

Impact of *Aeromonas* and diarrheagenic *Escherichia coli* screening in patients with diarrhea in Paraná, southern Brazil

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Abstract

Introduction: A wide diversity of bacterial agents may cause diarrhea, presenting challenges to clinical laboratories to define a diagnosis. Considering that most stool cultures are negative, we screened stool samples from patients with diarrhea for the presence of 14 bacterial enteropathogens, aiming to establish which of them should be included in routine stool analysis.

Methodology: Stool samples from 400 patients with diarrhea were analyzed for the presence of *Salmonella*, *Shigella*, *Campylobacter*, *Aeromonas*, *Plesiomonas shigelloides*, *Vibrio*, *Yersinia enterocolitica*, and diarrheagenic *Escherichia coli* using conventional microbiological methods and PCR. Two distinct samples were studied; one included predominantly patients involved in outbreaks, and the other patients of low socioeconomic status presenting sporadic cases of diarrhea.

Results: In total, 86 cultures (21.5%) were positive. Mixed infections were found in five patients, leading to recovery of 91 strains of enteropathogenic bacteria: *Salmonella* Enteritidis (9.2%), *Aeromonas* (7.2%), diarrheagenic *E. coli* (5.2%), and *C. jejuni* (1%). However, *Salmonella* predominated, with 11.5% frequency in diarrhea outbreaks, while *Aeromonas* predominated among patients of low socioeconomic status, with 14.6% frequency.

Conclusion: *Aeromonas* and diarrheagenic *E. coli*, which are not routinely screened for, deserve to be included in laboratory screening panels.

Key words: diarrhea; bacterial enteropathogens; prevalence; laboratory diagnosis; gastroenteritis agents.

J Infect Dev Ctries 2014; 8(12):1609-1614. doi:10.3855/jidc.4434

(Received 20 November 2013 – Accepted 21 March 2014)

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Introduction

Diarrheal diseases represent a major health problem in developing countries and are also a risk to travellers who visit these countries [1]. It is estimated that, worldwide, more than one billion episodes of diarrhea occur annually, causing about two million deaths per year, ranking third among all causes of infectious diseases-related deaths [1,2]. Furthermore, some bacterial agents of infectious diarrhea may cause serious long-term sequelae such as hemolytic uremic syndrome, Guillain-Barré syndrome, and malnutrition, in addition to acute morbidity and mortality. The wide diversity of bacterial agents that may cause diarrhea complicates accurate surveillance and diagnosis [1-3]. Moreover, defining etiology of acute diarrhea is critical to disease therapy and prevention [4].

Laboratory analysis of stool specimens and identification of enteric pathogens are dependent upon numerous conditions, and no one set of conditions is

ideal for all bacterial enteropathogens. Stool analysis in many cases can be extremely challenging, especially when enteropathogens other than *Campylobacter*, *Salmonella*, and *Shigella* are sought. Furthermore, stool cultures are expensive to perform, and decisions need to be made about how much effort and expense should be allowed to establish a definitive diagnosis [3]. We undertook this study to determine the presence of 14 bacterial enteropathogens in stool samples from patients with diarrhea, aiming to establish which of them should be included in routine stool analysis in our geographic region.

Methodology

Study design

This prospective study screened for 14 bacterial enteropathogens in stool samples from patients with diarrhea in Paraná state, southern Brazil. The criteria used for inclusion of patients with diarrhea was the

emission of three or more liquid or loose stools within a 24-hour period.

Sample collection and classification

Two groups were established. Group 1 (n = 305) included samples from patients involved in 11 foodborne outbreaks that occurred in several localities of Paraná state, and serious cases of sporadic diarrhea. The samples of group 2 (n = 95) were exclusively from patients of low socioeconomic status with sporadic diarrhea who attended the county laboratory of Curitiba, Paraná, southern Brazil. These patients presented mild to moderate diarrhea and complaints of abdominal pain; some complained of persistent diarrhea.

The stool samples of the 400 patients with diarrhea, who included adults and children, were collected between September 2010 and December 2011. Stool samples were transported in Cary-Blair medium at 4°C and maintained under refrigeration until the moment of analysis.

Laboratory procedure

The 400 stool samples were analyzed for the presence of *Salmonella*, *Shigella*, *Aeromonas*, *Plesiomonas shigelloides*, *Campylobacter*, *Vibrio*, and *Yersinia enterocolitica* using conventional microbiological methods [5-10], and for diarrheagenic *Escherichia coli* using a two-system multiplex polymerase chain reaction (PCR). *Salmonella* serotyping was performed at the national reference laboratory Fundação Oswaldo Cruz (Fiocruz), using antisera prepared at the same institution.

Multiplex PCR

The procedure described in [11] was followed. Briefly, for screening of diarrheagenic *E. coli* (DEC), the stool samples were inoculated in MacConkey agar. After overnight incubation at 36°C, a loopful of the area of confluent growth was resuspended in 500 µL of sterile water and used for DNA extraction by the boiling method. The extracts were centrifuged at 14,000 rpm for two minutes, and 3 µL of the supernatants were used in the two-system multiplex-PCR method developed by Fialho *et al.* [11]. The multiplex-PCR system 1 contains primers for detection of Shiga-toxin producing *E. coli* (STEC; *stx1*, *stx2*), enteropathogenic *E. coli* (EPEC; *eae*, *bfpA*), atypical enteropathogenic *E. coli* (aEPEC; *eae*), enterotoxigenic *E. coli* (ETEC; *lt*, *st*), enteroinvasive *E. coli* (EIEC; *ial*), and the internal control *16S* rRNA to validate the negative results. The multiplex-PCR

system 2 contains primers for detection of EIEC (*ipaH*), enteroaggregative *E. coli* (EAEC, CVD432), diffusely adherent *E. coli* (DAEC, *daaE*), and the internal amplification control *16S* rRNA. Samples positive in this step then had up to 50 isolated colonies tested individually by single PCR for the specific DEC marker. PCR reactions were performed in a final volume of 25 µL, containing Taq DNA polymerase buffer 1× (Invitrogen, Waltham, USA), 1.5 mM MgCl₂, 0.2 mM dNTP, 1 U of Taq DNA polymerase platinum (Invitrogen), and 3 µL of template DNA. The concentration of each pair of primers was adjusted empirically to obtain DNA bands of similar intensities with controls. The cycling programs used were 1 cycle at 94°C (4 minutes), 35 cycles at 94°C (1 minute), 55°C (1 minute), 72°C (1 minute), and a final cycle at 72°C (5 minutes) for multiplex-PCR system 1; for system 2, the conditions were the same except the annealing temperature that was 58°C (1 minute). Detection of PCR products was by electrophoresis in 2% agarose gel stained with ethidium bromide and visualized under UV light.

The antimicrobial susceptibility test was performed using the disk diffusion method, and the E-test was also used for *Campylobacter* [12,13].

Statistical analysis

Statistical analysis was performed with Statistica version 8.0 software (StatSoft Inc., Tulsa, USA), using Fisher's exact test (two-tailed) with Bonferroni correction. A p-value < 0.05 was considered significant.

Results

Bacterial enteropathogens were recovered from 86 (21.5%) stool cultures, of which 61 (20%) and 25 (26%), respectively, were from groups 1 and 2. No statistically significant difference was observed in the number of positive cultures between samples from groups 1 and 2 (p = 0.199). Mixed infections were found in five samples, one from group 2 in which *A. hydrophila* and aEPEC were recovered. In the four other samples, *Salmonella* was isolated together with aEPEC, DAEC, *A. caviae*, or *Aeromonas* spp. In total, 91 bacterial enteropathogenic strains were recovered, including *Salmonella* ser. Enteritidis (9.2%), *Aeromonas* (7.2%), diarrheagenic *E. coli* (5.2%), and *C. jejuni* (1%) (Table 1). These bacteria were isolated from patients of all ages; no significant difference was found in their frequencies among patients of different age groups. ETEC, EIEC, *Shigella*, *P. shigelloides*, *Vibrio*, and *Y. enterocolitica* were not found.

Antimicrobial susceptibility testing showed that most *Salmonella* (33/37) were resistant to nalidixic acid. Resistance was also observed to tetracycline (five strains), chloramphenicol (four strains), ampicillin (three strains), trimethoprim-sulfamethoxazole (three strains), and cefalotin (two strains); four strains were multiresistant. All *Salmonella* strains were susceptible to the following antimicrobials: amoxicillin-clavulanic acid, aztreonam, cefoxitin, ceftazidime, ceftriaxone, ciprofloxacin, ertapenem, gentamicin, imipenem, levofloxacin, and meropenem. Among *Aeromonas*, only one strain was susceptible to all the antimicrobials tested, and all but this strain were resistant to ampicillin. Most *Aeromonas* (23/29) were also resistant to cefalotin and cefazolin. This was expected since most of *Aeromonas* are resistant to these antimicrobials [10]. Resistance to the following antimicrobials was also observed: amoxicillin-clavulanic acid (six strains), cefoxitin (five strains), trimethoprim-sulfamethoxazole and nalidixic acid (four strains), tetracycline (two strains), and chloramphenicol (one strain). Six strains were multiresistant. All *Aeromonas* strains were susceptible to amikacin, aztreonam, cefepime, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, imipenem, levofloxacin, and meropenem. Among the 21 DEC strains, 10 were susceptible to all antimicrobials tested (amoxicillin-clavulanic acid, ampicillin, aztreonam, cefalotin, cefepime, cefotaxime, cefoxitin, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, imipenem, piperacillin-tazobactam, tetracycline, and trimethoprim-sulfamethoxazole). Ampicillin resistance was observed in only ten strains (four aEPEC, three

EAEC, and three DAEC), and one aEPEC was resistant to ampicillin and cefalotin. For *C. jejuni*, the antimicrobials tested were tetracycline, erythromycin, and ciprofloxacin. One strain was susceptible to all of them, two were resistant to ciprofloxacin, and one was resistant to tetracycline and ciprofloxacin.

Discussion

Over the past 20 years, the number of bacterial species involved in diarrheal syndromes has increased, challenging the laboratory diagnosis of bacterial diarrhea [3].

In our geographic region, most stool cultures are negative. Therefore, we conducted a prospective study screening stool samples from patients with diarrhea for the presence of 14 bacterial enteropathogens to establish their prevalence.

Overall, *Salmonella enterica* ser. Enteritidis was the bacteria recovered most frequently (Table 1). However, the frequency of *Salmonella* in stool cultures of patients from group 1 was significantly higher ($p = 0.004$) than that found in patients from group 2 (Table 1). Since group 1 contained mainly patients involved in outbreaks, this indicates that *Salmonella* is the most common bacteria involved in outbreaks of diarrhea. In contrast, *Salmonella* (2.1%) only ranked fourth, together with EAEC and behind *A. caviae* (10.5%), aEPEC (4.2%), and *A. hydrophila* (3.1%), among patients of low socioeconomic status with sporadic diarrhea (group 2). No significant difference was found in the distribution of the other enteropathogens between both groups (Table 1). However, if all strains of *Aeromonas* are taken

Table 1. Frequencies of bacterial enteropathogens found in stool cultures of patients from groups 1 and 2

| Bacteria (N = 91) | Group 1 | Group 2 | P* |
|--|------------|------------|-------|
| <i>Salmonella enterica</i> ser. Enteritidis (9.2%) | 35 (11.5%) | 2 (2.1%) | 0.004 |
| <i>Aeromonas caviae</i> (5.2%) | 11 (3.6%) | 10 (10.5%) | 0.015 |
| <i>Aeromonas hydrophila</i> (1.5%) | 3 (1.0%) | 3 (3.1%) | 0.149 |
| <i>Aeromonas</i> spp. (0.5%) | 1 (0.3%) | 1 (1%) | 0.419 |
| aEPEC (3.2%) | 9 (2.9%) | 4 (4.2%) | 0.517 |
| DAEC (0.7%) | 2 (0.6%) | 1 (1.0%) | 0.558 |
| EAEC (0.7%) | 1 (0.3%) | 2 (2.1%) | 0.142 |
| STEC (0.2%) | 0 | 1 (1.0%) | 0.237 |
| tEPEC (0.2%) | 0 | 1 (1.0%) | 0.237 |
| <i>Campylobacter jejuni</i> (1.0%) | 3 (1.0%) | 1 (1.0%) | 1.000 |

The values are number of enteropathogens isolated, n (%).

Group 1 (n = 305) patients from outbreaks and serious cases of sporadic diarrhea, 61 positive cultures, 4 of which were mixed infection (a total of 65 enteropathogens isolated); Group 2 (n = 95), patients of low socioeconomic status with sporadic diarrhea, 25 positive cultures and 1 mixed infection (total of 26 enteropathogens isolated).

*P: Fisher's exact test (two-tailed). Significant p-value with Bonferroni correction 0.005 (0.05/10).

together, their prevalence among patients in group 2 is three times higher than among patients in group 1 (14/95 vs. 15/305; $p = 0.001$). *Aeromonas* were the second most frequently isolated enteropathogens (Table 1), and were recovered both from children (15 strains) and adults (14 strains). It is worth remembering that most routine clinical laboratories do not screen for *Aeromonas*, and our results indicate that these enteropathogens were responsible for the majority of sporadic diarrhea cases (Table 1).

Aeromonas are causes of infectious diarrhea [2,3] and are also agents of traveler's diarrhea [3,14]. They are ubiquitous in aquatic environments and are transmitted to humans through contaminated water [3,15] and food [16].

Aeromonas have been reported as cause of diarrhea in several countries. Their incidence varies widely around the world, from 0.04% in Crete [17], 1.7% in Israel [18], 2% in Sweden [19], 4.8% in Switzerland [20], 3.1% to 6.5% in India [21], 3.9% to 25.9% and 3.2% to 32.3% in Bangladesh and Pakistan, respectively [22], and from 2.6% to 19.5% in Brazil [23-25].

Interestingly, the frequency of *Aeromonas* found here is significantly higher ($p = 0.003$) than that found in our previous study using stool samples from patients who attended private clinical laboratories, of whom 2.6% were found to have *Aeromonas* infections [23]. It is known that development of infectious diarrhea is related to environmental conditions including poor sanitation and hygiene, unsafe water supplies, poor education, and inadequate sewage disposal [2]. Since this sample comprised patients of low socioeconomic status, among whom a higher incidence of *Aeromonas* was found (Table 1), the difference in the prevalence of these bacteria between the two studies realized with patients of the same geographical area is probably related to the distinct living conditions of the people.

Considering our results, and also that *Aeromonas* has been found as a diarrhea agent in several studies, including some realized in developed countries [18-20], we suggest that these bacteria should be included in the panel of enteropathogens screened for in routine laboratory tests.

Detection of DEC requires specialized tests [3], since they are indistinguishable from *E. coli* commensal strains based on biochemical tests. In addition, serotyping is rarely sufficient to reliably identify a strain as diarrheagenic. In this study, we used multiplex PCR for detection of virulence genes that characterize the DEC pathotypes [11]. The DEC

strains ranked third among the enteropathogens recovered, with a frequency of 5.2%, with atypical EPEC being the predominant pathotype among them (Table 1). These strains are increasingly being implicated as important causes of gastroenteritis around the world [3]. In Switzerland, a frequency of 8% of DEC was found among children with diarrhea [20], while among Caribbean-Colombian children, a rate of 14.4% was reported [26], and in Brazil, rates from 7.6% to 48% [11,27-30] have been reported. In India, a frequency of 52% of DEC was found among children under five years of age with diarrhea [31], and in Ghana, these bacteria were recovered from 77% of children and 38% of adults with diarrhea [32]. These studies also showed that DEC are important causes of diarrhea. Based on these data and our results, we suggest that a search for DEC pathotypes is included in routine screening.

We also found *C. jejuni* as a diarrhea agent. Although these bacteria are among the most frequently isolated from patients with diarrhea [1,3], in our study, they only ranked fifth, after *Salmonella*, *A. caviae*, aEPEC, and *A. hydrophila*.

Also, since multiresistant strains were detected, we strongly recommend that antimicrobial susceptibility tests always be performed.

Conclusions

In summary, this study showed the prevalence of several enteropathogens in culture stools of people with diarrhea, some of which are not routinely screened by diagnostic laboratories. Based upon the frequency of *Aeromonas* and diarrheagenic *E. coli* recovery, which together represent 60% of the positive cultures, we suggest that these bacteria should be included in the panel of bacterial enteropathogens routinely screened for in stool cultures.

Acknowledgements

This study was supported by Fundação Araucária; the Brazilian Program of National Institutes of Science and Technology – INCT/Brazilian Research Council – CNPq/MCT; and CAPES/REUNI.

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Conflict of interests: No conflict of interests is declared.