Early Human Development 90 (2014) 579–585

Contents lists available at ScienceDirect

Early Human Development

journal homepage: www.elsevier.com/locate/earlhumdev

Gut microbiota in preterm infants with gross blood in stools: A prospective, controlled study

Mohamed Ben Said [a,b], Stephane Hays [a,c], Deplhine Maucort-Boulch [d,e,f], Abdallah Oulmaati [a], Stefanyia Hantova [g], Claire-Marie Loys [a], Estelle Jumas-Bilak [g,h], Jean-Charles Picaud [a,c,f,*]

[a] Department of Neonatology, University Hospital Croix Rousse, Hospices Civils de Lyon, F-69004 Lyon, France
[b] Faculty of Medicine of Tunis, University of Tunis El-Manar, 1007 Bab Saâdoun, Tunis, Tunisia
[c] Rhone-Alpes Human Nutrition Research Center, F-69310 Pierre Bénite, France
[d] Department of Biostatistics, Hospices Civils de Lyon, F-69003 Lyon, France
[e] CNRS, Laboratoire Biostatistique Santé, UMR 5558, F-69310 Pierre Bénite, France
[f] Lyon-Sud Charles Merieux Medical School, Claude Bernard University Lyon 1, F-69310 Pierre Bénite, France
[g] University Montpellier 1, Laboratoire de Bacteriologie-Virologie, EA 3755 UM1, Faculte de Pharmacie, F-34093 Montpellier, France
[h] Department of Hospital Hygiene, CHU de Montpellier, F-34000 Montpellier, France

ARTICLE INFO

Article history:
Received 21 March 2014
Received in revised form 6 July 2014
Accepted 8 July 2014

Keywords:
Prematurity
Gut microbiota
Staphylococcus
Infection
Rectal bleeding
Human milk

ABSTRACT

Objective: Gross blood in stools is a peculiar entity in preterm infants, but little is known about its etiology. As gut microbiota can be distorted in preterm infants, we aimed to evaluate the gut microbiota in infants with gross blood in stools.
Study design: In a prospective, controlled, single-center study, we enrolled all infants born before 34 weeks of gestational age presenting gross blood in stools that was either completely isolated or associated with mild clinical symptoms or radiological signs. Each case was paired with two controls who were hospitalized in the same unit and were matched for gestational age and birth weight. The diversity of the gut microbiota was analyzed using 16S rRNA gene PCR and temporal temperature gel electrophoresis. We calculated a diversity score corresponding to the number of operational taxonomic units present in the microbiota.
Results: Thirty-three preterm infants with gross blood in stools were matched with 57 controls. Clinical characteristics were similar in cases and controls. There was no statistically significant difference in the diversity score between the two groups, but microbiota composition differed. The proportion of infants with *Escherichia coli* was significantly higher in cases than in controls ($p = 0.045$) and the opposite pattern occurred for *Staphylococcus* sp. ($p = 0.047$).
Conclusion: Dysbiosis could be a risk factor for gross blood in stools in preterm infants. Additional, larger studies are needed to confirm the implications of the presence of different genotypes of *E. coli* and to evaluate preventive actions such as the prophylactic use of probiotics and/or prebiotics.

1. Introduction

In premature infants gross blood in stools may be related to severe diseases (necrotizing enterocolitis (NEC), colitis in Hirschsprung's disease, infectious colitis, or hemorrhagic disease of the newborn) or to milder diseases (allergic colitis, anal fissure, or swallowing blood syndrome) [1,2]. Apart from these conditions, gross blood in stools is a peculiar entity either completely isolated or is associated with mild clinical symptoms or radiological signs (gastric residuals, vomiting, mild abdominal distension, and mild radiological intestinal dilation). Isolated rectal bleeding has been related to ecchymotic colitis [3].

Occurrence of gross blood in stools has a significant impact on neonatal care, as the management of this disorder often requires a fasting period to start or to extend parenteral nutrition, which increases the risk of catheter-related sepsis [4]. However, published data on gross blood in stools in preterm infants are scarce.

While the predisposing factors of severe NEC (≥stage II) are well-known, including colonization by potentially pathogenic bacteria or gut dysbiosis [5–8], it is not the case for gross blood in stools. Maayan-Metzger et al. reported that a feeding regimen that did not include breast milk was the only variable that predicted isolated rectal bleeding and emphasized the benign nature of this bleeding [9]. Luoto et al. did not identify any risk factors in a small number of very-low-birth weight infants fed human milk supplemented with probiotics [10]. Luoto et al.

Abbreviations: NEC, necrotizing enterocolitis; OTUs, operational taxonomic units; TTGE, temporal temperature gel electrophoresis.

* Corresponding author at: Service de Néonatologie, Hôpital de la Croix Rousse, 103 Grande rue de la Croix Rousse, 69004 Lyon, France. Tel.: +33 472 001 550.
E-mail address: jean-charles.picaud@chu-lyon.fr (J.-C. Picaud).

http://dx.doi.org/10.1016/j.earlhumdev.2014.07.004
0378-3782/

did not detect significant changes in the composition of the gut microflora in preterm infants with gross blood in stools, but they focused their investigation on a few main bacterial genera and species [10].

In the present investigation, we evaluated the relationship between the composition of the intestinal microbiota and gross blood in stools in preterm infants.

2. Methods

2.1. Study design and population

We performed a prospective, controlled, single-center study. All preterm infants hospitalized between January and October 2011 in our tertiary care unit at the University Hospital Croix Rousse, Lyon, France were eligible for study enrolment if they met the inclusion criteria of birth gestational age ≤34 weeks, absence of congenital malformation, and gross blood in stools. The nurses in charge of daily care for cases and controls collected systematically few times per day information about digestive tolerance as routinely recommended in our unit: gastric residuals, vomiting, abdominal distension, stool consistency and gross blood in stools. Diagnosis was confirmed by physicians by clinical observation. Gross blood in stools was either completely isolated or associated with mild clinical symptoms or radiological signs (gastric residuals, vomiting, mild abdominal distension, and mild radiological intestinal dilation). We did not include infants who developed NEC ≥stage II according to the modified Bell classification [11] or exhibited spontaneous intestinal perforation. The study protocol was approved by the ethics committee of Lyon (*Comité de protection des personnes Sud Est IV Lyon*).

Each case was matched with two controls. Controls were the first two preterm infants hospitalized in the same unit during the same period, and whose birth weight and gestational age were similar (±100 g and ±1 week, respectively) to those of the cases. These infants were selected by research nurses among hospitalized infants fulfilling the above criteria, independently from clinicians who took care of these infants. Infants included as controls were monitored as routinely recommended in our unit: daily monitoring of gastric residuals, vomiting, abdominal distension, stool consistency and blood in stools. When infants included as controls later presented rectal bleeding they were excluded from the control group and not replaced by another one, then included in the group of cases and paired with two controls.

2.2. Routine care protocol

The feeding regimen of the very-low-birth weight infants was not changed during the study period. Cases and controls were fed according to the same NICU protocol. Enteral feeding was started at day 1 or 2 and complementary parenteral feeding was administered until the enteral intake reached 100 mL/kg/day. All infants were fed pasteurized human milk (their own mother's milk or donor milk) according to French regulations [12] until their body weight was ~1500 g. Then, if the mother had no milk, feeding with the same preterm formula (PreMilumel, Milumel, Torce, France) was commenced. Human milk was supplemented with a cow's milk protein based multicomponent fortifier (Eoprotine, Milumel, Torce France). None of the infants included in our study received probiotics. Full enteral feeding was 160 mL/kg/day and was achieved by increasing the feeding by 10–20 mL/kg/day depending on the feeding tolerance of the infant. According to the NICU protocol enteral feeding was started as continuous feeding followed by bolus feeding when full enteral feeding was reached and digestive tolerance was satisfactory. After full enteral feeding was achieved, a thickener was used when infants presented signs of gastro-esophageal reflux (carob-based when the infant was fed human milk and starch-based when the infant was fed preterm formula). If the signs of gastro-esophageal reflux persisted, we administered domperidone with or without a proton pump inhibitor (omeprazole or esomeprazole) depending on the severity of the signs. Infants who presented clinical and ultrasound signs of persistent ductus arteriosus were treated with ibuprofen (10, 5, and 5 mg/kg/day at days 1, 2, and 3, respectively).

2.3. Management of gross blood in stools

When gross blood in stools occurred, we systematically assessed blood count cells, serum C-reactive protein levels, serum procalcitonin levels, and blood culture (aerobic and anaerobic). Stool samples were collected for bacteriological culture and for the identification of rotavirus and adenovirus. Abdominal x-rays were also performed. Then, infants received no enteral feeding for a few days, depending on the clinical and radiographic data collected at the time of rectal bleeding and in the following days. For each case with gross blood in stools, stool samples were collected from the two matched control infants.

2.4. Data collection

We collected data on the pregnancies, the deliveries (antenatal steroids, mode of delivery, maternal diseases), the infants' characteristics at birth (gestational age, birth weight, gender, Apgar score), growth restriction (body weight less than −2SD for gestational age) [13] treatments during hospitalization before gross blood in stools (ventilation, antibiotics, ibuprofen treatment, postnatal steroids, anti-reflux treatment, breast milk or formula), treatments at the time of gross blood in stools (body weight, clinical examination, x-ray results, C-reactive protein levels, procalcitonin levels, complete blood count, blood culture), and information about the infant after gross blood in stools (fasting period, recurrence of rectal bleeding).

2.5. Analysis of gut microbiota

The qualitative composition of the gut microbiota was assessed via extraction of bacterial DNA from stool samples, PCR, temporal temperature gradient electrophoresis (TTGE), and identification of amplified sequences. Stool samples collected from cases and controls were stored at −80 °C until analysis. Approximately 50 mg of each homogenized stool specimen was placed in 1 mL of sterile DNA-free water in a 1.5-mL tube. The suspension was centrifuged for 10 min at 10,000 ×*g*. DNA was extracted from the pellet using the MasterPure Gram Positive DNA Purification Kit (Epicentre, Madison, WI, USA) according to the supplier's instructions and optimized by Roudière et al. [14].

The V2–V3 region (233 bp) of the 16S rRNA gene was amplified with primers HDA1 (with a GC-clamp) and HDA2 [14]. The reaction mixture (50 μL) contained 200 μM of deoxynucleoside triphosphate mix, 10 pmol of each primer, 2.5 U of Taq DNA polymerase in the appropriate buffer (FastStart High Fidelity PCR system, Roche, Basel, Switzerland), and 1 μL of template DNA. The amplification program, which was carried out with a Mastercycler apparatus (Eppendorf, Le Pecq, France), was 95 °C for 2 min, 30 cycles of 95 °C for 1 min, 62 °C for 30 s, and 72 °C for 1 min, with a final extension of 72 °C for 7 min. PCRs were checked by electrophoresis migration on a 1.5% agarose gel stained with ethidium bromide (500 μg/mL) and visualized on an ultraviolet transillumination system.

The DCode universal mutation detection system (Bio-Rad Laboratories, Marne La Coquette, France) was used for TTGE. Five microliters of the PCR product was added to 5 μL of loading buffer. The gels were prepared with 8% (wt/vol) bisacrylamide (37.5:1) and 7 M urea and were run with 1X Tris–acetate–EDTA buffer. Denaturating electrophoresis was performed at 46 V for 16 h with a temperature gradient of 63–70 °C (0.4 °C/h). Gels were stained with 10 mg/mL ethidium bromide and photographed with an ultraviolet transillumination system.

Identification of the TTGE bands was performed as described previously, via comparison of migration distances to the “gut microbiota diversity ladder” and sequencing on an ABI 3730XL sequencer (Takeley, United Kingdom) [14,15]. The resulting DNA sequences were compared