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Research Article

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Prevalence of Antibiotic-Resistant Bacteria Isolated From Infant Stools Aged Less Than 2 Years after Antibiotic Therapy Treatment

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Abstract

65 stool samples were analyzed for insulation, identification and multiresistant bacteria becoming from infant aged less than two years of the pediatric ward of Khalil Amrane Hospital-Bejaia. The study of antibiotic administration by type, revealed that amoxicillin was the most commonly administered antibiotic with a rate of (33.33%) followed by gentamicin at (20.43%) and ampicillin at (17.20%). As well, the distribution of antibiotics administered by family and group revealed the predominance of B-Lactamines with a percentage of (72.04%) of which the group of penicillins of group A is predominant (50.54%). The identifications and antibiotic susceptibility tests of the various isolated pathogenic strains were carried out. From all samples, 35 bacterial isolates were identified by macroscopic, microscopic observations, and physiological and biochemical tests. According to which it can be seen clearly that the most frequent species isolated was Clostridium perfringens with 12 (34.28%) isolates. Followed, by Escherichia coli with 11 (31.43%) isolates. Enterobacter sp were represented by 8 (22.86%) isolates. The least frequent species was Staphylococcus aureus with 4 (11.83%) isolates. Furthermore, antibiogram method showed that all bacteria tested were multiresistant to 1 to 6 antibiotics.

Keywords: Multiresistant Bacteria, Antibiotics, Infants and Stool Samples.

Introduction

Infectious diseases are a global concern. Among the concerns raised by this issue, bacterial resistance to antibiotics requires particular attention and specific measures [1]. In the other hand, the intestinal flora or microbiota is the set of bacteria that colonizes the digestive tract. A human being harbours 1014 bacteria in his digestive tract whereas it consists of only 1013 eukaryotic cells [2-4]. Also, for many years, the digestive microbiota has been poorly or not studied, as more than 70% of the bacteria in it cannot be cultivated by conventional methods [5]. Whereas, in the fight against infectious diseases, vaccines and antibiotics are the most valuable therapeutic tools available. However, the target bacteria develop new mechanisms to become more resistant to the action of antibiotics. Here and there pathogenic strains that are resistant to them develop [6]. Furthermore, multi-resistant bacteria as strains of a bacterial species found resistant to at least two classes of antibiotics to which strains of the same species are usually susceptible. The consequences of multi-resistant bacterial infections are multiple; the severity of disease, due to the ineffectiveness of antibiotics, and the increase in mortality [7]. As given this resistance problem that accompanies the massive use of antibiotics, important scientific and economic interests have emerged for metabolites or other molecules with antibacterial or probiotic activities [8-10]. Particularly, bacteria are considered resistant or multi-resistant (MRB) when, due to the accumulation of natural or acquired resistance, they are only sensitive to a small number of antibiotics usually active in therapy. BMRs are no more pathogenic or virulent than susceptible species, but they are more difficult to treat and cause treatment failures [11]. Those MRB are primarily concerned with hospital infections; enterobacteriaceae producing extended spectrum beta-lactamases, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci, multi-resistant *Acinetobacter baumanii* and multi-resistant *Pseudomonas aeruginosa* [12].

This study was focused on the isolation and identification of multi-resistant bacteria isolated from hospital in Bejaia region from the stools of infants less than two years of age, who had developed diarrhea after antibiotic treatment.

Materials and Methods

Stools Sampling

65 stool samples from infants aged from 1 to 24 months were taken from Boukhalfa Hospital, Amizour-Bejaia (36°38'41.3 "N 4°53'42.0

"E). Diarrhea was observed in all infants that had previously received antibiotic treatment for different diseases. An aliquot of each stool samples equivalent to a nut, were collected sterilely and released into coproculture boxes. Data as age and antibiotic administrated were collected.

Distribution percentage of each antibiotic administered was calculated according to the following formula:

$$ADP = \frac{n}{N} \times 100$$

Where: ADP (%): Distribution percentage; n: number of each antibiotic administered, N: total number of antibiotics administered.

Isolation and Purification of Isolates

The isolation of different multiresistant bacteria was carried out using the traditional microbiolgy method; one tube containing nutrient broth was used to enrich *E. coli* and *Enterobacter* sp. A second tube of nutrient broth was added with a layer of vasline oil, to ensure anaerobiosis, and for the enrichment of *Clostridium peringens*. Also, *Staphylococcus aureus* were enriched in a third tube containing Giolitti Cantoni broth. The three tubes were incubated at 37°C/24h. After incubation, isolation of each bacteria was carried out on selective media corresponding to each bacteria: EMB agar for Enterobacter sp and *E. coli*. Chapman medium for *Staphylococcus aureus*; liver meat agar for *Clostridium peringens*. Then, isolates were purified by 2 to 3 successive inoculation on the same selective medium as that of the isolation [13].

Identification of isolated bacteria

Macroscopic Identification

The identification of pathogenic bacteria through the microscopic aspect of colonies on selective agars is an essential step for the orientation of Genera and species [14]. Pure colonies are characterized by their macroscopic aspects on the corresponding selective agars for each species.

Microscopic Identification

Microscopic identification of isolated strains is performed by Gram staining [15]. Cell shapes and their modes of association are noted as shell, bacillus, isolated grouped in two, chain or cluster [1].

Biochemical and Physiological Identification

The identification of each isolate was based on mini specific biochemical galleries for genera or species including *Clostridium perfringens*, *E. coli*, *S. aureus* and Enterbacter sp according to [16].

Distribution percentages of each species were calculated according to the following formula:

$$SDP = \frac{m}{M} \times 100$$

Where: SDP (%): species distribution percentage; m: number of isolates of each species, M: total number of isolates bacteria.

Antibiogram

Antibiogram Standardization

Bacterial concentration inocula of each isolate were determined in order to perform the antibiogram technique. Bacterial suspensions were prepared in saline buffer solution and the standardized concentrations used were 10^8 CFU/ml for Gram-positive (*S. aureus*) bacteria and 10^7 CFU/ml for Gram-negative bacteria (*E. coli* and *Enterobacter* sp) equivalent to standard 0.5 McFarland. Except for *Clostridium perfringens* consisting of concentration of 10^9 CFU/ml [17].

Antibiogram Technique

The antibiotic susceptibility test was performed according to the recommendations of the Antibiogram Committee of the French Society of Microbiology [18]. The method consists of inoculum seeding of each bacterial isolate on Muëller Hinton medium. Inoculation was carried out by the swabbing method on the howl agar surface of the Petri plates. This operation was repeated three times by turning by turning the petri dishes by an angle of 60°. Discs of antibiotics were then placed on the surface of the agar. After incubation at 37°C/24h the diameters of the inhibition zones were measured [19].

Resistance percentages of each species against antibiotics ware calculated according to the following formula:

$$RP = \frac{l}{L} \times 100$$

Where: RP: Resistance percentage; l: number of resistant isolates for each species,
L: total number of isolated bacteria for each species.

Statistical Analysis

Resistance percentages (RP) of each species for antibiotics group were compared by comparison test of two proportions using STATISTICA 5.0.

Results

Frequency of Antibiotics Administered

The study of antibiotic administration by type revealed that amoxicillin was the most commonly prescribed with a percentage of 33.33% followed by gentamicin at 20.43% and ampicillin at 17.20%. The lowest administration percentages noted were for colistin and oxacillin at 1%; cefotaxime and cefatriaxone at 5% (Figure 1).The study of antibiotic administration by family and group revealed the predominance of B-Lactamines with a percentage of (72.04%) of which the group a penicillins group was predominant (50.54%). Followed, by the family of aminosides with (20.43%). aminosides avec (20,43 %). des aminosides avec (20,43 %).

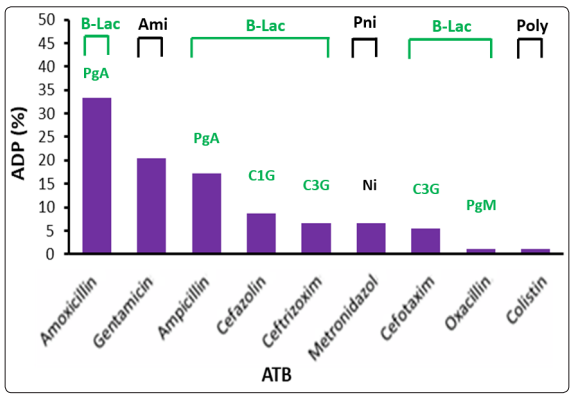

Figure 1: Antibiotic distribution percentage by family, group and type

(ADP: distribution percentage, ATB: antibiotic). B-Lac: Beta-Lactamine; Poly: polymixin; Np: nitriated products; Am: Aminosides; PgA: Group A penicillins, PgM: Group M penicillins; C1G: 1st generation cephalosporins; C3G: 3rd generation cephalosporins; Ni: Nitro-imidazoles A

Identification of Isolated Strains

Macroscopic and Microscopic Identification

Isolation on different selective media allowed 35 bacterial isolates to four species. The macroscopic and microscopic characteristic aspects of the colonies of each species are grouped in Table 1.

Table 1: Macroscopic and Microscopic Identification of Isolated Bacteria

ID	A	B	C	D
Mac	1-2 mm diameter creamy opac golden-yellow	black; large colonies	small flat green metal shine	medium bulging mucoids
Mic	Gram positive ; cocci; grape bunches and/or isolated and/or pairs and/or short chain	Gram positive ; bacilli	Gram negative; coccobacilli	Gram negative; bacilli

ID: identification, Mac: macroscopic identification; Mic: macroscopic identification; A: Staphylococcus aureus; B: *Clostridium perfringens*; C: *E. coli*; D: *Enterobacter* sp.

Biochemical and physiological identification

The identification of each species was confirmed by mini biochemical galleries regrouping specific tests for each species. *S. aureus* isolates produced coagulase, DNAase and catalase enzymes. Also, *C. perfringens* was characterized by particularly motility; growth at 44°C and formation of subterminal ovoid spore formation. Also, *E. coli* isolates were determined by growth at 44°C and indol production. Whereas, *Enterobater* sp isolates were identified by characteristics test like lactose assimilation; indole production.

Table 2: Biochemical and physiological identification of isolated bacteria

Species/test	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
S. aureus	+	+	+														
C. perfringens			+	+	+	+	+										sos
E. coli								+	+								
Enterobater sp					-	-	+		+	+	+	+	+	-	+	+	

+ positive reaction; - : negative reaction; 1. coagulase; 2. DNAase; 3. catalase; 4. growth at 50°C; 5. Gas production; 6. H2S production; 7. motility; 8. growth at 44°C; 9. indole production; 10. Urea production, 11. TDA (Tryptophan deaminase); 12. Citrate assimilation; 13. VP (Voges proskaer) 14. RM (methyl red); 15. Glucose assimilation; 16. Lactose assimilation; 17. sos: subterminal ovoid spore.

The results of identification are summarized in Table 3.

Table 3: Identification of Bacterial Isolates

Code	Species	Code	Species
P1	*Staphylococcus aureus*	P19	*Clostridium perfringens*
P2	*Staphylococcus aureus*	P20	*Clostridium perfringens*
P3	*Staphylococcus aureus*	P21	*Clostridium perfringens*
P4	*Staphylococcus aureus*	P22	*Clostridium perfringens*
P5	*Enterobacter sp*	P23	*Clostridium perfringens*
P6	*Enterobacter sp*	P24	*Clostridium perfringens*
P7	*Enterobacter sp*	P25	*Escherichia coli*
P8	*Enterobacter sp*	P26	*Escherichia coli*
P9	*Enterobacter sp*	P27	*Escherichia coli*
P10	*Enterobacter sp*	P28	*Escherichia coli*
P11	*Enterobacter sp*	P29	*Escherichia coli*
P12	*Enterobacter sp*	P30	*Escherichia coli*
P13	*Clostridium perfringens*	P31	*Escherichia coli*
P14	*Clostridium perfringens*	P32	*Escherichia coli*
P15	*Clostridium perfringens*	P33	*Escherichia coli*
P16	*Clostridium perfringens*	P34	*Escherichia coli*
P17	*Clostridium perfringens*	P35	*Escherichia coli*
P18	*Clostridium perfringens*		

According to the identification results, it can be seen clearly that the most frequent species isolated was Clostridium perfringens with 12 (34.28%) isolates. Followed, by Escherichia coli with 11 (31.43%) isolates. Enterobacter sp were represented by 8 (22.86%)

isolates. The least frequent species was Staphylococcus aureus with 4 (11.83%) isolates (Figure 2).

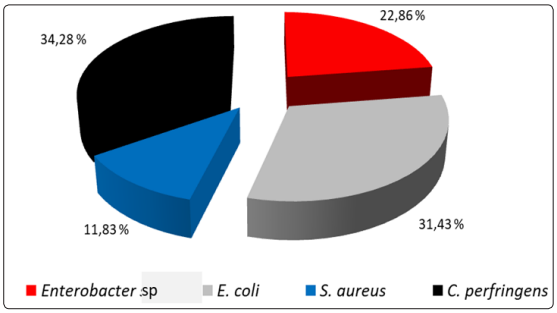

Figure 2: Species Distribution Percentage (SDP) of the Isolated Bacteria by Genera and Species

Antibiogram

The results of the antibiogram revealed that resistance varied according to antibiotic and the bacterial species. Also, multi resistance of different isolates resulted in variable inhibition zones (Figure 3).

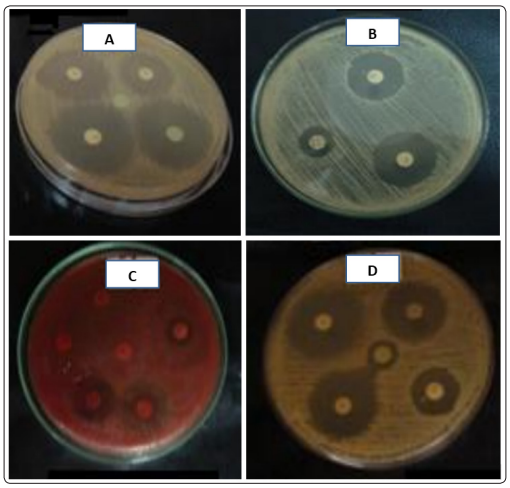

Figure 3: Antibiograms of Some Strains Tested

(A: P10 Enterobacter sp; B: P2 *S. aureus*; C: P13 *C. perfringens* and D: P33 *E. coli*).

Antibiogram of *S. aureus*

In this study we observed 100% resistance to penicillin, followed by cephotaxime and cephoxitine for 25% resistance each. All strains were not resistance to erythromycin, vancomycin, gentamycin and chloramphenicol (Figure 4)

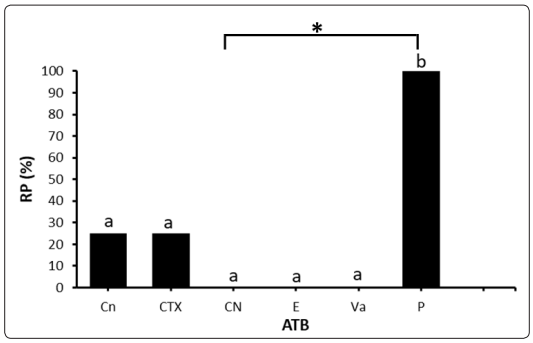

Figure 4: Resistance Percentage of *S. aureus Strains*

(PR: percentage resistance; E: Erythromycin; Va: Vancomycin; Cn: cefoxitin; CN: gentamycin; CTX: cefotaxim; P: Penicillin and C: Chloramphenicol). Bars designated by the same lowercase letter are statistically identical (P>0.05); *: significant difference.

Antibiogram of C. perfringens

The *Clostridium perfringens* strains showed 100% resistance to chloramphenicol and ampicillin, 8.33% to imipenem and 6.66% to colistin (Figure 5).

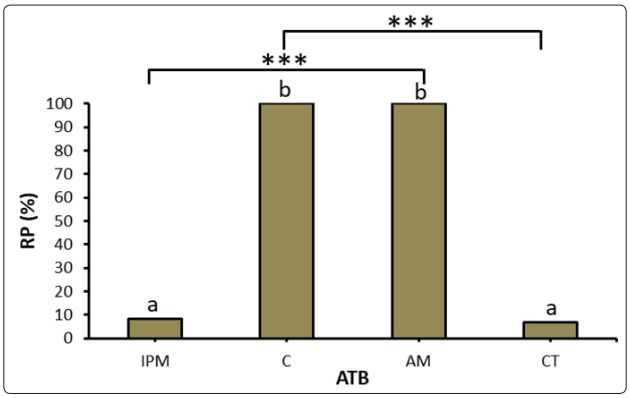

Figure 5: Resistance Percentage of *C. perfringens* Strains

(PR: percentage resistance; IPM: imipenem; C: Chloramphenicol; AM: ampicillin; CT: Colistin). Bars designated by the same lowercase letter are statistically identical (P>0.05); ***: high significant difference.

Antibiogram of E. coli

Based on antibiotic susceptibility test results, *E. coli* isolates showed 54.54% resistance to the combination of trimethoprim/ sulfamethoxazole antibiotics, 9.09% resistance to cefotaxime, ceftriaxone and gentamycin. All isolates were sensible to imipenem and chloramphenicol (Figure 6).

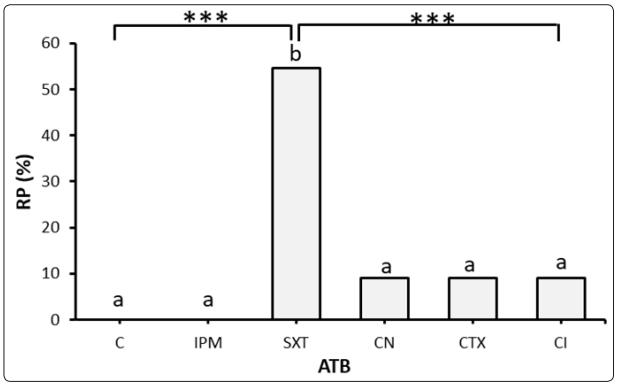

Figure 6: Resistance Percentage of *E. coli* Strains

(PR: percentage resistance; NC: gentamycin; IPM: imipenem; C: Chloramphenicol; CTX: Cefotaxime; CI: ceftriaxone and SXT: trimethoprim / sulfamethoxazole). Bars designated by the same lowercase letter are statistically identical (P>0.05); ***: high significant difference.

Enterobacter sp Antibiotic Susceptibility Test

The maximum resistance rate of Enterobacter sp isolates was noted for amoxicillin/clavulanic acid of 80%, followed by amoxicillin of

70%. Resistance to cefotaxime, kanamycin and gentamycin was 40% for all the isolates tested. While, the resistance to ceftriaxon was 10%. All isolates showed no resistance to chloramphenicol, amikacin and imipenem (Figure 7).

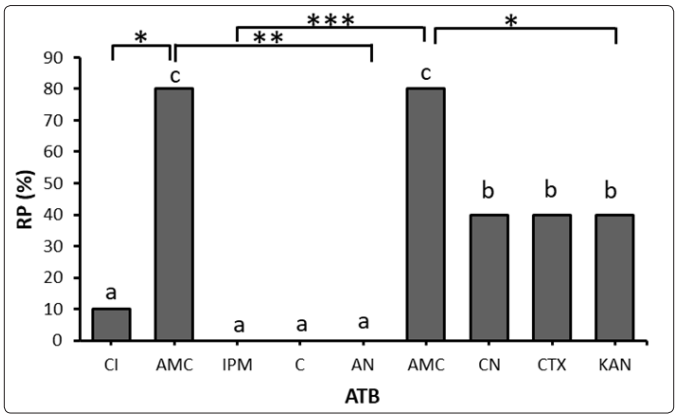

Figure 7: Resistance Percentage of *Enterobacter* sp Strains

(PR: resistance percentage; C: Chloramphenicol; AN: amikacin; Am: amoxylline, CI: ceftriaxon; CN: gentamycin; IPM: imipenem; CTX: Cefotaxime; KAN: kanamycin; AMC: amoxicillin/clavulanic acid). Bars designated by the same lowercase letter are statistically identical (P>0.05); *: significant difference; ** and ***: high significant difference.

Discussion

The phenomenon of antibiotic resistance has become a major concern, especially with regard to nosocomial infections, following increased and uncontrolled administration, especially of broad spectrum antibiotics. Hence a major interest in the study of antibiotic resistance and the understanding of this global scourge since its origin; namely in infants of young age.

In this current study, different bacteria were isolated from infants less than two years of age after antibiotic therapy; *Clostridium perfringens* 12(34.28%), *E. coli* 11(31.43%), *Staphylococcus aureus* 8 (22.86%) and *Enterbacter* sp 4 (11.83%). According to [5], analysis of the composition of the intestinal flora in taxa (bacterial genera and/or phylogenetic groups) reveals the existence of recurrent components, found in all individuals. Three bacterial Phyla, Firmicutes, Bacteroidetes and Actinobacteria account for the largest share of the dominant faecal bacteria. In particular, the phylum of Firmicutes (Gram-positive bacteria) is still highly represented. It first includes the group called "*Eubacterium* rectal-*Clostridium* coccoides" which is often the most important representing 14 to 31% of total bacteria on average depending on the studies [20,21]. Recent studies reported by [22], stated that antibiotics are known to affect the intestinal microbial flora, and the following changes may result in diarrhea associated with antibiotics. Also, occurrence of Staphylococci in fecal samples obtained from 50 infants at 1, 2, 4 and 8 weeks of age that colonization increases by 20% in infants aged 3 days, 40% in infants aged 1 week, 52% in infants aged 2 weeks 60% in infants aged 4 weeks and 64% at 8 weeks of age [23]. Furthermore, the faecal flora of *Escherichia coli* was characterized in 70 Swedish children, followed during the first year of their lives. Revealing that 42% of the 70 children were colonized by *Escherichia coli* as early as 3 days after birth [24].

In this work, investigation of the frequency of antibiotic administration in young infants clearly revealed the dominance of antibiotics of the B-Lactamines family, particularly, group A (amoxicillin and ampicillin), followed by aminosides (gentamycin). However, adult antibiotic administration percentages are 5 to 10% for ampicillin, 10 to 25% for amoxicillin/clavulanic acid, 15 to 20% for 3rd generation cephalosporins (cefotaxime), and 2 to 5% for fluoroquinolones (pefloxacin, ofloxacin), macrolides (erythromycin) and tetracyclines (metacyline) [25].

Current findings revealed that resistance of the isolated bacteria species in infant's stools were variable depending on antibiotic tested. Especially, since the introduction of antibiotics into the therapeutic arsenal of infectious diseases, microorganisms have developed defenses that make them insensitive to antibacterial agents. These antibiotic resistance to therapeutic doses appear more or less rapidly depending on the chemical complexity of the antibiotics and the genetic make-up of the bacteria [26]. All bacterial species or genera are concerned by the phenomenon of antibiotic resistance, which sometimes poses real therapeutic problems [27-29]. However, the misuse of these compounds, whether in the medical or agri-food sectors, has resulted in the progressive emergence of a multitude of resistance mechanisms [30]. In addition, resistant mutants spontaneously appear and are then selected. Mutants are not created directly by exposure to an antibiotic [31].

The frequency of antibiotic resistance varies from country to country. Differences in antibiotic therapy practice are undoubtedly among the possible causes of this difference [32,33].

First, the *S. aureus* isolates tested in this work were resistant to cefoxitin (25%); cefotaxim (25%) and penicillin (100%). Paerticularly, *Staphylococcus aureus* is mainly resistant to penicillin G by penicillinase production. Methicillin-resistant staphylococci are also resistant to many other antibiotics. Thus, resistance to aminosides, which concerned only streptomycin and kanamycin in the past, has been modified by the appearance of two new plasmid resistance phenotypes [34].

Second, the isolates of *C. perfringens* tested in this current study were resistant to chloramphenicol and ampicillin (100%); imipenem (8.33%) and colistin (6.66%). For example, [35] reported that multidrug-resistant strains of *Clostridium* were found only in the hospital setting in subjects treated with antibiotics. Ampicillin was the antibiotic to which most anaerobic strains were resistant; and no strain was found to be resistant to imipenem. Furthermore, significant growth in the number of ampicillin-resistant strains were observed among hospitalized patients with a recent history of unspecified antibiotic treatment [36]. A distinct correlation between antibiotic consumption and the local prevalence of resistant anaerobic fecal microorganisms has been reported for ampicillin and doxycycline [37,38]. The most important factor in Clostridium's resistance to B-lactamines appears to be the production of B-lactamases [39,40]. These mechanisms of resistance to B-lactam antibiotics in anaerobic bacteria are developed and attributed to B-lactamases that inactivate the antibiotic by changing the number or type of penicillins binding proteins (PLP), and that affect the affinity of proteins for antibiotics; as well as the penetration of the antibiotic will be blocked in the active site through the change in the external membrane porins of the target bacteria [41-43]. Resistance to carbapenems such as imipenem is very rare [44,45].

Third, the isolates of enterobacteriaceae tested in this study are resistant to different antibiotic tested. *E. coli* showed multi resistant to the combination of trimethoprim/sulfamethoxazole (54.54%), and (9.09%) for cefotaxim, ceftriaxone and gentamycin. Whereas, Enterobacter sp isolates revealed resistance to amoxicillin/clavulanic acid (80%), amoxicillin (70%), and resistance of (40%) to cefotaxim, kanamycin and gentamycin, and ceftriaxon (10%). In addition, resistance of enterobacteriaceae to the combination of amoxicillin-clavulanic acid and first-generation cephalosporins (cefalotin) is between 40% and 60%, with a large number of intermediate strains probably strong producers of penicillinase. Resistance frequencies are lower for second generation cephalosporins (cefoxitin) at 23%, third generation cephalosporins at 7 to 10% [46]. Also, imipenem remains active on almost all enterobacteriaceae, despite the description of some resistant strains, either because of a significant decrease in permeability associated with cephalosporinase hyperproduction or because of carbapenemase production [47]. Furthermore, with regard to aminoglycosides, the most significant development was the emergence in 1969 of plasmid resistance to gentamicin, which was often associated with resistance to kanamycin. In the other fand, there was a rapid increase in the frequency of this resistance until 1974, followed by a decrease and stabilization around 10% [48]. Also, resistance to amikacin, which is more stable to enzymatic inactivation, remains below 5%. Resistance to sulfonamides is stable, around 35% of the strains, while trimethoprim resistance is slightly lower. For chloramphenicol, resistance is 20%, the trend is towards decreasing and then stabilizing resistance [49].

In *E. coli*, resistance to aminopenicillins gradually increased to more then 50%. A large part of these strains appear intermediate, even resistant, and even to the combinations of amoxicillin (or ticarcillin)-clavulanic acid due to the production of either a high level of penicillinase or TRI (TEM resistant to β-lactamase inhibitors) enzymes [50-52]. Furthermore, for other antibiotics, resistance frequencies appear relatively stable, with the exception of those for cotrimoxazole, but also quinolones where resistance has increased over the past ten years with a current frequency of more than 15% for nalidixic acid and more than 10% for ciprofloxacin [53]. Also, in *E. coli* resistance is frequent to ampicillin (29.8%, varying from 15.5% to 53.9% depending on the country) where the only antibiotics for which resistance is less than or equal to 10% are imipenem (0%), second and third generation cephalosporins (1 to 5%), (10%), imipenem (0%), aminosides (1 to 7%) except kanamycin [49].

Strains belonging to the genus *Enterobacter* almost constantly combine natural resistance with various acquired resistance traits. These are typically hospital bacteria. Natural resistances are resistance to first generation aminopenicillins and cephalosporins by cephalosporinase production [54,55]. The frequency of acquired resistances appears to be relatively lower today than in the past for *Enterobacter cloacae*.

Conclusion

The spread of antibacterial-resistant pathogens is one of the most serious threats to the effective treatment of a disease. This current study described isolation and identification of multi-resistant antibiotic bacteria from infant stools less than two years old, which showed the high frequency of high resistance for 3 to 6 antibiotics in bacteria belonging to *C. perfringens*, *S. aureus*, *Enterobacter* sp and *E. coli*. Antibiotic resistance is still a topical issue as this phenome is evolving more and more, causing many problems in the treatment of infections. Particularly, this study was focused on the detection of multi resistant bacteria in infant stools. But, it is important to determine the modes of delivery of these bacteria to young infants.

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