



Virology

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

Gary N. McAuliffe^{a,*}, Susan L. Taylor^b, Sharon Moore^a, Joanne Hewitt^c, Arlo Upton^a, Anna S. Howe^d, Emma J. Best^e

^a Microbiology Department, Labtests and Northland Pathology Laboratories, Auckland, New Zealand

^b Microbiology Department, Middlemore Hospital, Auckland, New Zealand

^c Enteric, Environmental and Food Virology Laboratory, Institute of Environmental Science and Research Limited, Porirua, New Zealand

^d Department of General Practice and Primary Health Care, School of Population Health, University of Auckland, Auckland, New Zealand

^e Department of Paediatrics, Child and Youth Health, University of Auckland, Auckland, New Zealand



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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 fecal 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% confidence interval [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.

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1. Introduction

Rotavirus vaccine has dramatically altered the epidemiology of gastroenteritis worldwide. In countries where the vaccine has been introduced, 89–100% reductions in emergency department visits, 74–90% declines in hospitalizations for rotavirus gastroenteritis, and 29–50% declines in “all-cause” acute gastroenteritis hospitalizations have been observed (Kollaritsch et al., 2015). Indirect vaccine benefits have extended to unvaccinated older children and adults (Mast et al., 2015). In New Zealand (NZ), rotavirus vaccine (Rotateq™, Merk & Co.) was instituted on the National Immunisation Schedule (NIS) from July 2014 as a 3-dose schedule for all infants at 6 weeks, 3 months, and 5 months of age, and in common with other countries, national rotavirus hospitalizations declined by 87% within 2 years of vaccine introduction (Kelly et al., 2017). As a Southern Hemisphere nation, in NZ, prior to vaccine introduction, annual rotavirus peak activity was June–October (winter–spring); cases uncommonly occurred in the summer months (Grimwood et al., 2006).

Following these changes in disease prevalence, the reliability of diagnostic testing for rotavirus has been questioned, with reports that between 20 and 42% of rotavirus antigen tests cannot be confirmed by molecular methods (McAuliffe et al., 2018; Roczo-Farkas et al., 2015; Ye et al., 2013). These findings suggest that a review of existing testing algorithms should be performed by diagnostic laboratories in order to avoid errors and that confirmatory testing should be considered (Australian Government Department of Health; McAuliffe et al., 2018). However, international guidelines differ in their recommendations around the need for this (Payne and Parashar). Further studies, particularly evaluating which patient- and season-specific factors influence the reliability of results, may help inform testing protocols for clinicians and laboratories at both a local and national level (Lopez-Lacort et al., 2016). In New Zealand, laboratories employ a range of tests, such as enzyme immunoassays (EIAs), immunochromatography (ICT), and polymerase chain reaction (PCR) to detect rotavirus antigen or nucleic acid. Prior to vaccine introduction, confirmatory testing was not performed prior to reporting of results, and rotavirus infections are not notifiable to public health 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)

* Corresponding author. Tel.: +6495747200.

E-mail address: Gary.McAuliffe@labtests.co.nz (G.N. McAuliffe).

people in northern NZ (Statistics New Zealand, 2013). 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) 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 postvaccine introduction, LTA referred fecal 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 (McAuliffe et al., 2018), we undertook to retrospectively evaluate the reliability of our rotavirus immunoassay in our community-based population over a 14-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 (New Zealand Ministry of Health).

2. Methods

2.1. 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. Fifty 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.

2.2. 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 fecal samples submitted to the laboratories from those aged <3 years were automatically tested for rotavirus at LTA.

2.3. Confirmatory laboratory testing

In the period 22 October 2015–22 August 2016 (10 months), aliquots of 62/62 fecal samples testing positive by rotavirus EIA at LTA were refrigerated, batched, and sent weekly at ambient temperature to the ESR (New Zealand) for confirmatory rotavirus reverse transcription real-time quantitative PCR (RT-qPCR) as previously described (Pang et al., 2004). 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 (Banerjee et al., 2007; Gentsch et al., 1992; Gouvea et al., 1990; Iturriza-Gomara et al., 2004; Simmonds et al., 2008). Sequence analysis of partial VP6 region was used for the identification of the bovine backbone component of RotaTeq™ (Donato et al., 2012). For the sequence analysis, Bionumerics software (v7.6 Applied Maths, Sint-Martens-Latem, Belgium) was used for sequence alignment, and the identification was determined using

the RotaC v2.0 typing tool (<http://rotac.regatools.be>) and/or BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

From 23 August 2016 to 31 December 2016 (4 months), aliquots of 27/27 fecal samples positive by rotavirus EIA were sent, that day, at ambient temperature to Middlemore Hospital laboratory, NZ, and tested immediately by a rotavirus ICT (RIDA QUICK Rotavirus/Adenovirus Combi, Germany). Samples that tested negative with the ICT assay were reported as rotavirus negative. Samples that tested positive using both the EIA and ICT assays were then sent to ESR for RT-qPCR and genotyping where applicable. Samples that were EIA positive but not confirmed by either RT-qPCR or ICT were considered false positives for the purposes of this study.

2.4. Data

Rotavirus test data (including Middlemore confirmatory test results) were extracted from the information systems of LTA/NPL. Prior to 23 August 2016, samples testing positive at LTA were considered true positives for the purposes of reporting in the laboratory information system. RT-qPCR and genotyping results were provided retrospectively by ESR.

Immunization and ethnicity data were obtained via accessing the National Immunisation Register. Study months were allocated to southern hemisphere seasons: September–November (spring), December–February (summer), March–May (autumn), and June–August (winter).

2.5. Statistical analysis

Categorical variables were compared using either χ^2 or Fisher Exact where appropriate. The Mann–Whitney test was used for comparing medians. Positive predictive values (PPVs) for initially reactive rotavirus tests were determined by vaccine status and demographic and seasonal variables.

2.6. Ethics

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

3. Results

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

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

Eighty-nine of 2873 samples (3.1%) were reactive by EIA. None were duplicates from the same patient. Rotavirus RT-qPCR was performed on 59 of these samples, of which rotavirus was detected in 29 (49.1%). Twenty-seven samples had ICT performed as the second test; of these, rotavirus antigen was detected in 20 (74.1%) samples; subsequent RT-

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 ^a (3.1)	49 (1.7)	57 (46–67)
Age group (years)					
<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)

^a In total, 3 EIA reactive samples were not available for testing by a second assay (from 3 children: vaccinated Asian 1-year-old submitted in winter, vaccinated Asian 2-year-old submitted in winter, and unvaccinated Maori/Pacific 2-year-old submitted in spring). These are included in the total positive samples but are excluded from PPV calculations.

qPCR on 15 of these samples confirmed the presence of rotavirus; 5 were not tested as insufficient sample remained. Three (3.4%) of the 89 reactive samples were not tested by a second method due to insufficient sample.

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

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

In our cohort of patients, a quarter (785, 27%) had received 3 doses of RotaTeq™ vaccine prior to the time a sample was submitted for rotavirus testing. This proportion differed by age group (Table 1). Of the 89

patients with reactive EIAs, 32 (36%) had completed 3 doses of vaccine a median of 350 days (range 18–781) prior to testing.

The genotypes from those patients who had received 3 doses of vaccine were G9P [8], $n = 5$; G12P [8], $n = 4$; G2P [4], $n = 3$; G12P [8] vaccine component, $n = 1$; and G3P [8], $n = 1$. One sample was insufficient for genotyping.

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

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

When the initial rotavirus results using EIA were considered, positivity rates were lowest in those ≥ 5 years of age (1.9%); however, when only confirmed results (through ICT or RT-qPCR) were considered, rates were lowest in those <1 year of age (0.6%) (Table 1). Positive

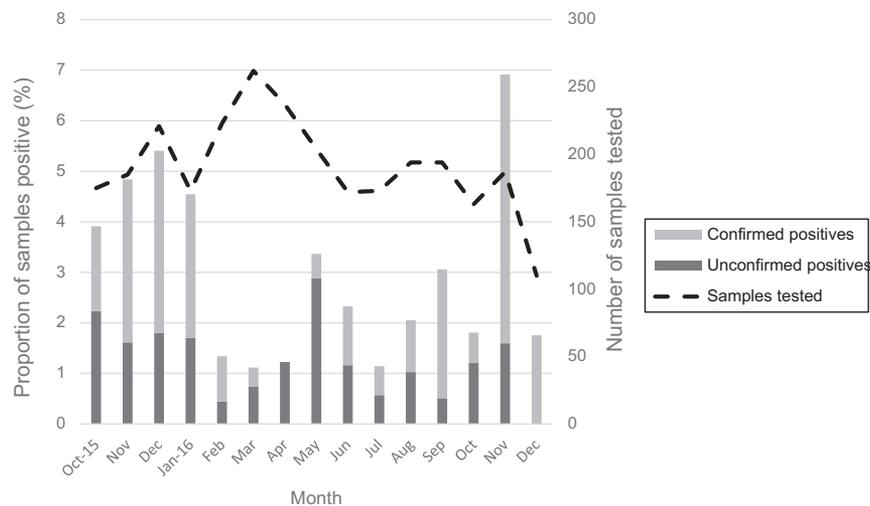


Fig. 1. Confirmed and unconfirmed rotavirus positive rates by study month. Proportion of samples with confirmed (Rotascreen II positive; ICT/RT-qPCR positive) and unconfirmed positives (Rotascreen II positive; ICT/RT-qPCR negative) per month. For comparison, the number of samples tested by Rotascreen II EIA per month is included on the secondary axis.

rotavirus EIAs were more likely to be confirmed in those ≥ 1 year of age (61%, 95% CI 50–72%) compared with those < 1 year (27%, 10–56%, $P = 0.049$).

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

Tests performed in the unvaccinated group were more likely to be confirmed (PPV 59%, 95% CI 46–71%) than those in the vaccinated group (PPV 53%, 95% CI 36–70%), but this finding was not statistically significant. Tests performed in New Zealand European (PPV 58%, 95% CI 42–72%) and Asian individuals (44%, 95% CI 19–73%) were more likely to be confirmed than from those of Maori/Pacific ethnicity (44%, 95% CI 42–72%), but this finding was not statistically significant.

4. Discussion

We found that 43% (37/86) of initially reactive rotavirus tests performed over a 14-month period in our community-based population could not be confirmed by a second assay (either ICT or RT-qPCR). Our findings are similar to reports elsewhere following vaccine introduction which have reported that up to 50% of samples positive by EIA or ICT are not confirmable (McAuliffe et al., 2018; Roczo-Farkas et al., 2015; Ye et al., 2013).

We believe that this low PPV is multifactorial in our community population, with the falling prevalence of rotavirus playing a major role in our findings. Historically, annual rotavirus positivity rates for our laboratories have been 7–18%; however, the vaccine introduced in 2014 has led to a substantial reduction in rotavirus prevalence both regionally (McAuliffe et al., 2018) and nationally (Kelly et al., 2017). Consequently, the 1.7% true positivity rate during the study period (more than 90% reduction compared with our prevaccine annual prevalence) approached the specificity limits of our assay. However, this finding is not attributable to poor test performance, as the calculated specificity of the Rotascreen II EIA (Microgen, UK) over this period was relatively high (98.7%) and within the reported parameters of this and other rotavirus EIAs and ICTs used worldwide (Kaplon et al., 2015; Tate et al., 2013; Ye et al., 2015).

Another important factor is the seasonal mismatch in testing and prevalence of rotavirus. Rotavirus activity follows seasonal patterns, and in NZ, prior to the introduction of vaccine, annual peak activity was June–October (winter–spring); cases uncommonly occurred in the summer months (Grimwood et al., 2006). In contrast, we found that confirmation rates for initially reactive tests were highest in spring and summer, consistent with shifting seasonality toward later rotavirus seasons as experienced in other countries following vaccine introduction (Aliabadi et al., 2015). We also saw that, in contrast to the seasonality of rotavirus activity, testing was performed year-round, with highest test numbers performed in late summer/autumn, when prevalence of true positives was the lowest. The higher testing rates in late-summer and autumn of 2016 are notable; they do not correspond to increased requesting for nor prevalence of bacterial or parasitic enteropathogens but may represent increased community prevalence of other viral enteropathogens for which we do not test. Year-round testing despite low prevalence of disease accounts for the low PPV of tests performed in autumn–winter and is likely to be due to tests performed on individuals with other causes of infectious and noninfectious diarrhea (Guarino et al., 2014; Polage et al., 2012).

We found that PPV also differed according to the age of the individual tested, with reactive results less likely to be confirmed in infants, a group who had recently received vaccine compared with older individuals, who had either not received vaccine or where vaccine had been administered more remotely with diminishing effect (Vesikari et al., 2010). Unfortunately, the small number of reactive rotavirus tests in

our population over this period limited our ability to analyze other factors which may have influenced the reliability of initially reactive tests. Nevertheless, though we found that age and season did influence the reliability of results, taking into account vaccine status, demographics (age, gender, ethnicity), and season did not enable selection of samples where initially reactive results would have $> 70\%$ PPV.

We did not perform evaluations of the sensitivity of the assays used over the study period, so it is possible that the known suboptimal sensitivity of ICT compared with RT-qPCR may have impacted on confirmation rates where ICT was used as the second assay, particularly where viral loads were low (Kaplon et al., 2015). However, generally, patients symptomatic with rotavirus have higher viral loads which are detectable by ICT (Tate et al., 2013), and it is reassuring that confirmation rates were higher during the period that ICT was used as the second assay.

Local assessments of existing testing algorithms should be performed by diagnostic laboratories in order to assess the reliability of results, which may be dependent upon the initial assay used, and the prevalence of disease in the population, but given that many commercial EIAs and ICTs have similar performance with comparable reported specificities to Rotascreen II (Kaplon et al., 2015; Ye et al., 2015), our findings are likely to be generalizable for community populations with high levels of vaccine coverage, particularly in years of low rotavirus activity.

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

There are several options available to attempt to address the poor PPV of testing. Foremost among these are changes in laboratory protocols to reduce unnecessary testing (such as performing testing only where rotavirus requested) which may minimize these preanalytical errors (Hawkins, 2012), as well as saving cost and time at the diagnostic laboratory. For example, in 2012–2014, when rotavirus testing was performed for all submitted feces samples for those < 3 years old, an average of 8200 samples were tested per year. This reduced to 2300 samples in 2016, mostly due only performing testing on request, with similar declines noted due to changes in laboratory practice predating introduction of vaccine at hospital laboratories in the Auckland region (McAuliffe et al., 2018). Though changes to algorithms such as these may ameliorate inappropriate requests, as we and others have demonstrated, they will not entirely prevent them, and education for the clinicians to assist with appropriate use of diagnostic tools is also warranted (McAuliffe et al., 2018).

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

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

Other considerations for community laboratories reconsidering their approach to diagnostic rotavirus testing include the following: rotavirus is no longer the most common cause of childhood gastroenteritis (Payne et al., 2013); children are often co-infected with multiple viruses (Guarino et al., 2014); rotavirus vaccine may be detected for variable periods following vaccination (Tate et al., 2013); and due to rotavirus vaccine implementation, there are shortages in the availability of diagnostic tests. Though highly sensitive RT-qPCR/PCR assays (Buss et al., 2015) 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 (Tate et al., 2013; Ye et al., 2017). 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.

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Competing interests

None declared.

Ethical approval

Not required.

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