	%
Enterobacteriaceae	3	30	3	15%
No microorganism	7	70	16	80
Other Gram-negative microorganism	0	0	1	5
Total of subjects of both genders	**10**	**100%**	**20**	**100%**

*N = Number of subjects % = Percentage of subjects infected according to the sex

Table 3 : Microorganism isolated from saliva of 30 adolescents

Specie	Number of isolated
Serratia liquefaciens	7 (35%)
Enterobacter cloacae	4 (20%)
E Scherichia coli	3 (15%)
Enterobacter aerogenes	2 (10%)
EEnterobacter gergoviae	1 (5%)
Serratia marcescens	1 (5%)
C Citrobacter freundii	1 (5%)
Proteus mirabilis	1 (5%)
Total	20 (100%)

Charts

Chart 1 : Antimicrobial Susceptibility of *Enterobacteriaceae* Isolated from the Saliva of High School Students

( ) = Number of strains isolated S = Sensitive; I = Intermediate sensitivity; R = Resistant

The values of sensitivity (μg/ml) were interpreted according to the CLSI (2015) standards.

AMC(Amoxicillin + Clavulanic Acid): S = ≥18; I = 14-17; R = ≤13; IPM (Imipenem): S = ≥23; I = 20-22; R = ≤19 CTX

Microorganism	AMC	IPM	CTX	TOB	DOX	TET	CIP
Serratia liquefaciens (7)	S(4) I(1) R(2)	S(1) I(4) R(2)	S(1) I(4) R(2)	S(7)	S(5) I(1) R(1)	S(4) I(2) R(1)	I(7)
Enterobacter cloacae (4)	S(1) R(3)	I(4)	R(4)	S(3) R(1)	S(1) I(1) R(2)	I(1) R(3)	I (3) R(1)
Escherichia coli (3)	S(2) R(1)	S(1) I(1) R(1)	S(2) I(1)	I(2) R(1)	S(1) R(2)	S(1) I(1) R(1)	I(3)
Enterobacter gergoviae (2)	S(1) R(1)	I(2)	I(1) RI(1)	S(2)	I(1) R(1)	S(1) I(1)	I(2)
Enterobacter aerogenes (1)	R(1)	I(1)	I(1)	S(1)	S(1)	S(1)	I(1)
Serratia marcescens (1)	R(1)	S(1)	S(1)	S(1)	I(1)	I(1)	I(1)
Citrobacter freundii (1)	R(1)	S(1)	S(1)	S(1)	R(1)	R(1)	S(1)
Proteus mirabilis (1)	R(1)	I(1)	I(1)	S(1)	I(1)	S(1)	I(1)
Total (20)	S(8)=40% I(1)= 5% R(11)= 55%	S(4)=20 % I I (13)=65% R(3)= 15%	S(5)=25 % I(8)=40% R(7)= 35%	S(16)=80 % I(2)=10% R(2)= 10%	S(8)=40 % I(5)=25% R(7)= 35%	S(7)=35 % I(6)=30% R(7)= 35%	S(1)=5 % I(18)=90% R(0)= 0%

(Cefotaxime): S = ≥26; I = 23-25; R = ≤22; TOB (Tobramycin): S= ≥15; I = 13-14; R = ≤12; DOX (Doxycycline): S = ≥14; I = 11-13; R = ≤10; TET (Tetracycline): S = ≥15; I = 12-14; R = ≤11 (Ciprofloxacin): S = ≥31; I = 21-30; R = ≤20.