Full Length Research Paper

Evaluation of rotavirus and intestinal parasite infection in a paediatric population in West Cameroon: Clinical characteristics and virus genotyping

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The prevalence of Rotavirus (RV)-related gastroenteritis in Cameroon was monitored in this study, identifying the circulating G-P types, and the presence of co-infection with intestinal parasites. The stool samples were examined for rotaviruses using chromatographic and molecular techniques, and parasites were identified by macroscopic and microscopic examination. 38% of samples were RV positive. Data revealed the presence of three different G-genotypes (G1, G2 and G8) and three P-genotypes (P [8], P[6] and P[4]). The main G-P combination was G1+G2P[8]. *Entamoeba histolytica* was identified in 44.4% of stool samples. The G2P[8] genotype identified is an unusual strain with the G1P[8] or G2P[4] more common. Genotype G8 strains, also associated with animals, have been sporadically recovered from humans and have been considered for inclusion in bovine-human reassortant vaccine. Epidemiological RV strain surveillance should be continued in order to support RV vaccination campaigns. The results also show a high prevalence of intestinal parasite infections.

Key words: Gastroenteritis, rotavirus, G/P genotype, semi-nested multiplex PCR, intestinal parasites, Cameroon.

INTRODUCTION

Acute diarrhoea is one of the most common childhood illnesses in both developing and developed countries. The etiological agents of diarrhoea include viruses, bacteria and parasites (Huh et al., 2009).

It has been estimated that worldwide, each year, diarrhoea attributable to rotavirus infection results in 453 000 deaths in children younger than 5 years which accounts for 37% of deaths attributable to diarrhoea and 5% of all deaths in this age group (Tate et al., 2012). 90% of these deaths occur in sub-Saharan Africa and South East Asia (Waggie et al., 2010).

The genes encoding the outer capsid proteins, VP7 and VP4, form the basis of classification of group A rotaviruses into G and P genotypes, respectively. Currently,
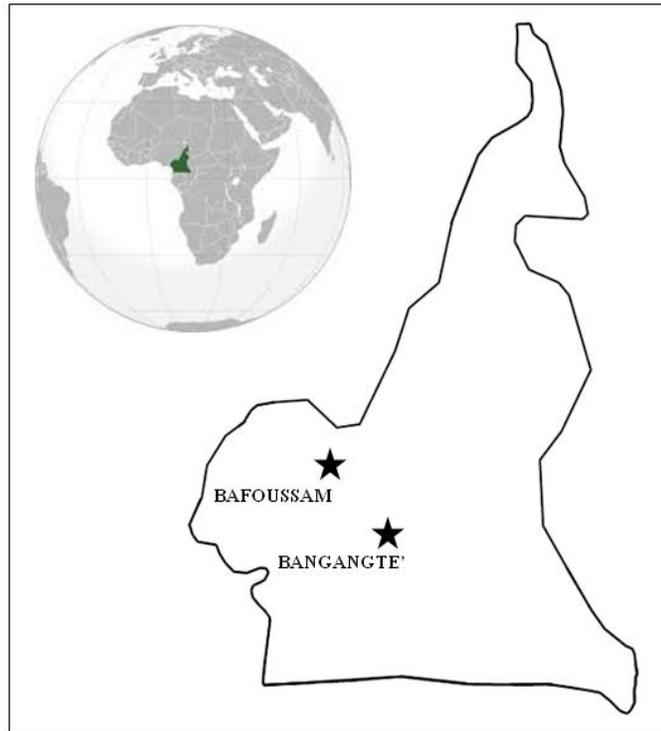
Figure 1. Map of Cameroon: localization of the hospitals included in the study.

27 G genotypes (G1-G27) and 35 P genotypes (P[1]-P[35]) have been described for RVs (Matthijnssens et al., 2011). Five combinations of these are common world-wide (G1P[8], G3P[8], G4P[8], G2P[4], and G9P[8]), accounting for 70% of globally identified strains (WHO, 2011; Cashman et al., 2011). However, these fully iden-tified combinations account for only 36.5% of strains circulating in Africa (Todd et al., 2010). In recent years, other G/P combinations have been found to be highly prevalent in various areas of the world (De Donno et al., 2009; Petrinca et al., 2010; Iturriza-Gómara et al., 2011; Patton, 2012). In Cameroon, in particular, has documen-ted the presence of novel human rotavirus genotypes, such as G5P[7], G5P[8], G8P[8] (Esona et al., 2004, 2009). This data reinforce the need to continue with sur-veillance programs in Cameroon where a high diversity of rotavirus strains has been reported.

Intestinal parasite infections are also common, with the greatest incidence and intensity occurring in developing countries (Mbuh et al., 2010). Roughly two billion people worldwide are estimated to be affected by geohelmin-thiasis, caused mainly by *Ascaris lumbricoides*, *Ancylostoma/ Necator* spp. and *Trichuris trichiura*. A further 50 million are infected by *Entamoeba histolytica* and 2.8 million by *Giardia duodenalis* (Kongs et al., 2001; Kouontchou et al., 2002; Guidetti et al., 2011).

So far, epidemiological surveillance of RV infection is lacking in almost all areas of Cameroon, and the avai-lable data is not sufficient to assess the real impact of RV infections as a public health issue in the country.

The aims of the present study were to assess the pre-valence of RV-related gastroenteritis in West Cameroon, and to identify the circulating G-P types. Using the same stool samples, we also assessed the presence of intes-tinal parasites in order to describe any clinical implica-tions resulting from the presence of co-infections.

MATERIALS AND METHODS

Study area and population

This research was performed in the West Region of Republic of Cameroon, which is a 13.872 km² territory, the smallest of the country's ten regions, with the highest population density (124/km²) and with a total population of 1.720.047 (census 2005). The West consists of eight divisions or departments. Our study took place in the MIFI department, particularly in its capital city, Bafoussam, and in the main city of the Ndé division, Bangangté (Figure 1).

Bafoussam, with a total population of 290,768 inhabitants, is characterized by two prevalent seasons: the dry one, from November to February and the rainy one, from March to October. The other town included in this study is Bangangté with a total population of 65,385 inhabitants. This area is instead characterized by four prevalent seasons: the short rainy season, from March to

May, the short dry season, from May to June, the long rainy season, from June to October and the long dry season, from October to March.

This pilot research took place in urban settings in the West of Cameroon, during the rainy season, to improve our knowledge on the burden of rotavirus disease for imminent (is expected in 2013) introduction of a rotavirus vaccine.

Enrolment and patients

Female and male patients aged 0 to 10 years presenting with acute diarrhoea (defined as at least 3 episodes of soft or liquid stools in the last 24 h) and/or other signs/symptoms suggesting intestinal infection (vomiting, fever, dehydration and abdominal pain) were included in the study.

Children were recruited from patients coming to the "Hôpital de District de Bangangtè" in Bangangté and the "Hôpital de District de la Mifi" in Bafoussam, Cameroon. The severity of gastroenteritis was evaluated using the Ruuska Vesikari Scale (Ruuska and Vesikari, 1990).

The study was approved by the ethics committees of the "Hôpital de District de Bangangté" in Bangangté and of the "Hôpital de District de la Mifi" in Bafoussam, in accordance with the Helsinki Declaration on ethical principles for medical research involving human subjects. Written informed consent was obtained from one of the parents of each patient in accordance with the local Ethics Committees's requirements and Cameroonian law.

Rotavirus investigations

Stool sample collection

From June to August 2010, stool specimens were collected from children with acute gastrointestinal infections in the first 48 h after recruitment.

The specimens were collected anonymously according to the Helsinki Declaration.
Samples were cryopreserved, stored at –20°C and transferred in a frozen state to laboratories at the University of Salento, where immunological analyses by chromatographic assay and genotype virological analyses by molecular techniques were performed.

Chromatographic assay

Each stool sample was analysed using the Cer*Test*® Rota-Adeno Card (CerTest, Biotec, Spain), which is a one-step chromatographic immunoassay for qualitative detection of RV and/or adenovirus in stool samples. This assay has a reported manufacturer's sensitivity of 100% for RV and 90% for adenovirus, and a specificity of 99% for RV and 100% for adenovirus.

Molecular techniques

Stool samples (0.5-1.0 ml) were suspended in 5 ml of 0.89% NaCl solution, centrifuged for 20 min at 3,000 rpm and filtered through a 0.22 µm filter. The double-strand viral RNA was extracted using the QIAamp Viral RNA kit™ (QIAGEN AG, Basel, Switzerland) in accordance with the manufacturer's instructions.

RNA was retro-transcribed and amplified by real-time PCR (Fastset RV™, Arrows Diagnostics, Italy).

Positive samples were further analyzed to determine G and P types using semi-nested multiplex PCR as described by Iturriza-Gómara et al. (2004).

All the RT-PCRs were performed with viral RNA extracted from reference samples as G/P positive controls and RNase-free water as a negative control.

The RT-PCR products were analyzed by electrophoresis in Tris-borate-EDTA buffer and 2% agarose gel, stained with ethidium bromide and visualized under UV light.

Parasite identification

Intestinal parasites were identified by macroscopic and microscopic examination of fresh stool samples at the Université des Montagnes laboratory, Cameroon. Samples were processed and examined within 12 h of collection. A sample of 2 mg (or mucous in the case of dysentery) was placed on a slide, mixed with a drop of saline solution, covered with a 20 x2 4 mm slide cover and examined with 10x and 40x objectives. A part of each stool sample was fixed in 10% formalin and sent to the Parasitology Laboratory of the Infectious Disease Institute, University of Ferrara, Italy, where it was microscopically re-examined, with and without Lugol solution (5% iodine, 10% potassium iodide in distilled water). In order to increase sensitivity, samples were also concentrated in accordance with a modified version of the Ridley-Allen method (Allen and Ridley, 1970), using ethyl acetate instead of ether.

RESULTS

A total of 56 patients were recruited from June to the middle of August 2010, 41 (73.2%) in the "Hôpital de District de Bafoussam" in Bafoussam, and 15 (26.8%) in the "Hôpital de District du Bangangté" in Bangangté.

Two patients did not meet the inclusion criteria: one because the clinical form was filled in, but the stool sample was not collected, the other because the stool sample was not collected within 48 hafter recruitment. Despite a target range of 0 to 10 years, 92.6% (50/54) of the patients enrolled were <5. The main reported symptoms were fever (80%), diarrhoea (70%), vomiting (46%) and dehydration (20%). A total of 22.2% (12/54) patients had a Vesikari score ≥11 and 77.8% (42/54) a Vesikari score <11.

Rotavirus detection

Chromatographic screening revealed four of 54 stool samples (7.4%) positive for rotavirus. Real-time RT-PCR was performed on 50 samples with four insufficient for further analysis. Of these 50, which included the four positives from the screening test, 19 (38%) were positive. Real Time PCR thus confirmed the positives found in the screening test and detected RV in a further 15 samples, equivalent to 32.6% of the 46 samples that were negative in the screening test.

The 19 positive samples were further investigated to define the VP7 and VP4 genotypes using semi-nested multiplex PCR. Of these, 13 samples were partially typed (only the VP7-type was obtained but not the VP4-type). A total of 6 specimens were characterized in terms of both

Table 1. Age distribution of patients for the rotavirus genotypes circulating in the Western Province of Cameroon.

Age Group (month)	samples collected (N)	RV positive number (%)	Genotypes identified
0-12	18	8 (42,1)	G2 (4 times) - G8 (1 time) - G1P[8] (1 time) - G2P[4] (1 time) - G2P[6] (1 time)
13-24	16	2 (10,5)	G2 (2 times)
25-60	12	5 (26,3)	G2 (3 times) - G1 (1 time) - G2P[8] (1 time)
61-120	4	4 (21,1)	G2 (2 times) - G1+G2P[8] (2 times)
0-120	50	19 (100)	

G and P specificities (4 = single G&P and 2 = mixed G&P).

Molecular analysis of the VP7 gene revealed the presence of three different genotypes (G1, G2 and G8). Among these, genotype G2 was predominant (73.7%; 14/19) whereas G1 was identified in 10.5% (2/19) of positive samples and G8 in 5.3% (1/19). Furthermore, G1 + G2 co-infection was observed in 2 samples (10.5%).

Among the VP4 genotypes, P[8], P[6] and P[4] accounted for 66.6% (4/6), 16.7% (1/6) and 16.7% (1/6) of positive samples respectively.

Overall, five G/P combinations were identified: G1+G2P[8] was identified in 33.3% (2/6) of positive samples, while G1P[8], G2P[8], G2P[4] and G2P[6] were each identified in 16.7% of samples (1/6).

Of the 19 patients that were positive for RV infection, 79% (n=15) were <5 years of age, 16% (n=3) had a Vesikari score ≥ 11 and 63% (n=12) were hospitalized and rehydrated.

Table 1 shows the age distribution of patients for the RV genotypes circulating in the West Cameroon. RV infection was found in all the age groups at different frequencies. Among the globally common strains, G2 was found circulating in all age groups, while G1P[8] and G2P[4] were identified only in younger children (<12 months). G8 and G2P[6] were also identified in the first age group while G2P[8] strain was found circulating among children 25-60 months of age. Mixed infections, instead, were identified only in older children (>60 months).

Parasite detection

Cysts of *E. histolytica* were identified in 44.4% of samples (n=24) while co-infections of *E. histolytica* and *A. lumbricoide)* were observed in 13.0% (n=7). In 27.8% (n=15) of samples, there were other, non-pathogenic *Endamoebidae,* such as *Entamoeba coli (E. coli)*, *Entamoeba hartmanni (E. hartmanni)* and *Endolimax nana (E. nana)*. Negative results were obtained in 14.8% of samples (n=8).

In the present study, among the 19 RV positive cases, only one of them was determined to have co-infection with *E. histolytica*, with co-infection rate of 5.26% (1/19); ten were determined to have co-infections with other non-

pathogenic *Endamoebidae*, with co-infection rate of 52.63% (10/19) and eight were determined not to have co-infections by intestinal parasites.

Of the 42 stool samples from patients with a Vesikari score <11, the most frequent diagnosis was *E. histolytica /E. dispar* (35.7%; 15/42), followed by *E. coli*, *E. hartmanni* and *E. nana* (30.9%; 13/42), and co-infection involving *E. histolytica* and *A. lumbricoides* (16.7%; 7/42). In this group, 16.7% of samples (7/42) were considered negative and no co-infection involving RV and *E. histolytica* was observed (Figure 2A). In this group, no case of co-infection involving RV and pathogenic intestinal parasites was documented.

In the 12 patients with a Vesikari score, ≥11 parasitic organisms identified included *E. coli*, *E. hartmanni E. nana* (16.7%; 2/12), and *E. histolytica* (75.0%; 9/12) while one sample (8.3%) was negative (Figure 2B). In this group was documented the only case of co-infection involving RV and *E. histolytica*. This patient was older than the standard age of patients with RV gastroenteritis, and presented a more severe clinical syndrome than the other RV-positive subjects.

DISCUSSION

Worldwide, circulating wild-type RV strains exhibit great diversity (Grassi et al., 2012). Current oral live attenuated vaccines against RV have the potential to greatly reduce the burden of severe RV disease in Africa. They include a monovalent human P[8]G1 RV vaccine (Rotarix™, Glaxo-SmithKline) and a pentavalent G1, G2, G3, G4 and P[8], bovine–human, reassortant vaccine (RotaTeq™, MERCK). Clinical trials of each vaccine have been conducted in Africa (Todd et al., 2010). Both vaccines have proved to be effective in preventing severe RV gastro-enteritis (Kirkwood, 2010).

Most of the rotavirus cases were identified in the age group 0-24 months, in particular in the age group 0-12 months, in accordance with other studies which also showed that rotavirus infection decreases with increasing age, due to acquired immunity that comes with age or asymptomatic infections (Ndze et al., 2012). Caution should be taken in interpreting these results as data was not collected for a sufficiently longer period.
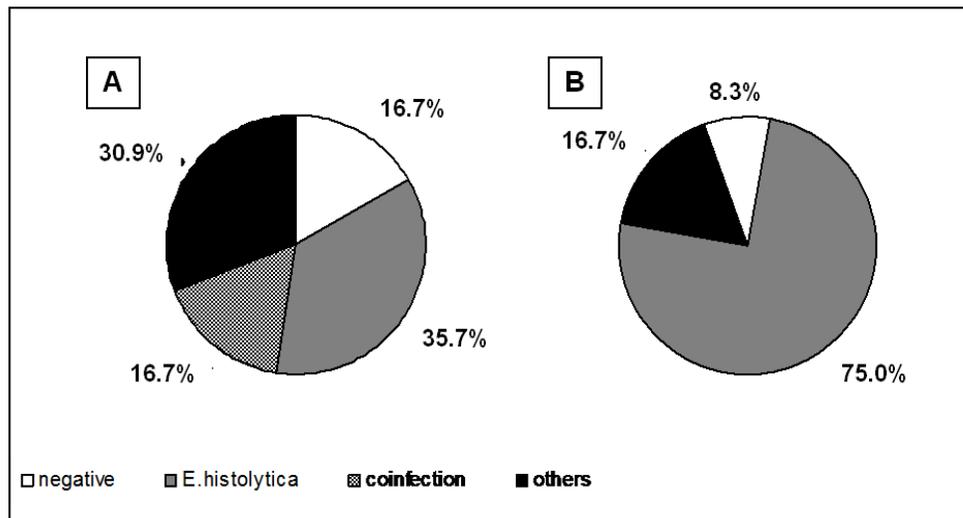
Figure 2. Distribution of parasitic infections in patients with a Vesikari score <11 (**A**) and ≥11 (**B**).

Molecular analysis of the VP7/VP4 genes revealed the presence of five different G/P combinations in the studied area. In this study, only 2 of the most common G/P combinations were identified (G1P[8] and G2P[4]), representing 33.4% of the typeable RV samples. However, the G2 genotype was also detected in association with P[6] and P[8]. Several studies in Africa have documented the association of G2 with P[6], in addition to the more typical P[4] allele (Page and Steele, 2004), while G2P[8] is a new and unusual G/P combination which, like G1P[4] and G4P[4], has been detected at relatively high frequency in various parts of the world in recent years (De Donno et al., 2009). Mixed RV infections (comprising >1 G type) were found in 33.3% of the typeable RV samples (G1+ G2P[8]). These mixed infections most likely represent naturally occurring reassortment among RV strains (De Donno et al., 2009) and were identified only in older children (>60 months).

Furthermore, this study documents the detection of the G8 genotype in West Cameroon. This type (also associated with animal infections) has been sporadically detected in humans in various geographical areas, first in Indonesia (Matsuno et al., 1985) and later in Finland, Italy, Nigeria, Brazil (Gerna et al., 1990; Adah et al., 1997) and other parts of the African continent, especially Malawi (Cunliffe et al., 1999; Matthijnssens et al., 2006). In this study, the detection at low levels of unusual G8 genotype was quite similar to what has been reported in studies conducted by Esona and co-workers during a 1999-2000 study in Western Cameroon (Esona et al., 2010).

In addition, two G genotypes (G1 and G2) identified in this study are present, in association with P[8], in the RV vaccine formulations currently used in many parts of the world. It has recently been proposed (Kapikian et al.,

2005) that the third genotype identified in this study (G8) be included in a bovine-human hexavalent reassortant RV vaccine (together with G types G1-G4), making it potentially suitable for use in developing countries.

Finally, there was a high number of untypeable P-type RV strains. It is possible that these represent unusual RV strains or strains of animal origin. Alternatively, the quality of the primers used for RT-PCR could be blamed because they do not possess consensus sequences, due to strains that have mutated in the primer binding site (Iturriza-Gómara et al., 2004; Mwenda et al., 2010).

It is noteworthy that PCR enabled detection of RV in 15 samples that were negative with the CerTest® Rota-Adeno card. The differing detection numbers are due to the different detection limits of the tests. Typically, antibody-based detection assays are 1000 - 10 000 fold less sensitive than real-time PCR; consequently the discrepancy observed in this study could be related to a low viral load in stool specimens.

Regarding the search for intestinal parasites, our findings show that the incidence of intestinal protozoan infections is relatively high in our study area, in line with the literature. Indeed, the prevalence of intestinal parasites in developing countries (the main causes of which are inadequate hygiene and sanitation) has been widely documented (Guidetti et al., 2011; Noormahomed et al., 2003; Norberg et al., 2003; Adeoye et al., 2007; Nyarango et al., 2008).

E. histolytica, A. lumbricoides and *E. coli* were the most commonly observed intestinal protozoa in the study area. These results are similar to those reported in other regions of Cameroon (Mbuh et al., 2010; Kouontchou et al., 2002), but they are not in line with studies in other countries, which found that the most prevalent protozoan species was *G. lamblia* (Beltran et al., 2004; Daryani et al.,

2003).

In the present study, only one case of co-infection involving RV and *E. histolytica* was documented: this patient was older than the standard age of patients with RV gastroenteritis and had a Vesikari score ≥11. He was the only one who was hospitalized, due to moderate / severe dehydration. This suggests that the presence of co-infections can result in major clinical complications, and thus it is recommended to perform surveillance not only of rotavirus but also of parasites.

Conclusions

This research is a pilot study for a project to be conducted in 2012-2013. Patients were recruited during the rainy season, which does not coincide with the annual peak in RV gastroenteritis cases. Data for the dry season are lacking. The preliminary data on RV infection were consistent with the literature and showed that RV is one of the major aetiological agents of paediatric diarrhoea in the studied areas also during the rainy season.

It is very important to document the rotavirus epidemiology and strain circulation before vaccine introduction and to continue monitoring after the vaccine is introduced.

This study also confirmed a high prevalence of human intestinal parasitic infection, mainly due to *E. histolytica*, and it assessed co-infection of intestinal parasites with RV. This preliminary evaluation could represent the starting point for improving organizational and technical aspects related to the implementation of larger studies of RV-intestinal parasite co-infection.

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