

## RAPID COMMUNICATIONS

# Evidence of false-positive results in a commercially available rotavirus assay in the vaccine era, Australia, 2011 to 2012

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**Concerns were raised about specificity of the VIKIA Rota-Adeno immunochromatographic kit. Only 28-37% of samples positive with the VIKIA kit could be confirmed using two real-time RT-PCR assays and three ELISA kits. On re-analysis of a subset of the positive samples, 86% remained positive with the VIKIA kit, however, 90% remained negative in the other assays. In a highly vaccinated population we found a high number of false-positive rotavirus tests with a widely-used commercial kit.**

We recently became concerned about the specificity of the VIKIA Rota-Adeno assay (bioMérieux, France) following an unexplained increase in positive results and feedback from clinicians.

Accurate detection of rotavirus is essential for prevention and control of rotavirus outbreaks and disease monitoring. There are two common methods used for routine diagnosis: immunochromatographic (ICT) assays and enzyme-linked immunosorbent assays (ELISA). ICT assays are relatively inexpensive, easy to use, rapid (results within 20 min) and with reportedly good sensitivity (96.6%) and specificity (92.9%) [1]. Many diagnostic laboratories in Australia use the VIKIA Rota-Adeno assay for detection of rotavirus in faecal specimens.

We therefore re-examined samples initially testing positive in the VIKIA Rota-Adeno ICT with other commercially available ELISA rotavirus assays and, for a subset of specimens, by RT-PCR.

## Methods

Ethics approval for this study was provided by the Children's Health Queensland Human Research Ethics Committee.

## Clinical specimens

We obtained a convenience sample set of 133 faecal specimens submitted for diagnostic rotavirus testing and collected between July 2011 and August 2012 from patients with symptoms of acute gastroenteritis. Specimens were from two laboratories in Queensland (n=113: Pathology Queensland, a publically funded laboratory, and Sullivan Nicolaides Pathology, a private laboratory) and from a private laboratory network in Victoria (n=20: Melbourne Pathology). The latter were submitted to the National Rotavirus Reference Centre (NRRC) in Melbourne, Victoria, for genotyping. All samples had been tested initially for rotavirus using the VIKIA ICT method according to the manufacturer's instructions (Queensland: 81 positive, 32 negative; Victoria: 20 positive).

### Real-time RT-PCR, Queensland samples only

All 113 Queensland specimens were tested initially in Queensland employing two real-time RT-PCR assays, using primers and TaqMan probe sequences described previously:

NVP3-PCR (NVP3-F1 ACCATCTACACATGACCCTC, NVP3-F2 ACCATCTTACGTAACCCCTC, NVP3-R GGTCACATAACGCCC, NVP3 probe ATGAGCACAATAGTTAAAAGCTAACACTGTCAA) [2,3], JVK-PCR (JVK-F CAGTGGTTGATGCTCAAGATGGA, JVK-R TCATTGTAATCATATTGAATACCCA, JVK probe ACAACTGCAGCTTCAAAGAAGWGT) [4].

RNA extraction was performed by homogenising ca. 25 µl of stool specimen with 225 µl of phosphate buffered saline to provide a concentration of ca. 10%. Then 200 µl of this suspension were extracted into a volume of 50 µl using the Roche High Pure Nucleic Acid extraction kit as per kit instructions (Roche Diagnostics, Australia). As described previously, specimens were

**TABLE 1**

Comparison of original VIKIA test results and repeat VIKIA test results for specimens with sufficient volume for re-testing, Queensland, July 2011–August 2012 (n=61)

|                     |          | Repeat VIKIA test |          |       |
|---------------------|----------|-------------------|----------|-------|
|                     |          | Positive          | Negative | Total |
| Original VIKIA test | Positive | 30                | 5        | 35    |
|                     | Negative | 1                 | 25       | 26    |
|                     | Total    | 31                | 30       | 61    |

spiked before extraction with 5 µl of equine herpes virus as an extraction and inhibition control [5].

All real-time RT-PCR reactions were performed using a Qiagen one-step RT-PCR kit. Each reaction mix contained in a total volume of 25.0 µl in RNase-free water: 0.4 µM of forward and reverse primers, 0.16 µM of Taqman probe, 1.0 µl of Qiagen one-step RT-PCR dNTP mix, 5.0 µl of Qiagen one-step RT-PCR buffer (5x), 1.0 µl of RT-enzyme and 2.0 µl of RNA extract or control. Cycling was performed on a Rotor-Gene instrument (Qiagen, Australia) or Applied Biosystems 7500 real-time PCR system (Life Technologies, United States) with the following cycling conditions: initial hold steps at 50 °C for 20 min and 95 °C for 15 min, followed by 45 cycles at 95 °C for 15 sec and 60 °C for 30 sec, with fluorescence signal read on green at 60 °C.

### ELISA testing

There were 103 samples available at the NRRC for further testing: 83 from Queensland (51 VIKIA-positive, 32 VIKIA-negative specimens) and 20 specimens

from Victoria. Thirty VIKIA-positive specimens from Queensland were not sent to the NRRC due to insufficient sample volume. Available specimens were retested using three commercial rotavirus ELISA assays: ProSpecT (Oxoid, United Kingdom), Premier Rotaclone (Bioline, United Kingdom) and Ridascreen (R-Biopharm AG, Germany). All three methods were performed as per the manufacturer's instructions.

### VIKIA retesting

To confirm initial VIKIA assay results, Queensland specimens with sufficient remaining sample (positive: n=35; negative: n=26) after PCR and ELISA testing, were retested using the VIKIA assay.

### In-house VP6 RT-PCR

At NRRC, any samples that gave a discordant result for the ELISA methods or appeared to be falsely positive in the VIKIA assay (n=55), were further tested using a rotavirus VP6-specific RT-PCR with primers ROT<sub>3</sub> AAAGATGCTAGGGACAAAATTG and ROT<sub>5</sub> TTCAGATTGTGGAGCTATTCCA [6,7].

## Results

### Samples from Queensland retested in a second VIKIA assay

Of the 81 VIKIA-positive and 32 VIKIA-negative Queensland samples, there was sufficient remaining specimen for VIKIA retesting on 35 and 26 specimens, respectively. Thirty of 35 initially VIKIA-positive and one of 26 initially VIKIA-negative Queensland specimens were positive on retesting (Table 1).

Twenty-seven of the 30 VIKIA twice-positive samples were negative in every other assay applied (Table 2). Of the 10 VIKIA retest-positive specimens with sufficient sample volume available for testing at the NRRC, seven were negative by all three ELISA assays (Table

**TABLE 2**

Test results for specimens with sufficient volume for VIKIA re-testing, in PCR and ELISA assays, Queensland, July 2011–August 2012 (n=61)

|                   |          | Queensland PCR (NVP <sub>3</sub> and JVK) |          |       | Melbourne ELISA assays (ProSpecT, Rotaclone, Ridascreen) |          |       | Victorian PCR |          |    |       |
|-------------------|----------|-------------------------------------------|----------|-------|----------------------------------------------------------|----------|-------|---------------|----------|----|-------|
|                   |          | Positive                                  | Negative | Total | Positive                                                 | Negative | Total | Positive      | Negative | NP | Total |
| Repeat VIKIA test | Positive | 2                                         | 29       | 31    | 3 <sup>a</sup>                                           | 7        | 10    | 0             | 7        | 3  | 10    |
|                   | Negative | 1 <sup>b</sup>                            | 29       | 30    | 0                                                        | 25       | 25    | 0             | 0        | 25 | 25    |
|                   | Total    | 3                                         | 58       | 61    | 3                                                        | 32       | 35    | 0             | 7        | 28 | 35    |

ELISA: enzyme-linked immunosorbent assay; Negative: negative in all assays; NP: specimen not tested in this assay; PCR: polymerase chain reaction; positive: positive in any assay.

<sup>a</sup> Two specimens positive in all three assays, one specimen positive in Rotaclone only.

<sup>b</sup> One sample positive in single RT-PCR assay, NVP<sub>3</sub>, only (cycle threshold: ca. 37 cycles).

**TABLE 3**

Restesting with different diagnostic assays of faecal specimens positive in the VIKIA assay, Queensland and Victoria, July 2011-August 2012 (n=133)

| VIKIA initial <sup>a</sup>                                                                                        | Queensland PCR assays |                      | ELISA assays          |                         |                         | VIKIA retest <sup>b</sup> | Melbourne PCR assay  | Number |
|-------------------------------------------------------------------------------------------------------------------|-----------------------|----------------------|-----------------------|-------------------------|-------------------------|---------------------------|----------------------|--------|
|                                                                                                                   | NVP3-PCR <sup>c</sup> | JVK-PCR <sup>c</sup> | ProSpecT <sup>d</sup> | Rotacclone <sup>d</sup> | Ridascreen <sup>d</sup> |                           | VP6-PCR <sup>e</sup> |        |
| Queensland specimens from Pathology Queensland and Sullivan Nicolaides Pathology, original diagnostic test: VIKIA |                       |                      |                       |                         |                         |                           |                      | 113    |
| Queensland specimens with insufficient material for sending to the NRRC                                           |                       |                      |                       |                         |                         |                           |                      | 30     |
| POS                                                                                                               | POS                   | POS                  | NP                    | NP                      | NP                      | NP                        | NP                   | 2      |
| POS                                                                                                               | NEG                   | NEG                  | NP                    | NP                      | NP                      | POS                       | NP                   | 21     |
| POS                                                                                                               | NEG                   | NEG                  | NP                    | NP                      | NP                      | NEG                       | NP                   | 5      |
| POS                                                                                                               | NEG                   | NEG                  | NP                    | NP                      | NP                      | NP                        | NP                   | 2      |
| Queensland specimens with sufficient material for sending to the NRRC                                             |                       |                      |                       |                         |                         |                           |                      | 83     |
| POS                                                                                                               | POS                   | POS                  | POS                   | POS                     | POS                     | POS                       | NP                   | 2      |
| POS                                                                                                               | POS                   | POS                  | POS                   | POS                     | POS                     | NP                        | NP                   | 14     |
| POS                                                                                                               | POS                   | POS                  | POS                   | POS                     | NEG                     | NP                        | POS                  | 1      |
| POS                                                                                                               | POS                   | POS                  | NEG                   | POS                     | POS                     | NP                        | NEG                  | 1      |
| POS                                                                                                               | NEG                   | POS                  | NEG                   | NEG                     | NEG                     | NP                        | NEG                  | 1      |
| POS                                                                                                               | NEG                   | POS                  | NEG                   | NEG                     | POS                     | NP                        | NEG                  | 1      |
| POS                                                                                                               | POS                   | NEG                  | NEG                   | NEG                     | NEG                     | NP                        | POS                  | 1      |
| POS                                                                                                               | NEG                   | NEG                  | NEG                   | POS                     | NEG                     | POS                       | NEG                  | 1      |
| POS                                                                                                               | NEG                   | NEG                  | NEG                   | NEG                     | NEG                     | POS                       | NEG                  | 6      |
| POS                                                                                                               | NEG                   | NEG                  | NEG                   | NEG                     | NEG                     | NP                        | NEG                  | 23     |
| NEG                                                                                                               | POS                   | NEG                  | NEG                   | NEG                     | NEG                     | NEG                       | NP                   | 1      |
| NEG                                                                                                               | NEG                   | NEG                  | NEG                   | NEG                     | NEG                     | POS                       | NP                   | 1      |
| NEG                                                                                                               | NEG                   | NEG                  | NEG                   | NEG                     | NEG                     | NP                        | NP                   | 6      |
| NEG                                                                                                               | NEG                   | NEG                  | NEG                   | NEG                     | NEG                     | NEG                       | NP                   | 24     |
| Specimens from Victoria provided to the NRRC for genotyping, original diagnostic test: VIKIA                      |                       |                      |                       |                         |                         |                           |                      | 20     |
| POS                                                                                                               | NP                    | NP                   | NEG                   | NEG                     | NEG                     | NP                        | NEG                  | 13     |
| POS                                                                                                               | NP                    | NP                   | NEG                   | POS                     | NEG                     | NP                        | NEG                  | 1      |
| POS                                                                                                               | NP                    | NP                   | POS                   | POS                     | POS                     | NP                        | POS                  | 6      |

ELISA: enzyme-linked immunosorbent assay; **NEG**: specimens negative in this assay; NP: test not performed on this specimen; NRRC: National Rotavirus Reference Centre; PCR: polymerase chain reaction; **POS**: specimens positive in this assay.

<sup>a</sup> Initial diagnostic test.

<sup>b</sup> VIKIA retest performed on specimens with remaining adequate volume after PCR/ELISA testing.

<sup>c</sup> Queensland PCR, not performed on Victorian samples.

<sup>d</sup> ELISA tests performed at the National Rotavirus Reference Centre, Melbourne.

<sup>e</sup> PCR performed at the National Rotavirus Reference Centre, Melbourne, on specimens discordant for any of the ELISA tests.

2). The initially negative, but retest-positive specimen was negative by the NVP3 and JVK PCR assays, and all three ELISA tests.

### Other specimens from Queensland and Victoria available for testing in other assays

There were further specimens from Queensland (n=52; 46 positive, six negative) and Victoria (n=20; all positive) which were not retested using the VIKIA assay, but for which PCR and ELISA results were available.

Of 20 VIKIA-positive specimens from Victoria, 13 were negative in all three ELISA assays and the VP6 PCR assay (Table 3). The six negative specimens from Queensland were negative in both NVP3 and JVK PCR assays, and all three ELISA assays. Four of the 46 positive specimens did not have sufficient specimen volume remaining for ELISA testing; two of these positive in both the NVP3 and JVK assays, and two negative in both (Table 3). Of the remaining 42 specimens, 14 were positive in both Queensland PCR assays and all three ELISA assays, and 23 were negative in each of these

assays as well as the VP6 PCR assay. The remaining five specimens provided mixed results.

## Discussion

The results of our study highlight the need to review the validity of diagnostic assays when disease incidence changes unexpectedly. Australia implemented a nationwide rotavirus vaccination programme in July 2007, and since that time notifications of laboratory-confirmed rotavirus infections and hospitalisations have fallen quickly in targeted and older age-groups [8-10]. Anecdotal feedback from clinicians and an unexplained increase in disease notifications prompted this investigation, which has identified a problem with false positivity in an ICT assay used widely in Australia and elsewhere.

Even though ours is a convenience sample, the results point towards inability to confirm by a variety of PCR and ELISA methods a substantial proportion of specimens twice positive using the VIKIA kit. These findings were reinforced by specimens from Queensland and Victoria which were tested only once using the VIKIA assay. As a sensitivity analysis, if we assume the remaining 46 initially VIKIA positive specimens with insufficient volume for retesting had all retested negative, there would still remain 27 of 81 Queensland specimens that were twice positive by the VIKIA assay, but were negative in two PCR assays (n=21) or three PCR assays and three ELISA assays (n=6) (Table 3).

Given the consistency of other methods it is unlikely that the ICT assay is detecting true positive results. Notably, our data suggest that between one and two thirds of VIKIA-positive samples may be actually false-positive results. Furthermore, of initially positive samples from Queensland with sufficient volume for repeat testing using the VIKIA kit, 86% remained positive on retest, with only three of these 30 specimens positive in one or more other assay, by PCR or ELISA. Given the consistency of the VIKIA retest values and our PCR and ELISA assay findings, conducted at different times in different locations, with all assays performed according to the manufacturer's instructions, we do not believe specimen degradation or test conditions are a logical or sustainable explanation for the apparent specificity issue. There were six Queensland specimens for which the VIKIA retest value differed from the original result, with five of these initially positive and negative in the repeat test. Possible reasons for these discrepancies include sample stability, human error in result interpretation, and specimens with low virus load.

The VIKIA ICT kit insert states that the method has 100% sensitivity and 100% specificity for rotavirus detection, based on testing of 103 positive and 290 negative stools [11]. In a prospective study of 57 samples from children younger than 36 months in Lyon in childcare centres during 2004-05, the reported sensitivity and specificity of this kit, compared to a PCR-based method, was 96.6% and 96.4% respectively

(PPV: 96.5%, NPV: 92.9%) [1]. Similar high specificity (100%) was reported by Bon et al. in 2006 [12]. Given this, it is difficult to know if our findings are due to recent changes in the assay or to specificity problems exposed by reduced disease incidence in a high vaccine coverage setting. We are therefore investigating further the specificity of this and other assays in a prospective study.

## Conclusion

We have shown a suboptimal test specificity using a commercially available rotavirus ICT assay. Assay-specific issues should be considered in the event of unexplained increases of rotavirus disease in the vaccine era.

## Conflict of interest

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None declared.

## Authors' contributions

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All authors collaborated on the design and conduct of the study. JR, CH, GN provided specimens. SY, SR-F performed PCR and ELISA testing on specimens. SY, DMW, SBL, KG analysed the data. SY wrote the first draft of the paper. All authors contributed to critical revision of the manuscript and have seen and approved the final version of the manuscript.

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