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## DNA Microarray for Detection of Gastrointestinal Viruses

### **This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/157330> since

*Published version:*

DOI:10.1128/JCM.01317-14

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1 DNA microarray for detection of gastrointestinal viruses

2

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22 Key words: gastroenteritis, virus, diagnostic, microarrays

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24 Running title:

25 DNA microarray for detection of viruses found in stools

26

27 **ABSTRACT**

28 Gastroenteritis is a clinical illness of humans and other animals characterized by vomiting and  
29 diarrhea, caused by a variety of pathogens including viruses. An increasing number of viral species  
30 have been associated with gastroenteritis or have been found in stool samples as new molecular tools  
31 are developed. In this work, a DNA microarray capable in theory of parallel detection of more than 100  
32 viral species was developed and tested. Initial validation was done with 10 different virus species and  
33 an additional 5 species were validated using clinical samples. Detection limits of  $1 \times 10^3$  virus particles  
34 of *human adenovirus C* (HAdV), *human astrovirus* (HAstV) and group A *rotavirus* (RV-A) were  
35 established. Furthermore, when exogenous RNA was added, the limit for RV-A detection decreased in  
36 one log. In a small group of clinical samples from children with gastroenteritis ( $n = 76$ ), the microarray  
37 detected at least one viral species in 92% of the samples. Single infection was identified in 63 samples  
38 (83%) and more than one virus was identified in 7 samples (9%). The most abundant virus species were  
39 RV-A (58%), followed by *anellovirus* (15.8%), HAstV (6.6%), HAdV (5.3%), *Norwalk virus* (6.6%),  
40 *human enterovirus* (HEV; 9.2%), *human parechovirus* (1.3%), *Sapporo virus* (1.3%) and *human*  
41 *bocavirus* (1.3%). To further test the specificity and sensitivity of the microarray, the results were  
42 verified by RT-PCR detection of 5 gastrointestinal viruses. The RT-PCR assay detected a virus in 59  
43 samples (78%). The microarray showed good performance for detection of RV-A, HAstV and  
44 calicivirus, while the sensitivity for HAdV and HEV was low. Furthermore some discrepancies in  
45 detection of mixed infections were observed, and were addressed by RT-qPCR of the viruses involved.  
46 It was observed that differences in the amount of genetic material favored the detection of the most  
47 abundant virus. The microarray described in this work should help to understand the etiology of  
48 gastroenteritis in humans and animals.

49

## 50 INTRODUCTION

51 Gastroenteritis stands among the five principal causes of mortality by disease and morbidity at  
52 all ages worldwide. The most affected population is children under 5 years of age, where it accounts for  
53 the second cause of post-neonatal death with approximately 2.6 millions deceased per year [1].  
54 Although the majority of deaths occur in developing countries, diarrheal disease is among the most  
55 common causes of illness worldwide, with approximately 4,620 millions episodes annually [1]. Besides  
56 humans, all vertebrate species suffer from enteric diseases. Infections in farm animals can lead to big  
57 economical losses, while household pets, like dogs and cats, are also affected. On the other hand, wild  
58 animals, like deer, monkeys, bats, foxes, wolves, and boars, among others, can act as potential  
59 reservoirs for pathogens [2]. Gastrointestinal (GI) infections are caused by a variety of pathogens  
60 including parasites, bacteria and viruses. The characterization of pathogens causing GI infections of  
61 viral etiology have led to the establishment of a main group of pathogens [*rotavirus A* (RV-A),  
62 *Norwalk virus* (NV), *human astrovirus* (HAstV), and *human adenovirus F* (HAdV-F)] [3] for which  
63 specific diagnostic tests were developed [4]. Tests for secondary or rare virus are available but usually  
64 restricted to experimental use. Routine diagnostic methods for viral gastroenteritis are nowadays based  
65 on the detection of virus components by immunoassays or by molecular methods [5,6,7,8], with the  
66 majority of these tests designed to evaluate only a single pathogen at a time.

67 The use of two or more specific primer sets (multiplexing) in polymerase chain reaction (PCR)  
68 allows the amplification of several targets in one test. Although multiplex tests are available for diverse  
69 viruses [9,10,11,12,13], facilitating rapid and sensitive detection of the main GI agents, these assays are  
70 still limited in number of viruses detected, and the results can be affected by mutations at primer  
71 binding sites. On the other hand, DNA microarrays represent an alternative to detect hundreds to  
72 thousands of potential pathogens in a single assay. Microarray detection is based on solid phase  
73 hybridization, in which specific probes are deposited on a surface and react with a mixture of labeled

74 nucleic acids. So far, different microarrays have been developed to detect causative infectious agents  
75 associated with a number of diseases: respiratory [14,15,16], hemorrhagic [17], blood borne [18,19],  
76 and central nervous system syndromes [20]. Other broad microarrays have been developed for virus  
77 discovery [21], however, diagnostic microarray specific for viruses found in GI tract is missing. Given  
78 the recent rise in the number of new viral species [22,23,24,25,26], diagnostic DNA microarrays  
79 represent the possibility to test their clinical importance and impact in human and animal health.

80 In this work, the development and validation of a DNA microarray designed to detect in  
81 principle more than 100 viral species associated with GI tract in vertebrates is presented. This  
82 microarray was successfully used to identify viruses in a small set of gastroenteritis clinical samples.

83

## 84 **MATERIALS AND METHODS**

85

### 86 **Cells, viruses, and clinical samples**

87 Viruses were either present in our laboratory or kindly provided by different partner laboratories  
88 (Table 1). Clinical samples from children presenting gastroenteritis during the winter season 2004-2005  
89 were obtained in Monterrey, Mexico, with the written consent of parent or guardian. Analysis of human  
90 clinical samples was approved by the Bioethics Committee of the Instituto de Biotecnologia. The initial  
91 screening of samples for RV-A was performed in Monterrey by silver staining of RV-A segmented  
92 double-stranded RNAs separated by SDS-PAGE electrophoresis. No previous screening for bacterial or  
93 parasitic agents was performed on group of samples. Triple layered particles of RV-A strain RRV were  
94 purified by cesium chloride density gradient as described previously [27].

95

### 96 **Microarray probe design**

97 All virus species that have been either associated with gastroenteritis or found in the  
98 gastrointestinal tract were identified by an extensive review of published literature, and selected to be

99 included in microarray. All available full-length genomes or complete gene sequences of the selected  
100 virus species were obtained from GenBank (up to February 2009) and the proper databases were  
101 created. For each virus species, sequence redundancy was eliminated according to a sequence similarity  
102 with cutoff values 95-99% using CD-HIT software [28]. One sequence for each species was selected as  
103 source for probes production and was processed as described [29]. Specifically, sequences were  
104 consecutively split into 70-mers with a shifting window of 3 nucleotides, with each 70-mer  
105 corresponding a potential probe. The 70-mer length probes have sufficient size to allow for stringent  
106 hybridization conditions while allowing for certain degree of mismatches, but are small enough to  
107 maintain species specificity [30,31,32]. Target probes were selected to be included in the microarray by  
108 analysis of BLAST results and calculation of hybridization thermodynamics ( $\Delta G$ ) calculated by the  
109 nearest-neighbor method [33]. To be considered good candidates for microarray, the  $\Delta G$  was required  
110 to be at least -70 kcal/mol for homologous sequence and higher than -40 kcal/mol for heterologous  
111 sequences. A minimum of 6 non-overlapping probes from conserved regions in virus genomes were  
112 selected for each virus, and each available genome sequence in target database for given species was  
113 recognized by at least two probes. When necessary, due to variability within a species, two or more  
114 source sequences were chosen and each single sequence was processed as described above.

115

116 Microarray probes *in-silico* analysis

117 Hybridization thermodynamics of RV-A selected probes was evaluated *in-silico* with VP1, VP2 and  
118 NSP5 segments of RV-A strains representing all full-genome G and P genotypes available.

119 Hybridization  $\Delta G$  (kcal/mol) between probe and target was calculated by nearest-neighbor method.

120 Best probe-target  $\Delta G$  was plotted in heatmap using R. Detection of a target is considered with  $\Delta G < -$   
121 50 kcal/mol.

122

123 **Microarray production**

124 Selected 70-mer probes were synthesized by Illumina Oligator (Illumina Inc, CA, USA).  
125 Oligonucleotides were resuspended to 400 pmol in 3X SSC buffer (0.45 M NaCl, 45 mM sodium  
126 citrate pH 7.0), and spotted onto epoxide-coated glass slides in the Microarray Facility of the Prostate  
127 Centre at Vancouver General Hospital, Vancouver, British Columbia, Canada. Each spot contained one  
128 specific probe to detect one virus species, and 4 pmol of spike70 (a 70-mer without biological  
129 complementary known sequence) [34] used to precisely identify probe spot locations on the  
130 microarray. Slides were maintained in humidity-free chamber until their use.

131

132 **Nucleic acid extraction, amplification and labeling**

133 Genetic material from virus lysates (cell culture supernatants from reference strains) was  
134 extracted by PureLink Viral RNA/DNA kit according to the manufacturer's instructions (Invitrogen,  
135 USA). For clinical samples, Norwalk and Sapporo virus positive controls, 100 µg of stool was added to  
136 conical screw cap tubes containing 100 mg of 150-212 µm glass beads (Sigma, USA), chloroform (100  
137 µl) and PBS up to 1ml. Samples were homogenized in a bead beater (Biospec Products, USA). After 10  
138 min centrifugation at 2000 x g supernatants were recovered and filtered in Spin-X 22 µm pore filters  
139 (Costar, NY) at 5000 x g during 10-20 min. Filtered samples were treated with Turbo DNase  
140 (Ambion, USA) and RNase (Sigma, USA) for 30 min at 37°C and immediately chilled on ice. Nucleic  
141 acids were then extracted from 200 µl using PureLink Viral RNA/DNA kit according to manufacturer's  
142 instructions (Invitrogen, USA). Nucleic acids from virus lysates or clinical samples were eluted in  
143 nuclease-free water, aliquoted, quantified in NanoDrop ND-1000 (NanoDrop Technologies, DE) and  
144 stored at -70°C until further use.

145 Sample processing and random-amplification of nucleic acids was performed essentially as  
146 described previously [21,35,36]. Briefly, reverse transcription was done using SuperScript III Reverse

147 Transcriptase (Invitrogen, USA) and primer-A (5'-GTTTCCCAGTAGGTCTCN<sub>9</sub>-3'). The  
148 complementary DNA (cDNA) strand was generated by two rounds of synthesis with Sequenase 2.0  
149 (USB, USA). The obtained cDNA was then amplified with KlenTaq polymerase (Sigma, USA) or Taq  
150 Polymerase (New England Biolabs, USA) using the primer-B (5'-GTTTCCCAGTAGGTCTC-3') by 30  
151 cycles of the following program: 30 sec at 94°C, 1 min at 50°C, 1 min at 72°C. As a last step,  
152 nucleotide-analogue aminoallyl-dUTP (TriLink, USA) in a 7:3 ratio with dTTP was incorporated  
153 during an additional 20 cycles of PCR using the same conditions described above and 5 µl of product  
154 from the previous PCR as starting material. The amplified products were purified with DNA Clean &  
155 Concentrator-5 kit (Zymo Research, USA). Coupling reactions of sample DNA with Cy3 and probe-70  
156 (70-mer complementary to spike-70) with Cy5 dyes (GE HealthCare, USA) were done as described  
157 elsewhere [31]. Fluorophore-labeled DNA was purified by the Zymo DNA Clean & Concentrator-5 kit  
158 and label incorporation was quantified by NanoDrop.

159

### 160 **Slides preparation, hybridization and scanning**

161 Microarray slides were treated just before their use with an ethanolamine wash solution (50 mM  
162 ethanolamine, 0.1 % SDS, 0.1M Tris pH 9) for 15 min at 50°C, followed by two washes in distilled  
163 water, and they were then dried by centrifugation for 5 min at 500 rpm. Processed slides were loaded  
164 with 30 µl of a combination of Cy3 and Cy5 labeled DNA in 3xSSC buffer, and the hybridization was  
165 left to proceed in a sealed chamber submerged in a water bath at 65°C for 8-12 h. After incubation the  
166 slides were washed consecutively in the following solutions: 2xSSC (65°C), 2xSSC, 1xSSC, 0.2xSSC  
167 and dried for 5 min at 500 rpm. Hybridization images were acquired with an Axon GenePix 4000B  
168 scanner (Molecular Devices, USA) synchronized with GenePix Pro 6.0 software to detect and measure  
169 spot intensities.

170



## 171 **Data Analysis**

172 Hybridization spot intensities were first filtered by the following spot-quality control  
173 parameters: spot size and shape (denoted as good/bad/absent), channel 532 foreground (F532) signal  
174 saturation (% F532 saturated < 5), and F532 signal proportion over channel 532 background (B532)  
175 signal [(% > B532 + 2 standard deviations) > 50%]. Spots showing good quality were used to generate  
176 microarray level background values. Normalization of intensity values was done with the formula:  
177  $(F532i/F532m)-(B532i-B532m)$  where F532i and B532i stands for foreground and background signal  
178 of spot “i” respectively, and F532m and B532m stands for the sum of all foreground or background  
179 spots respectively.

180 The statistical significance of probe intensities in the reference samples was obtained by the  
181 rank products algorithm [37] using a minimum of three technical replicates. Rank values from negative  
182 control samples were recorded and used to generate a 'spot rank value' included in subsequent spot-  
183 quality analysis. For clinical samples, z-score transformed intensities and their p-values were analyzed  
184 with fdr tool package [38] in R [39]. Positive virus species were defined as having at least two probes  
185 with p-values < 0.05 and a false discovery rate < 0.01.

186

187

## 188 **Limits of detection assays**

189 In order to determine the amount of virus particles detectable by the microarray, three reference  
190 viruses with different genome types were assayed: dsRNA RV-A, positive single stranded RNA  
191 (ssRNA+) HAstV and double stranded DNA (dsDNA) HadV-C. RNA was extracted from purified RV-  
192 A strain RRV, and MA104 cells. The RV-A genome molecular mass was calculated according to the  
193 following formula: [genome length (bp) x 325] /  $6.022 \times 10^{23}$  [40]. Decreasing dilutions of RV-A RNA  
194 corresponding from  $1 \times 10^8$  to 10 particles; were analyzed alone or mixed with an excess of MA104 cells  
195 RNA (50 ng). Similarly, decreasing dilutions of focus forming units titrated cell lysates of HAstV or

196 HAdV-C, corresponding from  $1 \times 10^7$  to 100 virus particles were extracted, amplified, labeled, and  
197 processed using the full microarray protocol as described above.

198

### 199 **Conventional diagnostic or confirmatory RT-PCR**

200 Nucleic acids extracted from clinical samples were used to perform diagnostic RT-PCR using  
201 Qiagen's One-Step RT-PCR kit (Qiagen, USA) or Super Script III One-step RT-PCR with Platinum  
202 Taq (Invitrogen, USA). For confirmatory RT-PCR, cDNA was generated with SuperScript III Reverse  
203 Transcriptase (Invitrogen, USA), and Taq Polymerase (New England Biolabs) was used for PCR  
204 reactions following the manufacturer's instructions. Oligonucleotide primers used in diagnostic or  
205 confirmatory RT-PCR are listed in Supplementary Table 1. PCR reactions for RV-A detection  
206 included a 5 min boiling followed by immediate ice-chilling step just before RT-PCR. Amplification  
207 conditions for RV-A, HAdV and calicivirus (CV) were: 30 min at 50°C, 15 min at 95°C; 40 cycles of  
208 30 sec at 95°C, 30 sec at 50°C, 1 min at 72°C, with final extension of 5 min at 72°C. RT-PCR  
209 conditions for human adenovirus (HAdV) were: 30 min at 50°C, 15 min at 95°C; 40 cycles of 30 sec at  
210 95°C, 30 sec at 55°C, 1 min at 72°C, with final extension for 5 min at 72°C. Human enterovirus (HEV)  
211 amplification program was: 30 min at 50°C, 15 min at 95°C; 40 cycles of 30 sec at 95°C, 30 sec at  
212 50°C, 30 sec at 72°C, with final extension for 5 min at 72°C. *Human parechovirus* (HPeV)  
213 amplification was: 30 min at 50°C, 15 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 48°C, 1 min at  
214 72°C, with final extension for 5 min at 72°C. *Anellovirus* (TTV) confirmation was performed as semi-  
215 nested PCR, conditions for the first round were: 2 min at 94°C; 35 cycles of 30 sec at 94°C, 30 sec at  
216 55°C, 30 sec at 72 °C, with final extension for 5 min at 72°C. The second round used the same program  
217 but only with 30 cycles. *Human bocavirus* (HBoV) was detected by Seeplex RV15 OneStep ACE  
218 Detection (Seegene, USA). PCR products were visualized in 2.0% agarose gels except for HEV, which  
219 required 3.5% gels due to small amplicon size.

220

## 221 **Semi quantitative RT-PCR/PCR detection of viruses**

222 One-step real-time RT-PCR and real-time PCR were performed using primers targeting  
223 conserved genomic regions (Supplementary Table 1). RV-A detection required previous sample boiling  
224 for 5 minutes and immediate ice-chill. For the RNA viruses (RV-A, HAstV, NV and HEV), detection  
225 was performed as a two-step process. First, 3 µl of RNA (5 ng) were reverse transcribed with 0.125 µl  
226 (50 U/µl)\_SuperScript III Reverse Transcriptase (Invitrogen, USA), 0.25 µl of RNase inhibitor (20  
227 U/µl), 12.5 µl of SYBR Green Master Mix 2X (Applied Biosystems, USA), 1 µl of the primer and  
228 DEPC treated water in a 24 µl final volume. Samples were incubated for 30 min at 48°C followed by  
229 enzyme inactivation for 10 min at 90 °C. In the second step 1 µl of second primer was added and PCR  
230 conditions were carried out as follows: HAstV and RV-A amplification program consisted of 10 min at  
231 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. NV amplification program was 5 min at  
232 95°C, 45 cycles of 10 sec at 95°C, 20 sec at 48°C, 45 sec at 60°C. HEV program was 10 min at 95°C,  
233 45 cycles of 20 sec at 95°C, 20 sec at 55°C, 1 min at 72°C and final extension of 5 min at 72°C. In the  
234 case of HEV, both specific primers were added before PCR, since the RT step was performed using  
235 random hexamers. HAstV amplification reactions consisted in 3 µl (5 ng) of DNA, 12.5 µl of SYBR  
236 Green Master Mix 2X and 1µl of each corresponding primer in a 25 µl volume reaction. Conditions  
237 were 95°C for 8 min, 45 cycles of 30 sec at 95°C, 20 sec at 55°C, 20 sec at 72°C, final extension of 5  
238 min at 72°C. Amplifications were carried out in an ABI Prism 7500 Sequence Detector System  
239 (Applied Biosystems). Dissociation curves were evaluated for non-specific products. Ct values  
240 corresponding to detection of specific virus sequences were obtained from triplicates of selected  
241 samples presenting co-infections and compared for the viruses detected. PCR primer sets for detection  
242 of CV, HAstV and HEV were designed to recognize the target at the genus level [[5,6,41](#)].

243

## 244 **RESULTS**

## 245 **Selection of viruses related to gastrointestinal infections**

246           An advantage of the microarray technology is the capacity to test hundreds, and even thousands  
247 of targets in a single assay. The main goal of this study was to develop an assay for detection of all  
248 viruses that have been found in stool samples from vertebrate, associated or not with gastroenteritis,  
249 what should facilitate clinical and epidemiological studies in humans and animals. A deep search of the  
250 scientific literature available in public databases resulted in a list of 128 viral species of viruses  
251 reported to be present in the gastrointestinal tract, representing 55 genera that belonged to 17 viral  
252 families (Supplementary Table 2). The list of virus species includes the well known human  
253 gastroenteritis viruses (calicivirus group, rotaviruses, human astroviruses, enteric adenoviruses),  
254 together with some recently described human viruses [*human adenovirus G* [23], *human bocavirus*  
255 [42], *cosavirus* [24], *Saffold virus* [43] and *salivirus A* [25,44]]. Classical, non-human gastrointestinal  
256 viruses (coronavirus, circovirus, and pestiviruses), and other new discovered viral agents (at the time of  
257 the microarray design) from different animal species, such as animal anelloviruses [45,46], bat  
258 astroviruses [47], and bovine kobuviruses [48], whose participation as pathogens is not well  
259 understood, are also included in the microarray. Thus, the virus species of interest comprehended a  
260 variety of viruses with different characteristics, such as RNA and DNA genomes, enveloped/non-  
261 enveloped virions, segmented or non-segmented genomes, single- or double-stranded genomes. All  
262 available complete gene or genome sequences were retrieved from a public database (GenBank) and  
263 were organized in a taxonomic hierarchical database following the ICTV classification at the date the  
264 microarray probes were design (ICTV, 2009) or, for novel species, as suggested by the discoverer.

265

## 266 **Probe selection and microarray validation**

267           A set of 1,256 70-mer microarray probes were selected from conserved regions and designed to  
268 identify 128 viral species associated with the GI tract, with at least 6 probes designed for each viral  
269 species and at least 2 probes corresponding to each sequenced viral genomes. To maintain stringent

270 experimental conditions (hybridization at 65°C) while allowing certain number of sequence variability,  
271 the probes were designed as 70-mers. The highest number of probes covered RV-A (42 probes),  
272 *alphacoronavirus* (28 probes), and mammalian *orthoreovirus* (25 probes) (Supplementary Tables 2 and  
273 3). For some viruses, the design of a complete set of 6 oligonucleotides was not possible due to the lack  
274 of enough complete sequences, nevertheless available probes were included for each viral species.

275 Reference strains for 10 viral species were available for probe validation. These species  
276 represent 6 viral families and include 4 main human pathogens (HAstV, NV, SV, RV-A), other human  
277 viruses (mammalian *orthoreovirus*, HAdV-C, *dengue virus 4*), and three non-human viruses (*feline*  
278 *calicivirus*, *bovine viral diarrhea virus 1*, and *bovine parainfluenza virus 3*) (Table 1). All reference  
279 strains tested were detected as expected, including four different RV-A strains (human strain Wa,  
280 simian strain RRV, porcine virus TFR-41, and bovine strain UK) and two different mammalian  
281 *orthoreovirus* strains (T1L and T3D) (Table 1). To test *in-silico* capacity of probes to recognize  
282 different and variable strains, 42 probes specific for rotavirus were analyzed with panel of all available  
283 G and P genotypes (Supplementary Figure 1). The only genotype that the microarray would not  
284 probably detect was G22P[35] belonging to a turkey rotavirus strain.

285

### 286 **Sensitivity and specificity of the assay**

287 To determine the sensitivity limits of the DNA microarray, the virus genetic material was  
288 extracted from lysates of HAstV- or HAdV-C-infected cells or from CsCl-purified simian strain RRV  
289 particles. In a series of cell lysate dilutions (corresponding from  $10^2$  to  $10^7$  viral particles), the  
290 microarray was able to detect as low as  $10^3$  HAdV-C or HAstV virus particles. Similarly, RV-A RNA  
291 (corresponding from 10 to  $10^8$  viral particles) was amplified before or after addition of a constant  
292 amount of cellular RNA (50 ng). In the absence of cellular RNA the detection limit of viral RNA was  
293  $10^3$  genome copies, however, when the complexity of the sample was augmented by adding cellular  
294 RNA, the detection limit was one logarithm lower, detecting  $10^4$  genome copies.

295 To evaluate the probe specificity, a rank products algorithm [37] was applied to the results  
296 obtained from technical replicates of reference viruses and mock-infected cell controls (MA104 cells,  
297 A549 cells, and C6/36 cells). Based on the false discovery rate test (FDR) included in the software, 16  
298 probes were identified as presenting nonspecific behavior (marked with \* in Supplementary Table 3).  
299 When analyzed, these nonspecific probes did not show any common feature, although some presented  
300 high GC content (>70%). In the following experiments the results obtained with these probes were  
301 excluded from analysis.

302

### 303 **Analysis of clinical samples**

304 To further test the capacity of the microarray to detect viruses, 76 samples from children under  
305 5 years of age, collected during the winter season 2004-2005 in Monterrey, Mexico, was analyzed. The  
306 collection of samples was originally screened for RV-A by polyacrylamide gel electrophoresis (SDS-  
307 PAGE), and stored at -70°C. Using the microarray developed in this study, a viral agent was detected  
308 in 70 out of 76 (92 %) samples tested; a single virus was found in 63 (83%) samples, while two or more  
309 viral species were detected in 7 (9%) samples (Fig. 1). Among the viruses detected, the most common  
310 was RV-A (44 samples), followed by TTV (12 positives), HEV (7), caliciviruses (6 positives – 5 NV  
311 and 1 SV), HAstV (5), HAdV (4 positive samples- 3 HAdV-F and 1 HAdV-A), HPeV (2) and one  
312 HBoV (Fig. 1). It is important to mention that only 6 (8%) samples remained negative after microarray  
313 detection, and that not all viruses found are known to be pathogenic. As mentioned above, after  
314 collection all samples were screened for the presence of RV-A by SDS-PAGE. Additionally, as  
315 described below, all samples tested with the microarray were tested for selected viruses by diagnostic  
316 RT-PCR, including RV-A. In thirty-four samples RV-A was identified by the three methods tested; 5  
317 additional samples were found positive by microarray and RT-PCR tests (Fig. 2). Another 8 were  
318 found positive either by microarray (n=5) or by RT-PCR (n=3) (Fig 2). Notably, the 3 samples that  
319 were only positive for RV-A by RT-PCR were found in mixed-infection samples.

320 To compare the results of the microarray with those of a routine diagnostic method for viral  
321 gastroenteritis, RT-PCR detection for a panel of 5 viruses [RV-A, HAstV, HAdV, CV (NV and SV)  
322 and HEV] was performed in all clinical samples. It is important to point out that the primer sets for  
323 HAdV, CV, and HEV are designed to recognize its target at the genus level [5,6,41].

324 The RT-PCR panel detected at least one virus in 59 samples (78%; Fig. 1B), a lower detection  
325 rate when compared to the DNA microarray when analyzing only these 5 viruses (n= 65, 85%). At the  
326 individual virus level, the RT-PCR panel confirmed the microarray results in all HAdV-positive  
327 samples (1 HAdV-A and 3 HAdV-F), having a positive predictive value (PPV) of 100%, all CV (5 NV  
328 and 1 SV) (PPV, 100%) , and 39 of 44 RV-A positive samples (PPV, 89%), while PPVs were lower for  
329 HAstV, with 3 of 5 positive samples identified (PPV 60%), and 5 of 7 HEV positive samples  
330 identified by microarray (PPV 71%) (Fig. 3).

331

### 332 **Detection of viruses in mixed infections**

333 The RT-PCR screening resulted in the identification of 16 mixed infections (MI), while the  
334 microarray identified only 7 MI (Fig. 1). The microarray detected up to 4 different viruses within one  
335 sample, with TTV found in all MI samples. The following viral combinations were found by  
336 microarray: HEV B/TTV (3 samples); and one sample of each NV/TTV; HEV-B/HAstV/TTV; RV-  
337 A/HPeV/TTV; and SV/HEV-B/HPeV/TTV (Fig. 1). Of interest, *human parechovirus* and *Sapporo*  
338 *virus* were detected only in co-infection. The MI combinations observed in RT-PCR were: RV-  
339 A/HAdV (8), RV-A/HEV (5), HAstV/HEV (1), RV-A/CV (1), and one triple infection  
340 HAdV/CV/HEV (Fig. 1B). Examining these 16 samples, we observed that RV-A was the only virus  
341 identified by microarray in all samples with RV-A/HAdV co-infection (n= 8), and in 4 out of 5 RV-  
342 A/HEV samples, while HAstV was the only virus identified in sample with HAstV/HEV co-infection  
343 (Table 2). In one sample NV was identified as the sole species by microarray, while RT-PCR results  
344 showed CV/HAdV/HEV triple co-infection (Table 2). Thus, in all of these 16 samples a single virus

345 was identified by the microarray, while at least two viral species were detected by RT-PCR.

346 One possible explanation for the discrepancies in the identification of mixed infections using  
347 microarrays and RT-PCR could be the variability in the relative amount of each virus genetic material  
348 in clinical samples, as it has been observed that individuals infected with some viruses, for example  
349 RV-A and NV, can shed large amount of viral particles in the acute stage of infection [49,50,51]. To  
350 explore this possibility, the amount of viral genetic material in selected samples with mixed infection  
351 was quantified by real-time RT-PCR. The use of equal quantities of starting material allowed us to  
352 compare directly the amplification Ct's of two viruses within a sample. The results showed that the  
353 single virus detected by microarray had, in most cases, a lower Ct value as compared to the second  
354 virus detected by qRT-PCR, with the only exception being the combination of RV-A/HEV, where RV-  
355 A was the only virus identified by microarray despite the fact that HEV had lower Ct values (Table 2).  
356 This indicates that MIs presenting large differences in the amount of the genetic material of the viral  
357 agents involved are prone to result in single virus detection by the microarray, generally, the one  
358 present more abundantly.

359 As consequence, when comparing the sensitivity and specificity of he microarray, as compared  
360 with the panel of individual diagnostic RT-PCR, the most prevalent or most frequently found viruses in  
361 single infections, such as RV-A, HAstV, and CV, showed good sensitivity and specificity (from 85-  
362 100%), while the sensitivity for viruses like HAdV and HEV was low, ranging from 30 to 42%, being  
363 clearly affected by other viruses present in the sample (Fig. 3, Table 2). For example, 4 samples that  
364 presented only HAdV were found positive by both microarray and RT-PCR, while in the remaining 9  
365 samples, which presented HAdV co-infection with RV-A (8 samples) and one with CV, only the  
366 second virus was identified by microarray (Table 2). It should be pointed out that most of these  
367 samples contained a low level of HAdV genetic material, with Ct values close to non-template control  
368 value (Ct 44.5) (Table 2).

369



370 **Detection of not common GI viruses**

371 Of note, the microarray found 3 viruses that usually are not evaluated in gastroenteritis samples.  
372 Two samples presented HPeV, both in co-infection (one with RV-A/ TTV, another with SV/ HEV B/  
373 TTV). An additional sample containing HBoV was identified (RV-A was identified by RT-PCR in this  
374 sample), and 12 samples presented TTV, 5 samples as single infection and 7 in co-infection with other  
375 viruses. As reference samples for these viruses were not available, confirmation RT-PCR coupled with  
376 capillary sequencing were performed, and the viruses detected by the microarray were confirmed in all  
377 these samples (results not shown). Fact that TTV single virus positive samples were found is not an  
378 indicator of causativeness.

379

380 **DISCUSSION**

381 Current routine viral testing is designed to detect only the most prevalent viruses, leaving  
382 frequently a 30-50% of cases without an agent identified [52]. In recent years, advances in molecular  
383 biology and the implementation of next generation sequencing has allowed the identification of several  
384 new viruses in intestinal samples [53,54,55,56,57]. The role of most of these viruses in diarrheal  
385 disease remains unclear (*Aichi virus, anellovirus, human bocavirus, human parechovirus, human*  
386 *picobirnavirus*, and some enteroviruses, among others) raising the need to study in detail their  
387 epidemiology. In order to gather information on GI virus diversity, proper tools are required for their  
388 monitoring. In this work a comprehensive and sensitive DNA microarray was developed and tested,  
389 which allows in principle the parallel detection of more than 100 gastrointestinal associated virus  
390 species.

391 Implementation of the microarray for detection of viruses is not an easy task. Design of probes  
392 and experimental conditions are two important parameters to consider. Resequencing microarrays  
393 permit identification of mutations but require high numbers of probes for a single agent, increasing its  
394 cost [58]. Arrays for sub-typing use less and shorter probes but are often designed for only one viral

395 species [59,60,61,62,63]. Microarrays used for virus discovery have proven very useful when usual  
396 suspects are discarded or in rare disease cases, but identification is not clear and require complex  
397 analysis [34].

398 Several DNA microarrays have been previously reported for identification of the main known  
399 gastrointestinal pathogenic viruses [59,60,61,62,63,64,65,66,67,68,69,70,71,72,73,74,75], however,  
400 they were mostly oriented to the identification or sub-typification of one viral species, and none had  
401 specifically addressed the list of viruses that can be found in stool samples.

402 The microarray platform described in this work has been validated with 14 reference viral  
403 strains, representing 10 different virus species. Importantly, 5 other viral species were identified using  
404 the microarray when analyzing clinical samples: HAdV-F, HAdV-A, HPeV , HBoV, and several TTV.  
405 The capacity of the microarray to identify correctly viruses whose probes were not validated in this  
406 work with cultured reference strains confirms that the methodology used to design probes is adequate,  
407 and increases the probability that the remaining probes will be also capable to identify their target  
408 viruses, this is additionally supported by *in-silico* detection of wide variety of RV-A strains using  
409 probes obtained from conserved genes; however, testing with other reference strains would be  
410 necessary. During the validation experiments some probes were found to react non-specifically with the  
411 amplified labeled DNA, regardless of its origin, in other words, they were found to be ‘sticky’, and  
412 they were excluded from further analysis. No common characteristic was found between these probes  
413 that could account for their non-specific binding behavior.

414 One of the critical parameters in virus detection is the sensitivity of the assay. There are several  
415 factors that can affect the sensitivity. In the case of a microarray, sample nucleic acids are generally  
416 processed by random primed amplification prior to hybridization, to ensure amplification of a wide  
417 variety of viruses. The product of random-PCR could be lower than that of specific PCR, decreasing  
418 the sensitivity of the assay as all genetic material is amplified, diluting the positive signal [76]. Limit of  
419 detection for three viruses with different genome types (dsDNA, dsRNA and ssRNA+) was established

420 in  $10^3$  virus particles, suggesting that genome nature does not affect the sensitivity of the assay.  
421 Moreover, testing the sensitivity of the microarray with purified RV-A RNA, we observed that addition  
422 of 50 ng of cellular RNA as a non-specific diluting RNA, decreased the sensitivity of detection ten-  
423 fold. To try to solve the sensitivity problem in complex clinical samples, agent-specific primers have  
424 been included in previous reports, together with random primers during amplification of the genetic  
425 material [14,15], with the disadvantage of narrowing the scope of targets for the microarray assay.

426 We subsequently analyzed a group of clinical samples collected from children with diarrhea.  
427 Initially, the clinical samples were screened by SDS-PAGE, which led to the identification of 34 RV-A  
428 positive samples, while the microarray presented in this work identified 44, suggesting that the  
429 microarray platform has a higher sensitivity than traditional methods. A similar sensitivity was  
430 obtained by RT-PCR, as 42 samples were found RV-A-positive. Even though our results indicate that  
431 the limit of detection of purified virus ( $1 \times 10^3$  viral particles) is similar to that reached with PCR assays  
432 [8], the microarray had a higher number of positive results when clinical samples were tested, possibly  
433 due to the natural genetic variation in primer binding regions of viruses found in sample viruses.

434 Although multiplexed assays are being developed, their use in routine testing is not generally  
435 implemented, and most studies use single pathogen tests. When RT-PCR screening for the most  
436 common viruses is performed, the percentage of clinical samples without a virus identified remains  
437 around 30-50% [13,77,78], while the microarray presented in this study detected a virus in 92% of the  
438 samples. This high detection rate could have been influenced by the time of sampling, since winter is a  
439 high season for viral gastroenteritis in the region and no pre selection for pathogens was performed. An  
440 additional advantage of the microarray test as compared to a set of different RT-PCR assays, is the  
441 capacity to identify viruses that are not commonly tested for, like those previously associated with  
442 diarrhea (like HPeV) and those of unclear clinical significance in GI disease (HBoV and TTV). In this  
443 work we found a wide range of circulating not typical viruses among children, similarly as observed in  
444 other studies [79,80] and their continuous surveillance should be considered. To our knowledge, this is

445 first report of description of HPeV, HBoV, and TTV in Mexican children.

446 As a consequence of the limited number of virus species routinely tested, prevalence of co-  
447 infections is a poorly explored issue. Usually, when a panel of up to 5 viruses is used, co-infection rates  
448 between 4-18% are observed, being the most common combination RV-A/NV [2,13,77,81,82,83].  
449 More recently, wide scope ranging metagenomic studies have shown that mixed infections are more  
450 common than previously thought [4,80], even in healthy individuals [79]. The analysis of the small set  
451 of clinical samples analyzed in this work showed that 30% (23 out of 76) contained more than 1  
452 gastrointestinal virus. The identification of individual viruses in co-infections presented some  
453 discrepancies when comparing the results from microarray and RT-PCR tests. Of seven samples with  
454 mixed infections identified by the microarray, five were confirmed by RT-PCR, while in 16 mixed  
455 infection identified by RT-PCR, a single virus was identified by the microarray, suggesting that the  
456 microarray may be less sensitive than RT-PCR for detection of mixed infections. To address this  
457 inconsistency, real time RT-PCR was implemented for the principal combinations of viruses that were  
458 missed by the microarray. This platform showed certain advantage for detection of RV-A over HAdV  
459 and HEV, as RV-A was identified even when the HEV genome was present in larger amounts. HEV  
460 was identified by microarray in samples co-infected with RV-A only when RV-A RNA was present in  
461 low amounts, close to negative control levels (Table 3). Preferential identification of RV-A by the  
462 microarray could be due to the large amount of virus particles excreted during the acute phase of  
463 infection, and by the large number of probes selected (42 oligonucleotides), as compared to 5 and 17  
464 probes for HEV A and HEV C, respectively, and 17 probes for HAdV. On the other hand, the two  
465 HEV samples positive for microarray that were missed by RT-PCR correspond to mixed infections,  
466 with HAdV/TTV and SV/HpeV/TTV, respectively. Despite several attempts to identify HEV in these  
467 samples by RT-PCR resulted in negative results, thus the possibility of a microarray false positive  
468 result cannot be discarded.

469 The number of virus species identified has increased considerably in the last decade with the

470 application of emerging genomic technologies such as microarrays and unbiased next-generation  
471 sequencing in studies of fatal or rare cases of disease in humans, wild, and domestic animals  
472 [25,56,84,85,86]. Adequate tools that allow detection of well-known pathogenic viruses while are  
473 capable of detecting the new or rare viruses in a single assay will contribute with useful  
474 epidemiological information about both kinds of viruses. This microarray includes viruses of different  
475 host origin in order to extend the range of use to veterinary studies. The oligonucleotide probes selected  
476 should allow the identification of target viruses despite the sequence variations that will occur in the  
477 following years, however, it will be important to update the microarray design on a regular basis to  
478 maintain the capacity to broadly detect pathogenic viruses and to include newly found viral species.

479         Parallel detection of gastroenterical viruses beyond the most common viruses should facilitate a  
480 better understanding of virus etiology, as it increases the rate of positive cases, closing the diagnostic  
481 gap, and allows inspection for mixed infections where secondary viral agents could represent an  
482 important factor. Adding data from case control studies and inclusion of other host parameters, as  
483 serological data, will help to provide evidence of virus pathogenicity. Furthermore, adequate and  
484 comprehensive epidemiological studies in wild and domestic animals should be considered.

485

## 486 **Acknowledgments**

487 The computational analysis was performed using the cluster of the Instituto de Biotecnología, UNAM,  
488 with the assistance of M.C. Jerome Verleyen. Authors thank Paul Gaytán Colín, Eugenio López-  
489 Bustos, and Santiago Becerra Ramírez from Unidad de Síntesis y Secuenciación de DNA, Instituto de  
490 Biotecnología, UNAM, for synthesis of the oligonucleotides used in RT-PCR assays. This work was  
491 supported by grant S0008-111593 from CONACYT. Miguel A. Martínez and María de los Dolores  
492 Soto-del Río were supported by a scholarship from CONACYT-Mexico.

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767 characterization of a novel adenovirus in the cloacal bursa of gulls. *Virology* 440: 84-88.

770 Figure 1. Prevalence of viruses in clinical samples. A group of 76 clinical samples from children  
771 presenting gastroenteritis was analyzed by the described microarray (A) or by diagnostic RT-PCR for 5  
772 most common gastrointestinal pathogens (B). Samples with co-infections are shown. NEG is for  
773 negative result (no virus identified).

775 Figure 2. Identification of rotavirus group A. A group of 76 gastroenteritis samples was analyzed by  
776 three methods for the presence of rotavirus. These included visualization of rotavirus dsRNA on SDS-  
777 PAGE gel, RT-PCR, and the microarray designed in this work. The circles represent numbers of  
778 rotavirus positive samples identified by one, two, or three methods used .

779

780 Figure 3. Microarray diagnostic sensitivity and specificity. A panel of 5 virus groups [rotavirus group  
781 A (RV-A), human astrovirus (HAstV), human adenovirus (HAdV), calicivirus (CV), and enterovirus  
782 (HEV)] was tested by RT-PCR in all 76 samples. Results were compared to those obtained by  
783 microarray analysis. The sensitivity, specificity, positive predictive value (PPV) and negative  
784 predictive value (NPV) of microarray (array), as compared to RT-PCR (PCR), for detection of  
785 particular pathogens are shown.

786

787 Supplementary Figure 1. *In-silico* hybridization of available RV-A G and P genotypes. Hybridization  
788 thermodynamic values ( $\Delta G$ ) were calculated for all 42 RV-A probes (labeled on the bottom) interacting  
789 “*in-silico*” with RV-A isolates representing different G and P genotype combinations. RV-A probes  
790 belong to VP1, VP2 and NSP5 segments (marked on top). G and P genotype combinations (marked on  
791 left) belong to RV-A strains isolated from different hosts (human, cow, dog, cat, simian, rhesus, pig,  
792 horse, goat, lamb, sheep, antelope, guanaco, mouse, rabbit, pigeon, chicken, turkey), marked on right,  
793 for whom complete genomes are available. From 27 G genotypes, complete genomes for 21 are  
794 available, while from 35 P genotypes only 23 are represented. Successful detection by RV-A probes is  
795 considered when  $\Delta G$  value is below -50 (shades of blue). RV-A reference strains (Wa, UK, RRV) used  
796 for validation are depicted as G1P[8]-human, G6P[5]-cow and G3P[3]-simian respectively.

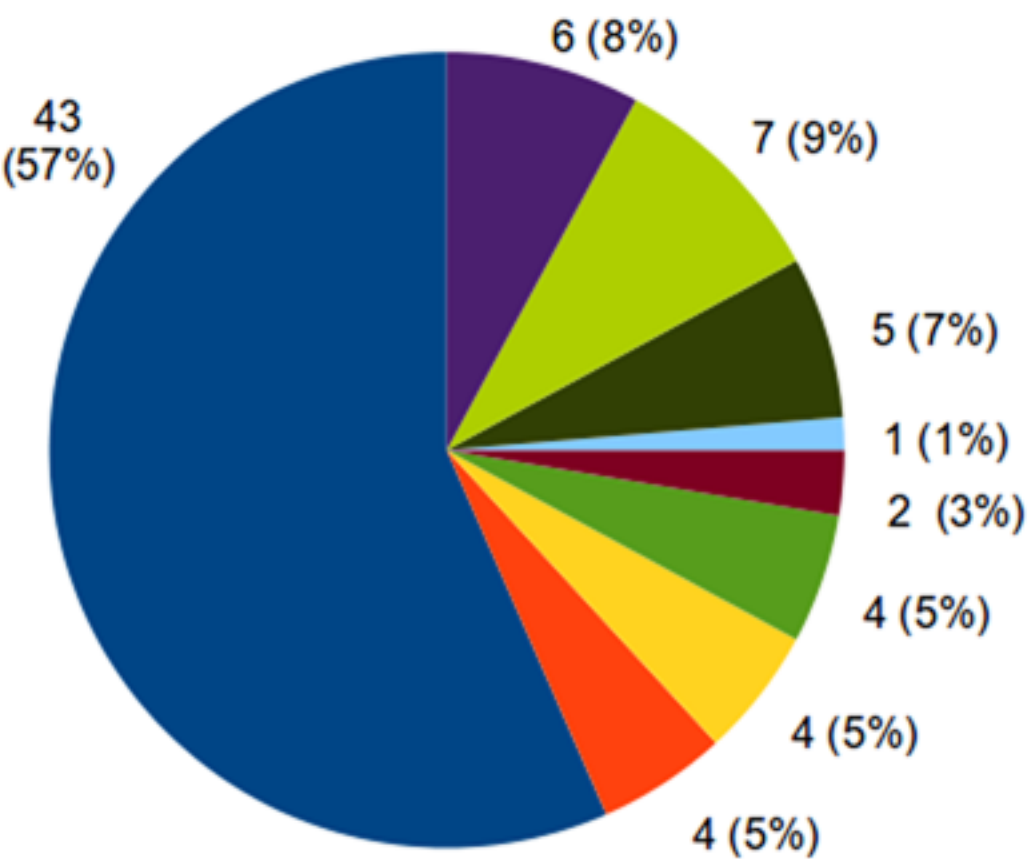
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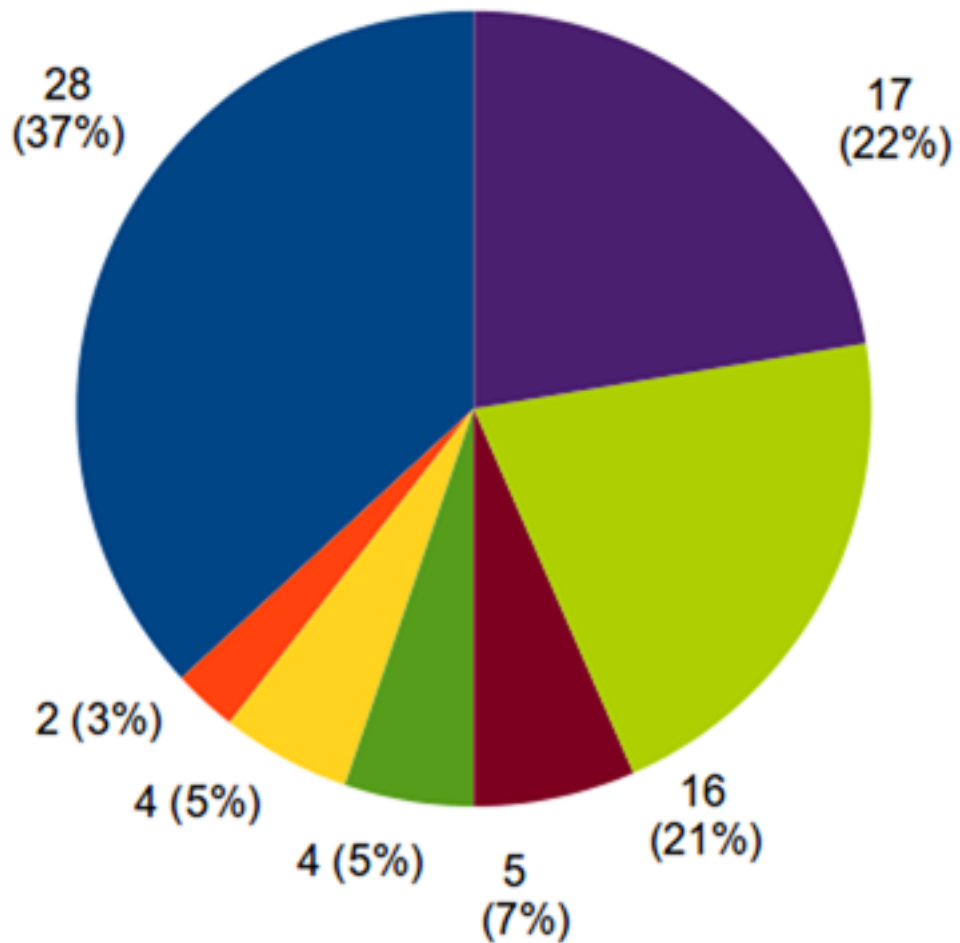
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Figure 1.

A) Microarray



B) RT-PCR panel



- Rotavirus-A
- Human astrovirus
- Human adenovirus
- Norwalk virus
- Human enterovirus
- Human bocavirus
- Anellovirus
- Co-infection
- Negative

Figure 2.

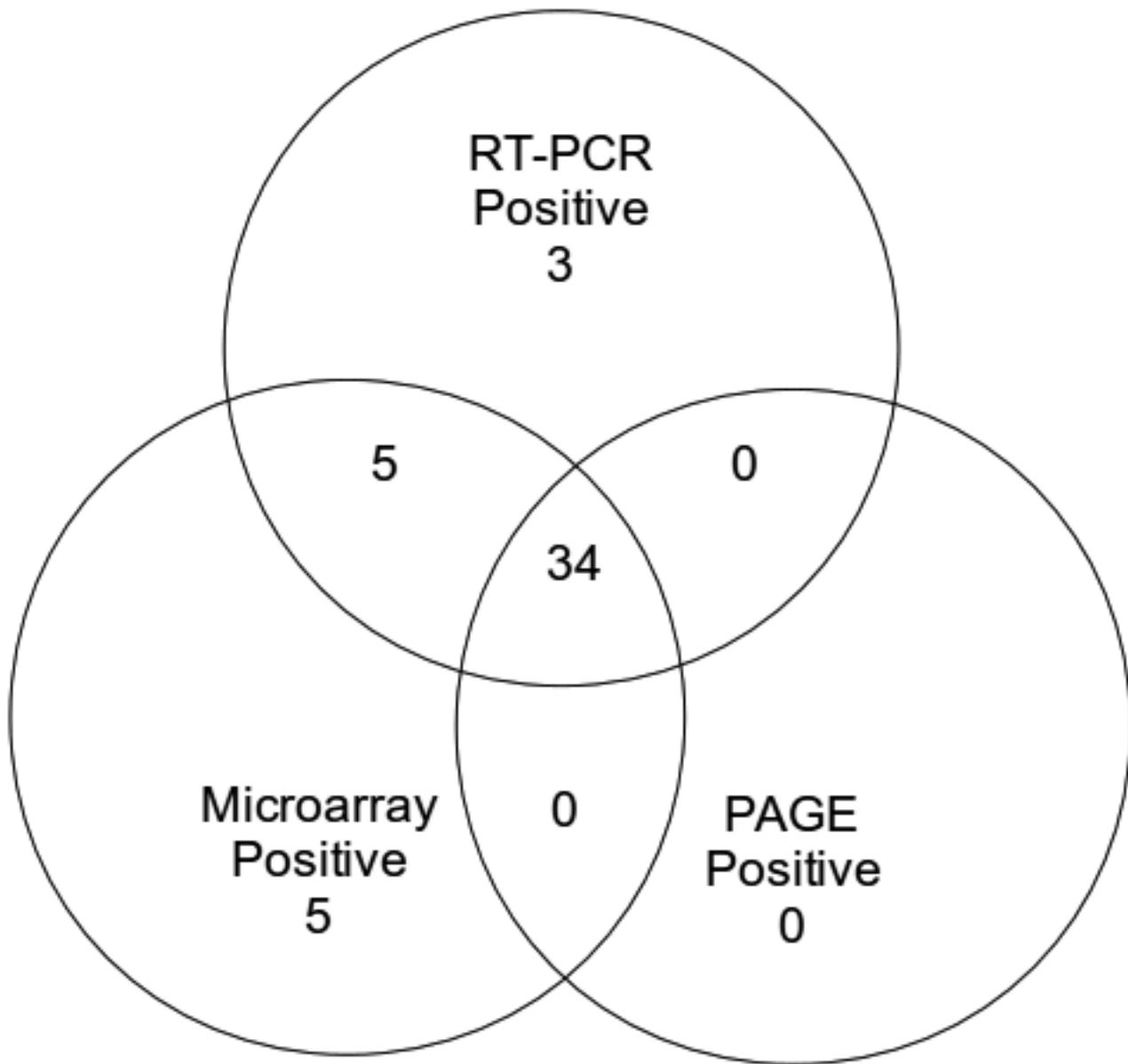


Figure 3.

<b>RV-A</b>				<b>HAdV</b>				<b>CV</b>				
	PCR+	PCR-		PCR+	PCR-		PCR+	PCR-		PCR+	PCR-	
array +	39	5	44	array +	4	0	4	array +	6	0	6	
array -	3	29	32	array -	9	63	72	array -	0	70	70	
	42	34	76		13	63	76		6	70	76	
Sensitivity 93%			Sensitivity 30%			Sensitivity 100%						
Specificity 85%			Specificity 100%			Specificity 100%						
PPV 88.6%			PPV 100%			PPV 100%						
NPV 90.6%			NPV 87.5%			NPV 100%						

<b>HAstV</b>				<b>HEV</b>					
	PCR+	PCR-		PCR+	PCR-		PCR+	PCR-	
array +	3	2	5	array +	5	2	7		
array -	0	71	71	array -	7	62	69		
	3	73	76		12	64	76		
Sensitivity 100%			Sensitivity 42%						
Specificity 97%			Specificity 97%						
PPV 60%			PPV 71.6%						
NPV 100%			NPV 89.9%						



Table 1. Reference virus species used in microarray validation

Family	Genus	Species	Strain <sup>a</sup>	probes positive/total <sup>b</sup>
Astroviridae	Mammastrovirus	<i>Human astrovirus</i>	Yuc8	4/4
Adenoviridae	Mastadenovirus	<i>Human adenovirus C</i>	Adv5	10/13
Caliciviridae	Vesivirus	<i>Feline Calicivirus</i>	F9	14/22
	Norovirus	<i>Norwalk virus</i> <sup>c</sup>	-	8/12
	Sapovirus	<i>Sapporo virus</i> <sup>c</sup>	-	5/14
Flaviviridae	Pestivirus	<i>Bovine Viral Diarrhea Virus 1</i>	NADL	6/6
	Flavivirus	<i>Dengue virus 4</i>	-	9/9
Paramyxoviridae	Respirovirus	<i>Bovine parainfluenza virus 3</i>	SF-4	9/9
Reoviridae	Rotavirus	<i>Rotavirus A</i>	RRV	22/42
			TFR-41	14/42
			UK	19/42
			Wa	21/42
			T1L	11/25
	Orthoreovirus	<i>Mammalian Orthoreovirus</i>	T3D	19/25

<sup>a</sup> Reference strains were provided by Dr. Ramon Gonzalez, FC-UAEM, (Human adenovirus C); Dra. Lorena Gutierrez, CINVESTAV-IPN, (Feline Calicivirus, Norwalk virus, Sapporo virus); Dra. Rosa E. Sarmiento, FMVZ-UNAM (Bovine Viral Diarrhea Virus 1, Bovine parainfluenza virus 3); Dra. Rosa Ma. Del Angel, CINVESTAV-IPN (Dengue virus 4); M.D. Terrence S. Dermody, Vanderbilt University School of Medicine (Mammalian orthoreovirus)

<sup>b</sup> number of oligonucleotide probes which recognized virus/ total number of oligonucleotide probes designed to bind viral species

<sup>c</sup> clinical reference samples



Table 2. Ct values of viral nucleic acid quantification in samples with co-infection

RT-PCR <sup>a</sup>	Microarray <sup>b</sup>	RV-A <sup>c</sup>	HAdV	HEV	NV <sup>d</sup>	HAstV
RV-A <sup>e</sup>	RV-A	21.9 <sup>f</sup>				
HAdV	HAdV		14.7			
HEV	HEV			23.8		
*NV	NV				19.6	
HAstV	HAstV					14.8
RV-A/HAdV	RV-A	<b>20.5<sup>g</sup></b>	37.6			
RV-A/HAdV	RV-A	<b>22.5</b>	28.4			
RV-A/HAdV	RV-A	<b>28.2</b>	41.3			
RV-A/HAdV	RV-A	<b>28.6</b>	44.5			
RV-A/HAdV	RV-A	<b>29.1</b>	43.8			
RV-A/HAdV	RV-A	<b>29.2</b>	43.4			
RV-A/HAdV	RV-A	<b>30.4</b>	30.6			
RV-A/HEV	RV-A	29.2		<b>25.4</b>		
RV-A/HEV	RV-A	29.2		<b>27.3</b>		
RV-A/HEV	RV-A	29.6		<b>28.5</b>		
RV-A/HEV	RV-A	30.8		<b>27.8</b>		
RV-A/HEV	HEV	38.4		<b>28</b>		
RV-A/NV	NV	30.1			<b>23.6</b>	
HAstV/HEV	HAstV			28		<b>23.2</b>
NV/HEV/HAdV	NV		34.1	28.4	<b>20.7</b>	
NTC <sup>h</sup>		36.4	44.5	33.7	33.7	29.9

<sup>a</sup> virus identified by diagnostic RT-PCR

<sup>b</sup> virus identified by microarray

<sup>c</sup> virus determined by real-time RT-PCR, RV-A (rotavirus group A), HAdV (human adenovirus), HEV (human enterovirus), NV (Norwalk virus), HAstV (human astrovirus)

<sup>d</sup> NV is detected at genus level as calicivirus

<sup>e</sup> Single infection samples were used as positive control

<sup>f</sup> Ct value detected by real-time RT-PCR

<sup>g</sup> lower Ct values are shown in bold

<sup>h</sup> NTC non template control