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Development of a Multiplex PCR Assay to Detect Gastroenteric Pathogens in the Feces of Mexican Children

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Abstract Acute gastroenteritis (AGE) is a major cause of childhood morbidity and mortality worldwide; the etiology of AGE includes viruses, bacteria, and parasites. A multiplex PCR assay to simultaneously identify human Astrovirus (HAstV), Calicivirus (HuCVs), *Entamoeba histolytica* (*E. histolytica*), and enteroinvasive *Escherichia coli* (EIEC) in stool samples is described. A total of 103 samples were individually analyzed by ELISA (enzyme-linked immunosorbent assays) and RT-PCR/PCR. HAstV and HuCVs were detected in four out of 103 samples (3.8 %) by RT-PCR, but ELISAs found only one sample as positive for HuCVs (2.5 %). *E. histolytica* was identified in two out of 19 samples (10.5 %) and EIEC in 13 out of 20 samples (70 %) by PCR, and all PCR products were sequenced to verify their identities. Our multiplex PCR results demonstrate the simultaneous amplification of different pathogens such as HAstV, EIEC, and *E. histolytica* in the same reaction, though the HuCVs signal was weak in every replicate. Regardless, this multiplex PCR protocol represents a novel tool for the identification of distinct pathogens and may provide support for the diagnosis of AGE in children.

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Introduction

Acute gastroenteritis (AGE) of infectious origin is a health problem in both developing and developed countries. AGE is responsible for approximately 3 million deaths per year, mainly affecting children under 5 years old [23, 33]. The causes of diarrhea include a wide range of viruses, bacteria, and parasites. Several epidemiological studies have shown that up to 50 % of AGE is caused by bacteria or parasites alone [25], whereas some estimates consider that almost 80 % of cases of AGE are due to viruses [4, 22]. The rotavirus is the most important agent responsible for diarrhea in children under 2 years of age. Currently, depending on the region, the human Astrovirus (HAstV) is the second or the third most common virus involved in AGE worldwide, as shown by ELISAs or RT-PCR in samples from different geographic regions in México or hospital samples collected in Madrid, Spain [24, 34]. The human Calicivirus (HuCV) is considered to be the most common cause of non-bacterial gastroenteritis outbreaks in all age groups [4, 37]. HuCV is highly infectious and frequently occurs in both developing and developed countries. Recently, it has been reported that the Norovirus (NoV) causes approximately 10 % of intestinal infections in developed countries, e.g., Holland and England, in a prospective population-based cohort study [9, 40].

HAstV and HuCVs are both single-stranded positive-sense RNA viruses with a similar genomic structure consisting of three open reading frames (ORFs) [3, 11, 23]. The NoV and *Sapovirus* genera belong to *Caliciviridae*, although *Sapovirus* is mainly associated with pediatric

AGE [36]. Based on their genomic sequences, both NoV and *Sapovirus* have been divided into three genogroups: I, II, and III (GI, GII, GIII) [12, 41]. Meanwhile, AGE caused by bacteria, which is known as diarrheagenic *Escherichia coli* (DEC), involves such specimens as *E. coli* and *Shigella* spp. The former represents a serious public health problem in developing countries [16] and, to a lesser extent, in the developed countries [7]. Of the six categories of the DEC, the most important is the enteropathogenic (EPEC) variant [20]. However, the enteroinvasive *E. coli* variant (EIEC) has recently been considered as an important factor that could be affecting diarrhea in children under 2 years of age in some developing countries [1, 16, 26, 27]. Upon indentifying DEC strains, an important problem is their differentiation from the non-pathogenic species that constitute normal intestinal flora [25]. Another important AGE pathogen is *Entamoeba histolytica*, which is a serious public health problem worldwide and a major contributor to childhood mortality and morbidity in the developing world [14]. In Mexico, *E. histolytica* is considered as an endemic health problem [32], and it should be differentiated from other non-pathogenic *Entamoeba* species, such as *Entamoeba dispar* [11, 32].

Until the 1970s, diagnostic techniques for characterizing infectious diarrhea were limited to bacteria and protozoa, and the etiologic agent was identified in a limited proportion of cases [21]. As a result of rapid progress in these areas, molecular techniques have recently facilitated the identification of other pathogens, such as viruses, because molecular techniques are more sensitive than classical methods. The detection of viruses has traditionally been based on techniques such as electron microscopy, which, although it is still useful, is limited to reference laboratories [37] and expensive. Similarly, cell culture is not considered useful for diagnostic purposes, mainly because propagation in cellular culture, for example calicivirus, has not yet been achieved [39]. Enzymatic immunoassays have also been used for the detection of these pathogens [10], but they have limitations. For example, the true incidence of HAstV has been underestimated using enzyme immunosorbent assays because the detection sensitivity is insufficient [34]. Nevertheless, the RT-PCR assay has recently been used as an extremely sensitive method to detect HAstV and HuCVs in fecal samples [5, 8, 19, 30, 38] because it allows rapid detection and characterization of both viruses. These results are more reliable in establishing the prevalence of the viruses than the classical methods as mentioned above [19].

Conventional methods to identify DEC strains include culturing stool samples in different culture media. However, several multiplex PCR assays have been recently developed to detect the DEC categories in human fecal samples [1, 2]. This detection effort revealed a major prevalence of the EPEC variety (6 %) [1], detected ten different specific virulence genes of the DEC pathogroups [25], and demonstrated that the multiplex PCR method is a faster, more sensitive, and more specific method to detect pathogens in children with AGE than the classical methods previously mentioned. Furthermore, two multiplex real-time PCR approaches have been reported by different groups. One of these approaches established a protocol for the exhaustive detection of DEC that was efficient in the detection of enterovirulent genes and is suitable for screening enrichment broths containing food samples [16]. In the second study, a multiplex real-time PCR method was developed to determine the sensitivity and specificity of this assay using a pool of five colonies rather than analyzing individual colonies, thereby producing a less expensive assay suitable for use in developing countries [2].

In the case of *E. histolytica* infections, multiplex PCR is considered a valuable method for distinguishing between pathogenic and non-pathogenic species, mainly in areas where *E. histolytica* is considered endemic [6, 35]. Several studies have shown that PCR, when used as a diagnostic method, is more specific than conventional methods, and it is possible to establish between 15 and 25 % of positive samples using this technique and thereby rule out the non-pathogenic *Entamoeba* [29].

The aim of the study was to develop a multiplex PCR assay to detect distinct AGE pathogens, such as HAstV, HuCVs, *E. coli* (DEC), and *E. histolytica*, in fecal samples to improve correct diagnoses, which may help to limit inappropriate antibiotic use, reduce costs, and reduce the risk of nosocomial transmission.

Materials and Methods

Multiplex PCR Assay

The references strains and positive samples of each pathogen were used to perform the multiplex PCR assays. The theoretical parameters of the annealing temperature were determined with Vector NTI Advance 11 software (Invitrogen), and the primer concentration was calculated as previously described (Table 1) [1, 3, 15, 17, 28, 29]. To distinguish the HAstV and HuCV RT-PCR products, we used the primers Mon-269 5′-CAA CTC AGG AAA CAG GGT GT-3′ and Mon-270 5′-TCA GAT GCA TTG TCA TTG GT-3′ to target the ORF2 region (449 bp) as described by Noel [28]. We performed an RT reaction before the multiplex PCR using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Germany) in the presence of the random primer (60 µM) and 10 µl of viral RNA to synthesize cDNA in a final volume of 20 µl

Table 1 Determination of optimized annealing temperature and primer concentration for the multiplex PCR assay

Pathogens	Tm value (°C)	Primer conc.
Astrovirus	55	10 μM
Calicivirus	56	50 pmol
E. coli ElEC	60	20 pmol
E. histolytica	55	20 pmol

μM Micromolar

E.coli ElEC, enteroinvasive *Escherichia coli*

E. histolytica, Entamoeba histolytica

following the manufacturer's instructions. A mixture was made with 5 μl each of cDNA (HAstV and HuCV) and PCR products (bacteria and parasites) with 4 μl of 10× PCR buffer, 1.5 mM $MgCl_2$ (BioTecMol), 250 μM each dNTPs (Roche), 1 U of Amplificasa (BioTecMol), and eight primers, the combinations of these parameters that were used are described in Table 1 (Mon269/270, p-289/p-290, IpaH1/IpaH2 and Psp5/Psp3), and free-nuclease water was added to a volume of 40 μl. The parameters for amplification were 95 °C for 15 min, followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 7 min and cooling at 4 °C. All the components except the cDNA/DNA templates were used in the mixture reaction for the negative controls. The multiplex PCR assay was performed in a Mastercycler personal Eppendorf (Germany). The expected sizes of all amplicons are HuCV (319 bp), HAstV(449 bp), *E. coli* EIEC (600 bp), and *E. histolytica* (876 bp). The amplified DNA was visualized by 1 % agarose gel electrophoresis and ethidium bromide staining.

Nucleic Acid Extraction

The genomic material was extracted following the manufacturer's instructions with the following commercials kits: QIAamp Viral RNA for viruses (Qiagen, Germany), ZR Fecal DNA Zymo Research for bacteria, and QIAamp DNA Stool for Entamoeba [13] (Qiagen, Germany).

RT-PCR

The samples were tested for the presence of HAstV and HuCVs by RT-PCR using primers Mon/340 5′-CGT CAT TAT TTG TTG TCA TAC T-3′ and Mon/348 5′-ACA TGT GCT GCT GTT ACT ATG-3′ targeting the ORF1a region (289 bp), as previously described [3], and p-289 5′-GAT TAC TCC AAG TGG GAC TCC AC-3′ and p-290 5′-TGA CAA TGT AAT CAT CAC CAT A-3′ (319 bp), as previously described [17], respectively. The reactions were performed using a One-Step RT-PCR kit (Qiagen, Germany) with 5 μl of the RNA extracted in the presence of 100 pmol of Mon340/Mon348 and 50 pmol of p289/p290, respectively. The parameters used were 50 °C for 30 min (RT), 95 °C for 15 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 7 min and subsequent cooling at 4 °C. The RT-PCR was performed in a Mastercycler personal (Eppendorf, Germany). In order to discard the presence of inhibitors, a test using a positive fecal sample to HAstV and HuCVs was performed prior to the RNA extraction.

PCR

In order to detect the presence of the DEC and *E. histolytica*, the DNA of each pathogen was used in a PCR reaction. Previously described primers were used for bacteria [1]: for EIEC, IpaH1 5′-GTT CCT TGA CCG CCT TTC CGA TAC CGT C-3′ and IpaH2 5′-GCC GGT CAG CCA CCC TCT GAG AGT AC-3′ (600 bp), and for EPEC, eae1 5′-CTG AAC GGC GAT TAC GCG AA-3′ and eae2 5′-CCA GAC GAT ACG ATC CAG-3′ (917 bp). As a positive control of each bacteria, we used a reference strain from the Public Health Department (Medicine Faculty, UNAM, México) growth at 37 °C for 18 h in selective media as previously described [1, 2, 27]. For *E. histolytica,* the primers used were Psp5 5′-GGC CAA TTC ATT CAA TGA ATT GAG-3′ and Psp3 5′-CTC AGA TCT AGA AAC AAT GCT TCT C-3′ (876 bp) [32]. The PCR reactions for DEC and *E. histolytica* were performed with Taq Polymerase Platinum High Fidelity (Invitrogen). For bacteria, 10 pmol of primers in the presence of 500 ng of DNA were used in a 20 μl reaction. The parameters were as follows: 95 °C for 5 min, followed by 40 cycles at 95 °C for 40 s, 60 °C for 1 min, and 72 °C for 2 min, with a final extension at 72 °C for 7 min and cooling at 4 °C. For *E. histolytica,* 40 pmol of primers were used, and the PCR conditions were as follows: 95 °C for 10 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1.5 min, and 72 °C for 2 min, with a final extension at 72 °C for 8 min and cooling at 4 °C. The PCR was performed in a Mastercycler personal (Eppendorf, Germany).

Nucleotide Sequencing

Prior to the sequencing analysis, the PCR products were excised from gels and purified using a QIAquick gel extraction kit (Qiagen) following the manufacturer's instructions. Sequencing was performed at Facultad de Estudios Superiores-Iztacala and Instituto de Fisiología Celular, UNAM (México). The nucleotide sequences were aligned with reference sequences from GenBank (NCBI Library) using Clustal W (2.1) version, (SDSC, Biology Work Bench, San Diego, USA).

Immunoenzymatic Assay

The stool samples were screened using standard ELISAs. The presence of the Astrovirus, Calicivirus, and Entamoeba antigens was detected with r-Biopharm (Germany) RIDA SCREEN Astrovirus, RIDA QUICK NoV, and RIDA QUICK *Entamoeba,* respectively, following the manufacturer's instructions.

Participants and Stool Specimens

Informed consent was obtained from parents/guardians before collecting the samples. A total of 200 fecal samples were obtained from Mexican children under 5 years old with diarrheic symptoms from distinct areas of Mexico City over a two-year period (November 2008–November 2010). The clinical symptoms included vomiting, abdominal cramps, and fever. After verifying the previously established inclusion and exclusion criteria, only 103 stool samples were diluted as described by Yuen [42] and the OPS manual [30]. In brief, a 30 % stool suspension was prepared in PBS (pH 7.4), and three aliquots were made. One of the aliquots was stored at −20 °C and was used for ELISAs and RT-PCR/PCR; the other aliquots were stored at −70 °C for further studies. The majority of the samples were from children between 6 months and 2 years old (Online Resource 1); the children were principally male. The clinical characteristics showed that the majority had a fever, semi-watery evacuations and low dehydration, but a considerable number of children showed no dehydration and had watery evacuations (Online Resource 2). The analysis of the clinical data revealed that some of the children received antibiotic or anti-parasite treatment, and some cases received the rotavirus vaccine (data not shown). These samples were not considered in the analysis by ELISA and molecular techniques.

Results

Establishment of the Multiplex PCR Assay

In order to establish a multiplex PCR assay, we first used the Vector NTI Advance 11 (Invitrogen, USA) bioinformatics software to discard hybridizations between the primers and to determine the optimal primer concentrations [15] needed to amplify all pathogen targets in one reaction tube (Table 1). In addition, published primer sets were selected with a long track record to validate the detection of viruses [3, 17], bacteria [1], and parasites [32]. Initially, we performed PCR assays using the nucleic acids of reference strains for each pathogen (viruses, bacteria, and parasites, data not shown). Four pairs of specific primers for each pathogen were pooled. The Astrovirus primers Mon-269/270 were used to amplify a 449-bp ORF2 fragment [28] to discern between Astrovirus and Calicivirus amplicons. All amplicons were obtained under the PCR conditions tested. We were able to individually amplify the four PCR fragments of the expected sizes (Fig. 1), both individually (lanes 1–4) and in one reaction tube (lane 5). The products were 319 bp for NoV (lane 1), 449 bp for HAstV (lane 2), 600 bp for EIEC lane 3), and 876 bp for *E. histolytica* (lane 4).

Detection of HAstV and HuCV by RT-PCR

Before performing the multiplex PCR assay on the samples collected from the study population, we obtained clarified extracts from 103 samples, which were screened by RT-PCR for the presence of HAstVs (primers Mon340/348, and Mon269/70) and HuCVs (primers p-289/p-290). Three specimens were positive for HAstV using primers Mon340/348 and one with primers Mon269/270 (Table 2). A previously characterized clarified stool sample [24] was used as a positive control. We also found four positive samples for HuCVs with primers p-289/p-290 (Table 2). The amplicons of the positive samples for both RNA viruses were sequenced (data not show), revealing two HAstV-1 serotypes, one HAstV-8 serotype, and one uninformative serotype despite two sequencing runs. Only one HuCVs sample was sequenced, which corresponded to the RdRp region of the Norwalk virus (NoV genogroup I). The sensitivity of the RT-PCR assays for each sample was compared to that of individual ELISAs (see below).

Identification of DEC

To identify the DEC specimens in the stool samples, we performed colony PCR assays with reference strains to

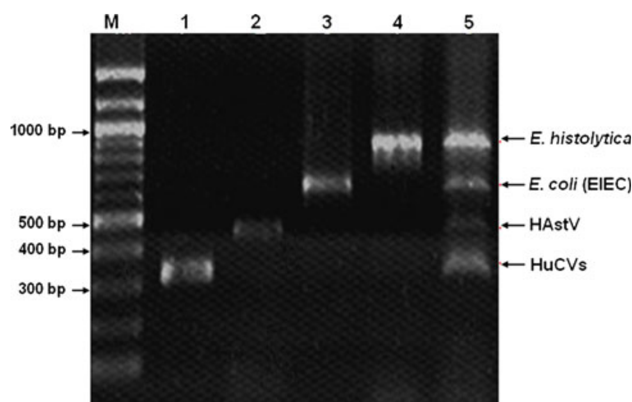

Fig. 1 Specificity of multiplex PCR assay with a mixture of eight primers for all pathogens studied. *Lanes 1–4* NoV (319 bp), HAstV (449 bp), *E. coli*, EIEC (600 bp), and *E. histolytica* (876 bp), respectively; *lane 5* the mixture of all PCR products of positive controls amplified in a single-reaction tube. *Lane M* 100-bp ladder DNA markers (New England, Biolabs)

establish the optimal amplification parameters (data not show). The reference strains were grown in selective media as previously described [1, 2]. From the collected specimens, 20 DNA samples were analyzed by PCR, excluding those that corresponded to children who received antibiotic treatment (data not show). Thirteen samples were positive for EIEC (Table 2) and were validated with the inclusion of the positive and negative controls in the reaction (DNA from healthy children, data not shown). The 600-bp amplicon was sequenced (data not show), and as expected, it corresponded to the *E. coli* strain HGZ12 ipaH6 gene. The same 20 samples were analyzed for EPEC, which is considered to be the principal producer of diarrhea in children of developing countries [26], and all were negative (data not shown), despite performing the analysis four times.

Detection of *E. histolytica* by PCR

To detect *Entamoeba* by PCR, we used the same samples tested by the ELISAs (Table 2) corresponding to children without previous anti-parasitic treatment. Initially, we characterized the samples using the RD5/3 primers [32] to amplify a 1,950-bp fragment that determines the presence of the *Entamoeba* genera in the DNA samples. However, we did not obtain any amplification, even when the presence of trophozoites was previously corroborated by light microscopy in the stool samples. With this result in mind, we used the Psp5/Psp3 primers, which are specific for *E. histolytica*, and generated an 876-bp fragment. We included a positive sample [31], and DNA samples from healthy children were used as a negative control. From the 19 samples analyzed by PCR, only two were positive for *E. histolytica*.

ELISA Analysis

In order to compare a conventional diagnostic method (Gold Standard) with the multiplex PCR assay, we tested the samples by ELISA using the r-Biopharm kits for HAstV, HuCV, and *E. histolytica*. Interestingly, all 103 samples tested for HAstV were ELISA-negative, including the four that were positive by RT-PCR. Because the indirect collection conditions (from diapers) of the stool specimens were difficult, resulting in possible HuCV viral antigen degradation [28], we decided to only analyze 40 samples. From these, just one sample was HuCV-positive by ELISA. Furthermore, all 19 samples tested for *E. histolytica* were negative by ELISA (Table 2). In order to discard Rotavirus as an AGE agent, ELISAs were performed on 90 samples from children not vaccinated against Rotavirus, and we found that all such samples were negative (data not shown). ELISA was not performed to test the presence of bacteria in stools samples.

Multiple PCR Assay Using Nucleic Acids Form Clarified Positive Samples

In order to validate our multiplex PCR assay, we combined the nucleic acids of each pathogen (the cDNA synthesized from viral RNA, and bacterial and parasite DNA) in one tube (Fig. 2a). We were able to amplify all the expected fragments except for the NoV product (lane 9). Control reactions for the amplifications were performed for individual amplicons with the same multiplex PCR conditions; the results showed four PCR fragments of the expected sizes (lanes 1, 3, 5, and 7, respectively). The multiplex PCR assay was repeated three times, and the NoV amplification was weak in every replicate. To discard a possible degradation of the NoV viral RNA, we performed an RT-PCR assay with the specific amplification parameters for NoV, and the expected 319-bp PCR product was observed (Fig. 2b), suggesting that the primer selection and/or conditions selected for the multiplex PCR assay were not optimal for the amplification of NoV sequences.

Table 2 Detection of pathogens by ELISA, RT-PCR, and PCR

Pathogens	ELISA		RT-PCR		PCR		Total samples	Positive samples (%)[c]
	+	−	+	−	+	−		
Astrovirus	0	103	4	99	–	–	103	3.8
Calicivirus[a]	1	39	4	99	–	–	103	3.8
E. histolytica[b]	0	19	–	–	2	17	19	10.5
E. coli ElEC	nd	nd	–	–	13	7	20	65

E coli EIEC, enteroinvasive *Escherichia coli*

+ positive, − negative, *ELISA* enzyme-linked immunosorbent assay, *RT-PCR* reverse transcription-polymerase chain reaction, *PCR* polymerase chain reaction, *nd* not determined

[a] Total samples analyzed to calicivirus by ELISA

[b] Total samples to *E. histolytica* analyzed by ELISA

[c] Final percentage the samples analyzed by RT-PCR/PCR

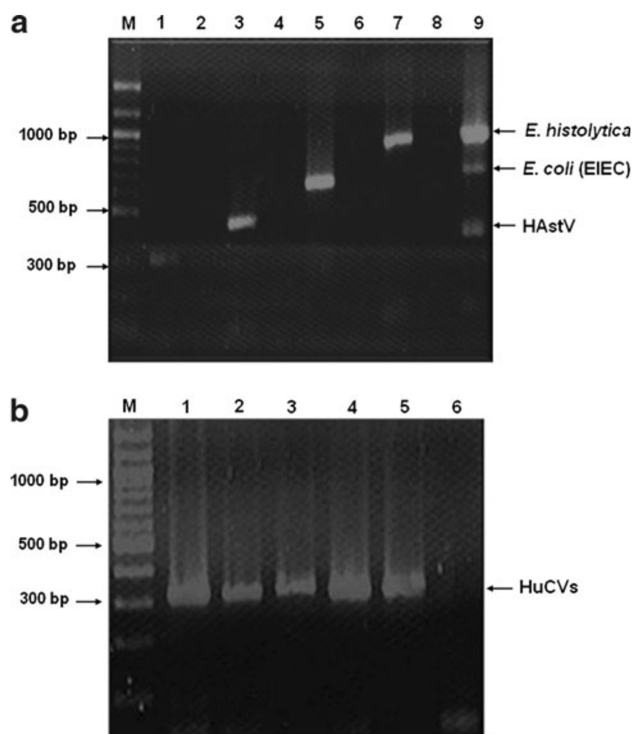

Fig. 2 **a** Simultaneous detection of viral RNA and DNA of bacteria and parasites by multiplex PCR; *lanes 1*, *3*, *5*, and *7* positive stool specimens correspond to NoV, HAstV, *E. coli* EIEC, and *E. histolytica,* respectively; *lanes 2*, *4*, *6*, and 8 represent a negative control for each sample. *Lane 9*, mixture of cDNA of HAstV and NoV, DNA of *E. coli* EIEC, and *E. histolytica*. **b** Individual analysis of NoV stool specimens with p289/p290 primers. Positive samples amplified by RT-PCR (*lanes 1–4*); *lanes 5* and *6* positive and negative controls, respectively. *Lane M*, 100-bp ladder DNA markers (New England, Biolabs)

Discussion

Infectious causes of pediatric gastroenteritis can be classified into three broad categories: viral, bacterial, and parasitic. All of these agents are important worldwide because they annually cause more than 700 million cases of acute diarrhea in children younger than 5 years old, particularly in developing countries [18]. A reliable diagnostic method that can be performed in the laboratories of pediatric hospitals to detect the AGE pathogen will be helpful for clinicians to provide better healthcare to the hospitalized children. With this goal in mind, we were able to establish a multiplex PCR assay to detect, in a single-tube reaction, the presence of three different pathogens: a virus (HAstV), bacterium (*Escherichia coli*, EIEC), and parasite (*E. histolytica)*.

Initially, we tested reference strains as positive controls to establish the specific PCR parameters and to eliminate possible primer–primer interactions among the primers (Table 1; Fig. 1). Subsequently, we proved that the nucleic acids isolated from the individual samples of the selected pathogens that tested positive by the initial screening were also amplified with the multiplex PCR conditions (Fig. 2a).

In the multiplex PCR, all products corresponding to each pathogen were amplified, although the amplification corresponding to the NoV genome was weak (Fig. 2a). Since the RT-PCR reaction in the presence of the p-289/p-290 was successful using in monoplex conditions, probably they do not work in multiplex PCR conditions (Fig. 2b), as suggested by Rohayem. For this reason, it is necessary to test other primer combinations (e.g., the Calman-29/Calman-32 primers that Rohayem [33] used to detect NoV genogroup I) to amplify the HuCVs in the multiplex PCR conditions. Furthermore, the sequencing of the amplicons for all pathogens found in this study demonstrated the high specificity of the multiplex assay with the selected primers.

Although the number of samples was limited to validate our multiplex method against conventional techniques used for the diagnosis of AGE, we examined the specimens by ELISA, and the results are summarized in Table 2. In agreement with previous studies performed with viruses, bacteria and parasites in which the molecular approach exhibited greater detection sensitivity for gastroenteric pathogens than ELISAs, our results are similar to those for the individual detection of each pathogen [1, 6, 8, 9, 16, 19, 25, 31, 34–36, 41, 42]. In the present study, the percentage of positive samples for both HAstV and NoV by RT-PCR was 3.8 % (Table 2), whereas only 2.5 % were positive for NoV by ELISA (although only forty samples were analyzed with this method). Further, all samples for HAstV were negative, demonstrating the increased sensitivity of the molecular technique. We suspect that the HAstV samples were negative by ELISA due to the sample collection conditions. The majority of the samples were recovered from diapers obtained from different childcare facilities, hospitals, and institutions. However, not all of the diapers were obtained immediately, which means that the viral antigen may have been degraded [28]. In addition, it is important to mention some differences with other works. A larger number of samples was previously analyzed in comparison to our work, the geographic conditions differed, and there were differences in factors such as economic status, weather, public health services, and the level of hygiene, which are all important factors that determine the presence of particular viral groups associated with AGE [36, 37].

Regarding the results for detection of DEC specimens, we did not test the samples by immunoenzymatic assays because the commercial kits available to detect combinations of other pathogens that may be involved in AGE but not considered in this study. In this study, we found that 65 % of the samples were positive for EIEC variants by the PCR assay, in comparison with the results obtained in Peru, where the EIEC strain was not detected, and the EPEC

strain was detected in 5.8 % by multiplex real-time PCR [2]. The results were different than we expected because EPEC is an important cause of infant diarrhea in developing countries [26]. However, EIEC was also not detected in Tanzania [25].

In this respect, our results suggest a special characteristic of the Mexican children's population regarding this *E. coli* variant, although we need to perform more experiments to prove this hypothesis because, even though EPEC is the most important *E. coli* diarrhea producer [20], the enteroinvasive variant (EIEC) has recently and frequently been found in children under 2 years old in some developing regions [1, 16, 26, 27], which is the main population included in this study (Online Resource 1).

In the cases of the negative results for the detection of *E. histolytica* through the ELISAs, the cause could be low sensitivity of the kit to analyze the samples compared with PCR. Recently, more specific and sensitive molecular methods, such as PCR, have been introduced as diagnostic tests [6, 13, 14, 29]. In one study, 127 samples (21 %) were positive for the *E. histolytica*/*E. dispar* complex by microscopy, and among these, 11 were positive by multiplex PCR, and only two were (1.57 %) corresponded to *E. histolytica* [6]. Our results were similar considering the total samples analyzed (103/1.94 %). However, we only analyzed 19 samples (10.5 %) that were previously analyzed by microscopy.

In summary, our results raise the possibility of using the novel multiplex PCR assay in developing countries, such as México, where there are regions with poor sanitation and populations with low socioeconomic conditions. This new molecular approach will be useful as a rapid and effective routine diagnostic tool for the detection of major pathogens causing diarrhea in an attempt to reduce the time lost at work by parents, which negatively impacts the economic development in developing countries. The cost to perform this assay may be high for some regions, but with the application of the diagnostic method in many samples that come from a pediatric hospital, we think this cost will be reduced. To our knowledge, this is the first study providing a new multiplex PCR approach to detect three out of four of the most important pathogens responsible for the AGE in fecal samples of Mexican children under 5 years old.

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Conflict of interest The authors declare that they have no conflicts of interest.

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