Diagnosing rotavirus A associated IID: using ELISA to identify a cut-off for real time RT-PCR

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Abbreviations

AUC	Area under the ROC curve
Ct	Cycle threshold
ELISA	Enzyme linked immunosorbent assay
IID	Infectious intestinal disease
IQR	Interquartile range
RT-PCR	Reverse transcription polymerase chain reaction
ROC	Receiver operating characteristic

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Abstract

Background

The use of RT-PCR for diagnosis of group A rotaviruses is increasing, but up to 14% of healthy individuals may be positive by RT-PCR. If RT-PCR is not well correlated with disease, rotavirus A may not always be the cause of illness in RT-PCR positive patients with infectious intestinal disease (IID).

Objectives

To describe the differences in faecal viral load between ELISA positive IID cases, RT-PCR positive cases and healthy controls. To develop a cut-off in faecal viral load for attributing illness to rotavirus A in RT-PCR positive IID cases.

Study design

Faecal viral load was measured, using real time RT-PCR, in 118 community IID cases and 65 healthy controls, previously tested by ELISA. Cycle threshold (Ct) values from the real-time RT-PCR were used as a proxy measure of viral load. A cut-off for attributing illness to rotavirus A was selected, using ROC analysis.

Results

There was little overlap in viral load between ELISA positive IID cases (median Ct 17) and healthy controls (median Ct 37), but ELISA negative, RT-PCR positive IID cases had viral loads similar to healthy controls (median Ct 37), indicating that RT-PCR is not detecting extra cases of group A rotavirus associated IID, only sub-clinical infections. The optimal cut-off in the real time RT-PCR was at Ct value 24 to 27.

Conclusion

ELISA is the best method for the laboratory diagnosis of rotavirus A associated IID. If RT-PCR is used, it is advisable to use a real time platform and to use a viral load cut-off equivalent to the detection limit of ELISA.

1. Background

Enzyme linked immunosorbent assay (ELISA) has traditionally been the method of choice for laboratory diagnosis of group A rotavirus associated infectious intestinal disease (IID)[1,2]. However, with the availability of reverse transcription polymerase chain reaction (RT-PCR) assays for rotavirus A [3,4] and the move towards multiplexing in clinical virology[5,6], the use of RT-PCR is increasing. Whilst RT-PCR does identify more rotavirus A infections than ELISA[7], up to 14% of healthy individuals may be positive by RT-PCR[8], indicating that in some RT-PCR positive IID cases, rotavirus A may not actually be the cause of illness.

Differences in faecal viral load between symptomatically and asymptomatically infected individuals have been demonstrated using real time RT-PCR[9] and histopathological studies indicate that damage to intestinal epithelial cells, caused by viral replication, may play a role in pathogenesis[10]. It may therefore be possible to use faecal viral load to indicate where rotavirus A is the cause of illness in RT-PCR positive IID cases.

2. Objectives

Research objectives were to describe the differences in rotavirus A viral load detected in IID cases positive by ELISA, IID cases negative by ELISA but positive by RT-PCR and healthy controls; to develop a cut-off in faecal viral load for attributing illness to rotavirus A in IID cases.

3. Study design

3.1 Specimens

Faecal specimens were collected from IID cases and healthy controls during the Infectious Intestinal Disease Study for England (1993-1996)[11]. IID cases were recruited from a community cohort, or at consultation with their general practitioner. IID cases had acute diarrhoea or vomiting lasting less than two weeks, with no known non-infectious cause, preceded by a symptom-free period of three weeks[12]. Healthy controls, with no history of IID for the preceding three weeks, were recruited concurrently to IID cases, from the community cohort or from the registration list of participating general practices (not after consultation for another condition)[12]. IID cases provided a faecal specimen during acute illness; controls provided a specimen at recruitment.

3.2 Testing

In the original study, faecal specimens from IID cases and controls were tested for rotavirus A using ELISA[11]. Specimens with sufficient volume remaining after testing were archived in frozen storage[13]. Subsequently, all archived specimens were retested for rotavirus A using RT-PCR[8]. In this study, a real time RT-PCR assay (method previously described[14]) was used to determine the viral load in specimens that were previously positive for rotavirus A by ELISA or RT-PCR.

The cycle threshold (Ct) values from the real time RT-PCR were used as a proxy measure of viral load. The Ct value is inversely proportional to the amount of virus present in the specimen, so the lower the Ct value the higher the faecal viral load. The real time RT-PCR assay was run for 45 cycles so the maximum possible Ct value for a positive specimen was 44.

3.3 Descriptive analysis

The median Ct value and inter-quartile range were calculated for IID cases and controls and comparisons were made between groups using the rank-sum test in Stata 10[15].

3.4 Receiver operating characteristic analysis

Receiver operating characteristic (ROC) analysis was used to select a cut-off in faecal viral load for attributing illness to rotavirus A. ELISA was used as the gold standard reference test to indicate where rotavirus A was the cause of illness in IID cases. The optimal cut-off was identified at the maximum value of the Youden index (sensitivity + specificity -1)[16-18]. The ROC analysis was repeated, using healthy controls in the reference negative group, to increase the sample size for the analysis in children aged less than five years. Healthy controls should serve as a suitable reference negative group because they have viral loads representative of rotavirus A infection without disease.

4. Results

4.1 Descriptive analysis

IID cases were aged up to 83 years and controls were aged up to 46 years; 60% of cases and 71% of controls were aged less than five years. The median Ct value in ELISA positive IID cases was substantially lower than in controls (Table 1) and there was very little overlap between the distribution of Ct values in these two groups (Figure 1). There was no evidence of a difference in Ct value distribution between the ELISA negative, RT-PCR positive IID cases and the controls (Figure1, Table 1), in all ages and when the analysis was restricted to children aged less than five years.

4.2 ROC analysis

Using ELISA as the gold standard, the optimal Ct value cut-off for attributing illness to rotavirus A in IID cases, for all ages, was in the range 25 to 28. There was a clear bimodal distribution of Ct values, with few observations in the range 25 to 28, so it was not possible to distinguish between these cut-off values. Using healthy controls as the reference negative group produced similar results (Table 2). The optimal cut-off for children aged less than five years was at Ct value 24, although the Youden index declined only slightly (difference less than 0.1) at Ct values of 25 to 27 (data not shown).

5. Discussion

We have used faecal viral load to demonstrate that ELISA diagnosis is highly correlated with disease in rotavirus A infection, in accordance with other community and hospital based studies[13,19-21], and that RT-PCR is probably only detecting additional infections in IID cases at levels not associated with illness. We have selected a cut-off in the real time RT-PCR assay to improve the specificity of diagnosing rotavirus A associated IID by this method.

A major strength of this study is the availability of specimens from healthy controls, which were essential for interpreting the RT-PCR results in IID cases. Degradation of the rotavirus A genome, during the prolonged storage of these specimens, is likely to be minimal, because the double stranded RNA is relatively stable. It is also unlikely that degradation will have occurred differentially across the specimen collection. Therefore, the patterns in faecal viral load described for IID cases and controls should

reflect relative levels at the time of specimen collection. However, the actual Ct value cut-off identified here should not be applied directly to real time RT-PCR results from fresh specimens, nor to results generated using a different assay protocol, because the Ct values may not necessarily equate to the same viral load per gram of faeces.

8. Conclusion

RT-PCR does not provide sufficient specificity for attributing illness to rotavirus A in IID cases. As the use of multiplex PCR assays for enteric viruses increases in routine diagnosis of IID, there will be a need to interpret the results of these sensitive tests to determine disease aetiology[22,23]. We have shown that ELISA positivity remains a good correlate of disease in rotavirus A infection, supporting the use of ELISA in the WHO protocol for surveillance of rotavirus associated gastroenteritis[24]. We have demonstrated that clinical laboratories can use real time RT-PCR testing of ELISA-positive and ELISA-negative specimens to define a suitable cut-off for their real time RT-PCR assays. Accurate diagnosis of disease aetiology is important both for individual patient care, where correct identification of the cause of illness is essential for clinical management, and at the population level, to ensure that estimates of rotavirus A disease burden are accurate, for assessing vaccine impact in immunized populations.

Conflict of interest

Table 1 – Distribution of rotavirus A real time RT-PCR Ct values in IID cases and healthy controls.

IQR is the interquartile range. ELISA indicates that the IID case was ELISA positive, RT-PCR indicates that the IID case was ELISA negative and RT-PCR positive.

Method of rotavirus A detection	IID Cases			Controls			Rank sum test p-value comparing cases to controls*
	Median Ct value	IQR	Sample size	Median Ct value	IQR	Sample size	
All ages							
All	18	15-30	153	37	33-39	65	<0.0001
ELISA	17	15-20	118				<0.0001
RT-PCR (ELISA negative)	37	32-39	35				0.96
<5 years							
All	17	15-22	92	37	33-40	46	<0.0001
ELISA	16	15-20	79				<0.0001
RT-PCR (ELISA negative)	35	32-38	13				0.37

* The rank sum test for ELISA and RT-PCR positive IID cases compare them to all controls.

Figure 1 – Percentage distribution of real time RT-PCR Ct values in IID cases and controls

Low Ct values correspond to high viral loads; the viral load decreases with increasing Ct value. 'ELISA cases' are IID cases positive by ELISA, 'RT-PCR cases' are IID cases negative by ELISA and subsequently positive by RT-PCR. Sample sizes: ELISA cases = 118, RT-PCR cases = 35, controls = 65.

Table 2 – ROC analysis results

AUC is the area under the ROC curve. CI is confidence interval.

	Ct cut-off	Youden Index	Sensitivity (95% CI)	Specificity (95% CI)	AUC	Sample size	
						Reference positive	Reference negative
All ages							
ELISA gold standard	25-28	0.96	0.96 (0.92-0.99)	1	0.99	118	35
Reference negative group controls	24-27	0.82	0.94 (0.90-0.98)	0.88 (0.80-0.96)	0.92	118	65
Aged < 5 years							
Reference negative group controls	24	0.84	0.95 (0.90-1.0)	0.89 (0.80-0.98)	0.95	79	46

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