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# Conventional and molecular methods in the diagnosis of community-acquired diarrhoea in children under 5 years of age from the north-eastern region of Poland

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SUMMARY

*Objectives:* The purpose of this study was to determine the main causative agents of community-acquired acute diarrhoea in children using conventional methods and PCR.
*Methods:* Stool samples were collected from 100 children under 5 years of age with acute diarrhoea during the autumn–winter period of 2010–2011. Rotaviruses and adenoviruses were detected by the stool antigen immunoassay, and *Salmonella spp*, *Campylobacter spp*, *Shigella spp*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Clostridium difficile*, enterotoxigenic *Bacteroides fragilis* (ETBF), and diarrhoeagenic *Escherichia coli* were detected by culture methods and PCR.
*Results:* Overall, enteropathogens were identified in 73% of the children. Bacteria, viruses, and mixed infections were noted in 37%, 24%, and 12% of diarrhoeal cases, respectively. The most common enteric pathogens were rotaviruses (31%), followed by *C. difficile* (17%), *Campylobacter jejuni* (13%), *Salmonella spp* (11%), and atypical enteropathogenic *Escherichia coli* (aEPEC) strains (10%). Compared with culture methods, PCR increased the overall detection frequency of the bacterial enteropathogens by 4%.
*Conclusions:* The high prevalence of *Campylobacter jejuni* suggests that the number of campylobacteriosis cases in Poland may be underestimated; this pathogen should be investigated routinely in children with diarrhoea. Moreover, *C. difficile* might be considered a causative or contributing agent of diarrhoea in 14.8% of children aged >1 year.


## 1. Introduction

Infectious diarrhoea is one of the most common childhood illnesses worldwide and is the leading cause of death among children aged <5 years, mainly in developing countries.[1] In urbanized countries, it is associated with significant morbidity-related socioeconomic costs.[2]

Our knowledge of infectious agents causing diarrhoea is not complete, and their variety and diversity make diagnosis problematic. So far, more than 20 species of bacteria, viruses, and parasites have been identified as definite or plausible enteropathogens, and molecular assays are necessary to identify several of them. Consequently, the aetiology of a substantial number of diarrhoea cases remains unrecognized.[3] In addition, several factors may contribute to the occurrence and proportion of particular enteropathogens, including age, birth weight, breast-feeding, season, geographic area or place of residence, etc.[4] Therefore, it is essential to monitor the local epidemiological situation with regard to infectious diarrhoea, to adapt diagnostic methods and introduce timely control measures.

Data regarding the aetiological agents of diarrhoea from our province are limited, particularly those related to the emerging or less common ones. For example, there are no records on the number of campylobacteriosis cases at all.[5] Thus, the major goal of the present study was to determine the main enteropathogens in children hospitalized for acute diarrhoea. A broad panel of bacterial enteric pathogens was tested, including (1) major enteropathogens such as *Salmonella spp*, *Campylobacter spp*, *Shigella spp*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*, (2) rare and/or plausible agents: toxigenic *Clostridium difficile* and

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enterotoxigenic *Bacteroides fragilis* (ETBF), and (3) diarrhoeagenic *Escherichia coli*: typical and atypical enteropathogenic *E. coli* (tEPEC and aEPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) strains. Moreover, rotaviruses and adenoviruses were also included.

## 2. Materials and methods

### 2.1. Patients and study design

The study was conducted on 100 children <5-years-old with acute diarrhoea (43 girls and 57 boys), who were admitted to the Department of Paediatric Infectious Diseases (Medical University of Bialystok, University Children's Hospital) between September 2010 and February 2011. The department is located in north-eastern Poland and provides inpatient care for children who reside in Bialystok city and Bialystok district. The estimated catchment population accounts for approximately 8200 children aged <5 years, representing about 50% of all children in this age group in our region.[6]

Acute diarrhoea was defined as the passing of three or more loose or watery stools within a 24-h period and/or vomiting with or without fever. Inclusion criteria were the following: age <5 years and written informed consent from the child's parents or legal guardians. Patients with diarrhoea lasting over 7 days and/or underlying illnesses were excluded. The children hospitalized up to 21 December (the first calendar day of winter) were assigned to the autumn group ($n$ = 59), and the remaining children were assigned to the winter group ($n$ = 41). In addition, the children were divided according to their place of residence into urban ($n$ = 75) and rural ($n$ = 25) subsets, i.e. areas where the population density is >150 or <150 inhabitants per square kilometre, respectively.[7] The place of residence was determined on the basis of the patient's address (verified by the child's parents or guardians) in conjunction with data from the National Official Register of Territorial Division of the Country.[7] The Bioethics Commission of the Medical University of Bialystok approved the study.

### 2.2. Specimens, culture methods, and immunoassays

Stool samples were collected on the day of admission and transported to the Department of Microbiology (Medical University of Bialystok). These were inoculated on standard media for bacteria and fungi: MacConkey, Salmonella–Shigella (SSA), Chapman, and Sabouraud agars and Selenite F (SF) enrichment broth. In addition, selective agar was used for *Yersinia spp* (Merck & Co, USA) and MacConkey agar with sorbitol (Oxoid, UK) for the isolation of EHEC strains. Plates were incubated overnight at 37 °C. SF broth was subcultured onto SSA after overnight incubation at 37 °C. Bacterial isolates were identified by standard microbiological methods and API tests (bioMérieux, France). *Salmonella spp* and *Y. enterocolitica* isolates were confirmed using slide agglutination tests with specific antisera (Biomed, Poland). *Campylobacter spp* were isolated on blood agar with selective supplement Campylosel (bioMérieux, France) under microaerophilic conditions for 48 h at 42 °C. The anaerobic pathogens, toxigenic *C. difficile* and ETBF strains were isolated on Cycloserine-Cefoxitin Fructose Agar (Oxoid, UK) and Wilkins–Chalgren agar with the selective supplement G-N Anaerobe (Oxoid, UK) under anaerobic conditions for 48 h at 37 °C, and confirmed by PCR with primers specific to *tcdA* (toxin A) and *tcdB* (toxin B) genes,[8,9] and the fragilysin gene.[10]

Rotavirus and adenovirus stool antigens were detected by immunochromatographic tests (VIKIA Rota-Adeno; bioMérieux, France).

### 2.3. PCR

Genomic DNA was isolated directly from the stool samples with a GeneMATRIX Stool DNA Purification Kit (EURx, Poland) and was used as a template for the PCRs.

Duplex and triplex PCRs were performed in 50-μl volumes containing the following components: 1× PCR buffer in deionized water, 200 mM dNTP, 2 U Taq polymerase (Fermentas, Lithuania), primers[8–14] at appropriate concentrations (Table 1), and 5 μl of the isolated DNA. Positive and negative controls were included. The PCR cycle conditions for all reactions were as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 60 s, 63 °C for 60 s, and 72 °C for 60 s, and a final extension at 72 °C for 5 min. Amplification was performed on a Mastercycler gradient (Eppendorf, Germany). All PCRs were done in duplicate. PCR products were visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide and documented by GelDoc 2000 system (Bio-Rad, USA). PCR was done blinded to culture results.

To exclude false-positive PCR results that could be a consequence of non-specific amplification of non-target genes, all PCR-positive and culture-negative samples for *C. difficile* and aEPEC strains, for which the discrepancies between PCR and culture results were the highest (see Results section), were further re-tested using confirmatory PCRs. For toxigenic *C. difficile*, the *tcdA* gene was used as the confirmatory marker.[8] For aEPEC strains, the second forward primer (5′-CCAGTATTCGCCACCAAT-3′) was designed to the *eae* gene (GenBank accession number **M58154.1**), producing with the primary reverse primer[11] an amplicon of 345 bp in size.

In addition, to differentiate EPEC strains from EHEC and *Escherichia albertii*, the samples were re-tested with primers for the *stx* gene[11] and *lysP* and *mdh* genes,[14] useful in the identification of EHEC and *E. albertii*, respectively. Moreover, all PCR-negative and culture-positive samples were amplified with 16S rRNA universal primers for bacteria,[15] to eliminate the possibility of PCR inhibition.

Bacterial strains from the American Type Culture Collection (ATCC) and clinical isolates from the microbial collection of the Department of Microbiology were used in the study as reference strains.

### 2.4. Statistical analysis

Potential correlations between diarrhoea and recorded variables (gender, place of residence – rural vs. urban, season of enrolment, previous infections and hospitalization, and antimicrobial therapy in the last 2 months) were studied using the Chi-square test, including the Fisher's exact and the Yates' correction tests. Moreover, the influence of these variables on the frequency of detection of specific enteropathogens was analysed using multivariate logistic regression. All analyses were performed using STATA ver. 11 software; $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Prevalence of enteropathogens

Overall, enteropathogens were detected in 73 (73%) of the children. Bacteria, viruses, and mixed infections were noted in 37%, 24%, and 12% of cases, respectively (Figures 1 and 2). In detail, viruses, predominantly rotaviruses, were identified in 36% of the children.

Furthermore, the most frequently detected bacterial enteropathogens in diarrhoeal samples were toxigenic *C. difficile* strains (17, 17%), *Campylobacter jejuni* (13, 13%), *Salmonella spp* (11, 11%), and aEPEC (10, 10%). However, aEPEC strains were found as the