DETECTION OF PATHOGENIC BACTERIA IN DIARRHEAL STOOL COLLECTED FROM CHILDREN IN NORTH LEBANON BY USING CONVENTIONAL STOOL CULTURE AND MICROARRAY TECHNIQUE « CLART® ENTEROBAC »

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ABSTRACT

Bechara, R., Hosny, M., AL-Kassaa, I., Dabboussi, F., Mallat, H. and Hamze, M. 2016. Detection of pathogenic bacteria in diarrheal stool collected from children in north lebanon by using conventional stool culture and microarray technique « clart® enterobac ». Lebanese Science Journal, 17(2): 233-239.

Illness caused by enteropathogens represents an important economic and health burden worldwide. The majority of enteropathogens causes gastrointestinal infections which have a great impact on public health both in developing and developed countries. The aim of this study is to detect and identify the main enteropathogens in Lebanese diarrheal stool from children under 15 years old. The detection was performed by using both conventional method and microarray technique CLART® EnteroBac (Genomica-Spain). Five enteric pathogens, Salmonella spp, Shigella spp, Clostridium difficile B, Enteropathogenic E. coli, Campylobacter jejuni were detected in 80 diarrheal stools, from children under 15 years old. The results showed that CLART® EnteroBac technique have detected enteropathogens in 19% of samples, whereas 1% returned positive using stool culture methods.

Keywords: Enteropathogens, CLART® EnteroBac, stool culture

INTRODUCTION

Infectious diarrhea is a major cause of infant morbidity and mortality worldwide. It caused around 800 000 fatalities annually in developed and developing countries (Liu et al., 2012). According to the World Health Organization (WHO), diarrhea is the third cause of infant mortality in the world after perinatal conditions and lower respiratory infections (Lassi et al.,

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2016). It was estimated that in the year 2010, the number of acute episodes of diarrhea was 1.7 billion per year in 139 countries (Fischer Walker et al., 2012). Diarrheal infections in children were caused mainly by four pathogens: Rotavirus, *Cryptosporidium* spp., Shiga-like Toxin *E. coli* (STEC) and *Shigella* spp. as reported by Kotloff et al. in 2013 (Kotloff et al., 2013). In 2015, an estimated 526,000 episodes of diarrhea and 922,000 cases of pneumonia in children, less than five years old, led to death (Liu et al., 2016).

In Lebanon, some diarrheal infections were considered as endemic infections such as Typhoid fever (Hamze and Vincent, 2004). Typhoid fever increased suddenly from 2003 to 2010 and the higher prevalence was in North Lebanon (Naji-Rammal and Bedrossian, 2010). The Lebanese Ministry of Public Health (MPH) reported that between January and septembre 2016 the number of typhoid fever and dysentery were 278 and 204 cases respectively (www.moph.gov.lb, consulted on 10-09-2016). Unfortunately in Lebanon, the stool culture protocol focused only on the detection of *Salmonella* spp and *Shigella* spp as target enteropathogen.

The main problem in such infections is the method of diagnosis. The conventional methods represented by stool culture and biochemical identification are not sensitive methods (Guerrant et al., 2001). In contrast, molecular diagnostics have an emerging role in the diagnosis of infectious diseases including infectious gastroenteritis (Platts-Mills et al., 2012; Tobias and Vutukuru, 2012).

Indeed, molecular methods based on the amplification of DNA/RNA can be a promising technique for enteropathogens identification and detection (Platts-Mills et al., 2012). CLART® EnteroBac (Genomica-Spain) is a molecular method based on microarray technique. This method can detect 11 different bacterial enteropathogen genus and species. All these enteropathogens can be detected in one run which saves time and cost.

The aim of this study was the identification of enteropathogens responsible of diarrhea, among 80 diarrheal stool samples collected from infected children aged between 9 months and 15 years old in North Lebanon, by using conventional stool culture methods and by the "CLART® EnteroBac technique (Genomica-Spain).

MATERIAL AND METHODS

Sample collection

Diarrheal stools with absence of rotaviral and adenoviral infections were chosen to proceed in this study. The detection of aforementioned viruses was realized using an immunochromatographic test kit (VedaLab®, France). The study took place in the "Laboratoire de Microbiologie Santé et Environnement (LMSE)" at Doctoral School of Sciences and Technology (DSST) / Faculty of Public Health (FSP) - Lebanese University between July 2 and September 5, 2015. In total, 80 stool samples from children were collected from the following hospitals and medical centers in North Lebanon: Nini Hospital, El-Youssef Hospital, Mazloum New Hospital and Hamidi medical center. Samples were transported in two ways, in sterile recipients and directly by swab (BBL ™ Port-A-Cul ™ (BD®, USA)). The swab sampling was realized to keep a good condition for *Campylobacter* spp. and *Clostridium difficile* (*C. difficile*) culture.

Stool culture

Immediately, after maximum of 2 hours, the samples were transported to the laboratory. The stool culture was performed according to the "Réferentiel en Microbiologie Médicale" (Remic) (Bonacorsi, 2015). For enteropathogenic *E. coli* (EPEC) and enterohemorragic *E. coli* (EHEC) detection, PCR reactions were performed according to Tobias and Vutukuru (Tobias and Vutukuru, 2012). The authors have targeted *CVD432, lt, sth, stp, eae, bfp, stx1, and stx2.* These genes can differentiate between EPEC and EHEC by two-step multiplex PCR (Tobias and Vutukuru, 2012). For children under 2 years old, the detection of EPEC strains was realized by using Nonavalent Antisera® (Bio-Rad, France) followed by PCR confirmation. All sorbitol negative *E. coli* strains were subjected to immunological test using *E. coli* O157 latex® test (Oxoid- UK) followed also by PCR confirmation. In this present study, the PCR reactions have targeted *eae, bfp, stx1* and *stx2* genes as described before by Tobias and Vutukuru (Tobias and Vutukuru, 2012).

Pathogen detection using CLART® Enterobac technique

The CLART® Enterobac testing was realized after completing the eighty stool sample's collection which were stored at -80 ° C until testing day. By this technique, the following enteropathogens can be detected: *Salmonella* spp, *Shigella* spp, *C. difficile* B, *Yersinia enterocolitica (Y. enterocolitica)*, enteropathogenic *E. coli* (EPEC) enterotoxicogenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorragic *E. coli* (EHEC), *Campylobacter jejuni* (*C. jejuni*), *Campylobacter* spp. and *Aeromonas* (aerolysin positive strain). The protocol of aforementioned technique was followed according to the manufacturer's instructions (http://genomica.es/en/documents/CLARTEnteroBacEnglishV4July2015.pdf)

RESULTS

The results of stool cultures revealed the absence of any enteropathogens unless of 8 sorbitol negative *E. coli* strains. The results of immunological test have showed the absence of EHEC strain, whereas the PCR reactions conducted on the sorbitol negative strains showed the presence of only one EPEC strain which carried the *eae* and *bfp* genes. The EPEC strain was found in 2 years old patient (Table 1). The retrospective implementation of Microarrays technique (CLART® Enterobac) allowed us to detect one or more enteropathogens in 15 samples of 80 samples analyzed (19%). Among these, CLART® Enterobac has identified: one strain of *C. difficile*, 2 strains of *Salmonella* spp, 2 strains of *C. jejuni*, 6 strains of *Shigella* spp. and 7 EPEC strains in 6 patients aged between nine months and three years old. In addition, co-infections were detected in 3 samples, which exhibit the following co-infectious agents: *Salmonella spp+ C. jejuni* (sample 19) and *Shigella* spp. + EPEC (samples 20 and 44). It is that patient 44 who carried EPEC strains is 3 years old only.

TABLE1

Comparison between Results Obtained using the Conventional Stool Cultures (with Conventional PCR Confirmation) and CLART® EnteroBac Technique

Sample number	**Age**	**Gender**	**Stool culture**	***CLART® EnteroBac***
2	3 years old	M	Negative	*Salmonella* spp.
7	3 years old	F	Negative	*Shigella* spp.
8	4 years old	F	Negative	*C. jejuni*
11	1 year old	M	Negative	*EPEC*
19	1 year and 6 months old	M	Negative	*C. jejuni* + *Salmonella* spp.
20	2 years old	F	Negative	*EPEC* + *Shigella* spp.
26	2 years old	M	Negative	EPEC
28	2 years old	F	Negative	EPEC
33	1 years and 6 months old	M	EPEC (PCR confirmed)	EPEC
34	3 years old	F	Negative	*Shigella* spp.
41	9 months	M	Negative	EPEC
43	5 years old	M	Negative	*Shigella* spp.
44	3 years old	F	Negative	EPEC + *Shigella* spp.
45	9 years old	M	Negative	*Shigella* spp.
46	9 years old	M	Negative	*C. difficile*

DISCUSSION

In Lebanon, the bacteriological test of stool remains very limited method because, on one hand, the test aims to detect only *Salmonella* and *Shigella* species. On the other hand, the majority of diarrheal patient, who are living under unfavorable condition, can buy all kind of drugs incliding antibiotics from pharmacies without medical prescriptions including antibiotics.

According to the literature, the study of bacterial populations using the culture media isolation method is not sufficient and allows only a very small proportion of species to grow (Nocker et al., 2007). Twenty years ago, several studies have showed that most species are fastidious and uncultivable on traditional media. Consequently, the importance of molecular biology in diagnosis of this type of bacteria greatly increased (Best et al., 2003; Gilmour et al., 2009; Houng et al., 1997).

The conventional methods are based on stool culture for bacterial enteropathogens, microscopic identification for parasites and antigen based kit for viral detection. For stool culture, the gut microflora may cover pathogen in the early stage of infection. Moreover, the use of antibiotic in uncontrolled way can lead to negative stool culture (Guerrant et al., 2001). The microscopic observation of parasites is the second conventional method. This method needs time and training and it lacks sensitivity (Haque et al., 1995). The third conventional method is an antigen based technique which was used often after 1970 to detect fastidious pathogens and viruses. However, these techniques are costly and apply only for limited enteropathogens (Kirby et al., 2010). The DNA based methods or molecular methods such as PCR (simplex or multiplex), real-time PCR, microarray and next-generation sequencing (NGS) have showed efficiency in all clinical diagnosis including the detection of enteropathogens (Maher et al., 2003; Tobias and Vutukuru, 2012).

In Lebanon, in spite of the unreliable results of conventional method, the stool culture is still prescribed as a standard protocol for gastroenteritis in children, adult and elderly. Maher et al. (2003) have reported that the 16S/23S PCR/DNA probe technique had a 94% sensitivity, and they have detected *Campylobacter* species in 41 culture-negative specimen. Furthermore, a study conducted by Lebanese researchers (Dabboussi et al., 2012) showed that among 90 culture-negative diarrheal stool samples collected in North Lebanon, 10 samples (11.1%) were positive for Campylobacteriosis using PCR. Another study conducted in 2015 by Amrieh et al., has showed that among 242 *E. coli* strains isolated from diarrheal stool collected from Lebanese people, 14 sorbitol-negative *E. coli* strains (5.7%) were isolated and one strain (0.4%) was confirmed as *E. coli* O157H7 by PCR reaction. The sorbitol test was considered as a differential way between normal and enteropathogenic *E. coli* (Ojeda et al., 1995). Unfortunately, after molecular method revolution, the sorbitol fermentation remains an insignificant indicator and has been replaced by detection of *stx1, stx2, bfp and eae* genes by PCR methods (Croxen et al., 2013).

In the present study, the CLART® Enterobac technique showed higher sensitivity than stool culture methods. This sensitivity is due to the technique conception which amplifies enteropathogen's DNA by multiplex PCR followed by probe hybridization. This technique was used in order to detect a large variety of enteropathogens in 80 diarrheal stool samples collected from Lebanese children aged from 9 months to 15 years. The conventional method was conducted in parallel in order to compare the sensitivity of both methods. As shown in Table 1, 15 patients were infected as following: 3 patients were infected by 2 different pathogens: two patients had the co-infection EPEC/*Shigella* and one patient had *C. jejuni/Salmonella.* This data is very important for treatment and for epidemiological study. However, the stool cultures have detected one infected patients which was a very low prevalence. Therefore, these results have confirmed that the stool culture lacks sensitivity and can lead to complicated situations especially in children or susceptible patients such as immunocompromised patients.

CONCLUSION

The conventional methods for gastroenteritis diagnosis are not efficient. The stool culture remains a non-sensitive method for enteropathogens detection. Thus, the use of molecular method seems to be an alternative for such diagnosis. Our results showed that among 79 culture-negative stool samples, 14 samples were positive by using CLART® EnteroBac technique. These results confirm that the conventional stool culture, despite its interest and importance for the isolation of entropathogens, revealed many limits (1.25% positivity); while

the CLART® EnteroBac method showed higher sensitivity (19%). Therefore, the molecular methods are very important for treatment and epidemiological studies. Furthermore, the culture-negative stool may lead to a complicated situation leading to the prescription of wrong drugs including even antibiotics. However, the expensive cost of the CLART® EnteroBac test remains a real obstacle.

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