Polymicrobial infections in children with diarrhoea
in a rural area of Jordan

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Abstract

Polymicrobial infections associated with diarrhoea are common in developing countries. Stool specimens were collected from 220 patient children and 100 controls. Potential pathogenic agents isolated from 143 (65%) children were identified by molecular and standard microbiological methods. Co-infections with two or more agents were detected in 50 (35%) cases. Escherichia coli, Shigella dysenteriae, Giardia and Entamoeba histolytica were found to be predominant. The etiologic agents could not be determined in 77 (35%) cases. The most significant risk factors were the age, the education level of the mother and the use of non-chlorinated water.

The high infection rate of diarrhoeal diseases is a strong indication that these pathogens circulate easily through the population.

Keywords: Bacteria; Diarrhoea; Jordan; Parasites; Polymicrobial infections

1. Introduction

Diarrhoeal diseases are the leading cause of morbidity and mortality among young children in developing countries [1,2]. Although oral re-hydration has been shown to reduce early child mortality, the diarrhoea-specific mortality in children contributes to the deaths of 2.5 millions per year [3]. Causes of diarrhoea in endemic areas include a wide variety of bacteria, viruses and protozoa. Poor food hygiene, contaminated water and low sanitation are common factors in communities with high rates of diarrhoeal disease.

In many low-income countries, it is more common to be infected than not to be. Indeed, a child growing up in an endemic community can expect to be infected soon after weaning and constantly re-infected for the rest of her or his life. Infection is most common among the poorest and the most disadvantaged communities and is typically most intense in children at school age [4]. Polymicrobial infections are also common in such communities and individuals harbouring such infections may suffer exacerbated morbidity, making children even more vulnerable. Thus, these infections pose a serious threat to the health and development of children in low-income countries. Use of an antibiotic is indicated only for more severe cases or in the presence of fever, dysentery or severe dehydration [5].

There is increasing evidence that polymicrobial infections in which microorganisms present specific pathologies may act in a synergistic or inhibitory fashion, impacting on either tissue, host cell destruction or the maintenance of health [6]. Among these, bacteria–bacteria-, virus–virus-, parasite–parasite- or virus–bacteria interactions have been well documented. For example, enteroaggregative Escherichia coli (EAEC) or enteropathogenic E. coli (EPEC) may provide a second
pathogen with better conditions to invade the intestine and cause diarrhoea [7].

This study was conducted to investigate the etiologies of acute and persistent diarrhoea in children of a rural area in Northern Jordan and the risk factors associated with their transmission.

2. Methods

2.1. Study area and patients

The study was conducted from September 1999 through September 2001 in Badia; a rural region located northeast of Jordan. The inhabitants are recently settled Bedouins and the majority are small-scale farmers of low-level socio-economic status. The study has been approved by the University Ethical Committee.

Stool specimens were collected from 220 pediatric patients, who were diagnosed with acute or persistent diarrhoea. Average age of the patients was 5.5 years (range, 1–10 years). Additional 100 stool specimens were collected from children, who had no diarrhoea or other gastrointestinal symptoms during four weeks preceding the entry to the study and serve as a control group. The control subjects were matched for age and sex with the cases and were recruited from children that came to the same health centres for other reasons, e.g., immunisation.

Malnourished children were determined by Z scores (weight-for-age, height-for-age and weight-for-height).

Parents provided verbal consent for the specimen collection and additional information that was recorded in the questionnaire for each patient. This information included demographic details, clinical history, physical signs, source of drinking water and educational level of the parents.

2.2. Bacteriology

Faeces were inoculated on blood, S-S agar (Biofilchem, Roseto D.A., Italy), MacConkey, Sorbitol MacConkey and cefsulodin-Irgasan novobiocin (Oxoid, Hampshire, England). They were incubated at 37 °C for 24–48 h. The classic pathogenic Enterobacteriaceae were investigated using bacterial cultures and PCR techniques. Various selective and differential media were used to isolate Salmonella (Selenite-F broth), Yersinia species (Yersinia selective agar and cold enrichment) and E. coli. Each plate was examined and colonies suspected to correspond to enteropathogenic bacteria were identified using the API 20E system (BioMe´rieux, Marcy l’Etoil, France). The Campylobacter blood-free medium was used to isolate Campylobacter species and was incubated under microaerophilic conditions at 42 °C for 48 h. Isolates of Campylobacter jejuni were confirmed by PCR.

Stools were screened for enterohemorrhagic E. coli by plating them on sorbitol-MacConkey agar and non-sorbitol-fermenting colonies were tested for E. coli 0157:H7 by PCR as indicated below.

The virulence factors for the different categories of E. coli were detected using a PCR technique. Subcultures of single colonies for all morphotypes of E. coli observed were grown on MacConkey agar as previously described [8]. PCR was performed to detect plasmid DNA of EAggEC and the ipah chromosomal gene for enteroinvasive E. coli strains; enteroaggregative stable toxin type 1 (EAST1 gene); VT1 and VT2 genes for verotoxigenic E. coli strains; heat-stable (ST) and heat-labile (LT) enterotoxins for ETEC strains; and the eae and bfp genes and the enteropathogen adherence factor (EAF) for enteropathogenic E. coli (EPEC) strains. EPEC strains were considered to be positive when at least one of these characteristics was found.

2.3. DNA extraction and amplification

DNA extraction of the specimens was performed using a commercial purification system (Wizard Genomic DNA Purification kit; Promega, Madison, WI) following the manufacturer’s instructions. Final pellets were resuspended in 50 ml of 10 mM Tris–HCl, 1 mM EDTA, pH 7.2 (TE).

Infections with E. coli O157:H7 were confirmed by PCR. In addition, co-infections with Salmonella spp., Shigella dysenteriae, C. jejuni and Yersinia enterocolitica, were confirmed by using the DNA Detect™ Multi-Enteric 4 PCR Kit (Vita-Tech International Inc., Canada). This kit includes genus- or species-specific primers designed to amplify a highly conserved region within the 16S rRNA gene of the four pathogens in stool samples.

Five colonies suspected to be infected with E. coli were transferred from MacConkey agar to Brain Heart infusion agar, Simmons’ citrate and lysine iron agar. Citrate-negative cultures were tested and further identified using the E. coli DNA Detect™ Kit (Vita-Tech International Inc., CAN), which is specific for E. coli O157:H7.

Positive controls provided with the kit were used in parallel with the samples. DNase-free water was used as a negative control to monitor possible cross contamination.

PCR amplifications were carried out in a thermal cycler (GeneAmp 9700, Perkin–Elmer, Cetus, Emeryville, CA) using the cycling conditions described by the manufacturer.

Fifteen microliters of the PCR product were run on a 3% agarose gel and stained with ethidium bromide.
2.4. Parasitology and virology

Saline and iodine solutions prepared from fresh specimens were examined under the microscope to detect living and/or moving trophozoite phase of the protozoa. Samples were processed using the formalin–ether sedimentation technique and stained with a modified Ziehl-Neelsen procedure for enteric coccidian and/or with trichrome stain for protozoon. Specimens were tested for rotavirus using the Amizyme rotavirus detection system and using a standard ELISA (AMICO Laboratory Inc., Nashville, TN).

2.5. Statistical analysis

Analyses were generated using statistical software package SPSS (SPSS Inc., Chicago, IL). The correlation between diarrhoea and potential risk factors as registered in the questionnaire was assessed. $P$ values were calculated using the two-tailed test and significance was measured at the $P<0.05$ level. The strength of the correlation of risk factors was estimated by odds ratio (OR) and their corresponding 95% confidence intervals (CI) were obtained. Averages, ranges and percentages of positive samples were calculated.

3. Results

Malnourished children (primarily undernourished), 55 (25%) cases, presented more risk of contracting persistent diarrhoea caused by multiple pathogens. Persistent diarrhoea was reported in 30 (21%) positive cases, they had diarrhoea over 14 days or longer and it was associated with infections by Entamoeba histolytica, EggEC strains and polymicrobial infections.

3.1. Bacteriology

Potential pathogens were found in 143 out of 220 (65%) cases, in 60 (42%) cases being less than five year old children. The main pathogenic bacteria identified (Table 1) and significantly associated with diarrhoea were members of the Enterobacteriaceae, 66 (46.2%) cases. Shigella spp. were detected in 15 (10.5%) cases, S. dysenteriae in nine (6.3%) cases. Salmonella spp. were isolated in 11 (7.7%) cases and Y. enterocolitica in eight (5.6%) cases of acute diarrhoea. ST-producing E. coli was detected in 20 (14%) cases, alone or associated with parasites, LT-producing E. coli five (3.5%) cases, EPEC five (3.5%) cases and EaggEC strains four (2.8%) cases. C. jejuni was detected in 12 (8.4%) subjects with acute diarrhoea.

None of the above pathogens was detected in the control subjects apart from the ST-producing E. coli which was detected in 8 (8%) asymptomatic control subjects.

Dehydration was detected in 11 patients (three with Shigella, four with rotavirus plus EAST1, two with ST ETEC, one with LT ETEC plus Giardia lamblia and one with EAST1). Severe and bloody diarrhoea observed in 30 (21%) cases were associated with E. histolytica, S. dysenteriae and E. coli O157:H7 infections. High counts of faecal leukocytes (more than five leukocytes per high power field (HPF)) were observed in those samples, but not in patients infected with other pathogens.

3.2. Parasitology and virology

The parasites observed in association with diarrhoea were G. lamblia 43 (30%), Blastocystis hominis 29 (20.3%), E. histolytica 15 (10.5%), Cryptosporidium sp. 14 (9.8%), Cyclospora cayetanensis 8 (5.6%) and Isospora belli 3 (2.1%). Other parasites were also identified, e.g., Ascaris and Hymenolepis nana, but were not associated with diarrhoea.

In the control group, in addition to the parasites G. lamblia 12 (12%), B. hominis 8 (8%), H. nana and Ascaris lumbricoides, non-pathogenic microorganisms were also identified.

Rotavirus was significantly associated with acute diarrhoea (OR=3; $P=0.001$) and detected in 58 (40.6%)

Table 1: Comparison of enteric pathogens that were detected in patients and controls

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of positive cases (%)</th>
<th>Patients ($n=143$)</th>
<th>Controls ($n=100$)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giardia lamblia</td>
<td>43 (30.0)</td>
<td>12 (12.0)</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Blastocystis hominis</td>
<td>29 (20.3)</td>
<td>8 (8.0)</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Entamoeba histolytica</td>
<td>15 (10.5)</td>
<td>0 (0.0)</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium spp.</td>
<td>14 (9.8)</td>
<td>0 (0.0)</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Cyclospora cayetanensis</td>
<td>8 (5.6)</td>
<td>0 (0.0)</td>
<td>0.040</td>
<td></td>
</tr>
<tr>
<td>Isospora belli</td>
<td>3 (2.1)</td>
<td>0 (0.0)</td>
<td>0.110</td>
<td></td>
</tr>
<tr>
<td>Members of Enterobacteriaceae</td>
<td>66 (46.2)</td>
<td>8 (8.0)</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>12 (8.4)</td>
<td>0 (0.0)</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>Rotavirus</td>
<td>58 (40.6)</td>
<td>5 (5.0)</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Polymicrobial</td>
<td>50 (35.0)</td>
<td>8 (8.0)</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>
cases compared to the 5 (5%) in the control group. Ten (17.2%) of the cases were co-infected with other pathogens such as EAST1, B. hominis, Giardia or H. nana.

3.3. Polymicrobial infections

Fifty (35%) of all the positive cases had more than one and up to three associated pathogens. These cases involved a member of the Enterobacteriaceae plus another pathogen (20 cases), rotavirus plus another pathogen (10 cases) such as Giardia, B. hominis, H. nana or EAST; E. histolytica plus Cryptosporidium; LT ETEC plus Giardia. These infections were strongly associated with prolonged disease and more severe clinical presentation e.g., diffuse abdominal pain, dysentery or persistent diarrhoea, anorexia that eventually caused a reduction in nutrient intake (odds ratio = 5.9; 95% confidence interval = 1.3–37.3), and with a reduced functional ability at presentation (P = 0.02).

Co-infections were observed in 8 (8%) of the control subjects involving mainly the mentioned parasites and bacteria or parasites and rotavirus, but none of these was associated with diarrhoea or any other symptoms. The etiologic agents could not be detected in 77 (35%) samples, since no potential pathogen was recovered.

3.4. Risk factors

Risk factors that significantly correlated with contracting diarrhoea in these children were the age of the child, OR: 3.9, 95% CI: 1.5–14.0, P < 0.001; the low socio-economic status of the parents, OR: 2.5, 95% CI: 1.7–2.5, P < 0.001; the level of education of the mother, OR: 3.7, 95% CI: 3.2–4.3, P < 0.001; and the use of poorly chlorinated or tank water, OR: 2.7, 95% CI: 2.3–3.9, P < 0.001.

4. Discussion

Diarrhoeal illnesses still remain an important cause of infectious morbidity in children and are exceeded only by respiratory tract infections [9]. Underlying conditions, such as malnutrition, etc, modify the risk of contracting diarrhoea and are also common in this area. Diarrhoea and malnutrition were present in 55 (25%) malnourished children. These two pathologies, which decrease the immunological resistance, are closely related and contribute significantly to infant mortality [10]. These factors combine to facilitate the spread of enteropathogens and epidemics are common in such environment [11].

The results of this study show that 50 (35%) children of the Jordan Badia had more than one and up to three associated pathogens. This is in agreement with other studies conducted in developing countries [9,12].

Co-infections with two or more agents and up to five associated pathogens were detected in more than one-third of the positive cases [9]. The characterisation of the polymicrobial interactions associated with carriage and invasions of pathogens is related to the host response patterns of single vs. multiple infections, required to elicit synergy or inhibition.

The main etiologic agents were exclusively found in cases and were significantly associated with diarrhoea, these included members of Enterobacteriaceae 66 (46.2%), C. jejuni, rotavirus, and parasites such as E. histolytica, Giardia and Cryptosporidium.

The infections reported here with S. dysenteriae 9 (6.3%) were similar to those (7%) reported in children from an urban area in Jordan [13], while higher infection rates were reported for the other Shigella spp.

Entamoeba histolytica, E. coli O157:H7, and S. dysenteriae infections were associated with cases of severe and bloody diarrhoea and were observed in 30 (21%) patients. The biochemical identification of the later two bacterial species was confirmed by PCR assay, which improved the diagnostic yields.

Yersinia enterocolitica was isolated in 8 (5.6%) cases of acute diarrhoea and was previously reported to be an uncommon cause of diarrhoea in children and adults in Jordan [14].

Giardia lamblia 43 (30%) and B. hominis 36 (25.2%) infections were previously reported in children with acute diarrhoea in urban areas of Jordan [14,15]. The infection rate with Cryptosporidium spp. (9.8%) in this area is higher than in urban areas (4%) [16]. Higher infection rates were reported in a study conducted in Bangladesh when a potential enteric pathogen was isolated from 74.8% of diarrhoeal children and 43.9% of controls; the infections with multiple pathogens were also common [12]. Some enteropathogens reported here (e.g., Campylobacter, Shigella spp., and Cryptosporidium) to be associated with diarrhoea were also described in children from other developing countries [9,12,15]. However, E. histolytica, Salmonella spp. and G. lamblia were not significantly associated with diarrhoea [12].

The low rates of intestinal nematodes and cestodes observed in children suggest a low prevalence of these worms in the Badia region and in the urban areas of Jordan [16].

The etiologic agents could not be detected in 77 (35%) patients since no pathogen or potential pathogen could be recovered. These putative agents might have included other potentially pathogenic species or non-infectious microorganisms. To identify an associated organism is difficult; even though in outbreaks of gastrointestinal illness, a pathogen is not identified in nearly 50% of the documented outbreaks. For example, in cases of foodborne illness in the US about 81% of all cases and 64% of the deaths were due to unknown or unidentified agents [17].
The infection rates are expected to decrease in the study area as a result of a general improvement of the quality of life. In addition, local programs for the control of the diarrhoeal disease were instituted; those included promotion of breast-feeding, oral rehydration therapy and specific health education. The highest infection rate (42%) recorded in children less than five years old is not surprising since the rate of gastrointestinal illness is always highest in children between one and four years. It is estimated that residents of developed countries experience one episode of gastrointestinal illness every two years, while residents of developing nations may experience five to ten episodes per year. However, the high endemic rate of gastrointestinal disease in both developed and developing countries receives still little attention [18].

The high prevalence of gastrointestinal disease is a strong indication that gastrointestinal pathogens circulate through this population with ease and that the environment, the food, and water are vehicles of transmission for these microorganisms. The polymicrobial infections registered in many children indicate a common source: poorly chlorinated water or tank water among them; risk factors that were significantly associated with the infections.

In conclusion, the results indicate the prevalence of polymicrobial infections among children in rural areas and emphasise the need to develop exploratory approaches to examine polymicrobial interactions and think beyond the one organism-one disease concept. The knowledge of the etiological agents of diarrhoea in children and the associated risk factors would set off future research involving inter-microbial interactions. This knowledge will also assist in defining the environmental/physiological conditions that contribute to the microbial synergy or inhibition within the same host and lead to an increase susceptibility. Such studies will provide useful information to investigators in the development of more effective diagnosis, prevention and treatment strategies.

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References