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Virus diversity of acute diarrhea in tropical highlands

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ABSTRACT. Infectious acute diarrhea (IAD) is an important health problem affecting a large number of Latin-American children. Several reports show that bacteria, parasites and virus are involved in the burden of this disease. Most reports reveal Rotavirus A as the responsible etiological agent, at the same time, there seems to be some correlation between IAD and seasonal weather changes. To learn about the type of microbial agents associated with IAD in children during mildly changing yearly climatic conditions, as found in a high altitude tropical city, and to identify the viral agents affecting this population, stool samples from 300 children under 5 years of age were studied throughout a one-year period. Bacteria and intestinal parasites were identified by routine methods, while viruses were detected and typed by EIA and PCR. 20.6% of the IAD studied was associated with bacteria; 9% with parasites and 40% with virus. Group C Rotavirus accounted for 20.2%, group A Rotavirus for 13% and Calicivirus 10%. During November-April ($p<0.007$) more virus associated IAD was found, while bacteria ($p<0.03$) or parasite ($p<0.00014$) related IAD was prevalent from May to October. The mild seasonal weather changes don't seem to be associated with any other microbial agent.

Key words: Infectious acute diarrhea, group A rotavirus, group C rotavirus, calicivirus, astrovirus, enteric adenovirus.

RESUMEN. La enfermedad diarreica aguda (EDA) es un problema importante de salud que afecta a un gran número de individuos en América latina, principalmente a los niños. Muchos reportes muestran que algunas bacterias, parásitos y virus pueden estar implicados con esta patología y se han señalado a los Rotavirus A como los responsables de un alto número de los casos. También se ha observado que en países localizados fuera de la zona ecuatorial, existe un comportamiento estacional relacionado con la aparición de las EDAs.
Con el objeto de conocer el comportamiento de las EDAs en regiones ecuatoriales altas (más de 2.000 mts. sobre el nivel del mar), donde los climas permanecen constantes todo el año, se analizaron en 300 muestras diarreicas de niños menores de cinco años, la presencia de virus, parásitos y bacterias. Estas muestras fueron recolectadas por un periodo de un año.
Las bacterias y parásitos fueron identificados con los métodos convencionales y los virus fueron identificados con pruebas de ELISA y PCR. 20.6% de los casos estudiados se asociaron con la presencia de bacterias, 9% con la presencia de parásitos y 40% con la de virus. El Rotavirus del grupo C fue hallado en 20.2% de los casos, Rotavirus del grupo A en un 13% y Calicivirus en un 10%. Durante el periodo entre noviembre y abril se encontró mayor presencia viral ($p<0.007$) mientras que bacterias y parásitos presentaron mayor prevalencia en los meses de mayo a octubre ($p<0.03$ y 0.00014 respectivamente). Con este estudio se dilucidó que la EDA, en climas de zonas ecuatoriales altas, no parece estar asociada con ningún otro agente microbiano.

Palabras clave: Enfermedad diarreica aguda, Rotavirus del grupo A, Rotavirus del grupo C, Calicivirus, Astrovirus, Adenovirus entérico.

INTRODUCTION

Infectious Acute Diarrhea (IAD) in children has been established as one of the most important causes of morbidity in developing countries. Each year diarrhea related disease cause almost 1 billion cases of episodes of diarrhea in developing countries in children under 5 years of age.[1,2] Estimated incidence rates range from 3,5 to 7 episodes/child/year during the first two years of life, and from 2 to 5 episodes/child/year for the first 5 years.[3]

In Latin America, the incidence of diarrhea among infants varies widely between different regions and localities. In the poorest periurban communities of Peru and Brazil, the average annual number of diarrhea episodes per children under 2 reaches 10 or more;[1] in Venezuela, the incidence is 2.2 episodes/child/year in children under 5.[5] The mortality rate is higher in children under one year of age, with a world estimate between 4.5 to 21.8 deaths per 1,000 live births. Between 1 and 4 years, the rate drops to 4.6 deaths per 1,000. Nevertheless, data from Pan American Health Organization (PAHO) indicated that the overall mortality from diarrhea related disease in Latin America has declined over the past years.[5]

Rotavirus A (RV A) and *E. coli* have been reported worldwide to be the principal cause of gastroenteritis. Rotavirus infections account for up to 60% of all diarrhea episodes in developing countries and an estimated 440,000 deaths in children every year.[6]

Its epidemic peak occurs in winter in temperate climates.[2] Enteric adenovirus (AdE), Calicivirus (CV), Rotavirus C (RV C) and Human Astrovirus (HAstV) have not been so extensively studied but there are some reports about their prevalence.[7-12]

Bacteria as *Campylobacter* spp, *Salmonella* spp, *Shigella* spp and *Yersinia* spp are also important etiologic agents

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in IAD. *Shigella spp* is the most important cause of acute[6] bloody diarrhea and account for about 15% of all deaths attributable to diarrhea in children under 5 years of age.[2]

Most studies report that viral diarrhea is epidemic, with bouts that can be related to humidity, rainfall and seasonal temperatures.[13]

Colombia is a developing country located across the equator in the northwestern corner of South America. The capital, Bogotá, is located on a plateau of some 600 km^2, 2,300 m. above sea level, known as Sabana, 4°38' north of the Equator. Facatativá, a town of some 70,000 people, is located in the Sabana, 30 km from Bogotá, with an approximate children population of 9,000 under the age of 5. There are no seasons and the temperature varies very little throughout the year with an average around 14°C. However, there are strong temperature changes during the day, with temperatures close to freezing around 3 a.m. and as high as 20-22°C at noon. Even though there are no marked rainfall seasons, there are periods of higher and lower rainfall through the year shown around April and October. Average humidity is 70% and it usually increases with higher temperatures. There are some industrial pig and cattle farms in the rural areas of the town, however, figures from the State Health Service shows that Facatativa has higher rates of IAD than similar size towns around Bogotá. (Personal communication).

In this article we describe i) the virus diversity involved in acute diarrhea in tropical highlands, where CV and RV C appeared as important and emergent agents, and ii) the relationship between microbial prevalence and environmental conditions found in the Colombian highlands.

MATERIAL AND METHODS

Location and description of study: Facatativa is a town with close to 70,000 inhabitants located in the Colombian Highlands, 2,300 meters above sea level. It has an average temperature of 14°C, without any marked differences in rainfall or temperature throughout the year.

Specimen collection: Stool samples were collected from children under 5 years of age who came to the emergency room at San Rafael Hospital, Facatativa, Colombia, with acute gastroenteritis between November, 1999 and October, 2000.

Detection of bacteria and parasite agents: Fecal samples were cultured routinely for presence of bacterial pathogens (*E. coli, Salmonella, Shigella, Edwarsiella*), and examined by direct microscopy for eggs and parasites. Strains of *E coli* were classified by aglutination test.

Detection of viruses: RV A, AdE (seotypes 40/41), and HAstV were detected with a Kallestad EIA kit (Pathfinder Kallestad Diagnostics, Chaske. Minn) for RV A; Adenoclone 40/41 (Meridian Biosciencie Europe) gently donated by CDC, Atlanta and Dako (Denmark) for Astrovirus, following the manufacturer's recommendations. CV were analyzed by RT PCR techniques using primers MON 431, 432, 433 and 434, previously reported by S. Monroe at CDC. RV C was detected by a homemade EIA kit. (Gutierrez et al. Under revision). Briefly, for RV C, 282 of 300 fecal specimens were diluted 10-fold in PBS and tested by EIA using reagents specific to the prototype porcine Cowden strain.[8]

Typing of RV A and HAstV: Samples positive for RV A and HAstV were further typed according to the method described by Gouvea for VP7[14] and Gentsch for VP4 RV genes[15] and Noel for HAstV.[9] Viral RNA was extracted from fecal samples that had been stored at –4°C using Trizol reagent (GIBCO BRL), following suspension of stool in PBS. Extracted RNA was used for reverse transcription and PCR.

Statistical analysis: Six parameters were considered: age, gender, period of the year for each diarrhea episode, humidity (%), temperature (°C) and rainfall (mm). Statistical analysis was performed with EPIINFO 6.1. Three different analyses were performed: 1) A descriptive statistical analysis to find the frequencies of each pathogen. 2) A bivariated analysis in order to clarify the relationship between pathogen and risk factors and 3) a hypothesis test to find out whether or not there was epidemic or endemic behavior of the pathogens.

RESULTS

163 of the 300 outpatients children studied were girls, and 137 boys. Mean age was 31 months. Definite IAD diagnosis was possible only in 55% of the stool samples. Bacteria were found in 20.6%, intestinal parasites in 9% and virus in 40% of the total samples. RV C was by far the most common agent found (20.2%), followed by RV A (13%) and CV (10%). (Table 1). When genotyping was attempted for RV A, 14 samples were found to be G1 and 11 P8 No other G or P types were found.

Five children were infected with bacteria, parasites and virus at the same time; 4 with both bacteria and parasites; 21 with virus and bacteria and 9 with parasites and virus. (Fig. 1) None were infected with *E. coli, Salmonella, Shigella, Edwarsiella* and *Campylobacter* at the same time, and only three had *Campylobacter - Edwarsiella* together. None were infected with the five virus. Eleven children had RV A and C together; five had RV C and CV; four children had RV A and CV; three had RV C and AdE, two children had RV A and AdE and, two children had HAstV and AdE.

Using bivariate analysis (Table 2), the only variable relationships found was humidity and *Giardia lamblia* (OR = 4.19 and CI=1.27-10,7). This suggests that high

Table 1. Distribution of frequencies by gender and type of pathogen. * Parasites were found as cysts and just two were trophozoites.

Pathogen	Positive cases	%	IC 95%	Positive cases in girls	Positive cases in boys
BACTERIA	62	20.6	16-25.2	40	22
E. coli enteropatogenica	26	8.6	5.3-12	17	9
E. coli hemolytic	0	0	0	0	0
Salmonella sp	6	2	0.2-0.37	4	2
Shigella sp	4	1.3	0-0.2	3	1
Edwarsiella sp	16	5.4	2-8	9	7
Campylobacter sp	13	4.3	1.8-6.8	9	4
VIRUS	120	40	34.2-45.7	64	56
Rotavirus A	39	13	9.0-16.9	23	16
Adenovirus40/41	7	2.3	0.4-0.42	5	2
Astrovirus	8	2.7	0.4-0.6	3	5
Rotavirus C	57	20.2	15-27	34	23
Calicivirus	30	10	1.1-5.5	13	17
PARASITE	26	9	5.3-12	11	15
Entamoeba hystolitic *	14	4.7	2-7	4	10
*Giardia lamblia**	14	4.7	2-7	8	6
Non identify agent	135	45			

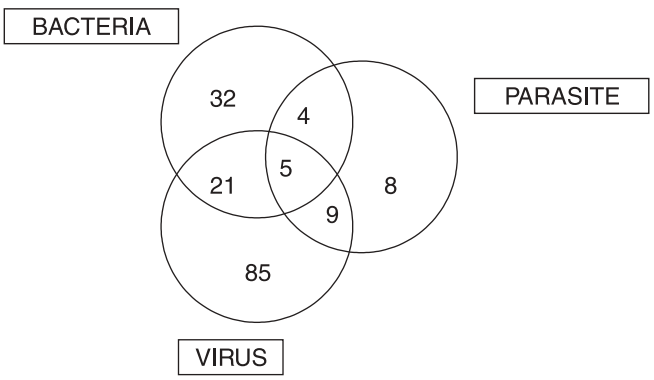

Figure 1. *Virus, Bacteria and Parasite Coinfection found in Facatativa children. There are five cases of virus, parasites and bacteria; 4 cases of bacteria and parasites; 9 cases of parasites and virus and 21 cases of virus and bacteria found in the same sample.*

humidity increase the risk for *Giardia lamblia*. (Data not shown)

As shown in Table 2, there is no significant statistical correlation between the pathogens and children's age. However, virus is the pathogen which infects children at an earlier age (Table 3).

The hypothesis test was carried out to ascertain whether or not there was a difference in the incidence of episodes between the periods November-April and May-October could be established. The results showed that during the period November-April there were more virus-associated cases (p=0.007), while during the same period fewer cases caused by bacteria and parasites were determined. (p=0.03 and p=0.00014 respectively). Fig. 2 shows a descriptive analysis of the behavior of all of the pathogens during the year of study (Fig. 2 part A) and part B of Fig. 2 shows only the behavior of each virus during the same time (Fig. 2 part B).

DISCUSSION

In Facatativá, an endemic behavior of IAD was observed throughout this study. There were viruses, bacteria and parasite co-circulating every month but only 55% of their pathogen agents were detected as being associated with this IAD. Epidemiological reports from several countries show that the etiological agent can be identified in only 50 to 70% of the cases of IAD.[2,4,16-18] There is still some doubt about the cause of the 45% missed. An interesting finding relates to the prevalence of parasite associated IAD. In fact, 9% of the cases were associated to *Hystolitic entamoeba*, and *Giardia lamblia*. In addition to the above, we also found *Entamoeba coli* and *Endolimax nana* and some other eggs, but they were not included in our analysis, since they do not seem to be associated with IAD in Colombia. This case should be reevaluated due to the fact that some reports describe outbreaks associated with these kinds of "non pathogenic agents"

Some of the main paradigms in IAD are that RV A is the main agent causing this disease in children under five while *E. coli* is considered the second most important pathogen. Nonetheless, with the new and more sensitive

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Table 2. A bivariated analysis in order to clarify the relationship between pathogen and risk factors OR: Odds ratio, IC: Confidence Interval, ind.: indeterminate. There was not significant impact in any result.

	Range of variables	RV A	Ad E	HAstV	RV C	CV
Age (months)	0-24 25-60	OR: 1.23 IC 0.59-2.55 p=0.54	OR: 0.36 IC: 0.05-2.14 p=0.2	OR: 3.07 IC:0.37-67.3 p=0.27	OR: 1.21 IC: 0.65-22.5 p=0.52	OR: 0.79 IC 0.35-1.78 p=0.57
Gender	Female/Male	OR: 1.24 IC: 0.59-2.56 p=0.5	OR: 2.14 IC: 0.36-16.39 p=0.35	OR: 0.49 IC 0.09-2.43 p=0.33	OR: 1.1 IC 0.59-2.07 p=0.74	OR: 0.61 IC 0.27-1.4 p=0.20
Temperature	12.1-14°C 14.1-17.4°C	OR: 0.63 IC: 0.29-1.36 p=0.19	OR: 0.43 IC:0.08-0.51 p=0.26	OR:2.38 IC:0.29-52.2 p=0.4	OR: 1.01 IC: 0.51-2 p=0.98	OR: 0.65 IC 0.28-1.54 p=0.28
Rainfall	0-8 mm 2 9- 24 mm 2	OR: 0.93 IC: 0.28-3.35 p=0.89	ind	OR: 0.63 IC 0.07-14.02 p=0.66	OR: 3.84 IC 0.85-24.1 p=0.05	ind
Humidity	51-70% 71-90%	OR: 1.16 IC:0.51-2.6 p=0.69	OR: 1.16 IC: 0.15-6.94 p=0.57	OR: 0.47 IC 0.02-4.06 p=0.48	OR: 0.81 IC 0.39-1.66 p=0.54	OR: 1.25 IC 0.52-2.98 p=0.58

Table 3. Frequencies of the pathogens by age groups. C.F: cumulated frequency. Remarked result means 50% of children are already infected. Cumulated frequencies of HAstV and AdE were not calculated because they had small numbers.

Age (months)	Bacteria	C. F.	Virus	C. F.	Parasite	C. F.		
0-3	3	4.61	7	5.84	2	7.8		
4-6	1	6.5	8	12.51	0	7.8		
7-12	13	27.5	15	25	4	23		
13-24	13	48.4	31	**50.85**	5	42.1		
25-36	12	**67.8**	24	70.85	5	**61.35**		
37-48	11	85.5	22	89.2	8	92.1		
49-60	9	100	13	100	2	99.9		
Total	62		120		26			
Age (months)	RV A	C. F.	AdE 40/41	HAstV	CV	C. F.	RV C	C. F.
0-3	2	5.2	0	1	1	3.3	5	8.8
4-6	5	18.3	0	0	2	9.9	3	14.07
7-12	4	28.8	2	2	4	23.3	5	22.87
13-24	10	**55.2**	0	2	7	46.6	18	**54.45**
25-36	6	70.9	3	2	8	**73.3**	11	73.75
37-48	6	86.7	1	1	7	96.6	9	89.55
49-60	5	99.9	1	0	1	99.9	6	100
Total	38		7	8	30		57	

techniques, some other pathogens have been taking into account.

Rotavirus C was described about 25 years ago, and its epidemiological behavior seems to be similar to that RV A but with less prevalence (0.3 – 6.8% in children under five years of age)[8,19] however, we report a prevalence of 20% which implies that RV C is more common than anticipated previously.[20] The question is whether RV C can be considered as an emergent agent. If we accept that an emergent agent is one which has been diagnosed with high prevalence for the first time, RV C could be an emergent agent among Facatativá's children, but if we consider as

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an emergent agent some agent which has first appeared in the population, we can not be sure, because this is the first time that RV C has been looked for. Whether RV C is emergent or not must be confirmed by continuing the study at least for two more years or longer. However, it must be pointed out that the most important result as of now is the high prevalence found among the population targeted.

Calicivirus has been reported as the most important agent causing outbreaks associated with food in people from all ages. In places which have seasons, this virus has been associated with outbreaks and only in a few regions it has been found as endemic agent, with a prevalence around 2 –14%.[21,22] Our results show a prevalence of 10% for the entire year, with a behavior tendency more similar to RV C than RV A, with a small increase of cases after July, but without any remarkable behavior which could help us to plan how to fight against it.

It is possible that RV C and CV could be responsible for at least a part of the increase in our detection rates, but still there are a missing 45% of the samples without any recognized agent. Even though there are episodes of diarrhea in which the etiological agents are yet to be diagnosed, 20% of prevalence for RV C and 10% for CV

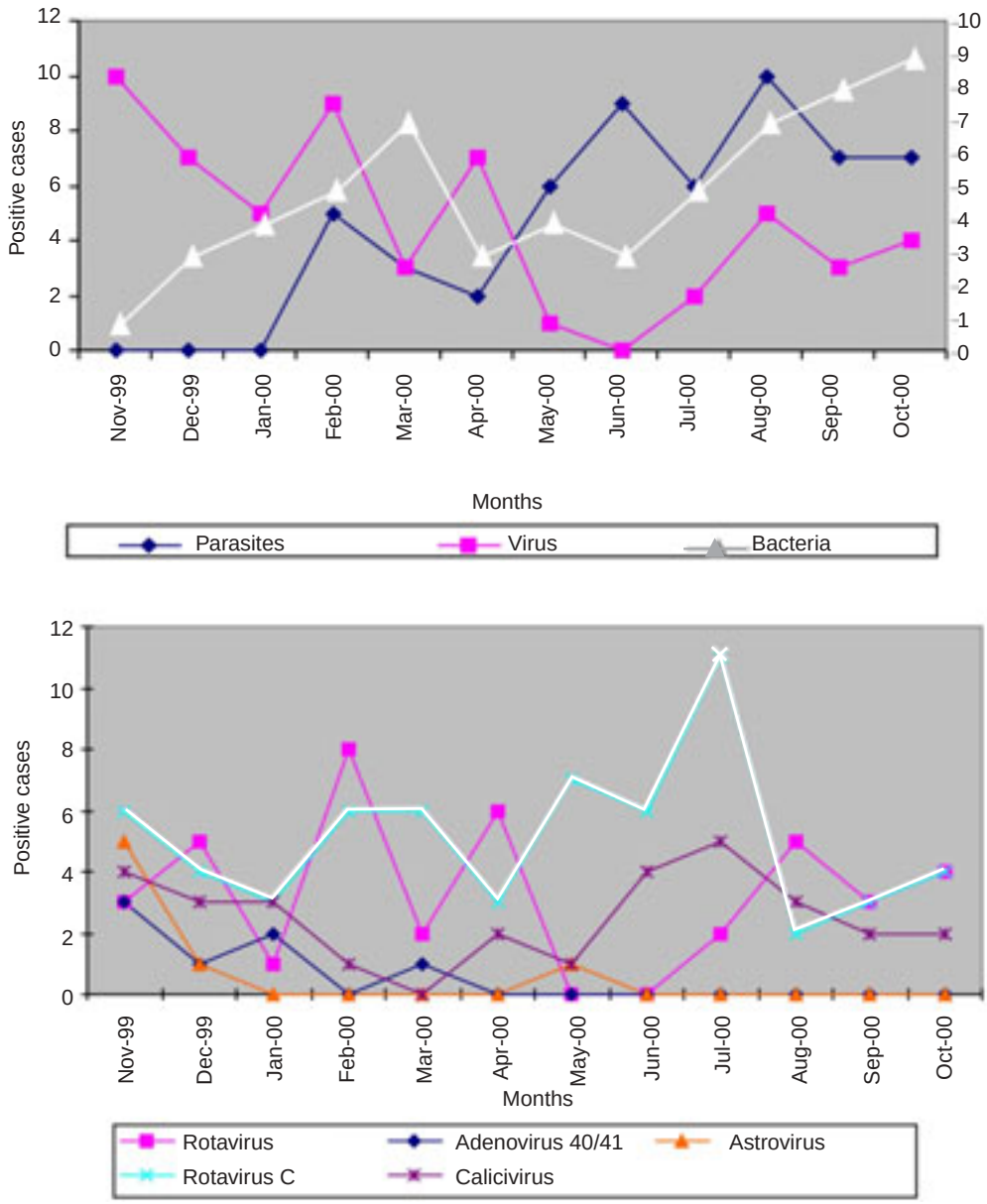

Figure 2. *A. Distribution of pathogens in different periods of the year. B. Distribution of virus in different periods of the year.*

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should alert sanitary authorities on the presence of pathogens not clearly recognized as associated to IAD.

It is generally accepted that risk factors for IAD are age, gender, and seasonal and climatic conditions such as temperature, rainfall and humidity. However, in this study no strong correlations were found between these factors and the IAD episodes. Suzuki et al showed a prevalence of 21% rotavirus infection in children of Quito, Ecuador, a location with similar geographical and altitude conditions to Facatativá and, likewise, found no correlation to seasonal weather changes.[23] Nevertheless, in our study there seems to be a greater prevalence of viral associated diarrhea in the period November-April, while in the period May-October more parasite and bacterial diarrheas were detected. (Fig. 2 part A and B). In fact, 100% of the AdE and 87.5% of the HAstV were found in the period November-April. Our findings should alert medical officers, so that routine checks for these viruses are performed during this yearly period.

IAD is a childhood related disease. Episodes are more frequent between the age of 3 months and five years. Children under five months still have passive maternal immunity. After the fifth year, children develop immunity and immune memory that decrease the risk of infection. A report from South Africa shows that IAD decreases as children become older.[25] Venezuelan and Peruvian studies also show that children under two are the ones with the highest risk of IAD.[4,16,24] Our results are not clear-cut in this sense, even though they show a tendency for children between thirteen and thirty-six months to be more susceptible.

Even though we had hope for more associations between studied variables and pathogens, there was only one that was statistically significant. There was that ambient humidity higher than 71% increases infection risk for *Giardia lamblia*. Even thought there was virus diversity throughout the year, some cases had an epidemic behavior. Most of the HAstV cases were found in the period November-December.[9]

IAD is not always caused by a single infectious agent, and we frequently find concomitant infection by two or even more agents. This fact should be kept in mind when treating children who might be over super infected.

In conclusion, there is high virus diversity of acute diarrhea in non-hospitalized children in the tropical highlands. Some not well pathogens like RV C and CV seem to play an important role in their association with IAD, and no rules influence their presence throughout the year.

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