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Characterization and Comparison of the Digestive Commensal Flora of Exposed and Non-Exposed Infants to HIV

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ABSTRACT

Background: The mother's HIV infection disrupts the gut microbiota of HIV-exposed infants (dysbiosis). Objectives: The aim of this study was to compare the intestinal microbiota from feces of infants exposed and not exposed to HIV in order to justify the hypothesis that HIV-exposed infants have a disturbed immune system. Methods: The bacteriological tests, May-Gram staining and biochemical tests were performed to identify bacterial groups. Results: A total of 18 infants stool samples were analyzed, 12 samples from non-exposed children and 6 samples from exposed children with an age ranch of 6 weeks to 6 months. The results showed that the fecal microbiota of HIV-exposed infants is more diverse than that of unexposed infants. In the fecal microbiota of unexposed infants, the genus *Klebsiella* (75%, 15005000 CF/Uml) in particular the species *Klebsiella Pneumoniae Pneumoniae* (42%, 5005000 CFU/ml) was predominant. However in the fecal microbiota of infants exposed the proportion of *Klebsiella* (50%, 2000000 CFU/ml) was in minority face to staphylococcus genus (83%, 17525CFU/ml) whose *Staphylococcus aureus* was largely dominant (67%, 7525 CFU/ml). *Escherichia coli* (83%, 224200 CFU/ml) and *Enterobacter* (83%, 842000 CFU/ml) were the dominant species of fecal flora in HIV-exposed infants. Conclusions: Infection with HIV from seropositive mother changes the microbiota of infants exposed to HIV and consequently may disrupts the immune system.

Keywords: Comparison, fecal microbiota, infants exposed to HIV.

1. INTRODUCTION

The human microbiota (commensal flora) represents the set of micro-organisms living in the interior or on the human body [1]. Depending on their location, four major types of commensal flora are defined including skin commensal flora, respiratory, genital and digestive [2].

Digestive commensal flora is the largest and most abundant in the body, it is a large and dynamic ecosystem, and contains about 1014 microorganisms including 500 to 1000 different species. [3] Indeed, it plays an important role in maintaining the health of the host as it is needed for nutrition, pathogenesis and immunology. [4] At the stomach flora is limited by the very high acidity of gastric environment and abundance of secretions in the small intestine. Streptococci, Staphylococci and lactobacilli are predominant. In the colon, the gut flora is the most diverse of the body and there are mainly anaerobic bacteria (Bacteroides, Bifidobacterium and Clostridium), as well as Enterobacteriaceae, Enterococci and Staphylococci [2].

From birth, began the colonization of the digestive tract of the infant by microbes that originate in the microbiota of the mother. The microbiota of the infant varies according to the mode of delivery [5]. It is dependent on genetic and environmental factors as well as the mode of feeding [6]. Breast milk plays a vital role in the growth of the intestinal microbiota because it contains organisms from the skin of the mother and elements that feed some microbes and protect against others [6, 7]. Breast milk also plays a direct role on the developing immune system in infants [6-8].

However, the HIV/AIDS pandemic is creating a dysbiosis in the microbiota of infants exposed to this terrible pandemic. [9] Indeed, with the increase of young women infected with HIV worldwide and seroprevalence which

continues to grow among young women in sub-Saharan Africa, infants quickly became another major victim of the HIV pandemic. Although these HIV-exposed infants are not infected with HIV, this growing population of children experiencing two times more morbidity and mortality than children born to HIV negative mothers [10, 11]. Indeed, studies have shown that pregnant women infected with HIV are in their microbiota dysbiosis [12] and that this dysbiosis and especially the composition of breast milk affects infant microbiota [6, 7]. Also, hypotheses state that the immune system of infants exposed to HIV would be disrupted which could be explained an imbalance of the microbiota of its infants. The objective of this study was to analyze and compare the digestive commensal flora of infants exposed to HIV from positive mothers and unexposed infants.

2. MATERIALS AND METHODS

2.1 Study site and population

The study took place at the University Hospital Joan Ebori Foundation (CHUFJE) from September to December 2018. Samples of feces were collected from infants 6 to 24 weeks without sex distinction. The origin of the samples was done at several health facilities in Libreville including Lalala Health Center, the London Health Center, the Louis Health Center, polyclinic EL RAPHA and University Hospital Mother and Child Foundation Jeanne Ebori. , the sampling was done in agreement with patients after informed consent. All the information collected has been treated in strict respect of the anonymity and confidentiality of the persons.

2.2 Bacteriological tests

The sampling was done by the mothers in sterile pots using a sterile spatula and each pot was identified (names, first names, date and time of sampling). After collection, the stools were immediately brought to the laboratory. If stool delivery or laboratory examination could not be done within six hours, the samples were immediately stored as follows: 2-8°C for 72 hours; and at - 20°C for one month.

Each sample was observed to note its purulent appearance, presence of mucus and blood and its liquid consistency, soft or solid. The microscopic study was performed with 1/10 dilution of the stool sample using physiological saline and 10.µl sample to drop on a sterile blade. The reading was done at objective x 40. This direct examination makes it possible to observe the shape of the bacterium (Cocci, bacillus, coccobacillus), the mobility of the bacterium and the presence of the cells.

2.3 Identification of Bacterial groups by Gram staining

The identification consisted to evaluate the percentage of Gram-positive and Gram-negative bacteria to determine the balance of faecal flora.

Gram staining was performed briefly as follow: dilution at 1/10 (stool sample – saline physiological) was performed, 10µl were collected, deposited and subsequently spread on a slide and fixed to the flame. A few drops of Gentian solution were made and one minute after the plate was rinsed with water to remove excess dye. Lugol allowed to set Gentian violet, a few drops were made on slides and one minute after the blade has been first rinsed with water to remove excess Lugol then with alcohol for discoloration. Few drops of the fuchsin solution were

poured onto the slide for counterstaining and one minute after the excess was removed with water and the slide was dried and then observed under a microscope at x100 magnification. Gram + (purple) and Gram - (pink) bacteria could be identified.

2.4 Search cells by staining May-Giemsa

The coloring of May-Giemsa has identified the presence or absence of cells in the stool. The protocol was briefly performed as follows: a dilution of 1/10th (stool sample – saline physiological) was performed, 10µl were collected, deposited and subsequently spread on a slide and fixed to the flame. Then, the entire blade has been introduced in the May solution for three minutes and was rinsed with water. Then it was introduced in the Giemsa solution for ten minutes and was then rinsed, dried and observed by a microscope at x 100.

2.5 Culture: seeding and isolation

Seeding and isolation was carried out as follow: A preparation of 10 to 20 mg of the stool sample mixed with physiological saline made it possible to obtain a 1/10 dilution (stock solution). From the stock solution, serial dilutions were performed. Each dilution was measured Mc Ferland and seeding each of the preparations was done by flooding. (Seeding technique for counting) on specific areas including Eosin Methylene Blue (EMB) selective medium favorable to the growth and multiplication of Enterobacteria, the agar medium Hektoen isolation of Salmonella and Shigella., Agar Chapman isolation medium bacteria of the genus Staphylococcus and Sabouraud agar selective medium favoring growth of the yeasts. Inoculated media were placed in an oven at 37 ± 2°C for 24 hours. The characterization consisted in determining for each crop the number, the germs, and for each type of colony, the color, the size, and the appearance. Colonies fewer and few identifiable were picked by the streak technique.

2.6 Germ identification tests

Three Api galleries were used including API 10S or 20E (Biomerieux, France) for the identification of Enterobacteriaceae, the API Staph for staphylococci and Api Candida for yeast. On a plate containing wells previously moistened wells were filled with bacterial inoculum and gallery was incubated at 37°C ± 2 for 18 to 24 hours and the reading could be made by following the catalog recommendations.

2.7 Catalase test

The catalase test distinguishes Streptococci and Staphylococci. The bacteria reduce hydrogen peroxide (H_2O_2) in water with release of O_2. The bacteria were catalase positive if there was formation of air bubbles which reflected the presence of Staphylococci. And if not, the bacteria were catalase negative so the presence of streptococci was detected.

2.8 Agglutination test

The agglutination test allows to distinguish *Staphylococcus aureus* from *Staphylococcus saprophyticus*. This test was carried out using a kit (Pastorex TM Staph plus Kit, Bio-Rad, France) composed of a reagent, a control, a test card containing wells and mixing sticks. The test was positive when agglutination was observed in presence of

reactive and negative when there was no reaction. The presence of agglutination indicated the presence of *Staphylococcus aureus* and the absence indicated the presence of *Staphylococcus saprophyticus*.

2.9 Blastosis test

This test makes possible to identify the species *Candida albicans*. 0.5 ml of serum was introduced into a hemolysis tube, then the strain to be tested is taken on solid medium with platinum loop and put in the tube to obtain a suspension of light opacity. The tube was then incubated in an oven at 37°C ± 2 for 2 - 3 hours then a drop of the suspension was removed for observation under the microscope. If a number of cells or blastospores were detected , strain was *Candida albicans*.

3. RESULTS

Samples of feces were collected from eighteen infants, all aged from 6 to 24 weeks of which six were exposed to HIV uninfected and twelve non exposed to HIV. For each sample the proportion of Gram + and Gram -, the presence or absence of cells was evaluated.

3.1 Gram and May Giemsa stain

Analysis of the results by Gram and May Giemsa staining made it possible to determine the proportion of bacteria and the presence of the cells in the microbiota. The proportion of Gram + and Gram - bacteria in the fecal microbiota of both populations showed that 67% of Gram + bacteria are present in HIV-exposed infants compared to 58% in non-exposed infants, but there are 42 % of Gram bacteria - in non-exposed infants vs 33% in HIV-exposed infants uninfected. Regarding the cells, the presence of cells in the fecal flora was higher in exposed infants (83%) compared to those not exposed (58%).

Comparing the composition of microbiota in infants exposed and unexposed to HIV

Several bacterial strains have been identified in the fecal microbiota of both populations. Some were present in both cases and others were present in only one of two populations (Figure 1).

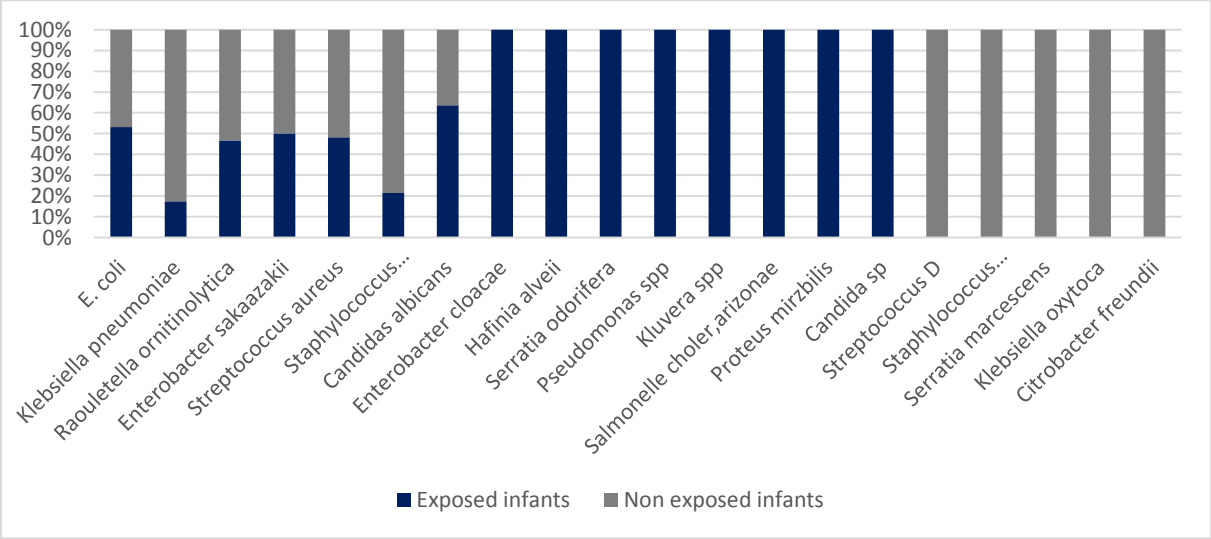

Fig. 1: Comparison and Diversity of the microbiota in exposed and non-exposed infants to HIV.

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A total of 29 bacterial species were isolated in the stools of 6 infants exposed to HIV against 26 bacterial species in the stools of 12 unexposed infants. *E. coli*, *Klebsiella pneunomiae*, *Raouletella ornitinolytica*, *Enterobacter sakaazakii*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Candida albicans* were the bacterial species present in the feces of both populations but in different proportions. *Enterobacter cloacae*, *Hafinia alveii*, *Serratia odorifera*, *Pseudomonas spp*., *Kluyvera spp*, *Salmonella choler. arizonae*, *Proteus mirabilis* and *Candida spp*. were bacteria found only in the microbiota of exposed infants but absent in unexposed infants. While *Serratia marcescens*, *Klebsiella oxytoca*, *Citrobacter freundii*, *Streptococcus D*, *Staphylococcus saprophyticus* were the only bacteria present in the microbiota of unexposed infants.

The isolated bacteria were grouped into families. Four microbial families were distinguished including Enterobacteriaceae, Staphylococci, Streptococci, and Candida (Table 1).

Table 1: Distribution of bacterial groups in the microbiota of exposed infants uninfected and not exposed to HIV.

Bacterial groups in microbiota	Exposed infants to HIV N (%)	Not exposed infants to HIV N (%)
Enterobacteria	20 (68.96)	16 (61.55)
Staphilococcus	5 (17.24)	8 (30.77)
Candida	4 (13.80)	1 (3.84)
Streptococcus	0 (0)	1 (3.84)
Total	29 (100)	26(100)

Table 1 shows the different groups of microorganisms present in the microbiota of exposed infants and not exposed to HIV. The microbiota of exposed infants was composed of a greater proportion of *Enterobacteria* (68.96%) and Candida (13.80%) while the proportion of *E. coli* was lower (61.55%) in the microbiota non-exposed infants with high levels of Staphylococcus (30.77%). In addition to the *Enterobacteriaceae*, the *Candida* and *Staphylococcus*, the microbiota unexposed infants contained *Streptococcus* (3.84%) totally absent in infants exposed to HIV.

The results of this study showed qualitative and quantitative differences between the faecal microbiota of exposed and unexposed infants. The table below (Table 2) shows the proportions and amounts of each microbial species depending on the status of HIV mother.

Table 2: Distribution of various microorganisms present in the fecal microbiota of infants

Taxonomies	Infants non exposed to HIV		Infants exposed to HIV	
	N. (%)	Quantity (UFC/ml)	N. (%)	Quantity (UFC/ml)
Klebsiella	9 (75%)	15005000	3 (50%)	2000000

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K.pneumeniae	5 (42%)	5005000	1 (17%)	1000000
Klebsiella oxytoca	2 (17%)	10000000	-	-
Raouletella ornithinolytica	2 (17%)	-	2 (33%)	1000000
Staphylococcus	7 (58%)	3600	5 (83%)	17525
Staphylococcus aureus	3 (25%)	100	4 (67%)	7525
Staphyloccocus epidermidis	3 (25%)	3400	1 (17%)	10000
Staphyloccus saprophyticus	1 (8%)	100	-	-
Escherichia	4 (33%)	5000050	5 (83%)	224200
E. coli	4 (33%)	5000050	5 (83%)	224200
enterobacter	1 (8%)	-	5 (83%)	842000
Enterobacter sakazakii	1 (8%)	-	2 (33%)	505000
Enterobacter cloacae	-	-	3 (50%)	337000
serratia	1 (8%)	-	2 (33%)	20000
Serratia marcescens	1 (8%)	-	1 (17%)	10000
Serratia odorifera	-	-	1 (17%)	10000
Candida	1 (8%)	-	4 (67%)	505550

Candida albicans	1 (8%)	-	2 (33%)	505000
Candida spp	-	-	2 (33%)	550
Citrobacter	1 (8%)	-	-	-
Citrobacter freundi	1 (8%)	-	-	-
Streptocoques	1 (8%)	1000000	-	-
Streptocoque D	1 (8%)	1000000	-	-
Hafria	-	-	1 (17%)	1000
Hafria alveil	-	-	1 (17%)	1000
Pseudomonas	-	-	1 (17%)	100
Pseudomonas spp	-	-	1 (17%)	100
Kluvera	-	-	1 (17%)	1000000
Kluvera spp	-	-	1 (17%)	1000000
Salmonella	-	-	1 (17%)	100
Salmonella choler. Arizona	-	-	1 (17%)	100
Proteus	-	-	1 (17%)	10000
Proteus mirabilis	-	-	1 (17%)	10000

N Number of infants

The analysis of fecal microbiota in exposed infants uninfected and not exposed to HIV shows a high diversity of microorganisms present in the fecal microbiota of infants exposed to HIV. Nevertheless there are microorganisms common to both microbiota but present in different quantities, specific to each infant and independent of HIV exposure.

Indeed, the most abundant bacteria in the fecal microbiota of infants not exposed to HIV were *Klebsiella*, *Staphylococcus*, *Escherichia*, *Enterobacter* and *Candida*. *Klebsiella* was predominant in the fecal microbiota of unexposed infants with an average of 75% of the number of colonized infants and an average of 15005000 CFU/ml per stool sample (75%, 15005000 CFU/ml) and dominated by the species *Klebsiella pneumoniae pneumoniae* (42%, 5005000 CFU/ml), followed by *Klebsiella oxytoca* (17%, 10000000 CFU/ml) and *Rauletella Ornithinolytica* (17%).

Then comes *Staphylococcus* (58%, 3600 CFU/ml), dominated by *Staphylococcus aureus* species (25%, 100 CFU/ml) and *Staphylococcus epidermidis* (25%, 3400 CFU/ml), followed by a rare occurrence of *Staphylococcus saprophyticus* (8%, 100 CFU/ml). *Escherichia genus* is the third most predominant bacterium, consisting mainly of *Escherichia coli* (33%, 5000050 CFU/ml). Finally there is the presence of *Enterobacter* sakazakii (8%), *Candida albicans* (8%) and *Serratia marcescens* (8%). However, in the case of HIV-exposed infants, the fecal microbiota was dominated by three strains of bacteria, *Staphylococcus* (83%, 17525 CFU/ml), of which *Staphylococcus aureus* is largely dominant (67%, 7525 CFU/ml), followed by *Staphylococcus epidermidis* (17%, 10000 CFU/ml), *Escherichia* composed only of the species *Escherichia coli* (83%, 224200 CFU/ml) and *Enterobacter* (83%, 842000 CFU/ml) of which the dominant species is *Enterobacter cloacae* (50%, 505,000 CFU/ml), followed by *Enterobacter sakazakii* (33%, 337000 CFU/ml).

Then comes the genus Candida (67%, 505 550 CFU / ml) consisting of *Candida albicans* (33%, 505 000 CFU/ml) and *Candida spp* (33%, 550 CFU/ml), Klebsiella strain (50%, 2000000 CFU/ml) consisting of *Klebsiella pneumeniae pneumonia* (17%, 1 million CFU/ml) and *Raouletella ornithinolytica* (33%, 1 million CFU/ml). Finally, the genus *Serratia* (33%, 20,000 CFU/ml), *Serratia marcescens* consisting of (17%, 10 000 CFU/ml) and *Serratia odorifera* (17%, 10 000 CFU/ml).

In addition to the common microorganisms in both microbiota, some bacterial strains are specifics to each microbiota. In unexposed infants to HIV, it is *Citrobacter freundii* (8%) and *Streptopococcus D* (8%) and among exposed infants, it is *Hafria alveil* (17%, 1000 CFU/ml), *Pseudomonas spp* (17%, 100 CFU/ml), *Kluvera spp* (17%, 1000000 CFU/ml), *Salmonella choler. Arizona* (17%, 100 CFU/ml), and *Proteus mirabilis* (17%, 10000 CFU/ml). Previous results showed that the intestinal microbiota of HIV-exposed infants is more diverse than that of unexposed infants.

4. DISCUSSION

Today with the Prevention of Mother to Child Transmission (PMTCT), the rate of HIV transmission from mother to child has dropped considerably. But despite this, this growing population of children experiences twice as much morbidity and mortality as infants born to HIV-negative mothers [9, 10].

The results of this study showed qualitative and quantitative differences between the faecal microbiota of infants exposed to HIV and the non HIV-exposed infants. The fecal microbiota of these two populations consisted of *Klebsiella*, *Staphylococcus*, *Escherichia Coli*, *Enterobacter* and *Candida* strains. In the microbiota of unexposed

infants, Klebsiella dominated by *Klebsiella pneumoniae pneumoniae* was predominant. However in the fecal microbiota of infants exposed the proportion of *Klebsiella* is minority.

On the other hand *Staphylococcus aureus* species is largely dominant, *Escherichia coli* and the *Enterobacter* strain are predominantly present in the faecal flora of HIV-exposed infants. Previous studies have shown the predominance of *Streptococcus*, *Enteroccoccus* and *Staphylococcus epidemis* and *S. aureus* in faecal microbiota of exposed infants to HIV. *Bifidobacterium*, *Clostridium* and *Lactobacillus* have not been identified, they also are representative of bacteria groups which colonize the fecal microbiota in exposed infants [13].

Treatment of HIV-infected mothers with antiretroviral drugs and antibiotic therapy are associated with changes in the microbiota of HIV-exposed infants may further contribute to the exposed infant's disrupted microbiome [9]. Indeed, studies have shown that antibiotics delay the colonization of beneficial and commensal bacteria such as *Bifidobacteria*, *Lacticobacilli* but favor the proliferation of pathogenic bacteria [14]. This could justify the proliferation of *Staphylococcus aureus* in the microbiota of exposed infants knowing that its infants are under BACTRIM which is a combination of two antibiotics including sulfamethoxazole and trimethoprim. Maternal HIV infection is associated with changes in the microbiota of HIV-exposed infants and maternal milk oligosaccharides in mothers have been associated with specific bacterial species in these infants [9].

Oligosaccharides, which constitute breast milk, have important direct associations with infantile stool bacteria, depending on whether the mother is seropositive or seronegative [13]. Indeed, previous studies carried out in 2016 on the microbiota of infants showed that in HIV-positive women, an increase of 3 'Siallilactose in oligosaccharides is observed and this increase was associated with a proliferation of Enterococcaceae and Fusobacteriaceae in the stool infants. In contrast, HIV-negative women increased lacto-N-fucopentaose was observed and reflected an increase in Bifidobacterium. [9].

Also, studies show that colonization of lactobacillus and Bifidobacteria in the digestive tract inhibits the growth of pathogenic microorganisms such as *Staphylococcus aureus* [16]. So a small amount of bacteria such as *Bifidobacteria* and *Lacticobacilli* may be responsible for disturbances in the microbiota of HIV-exposed infants. Many studies have shown the relationship between the gut microbiota and the immune system. Indeed, the intestinal microbiota is one of the first stimuli of the immune system [15], disruption of gut microbiota could therefore lead to a disruption of the immune system.

The results of this study showed that the microbiota of exposed infants was more diverse than that of unexposed infants. Several bacteria are absent in the microbiota of unexposed infants, particularly *pseudomonas spp* which in previous studies of the microbiota has been associated with HIV infection [17]. In addition to *Pseudomonas spp* we note the presence of *Salmonella choler Arizona* which is a potentially pathogenic bacterium.

5. CONCLUSION

Despite Prevention of Mother-to-Child Transmission Infants at Risk of HIV remain an important problem in our society. This study highlights qualitative and quantitative differences between fecal microbiota in exposed and unexposed infants. It is observed that the fecal microbiota of exposed infants in addition to being more diverse than

that of unexposed infants contains bacteria. Although potentially pathogenic, such as Salmonella choler Arizona and Pseudomonas spp., both microbiota present common bacteria but in different proportions and amounts depending on whether the infant is exposed or not.

These observed changes in the microbiota of exposed infants is the result of HIV infection in the mother who in previous studies is causing an imbalance in the gut microbiota. Indeed, the absence or late proliferation of beneficial bacteria to the host such as Bifidobacteria make immature and disrupt the intestinal microbiota. However, the intestinal microbiota is one of the first stimuli of the immune system and consequently a disturbance of the intestinal microbiota will therefore cause a disruption of the immune system and lead to an increase in the morbidity and mortality of infants exposed to HIV.

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