1 **Suboptimal performance of rotavirus testing in a vaccinated community population should prompt**
2 **laboratories to review their rotavirus testing algorithms in response to changes in disease**
3 **prevalence**

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ABSTRACT

Rotavirus vaccine has reduced disease prevalence in many countries. Consequently, we aimed to assess the reliability of a rotavirus immunoassay in the community population of Auckland and Northland, New Zealand.

Between 22 October 2015 and 31 December 2016, 2873 faecal samples were tested by enzyme immunoassay (EIA, Rotascreen II, Microgen, UK) from 2748 patients, (median age 8 years, range 0-101 years). Eighty-nine (3.1%) samples were reactive; 86 samples were tested by a second method. Rotavirus was confirmed in 49/86 (57%). Positive rotavirus EIAs were more likely to be confirmed in samples from cases ≥1 year of age (positive predictive value, PPV 61%, 95% CI 50-72%, $P = 0.049$), and in spring/summer (PPV 67%, 95% CI 55-78%, $P = 0.003$).

Reactive rotavirus tests required confirmatory testing regardless of demographic, vaccine or seasonal factors; a review of rotavirus testing algorithms may be necessary in other vaccinated community populations.

KEYWORDS

Rotavirus, community, diagnostic, test, false positive

HIGHLIGHTS

Rotavirus was detected in 3.1% of all samples in a community based setting

43% of reactive rotavirus tests were not confirmable

Seasonal mismatches occur in prevalence of disease and number of tests performed

Confirmation rates were highest in spring-summer and in children > 1 year of age

A review of rotavirus testing algorithms is necessary in community populations

45 INTRODUCTION

46 Rotavirus vaccine has dramatically altered the epidemiology of gastroenteritis worldwide. In
47 countries where the vaccine has been introduced, 89–100 % reductions in emergency department
48 visits, 74–90 % declines in hospitalisations for rotavirus gastroenteritis, and 29–50 % declines in 'all-
49 cause' acute gastroenteritis hospitalisations have been observed.[1] Indirect vaccine benefits have
50 extended to unvaccinated older children and adults.[2] In New Zealand (NZ), rotavirus vaccine
51 (Rotateq™, Merk & Co.) was instituted on the National Immunisation Schedule (NIS) from July 2014
52 as a 3-dose schedule for all infants at 6 weeks, 3 months and 5 months of age, and in common with
53 other countries, national rotavirus hospitalisations declined by 87% within two years of vaccine
54 introduction.[3] As a Southern Hemisphere nation, in NZ, prior to vaccine introduction, annual
55 rotavirus peak activity was June-October (winter-spring); cases uncommonly occurred in the summer
56 months. [4]

57 Following these changes in disease prevalence, the reliability of diagnostic testing for rotavirus has
58 been questioned, with reports that between 20-42% of rotavirus antigen tests cannot be confirmed
59 by molecular methods.[5-7] These findings suggest that a review of existing testing algorithms should
60 be performed by diagnostic laboratories in order to avoid errors, and that confirmatory testing
61 should be considered.[7-8] However, internationally guidelines differ in their recommendations around
62 the need for this.[9] Further studies, particularly evaluating which patient and season specific factors
63 influence the reliability of results may help inform testing protocols for clinicians and laboratories at
64 both a local and national level.[10] In New Zealand, laboratories employ a range of tests, such as
65 enzyme immunoassays (EIA), immunochromatography (ICT) and polymerase chain reaction (PCR) to
66 detect rotavirus antigen or nucleic acid. Prior to vaccine introduction, confirmatory testing was not
67 performed prior to reporting of results, and rotavirus infections are not notifiable to public health
68 authorities.

Labtests (LTA) and Northland Pathology laboratories (NPL) serve as the sole community laboratories for an urban and rural population of 1.6 million (60% NZ European, 23% Asian, 15% Pacific, and 11% Maori) people in northern New Zealand.[11] Prior to vaccine introduction, rotavirus testing was performed routinely on samples from children <3 years old, and otherwise on request, using Rotascreen II (Microgen, UK) enzyme immunoassay (EIA). Testing of samples submitted to both laboratories was performed at LTA. In response to vaccine introduction, from 2nd November 2015, samples from patients of all ages were only tested for rotavirus on request. From October 2015, as part of laboratory surveillance post-vaccine introduction, LTA referred faecal samples reactive for rotavirus for genotype analysis at the national public health laboratory (the Institute of Environmental and Scientific Research, ESR, New Zealand). From August 2016, due to high rates of initially reactive samples which were unable to be genotyped, samples testing positive for rotavirus were referred for confirmatory testing at Middlemore hospital laboratory, NZ (RIDA QUICK Rotavirus/Adenovirus Combi immunochromatography test, R-Biopharm, Germany), prior to reporting and referral for genotyping.

Given the issues with reproducibility of reactive rotavirus test results, and reports of unconfirmed results using other assays by laboratories in the region,[7] we undertook to retrospectively evaluate the reliability of our rotavirus immunoassay in our community based population over a fourteen month period following rotavirus vaccine introduction, and to determine the influence of patient demographics (age, gender, ethnicity), vaccine status and season. During this study period, regional vaccine coverage rates for infants aged up to 6 months were 72-83% and 90-95% by 12 months of age.[12]

METHODS

Study design

This was a retrospective observational study. The study period was 22 October 2015-31 December 2016 (14 months). Data for 2873 of the 2923 (98.3%) stool samples submitted to LTA/NPL for diagnostic purposes for rotavirus testing were included in this study. 50 samples were excluded from analysis as a National Health Index (NHI, a unique identifier assigned to NZ residents on contact with health services) was not available. The NHI was necessary to enable linkage to demographic data and vaccine status.

Initial laboratory testing

Stools were tested at LTA according to existing laboratory algorithms. All samples from Northland were referred by NPL for testing at LTA. Rotavirus testing at LTA was performed using Rotascreen II (Microgen, UK) EIA on neat stool samples submitted to the NPL and LTA with a specific request for rotavirus testing from 2 November 2015. Prior to this, all faecal samples submitted to the laboratories from those aged <3 years were automatically tested for rotavirus at LTA.

Confirmatory laboratory testing

In the period 22 October 2015-22 August 2016 (10 months) aliquots of 62/62 faecal samples testing positive by rotavirus EIA at LTA were refrigerated, batched and sent weekly, at ambient temperature to the Institute of Environmental Science and Research (ESR, New Zealand) for confirmatory rotavirus reverse transcription real-time quantitative PCR (RT-qPCR) as previously described.[13] Rotavirus G and P genotypes were determined on all RT-qPCR positives using type-specific VP7 and VP4 nested PCR assays, or by sequence analysis of partial VP7 and VP4 regions. [14-18] Sequence analysis of partial VP6 region was used for the identification the bovine backbone component of RotaTeq™.[19] For the sequence analysis, Bionumerics software (v7.6 Applied Maths, Sint-Martens-Latem, Belgium) was used for sequence alignment, and the identification determined using the

117 RotaC v2.0 typing tool (http://rotac.regatools.be), and/or BLAST

118 (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

119 From 23 August 2016 to 31 December 2016 (4 months), aliquots of 27/27 faecal samples positive by

120 rotavirus EIA were sent, that day, at ambient temperature to Middlemore Hospital laboratory, NZ,

121 and tested immediately by a rotavirus ICT (RIDA QUICK Rotavirus/Adenovirus Combi, Germany).

122 Samples that tested negative with the ICT assay were reported as rotavirus negative. Samples that

123 tested positive using both the EIA and ICT assays were then sent to ESR for RT-qPCR and genotyping

124 where applicable. Samples that were EIA positive but not confirmed by either RT-qPCR or ICT were

125 considered false-positives for the purposes of this study.

126 *Data*

127 Rotavirus test data (including Middlemore confirmatory test results) were extracted from the

128 information systems of LTA/NPL. Prior to 23 August 2016, samples testing positive at LTA were

129 considered true positives for the purposes of reporting in the laboratory information system. RT-

130 qPCR and Genotyping results were provided retrospectively by ESR.

131 Immunisation and ethnicity data were obtained via accessing the National Immunisation Register

132 (NIR). Study months were allocated to southern hemisphere seasons: September-November (spring),

133 December-February (summer), March-May (autumn), June-August (winter).

134 *Statistical analysis*

135 Categorical variables were compared using either χ^2 or Fisher Exact where appropriate. The Mann

136 Whitney test was used for comparing medians. Positive predictive values (PPV) for initially reactive

137 rotavirus tests were determined by vaccine status, demographic and seasonal variables.

138 *Ethics*

139 Ethical approval was sought but deemed unnecessary for this study (HDEC reference 17STH133).
140 Institutional level approval was obtained from University of Auckland Ethics Committee (reference
141 019915) for database access.

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143 RESULTS

144 Over the 14-month study period, 2873 faecal samples from 2748 patients were tested at LTA using
145 Rotascreen II (Microgen, UK) EIA. Over half (1534, 56%) of samples were from male patients. The
146 median age of patients tested was 8 years old (range 0-101 years). The majority of samples
147 (1571/2873, 55%) were from patients over 5 years of age (Table 1), and 45% (1292/2873) were from
148 patients over 15 years of age.

149 An average of 192 EIAs were performed per month (range 110-262 samples, standard deviation (SD)
150 35) over the study period. A mismatch in test volumes and test positivity was seen, with peak testing
151 (average 237 samples/month) occurring at times of lowest rotavirus activity (true positive
152 prevalence 4/946, 0.42%) in late-summer/autumn (February-May 2016) and low testing volumes
153 (average 183 samples/month) in spring (October-November 2015, September-November 2016)
154 corresponding with highest prevalence of true positives (true positive prevalence 25/915, 2.7%,
155 Figure 1). Most, (64/89, 72%) reactive tests were seen in spring-summer (September-February).
156 There was an additional peak of reactive results in May 2016, but 6/7 of these results were not
157 confirmed by RT-qPCR.

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167 **Table 1.** Patient demographics, initial rotavirus reactivity and confirmatory results

Demographic	Samples tested (%)	Completed 3 doses vaccine (%)	Positive (%)	Confirmed (%)	PPV (95% ci)+
All	2873 (100)	785 (27)	89* (3.1)	49 (1.7)	57 (46-67)
Age group (yrs)					
< 1	468 (16)	357 (76)	11 (2.4)	3 (0.6)	27 (10-56)
1-2	468 (16)	385 (82)	26 (5.6)	17 (3.6)	65 (46-81)
2-4	366 (13)	42 (11)	22 (6)	12 (3.3)	55 (35-73)
>5	1571 (54)	1 (<0.1)	30 (1.9)	17 (1)	57 (39-73)
Season					
Autumn	703 (24)	-	13 (1.8)	2 (0.3)	15 (4-42)
Spring	904 (31)	-	38 (4.2)	25 (2.7)	66 (50-79)
Summer	727 (25)	-	25 (3.4)	17 (2.3)	68 (48-83)
Winter	539 (19)	-	10 (1.9)	5 (0.9)	50 (24-76)
Vaccine status					
Vaccinated	785 (27)	-	30 (3.8)	16 (4.1)	54 (36-70)
Unvaccinated	2088 (73)	-	56 (2.7)	33 (1.6)	59 (46-71)
Ethnicity					
Asian	815 (28)	-	30 (3.7)	16 (2.0)	57 (39-73)
Maori/Pacific	402 (14)	-	10 (2.5)	4 (1.0)	44 (19-73)
New Zealand European	1277 (44)	-	38 (3.0)	22 (1.7)	58 (42-72)
Other	348 (12)	-	10 (2.9)	7 (2.0)	70 (40-89)
Unknown	31 (1)	-	1 (3.2)	0 (0)	0 (0-80)

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169 **Footnotes**

170 *In total 3 EIA reactive samples were not available for testing by a second assay (from three
171 children: vaccinated Asian one year old submitted in winter; vaccinated Asian 2 year old submitted
172 in winter; unvaccinated Maori/Pacific 2 year old submitted in spring). These are included in the total
173 positive samples but are excluded from PPV calculations.

174 +, Positive predictive value (95% confidence interval).

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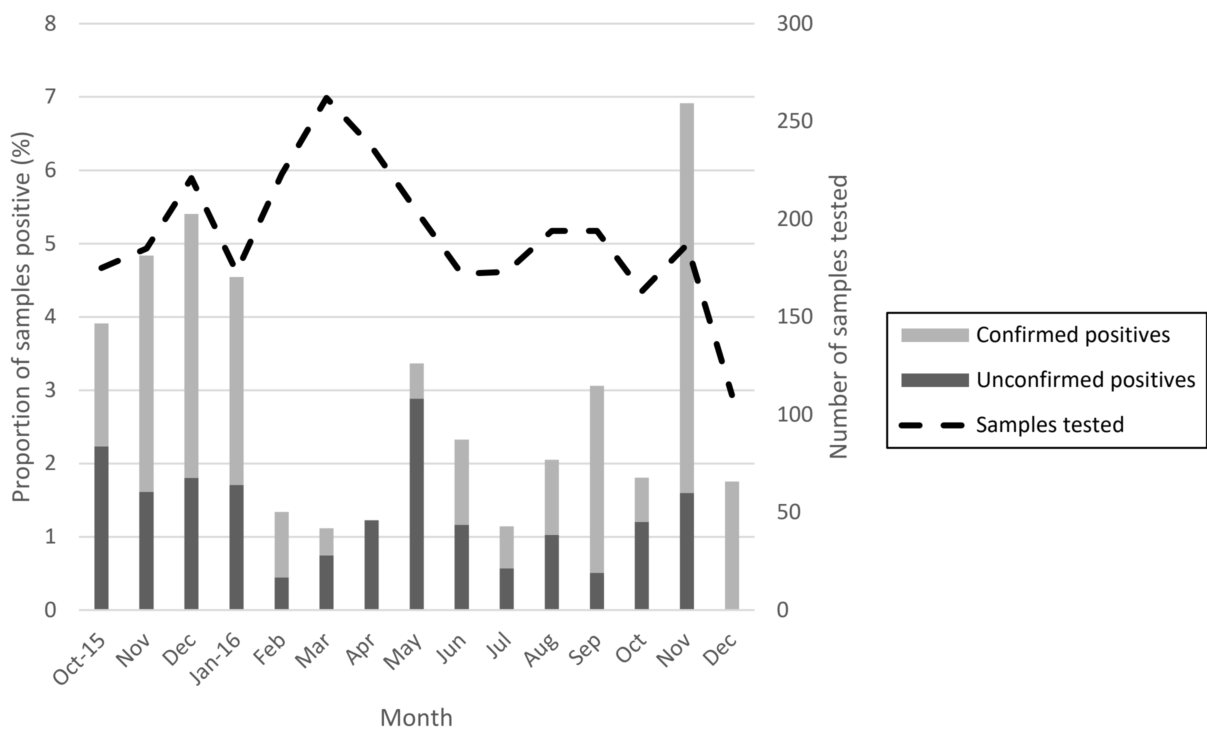
181 **Figure 1.** Confirmed and unconfirmed rotavirus positive rates by study month.



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183 **Footnotes**

184 Proportion of samples with confirmed (Rotascreen II positive; ICT/RT-qPCR positive) and

185 unconfirmed positives (Rotascreen II positive; ICT/RT-qPCR negative) per month. For comparison,

186 the number of samples tested by Rotascreen II EIA per month are included on the secondary axis.

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194 Eighty-nine of 2873 samples (3.1%) were reactive by EIA. None were duplicates from the same
195 patient. Rotavirus RT-qPCR was performed on 59 of these samples, of which rotavirus was detected
196 in 29 (49.1%). Twenty-seven samples had ICT performed as the second test; of these rotavirus
197 antigen was detected in 20 (74.1%) samples; subsequent RT-qPCR on 15 of these samples confirmed
198 the presence of rotavirus; five were not tested as insufficient sample remained. Three (3.4%) of the
199 89 reactive samples were not tested by a second method due to insufficient sample.

200 Overall, 49/86 (57%) of initially reactive tests by EIA were positive on subsequent testing using either
201 RT-qPCR or ICT, resulting in a true positivity rate of 1.7%, and an unconfirmed positivity rate of 1.3%.
202 The estimated specificity of the EIA was 98.7% (95% confidence interval 98.2-99.0%) with a positive
203 predictive value (PPV) of 57% (95% CI 46-67%) over the study period.

204 The G and P genotype was determined for 44/49 of the rotavirus-confirmed positives. The genotypes
205 identified were G12P[8], n=14 (29%); G9P[8], n=10 (20%); G2P[4], n=10 (20%); G3P[8], n=4, (8%);
206 G8P[8], n=2 (4%); G3P[9], n=2, (4%); G1P[8] n=1 (2%). One (2%) was G1P[8] with a RotaTeq™ vaccine
207 component identified. Further details of the patients from whom these genotypes were detected
208 are given below.

209 In our cohort of patients, a quarter (785, 27%) had received three doses of RotaTeq™ vaccine prior
210 to the time a sample was submitted for rotavirus testing. This proportion differed by age group

211 (Table 1). Of the 89 patients with reactive EIAs: 32 (36%) had completed 3 doses of vaccine, a
212 median of 350 days (range 18-781) prior to testing.

213 The genotypes from those patients who had received three doses of vaccine were: G9P[8] n=5,
214 G12P[8] n=4, G2P[4] n=3, G12P[8] vaccine component n=1, G3P[8] n=1, one sample was insufficient
215 for genotyping.

216 One hundred and four samples were submitted from patients who had received a dose of rotavirus
217 vaccine in the previous 28 days. Two were positive by EIA; one of these, submitted 14 days after the
218 patient's first vaccine dose was not confirmed by PCR, and one, submitted 21 days after the patient's
219 third dose of vaccine was confirmed as vaccine strain by sequencing.

220 The median age of patients with positive rotavirus EIAs was 2 years old (range 0-92 years) and did
221 not differ between confirmed and unconfirmed groups.

222 When the initial rotavirus results using EIA were considered, positivity rates were lowest in those ≥5
223 years of age (1.9%), however when only confirmed results (through ICT or RT-qPCR) were
224 considered, rates were lowest in those <1 year of age (0.6%) (Table 1). Positive rotavirus EIAs were
225 more likely to be confirmed in those ≥1 year of age (61%, 95% CI 50-72%) compared with those <1
226 year (27%, 10-56%, $P = 0.049$).

227 RT-qPCR/ICT rotavirus-confirmed positivity rates varied by season (Table 1) and were higher in
228 spring/summer (2.5%) than autumn/winter (0.6%, $P < 0.001$) whereas unconfirmed positives did not
229 vary by season (1.3% versus 1.3%) (Figure 1). Consequently, PPV of rotavirus EIA was higher in
230 spring/summer (PPV 67%, 95% CI 55-78%) than autumn/winter (PPV 30%, 95% CI 17-55%, $P = 0.003$)

231 Tests performed in the unvaccinated group were more likely to be confirmed (PPV 59%, 95% CI 46-
232 71%) than those in the vaccinated group (PPV 53%, 95% CI 36-70%) but this finding was not
233 statistically significant. Tests performed in New Zealand European (PPV 58%, 95% CI 42-72%) and

234 Asian individuals (44%, 95% CI 19-73%) were more likely to be confirmed than from those of
235 Maori/Pacific ethnicity (44%, 95% CI 42-72%) but this finding was not statistically significant.

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237 DISCUSSION

238 We found that 43% (37/86) of initially reactive rotavirus tests performed over a 14-month period in
239 our community-based population could not be confirmed by a second assay (either ICT or RT-qPCR).
240 Our findings are similar to reports elsewhere following vaccine introduction which have reported
241 that up to 50% of samples positive by EIA or ICT are not confirmable.[5-7]

242 We believe that this low positive predictive value is multifactorial in our community population, with
243 the falling prevalence of rotavirus playing a major role in our findings. Historically, annual rotavirus
244 positivity rates for our laboratories have been 7-18%; however, the vaccine introduced in 2014 has
245 led to a substantial reduction in rotavirus prevalence both regionally[7] and nationally.[3] Consequently,
246 the 1.7% true positivity rate during the study period (more than 90% reduction compared with our
247 pre-vaccine annual prevalence) approached the specificity limits of our assay. However, this finding
248 is not attributable to poor test performance, as the calculated specificity of the Rotascreen II EIA
249 (Microgen, UK) over this period was relatively high (98.7%), and within the reported parameters of
250 this and other rotavirus EIAs and ICTs used worldwide.[20-22]

251 Another important factor is the seasonal mismatch in testing and prevalence of rotavirus. Rotavirus
252 activity follows seasonal patterns, and in NZ, prior to the introduction of vaccine, annual peak
253 activity was June-October (winter-spring); cases uncommonly occurred in the summer months.[4] In
254 contrast, we found that confirmation rates for initially reactive tests were highest in spring and
255 summer, consistent with shifting seasonality toward later rotavirus seasons as experienced in other
256 countries following vaccine introduction.[23] We also saw that, in contrast to the seasonality of
257 rotavirus activity, testing was performed year-round, with highest test numbers performed in late

258 summer/autumn, when prevalence of true positives was the lowest. The higher testing rates in late-
259 summer and autumn of 2016 are notable; they do not correspond to increased requesting for, nor
260 prevalence of bacterial or parasitic entero-pathogens, but may represent increased community
261 prevalence of other viral entero-pathogens for which we do not test. Year round testing despite low
262 prevalence of disease accounts for the low PPV of tests performed in autumn-winter and is likely to
263 be due to tests performed on individuals with other causes of infectious and non-infectious
264 diarrhoea.[24,25]

265 We found that positive predictive value also differed according to the age of the individual tested,
266 with reactive results less likely to be confirmed in infants, a group who had recently received vaccine
267 compared with older individuals, who had either not received vaccine or where vaccine had been
268 administered more remotely with diminishing effect.[26] Unfortunately, the small number of reactive
269 rotavirus tests in our population over this period limited our ability to analyse other factors which
270 may have influenced the reliability of initially reactive tests. Nevertheless, though we found that age
271 and season did influence the reliability of results, taking into account: vaccine status, demographics
272 (age, gender, ethnicity), and season, did not enable selection of samples where initially reactive
273 results would have >70% positive predictive value.

274 We did not perform evaluations of the sensitivity of the assays used over the study period so it is
275 possible that the known suboptimal sensitivity of ICT compared with RT-qPCR may have impacted on
276 confirmation rates where ICT was used as the second assay, particularly where viral loads were
277 low.[20] However, generally, patients symptomatic with rotavirus have higher viral loads which are
278 detectable by ICT,[22] and it is reassuring that confirmation rates were higher during the period that
279 ICT was used as the second assay.

280 Local assessments of existing testing algorithms should be performed by diagnostic laboratories, in
281 order to assess the reliability of results, which may be dependent upon the initial assay used, and
282 the prevalence of disease in the population; but given that many commercial EIAs and ICTs have

283 similar performance with comparable reported specificities to Rotascreen II,[20,21] our findings are
284 likely to be generalizable for community populations with high levels of vaccine coverage,
285 particularly in years of low rotavirus activity.

286 Hence, we do not believe that use of a different EIA or ICT as the initial test would have altered our
287 findings. Our results also indicate that laboratory surveillance for rotavirus using community samples
288 will underestimate true vaccine effectiveness, particularly in infants.

289 There are several options available to attempt to address the poor positive predictive value of
290 testing. Foremost amongst these are changes in laboratory protocols to reduce unnecessary testing
291 (such as performing testing only where rotavirus requested) which may minimise these pre-
292 analytical errors,[27] as well as saving cost and time at the diagnostic laboratory. For example, 2012-
293 2014, when rotavirus testing was performed for all submitted faeces samples for those < 3 years old,
294 an average of 8200 samples were tested per year. This reduced to 2300 samples in 2016, mostly due
295 only performing testing on request, with similar declines noted due to changes in laboratory practice
296 predating introduction of vaccine at hospital laboratories in the Auckland region.[7] Though changes
297 to algorithms such as these may ameliorate inappropriate requests, as we, and others have
298 demonstrated, they will not entirely prevent them, and education for the clinicians to assist with
299 appropriate use of diagnostic tools is also warranted.[7]

300 Some laboratories may wish to consider performing confirmatory testing, prior to reporting results
301 by the use of another EIA, ICT, or PCR, as LTA did during the study period. However, this requires
302 additional expense, labour, and may confer logistical challenges if performed at a second site.

303 Another option is to reconsider the role of rotavirus testing in community laboratories. Subsequent
304 to our study, LTA has ceased testing for rotavirus in the community population, and now refers
305 public health requests for outbreak investigations and those from hospital specialists to hospital
306 laboratories for testing. This may not be a suitable option for all laboratories, particularly those that
307 are involved in epidemiological surveillance activities.

Other considerations for community laboratories reconsidering their approach to diagnostic rotavirus testing include: rotavirus is no longer the most common cause of childhood gastroenteritis,[28] children are often co-infected with multiple viruses,[25] rotavirus vaccine may be detected for variable periods following vaccination,[22] and due to rotavirus vaccine implementation there are shortages in the availability of diagnostic tests. Though highly sensitive RT-qPCR/PCR assays,[29] allowing detection of multiple enteric viruses are increasingly available, and represent an alternative to EIAs and ICTs for rotavirus detection, their advantages should be weighed against their potential to detect clinically insignificant virus following vaccine, or remote infection.[22,30] We would caution against their widespread use in unselected community patients for these reasons.

In conclusion: following the introduction of rotavirus vaccine, we have found high rates of false positive reactive rotavirus results in our community based population. We would encourage other laboratories to review their rotavirus testing algorithms in response to changes in disease prevalence.

CONFLICT OF INTEREST

Funding: JH received funding from the New Zealand Ministry of Health for RT-qPCR and genotyping

Competing interests: None declared

Ethical approval: Not required

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