Rotavirus detection in bulk stool and rectal swab specimens in children with acute gastroenteritis in Norway

Abstract

Background: Bulk stool specimens are traditionally used for rotavirus detection but may be challenging to obtain from young children. Immediate and easy sampling may however be required in different situations, such as outbreak investigation.

Objectives: We assessed the diagnostic performance of rectal swabs compared to bulk stools for the detection of rotavirus by Enzyme Immunoassay (EIA) and multiplex semi-nested reverse transcription PCR (semi-nested RT-PCR) in children recruited through active hospital-based surveillance of acute gastroenteritis in Norway.

Study design: We obtained 265 paired bulk stool and rectal swab specimens from children under 5 years of age hospitalized with acute gastroenteritis (AGE). Both types of specimens were analyzed for rotavirus by EIA and semi-nested RT-PCR. In addition, VP6-spesific real-time PCR was used to evaluate the detection performance in the two specimen types.

Results: Concordant results were obtained in 257 (97%) paired specimens by EIA and in 248 (94%) pairs by semi-nested RT-PCR. Results of VP6-specific real-time PCR obtained from 100 pairs of specimens showed concordance in 91% of the pairs. Sensitivity and specificity for rectal swab specimens were 95% and 100% by EIA; 95% and 92% by semi-nested RT-PCR, respectively.

Conclusion: Both EIA and semi-nested RT-PCR showed a high accuracy, and rectal swab specimens are appropriate for rotavirus diagnosis and may be used as an alternate specimen type when collection of bulk stool is not feasible.

Background

Rotavirus group A (RVA) is the most common cause of acute gastroenteritis (AGE) in infants and young children under 5 years. Before the introduction of universal rotavirus vaccination in Norway, approximately 4.0 hospital inpatient and 2.3 outpatient cases per 1000 children with AGE under age 5 years were associated with a rotavirus infection annually. In addition, rotavirus-associated mortality was estimated at 0.17 deaths per 100,000 children < 5 years old, corresponding to one death biennially [1]. Following the introduction of rotavirus vaccination, the disease burden has reduced substantially [2].

Rotavirus gastroenteritis is not clinically distinguishable from AGE caused by other viruses. To confirm infection by rotavirus, a number of different laboratory methods are available. Immunochromatographic tests, enzyme immunoassay (EIA), reverse transcription-polymerase chain reaction (RT-PCR) and electron microscopy can be used to diagnose rotavirus infection by direct detection of antigen, genomic RNA or particles in fecal specimens [3].

The limit of detection of each method depends on some factors like different sensitivity for different types of samples and not least the amount of virus or viral antigen, which can vary between different types of clinical specimens. Furthermore, sensitivity and specificity of detection methods might be different from one to another according to previous studies comparing this issue between antigen methods, rapid tests and PCR [4].

Bulk stool (BS) specimens are traditionally preferred over rectal swabs (RS) for detection of enteric viruses because of the assumed higher amount of virus present. However, for bacterial gastroenteritis diagnostic, RS are frequently used as an alternative specimen type to BS [5].

Only a few earlier studies have compared diagnostic performance of BS specimens versus RS for the detection of viral causes of AGE. Rodrigues and coworkers, as well as Arvelo et al., evaluated rotavirus detection rates in BS and RS [6-9], but a limited amount of samples were collected in these studies.

Studies conducted in some parts of the world, particularly in the USA and Asia, investigated detection performance in RS. In these studies, rotavirus detection rates were either not compared with detection rates in BS, or the RS were used for detection of other enteric viruses or bacteria [8, 10-14]. However, it may be more difficult and time consuming to collect BS specimens, particularly in infants and young children especially in case of a short hospital stay. RS on the contrary may be easier to obtain in a clinical setting; their transportation and further handling are also more feasible.

Thus, in this study, we aimed to compare the diagnostic performance of BS versus RS for the detection of rotaviruses by EIA and semi-nested RT-PCR in children under 5 years old recruited through active hospital-based rotavirus surveillance. Rotavirus hospital surveillance was established in four sentinel hospitals in Norway in 2014, prior to introduction of rotavirus vaccine into Norwegian childhood immunization program in October 2014.

Objectives:

The aim of this study was to assess the diagnostic performance of bulk stools versus rectal swabs for the detection of rotavirus by EIA and semi-nested RT-PCR in children recruited through active hospital-based surveillance for acute gastroenteritis.

Study design

Study population

The study population included children under age 5 years (54 % male) residing in the catchment areas of four surveillance hospitals; (Oslo University Hospital Ullevål in Oslo, Østfold Hospital in Fredrikstad, St. Olav's University Hospital in Trondheim, and Stavanger University Hospital in Stavanger). Rotavirus surveillance, initiated in February 2014, included active ascertainment

of all acute gastroenteritis cases in children <5 years of age seeking care in participating hospitals within 10 days of illness onset.

Cases with nosocomial gastroenteritis were not included. Detailed description of surveillance protocol is provided elsewhere [1]. Clinical and demographic data were collected from all included children using a standard questionnaire. Collected information included hospital name, patient's age and sex, date of specimen collection, presence and onset of gastroenteritis symptoms at admission, length of hospital stay, and outcome of hospitalization. To assess severity of gastroenteritis cases, we applied the Vesikari severity scale [15, 16] to classify cases as severe (score of ≥11), moderate (7-10), or mild (<7).

Specimen collection and preparation

Paired specimens (BS and RS) were collected by a hospital nurse from 265 children enrolled within the first 48 hours of hospitalization consecutively from 15th April 2014 to 9th December 2015. BS specimens were gathered in a 25 ml container, whereas RS were collected by using Copan Regular fecal swab (470CE, Copan Italy S.p.a, Brescia, Italy) containing 2 ml of Cary-Blair Transport Medium[1]. RS were taken by gently inserting the swab into the rectum and rotating it before retreat, which allowed stool to attach to the swab.

The BS specimens were initially tested for the presence of rotavirus antigen in the participating hospitals by commercial immunochromatographic tests (RidaQuick, R-Biopharm, Darmstadt, Germany; Vikia, bioMérieux, Marcy l'Etoile, France) or semi-nested RT-PCR (commercial or in-house assay). All BS specimens were then transferred to the national rotavirus reference laboratory at the Norwegian Institute of Public Health (NIPH) together with the corresponding RS for further testing.

All specimens (BS and RS) were pre-processed by making a 10% suspension in dilution buffer supplied with the rotavirus RIDASCREEN kit (R-Biopharm AG, Darmstadt, Germany) by

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[1] This medium is recommended for use in the transport and preservation of clinical specimens, primarily stool and rectal swabs.

adding approximately 100 µl of thin stool (or approximately 50-100 mg of solid stool) to a labelled test tube with 900 µl sample dilution buffer and then blended well in a Vortex mixer for homogenizing and subsequently centrifuged at 2000 G (13500 rpm) for 3 minutes before further testing.

Rotavirus detection

Rotavirus detection by EIA

Presence of rotavirus antigen in 100 µl 10% suspension of BS and RS specimens was analyzed by an EIA assay from RIDASCREEN kit according to the manufacturer's protocol. The test was carried out in an automated EIA system, DS2® (Dynex Technologies Inc, Chantilly, USA).

Nucleic acid extraction and multiplex semi-nested RT-PCR

For total nucleic acid extraction, 200 µl of 10% fecal suspension was used with Viral NA Small Volume kit in the MagNA Pure 96 automated nucleic acid extraction instrument, and eluted into 50 µl according to the manufacturer protocol (Roche Applied Science, Penzberg, Germany). The suspension was used consecutively to avoid freezing and thawing of the samples.

All extracts were then subjected to semi-nested RT-PCR to confirm and genotype the RV positive results using previously described protocols [17, 18].

The amplification products were analyzed by gel electrophoresis using a 2% agarose E-gel with E-Gel® Precast Agarose Electrophoresis System (Thermo Fisher Scientific Corporation, Waltham, USA).

Rotavirus detection and quantification by VP6-specific real-time PCR

In order to evaluate the detection performance in the two specimen types, a VP6-specific real-time PCR with consensus primers was performed. VP6 is one of RVA structural proteins located on the Inner Capsid. Of the 265 paired samples, 100 (38%) were analyzed. Since no international standard for RVA viral load is available, the cyclic threshold (Ct) value was used as an expression of the amount of virus present. As the Ct value without the usage of a standard or calibrator does not allow an estimation of the total viral load (copies/ml or IU/ml) quantitation, the estimates of virus content in this study therefore can be considered relative or semi-quantitative. The cut-off value of the VP6-specific real-time PCR was chosen to be at Ct 38 according to the conclusion during validation of the method. This cut-off value was selected due to a high number of background signals appearing after 37 cycles in negative controls [19, 20].

Data analysis

We explored association between rotavirus detection rates (BS and RS rates) and a number of covariates such as age, sex, the Vesikari score, and patient type (inpatient vs. outpatient) using logistic regression models. All analyses were performed using the statistical software STATA, version SE13 (StataCorp LP, College Station, TX, USA).

Results

A total of 265 children with acute gastroenteritis were included in the study, of which 220 (83%) were inpatient and 45 (17%) outpatient cases, defined as patients whose hospital stay did not exceed 5 hours. Based on the Vesikari score, 73% (n=153) of enrolled children were categorized having a severe AGE, 20% (n=42) as moderate and 7% (n=15) as mild. Both BS and RS were available from all enrolled patients and all 265 pairs of specimens were tested by EIA and semi-nested RT-PCR for rotavirus. The RV-positive cases were defined as being EIA positive and

confirmed by semi-nested RT-PCR. The highest percentage of rotavirus-positive cases 86% (n=121) was observed among children aged 6-35 months.

As shown in Table 1, 149 (56.2%) pairs were positive for rotavirus in both specimen types by EIA, whereas 147 (55.5%) pairs were positive by semi-nested RT-PCR. Concordant results were obtained in 257 (97%) paired specimens by EIA, and in 248 (94%) pairs by semi-nested RT-PCR. Furthermore, 108 pairs were negative in both specimen types by EIA, while 101 pairs were negative using semi-nested RT-PCR. Only 3.4% of paired specimens had discordant results (Table 1).

Table 1 | Comparison of the results for rotavirus detection in paired specimens (n=265 pairs of bulk stool and rectal swabs) by EIA and semi-nested RT-PCR.

|	Enzyme Immunoassay			Semi-nested RT-PCR		
	BS Pos.	BS Neg.	Total	BS Pos.	BS Neg.	Total
RS Pos.	149	0	149	147	9	156
RS Neg.	8	108	116	8	101	109
Total	157	108	265	155	110	265

To test the validity of results obtained from rectal swabs, we estimated the sensitivity and specificity of rotavirus detection in RS by using bulk stool as "the gold standard". The sensitivity was 95% for the RS specimens with both EIA (149/157) and semi-nested RT-PCR (147/155), while the specificity of the RS was 100% by EIA (108/108) and 92% by semi-nested RT-PCR (101/110).

The results of VP6 specific real-time PCR obtained from 100 pairs of specimens—BS and RS—showed concordance in 91% of pairs where 51% pairs were positive, and 40% pairs were negative for RVA (Table 2).

Table 2 | VP6-specific real-time PCR results for 100 pair samples.

	BS Pos.	BS Neg.	Total
RS Pos.	51	6	57
RS Neg.	3	40	43
Total	54	46	100

Among the 51 positive pairs by VP6-specific real-time PCR, RS had higher Ct-values compared with BS: 30 RS (59%) had Ct-values more than 21 versus 12 BS (24%) in the same Ct-value category (Figure 1).
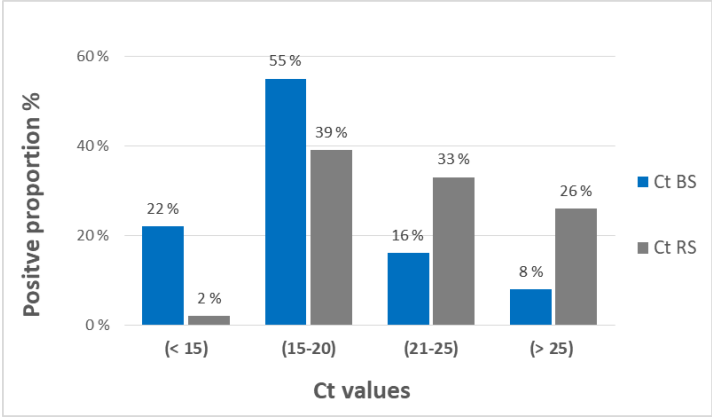
Figure 1 | Association between Ct-values in rectal swab versus bulk stool RV positive specimens obtained by VP6-specific real-time PCR.

Furthermore, VP6-specific real-time PCR, showed the mean (range) Ct-values for positive RS (Ct_{RS}) and positive BS (Ct_{BS}) specimens were 21.97 (13.42-32.70) and 18.20 (11.64-32.58), respectively. The median calculated to be 17 for Ct_{BS} and 20 for Ct_{RS}. Only three positive BS specimens of 51 were negative in the semi-nested RT-PCR.

The multiple logistic regression analyses indicated that there were significant differences in the numbers of RV-positive between severe and mild cases and between hospitalized and outpatient cases for both BS and RS detection rates.

Discussion

This study compared the diagnostic performance of rectal swab with bulk stool specimens for rotavirus detection by using two methods (EIA and semi-nested RT-PCR) in children under 5 years of age hospitalized with acute gastroenteritis. The two specimen types obtained simultaneously from children enrolled in this study had comparable sensitivity and specificity for rotavirus detection, despite the low quantities of virus present in the RS [21, 22]. The high concordance between BS and RS by using both EIA and semi-nested RT-PCR indicated that RS were equally appropriate for rotavirus detection in a clinical setting. There were only 5.1% of paired specimens that demonstrated discordant results by these two detection methods.

The most obvious explanation for negative results is a low viral load in the specimens leading to results under the detection limit of the EIA or PCR methods. This may be the main reason for negative results in the RS samples, which collect only a small quantity of fecal matter, especially in cases of watery diarrhea. Another reason that might explain the RS negative samples is the ability of Cary-Blair transport medium used in RS specimens to preserve GE viruses although many reports suggest that the usage of Cary-Blair medium is appropriate for pathogen detection even with a decreased specimen volume [23-25]. In addition, negative results may be due to the insufficient amount of fecal matter collected because of technical difficulties during the sampling procedure.

On the other hand, the false negative results could be explained by inhibition in the PCR analysis due to the inhibitory substances (e.g. salts) which may be present in stool, thereby influencing the assay's sensitivity. This could be prevented by diluting the extracted RNA, which in turn will cause a reduction in the sensitivity corresponding to the dilution factor. Another method is to use a purification step in addition to the RNA extraction which has been proven to be very effective [26]. One limitation of the study is that internal control was not implemented in the method, which could have revealed possible inhibition of the PCR. Another

limitation is that data was lacking on the exact time of onset of the illness. The clinical information included only categorized data on symptoms e.g. if the child had diarrhea for "1-4 days", "5 days", or "6-10 days". Therefore, the date of onset data could not be plotted against the Ct values which might have helped in explaining the negative results.

In 80.4 % of the positive paired samples (among the 100 paired) analyzed by VP6-specific RT-qPCR had Ct_{BS} lower than Ct_{RS}. In addition, more than half of RS versus one fourth of the BS had moderate to weak positive results ($Ct \geq 21$). This, as expected, suggests a lower viral load in RS than in BS, which is in line with reports from other studies [6, 7, 27].

Applying the Vesikari severity scale resulted in 73% of included patients being categorized as severe gastroenteritis cases. This corresponds well with 76.5% of BS samples having Ct-values below 20 in the VP6-spesific real-time PCR, suggesting a relatively high amount of virus present in the samples. The negative correlation between Vesikari severity scale and the Ct-values matches well with previous studies [19].

We found no significant associations between rotavirus detection rates in paired specimens (BS and RS) and sex and age of the child. However, as expected, there was a significant difference in the number of positive specimens between inpatients and outpatients (by both EIA and PCR) likely due to severity of cases.

To our knowledge, this is the first study in Europe that compared the diagnostic performance of RS versus BS for detection of RVA. However, studies by Rodrigues et al., 2007 and Arvelo et al., 2013 [8, 9] showed similar correlations of rotavirus detection by rectal swab in comparison to bulk stool. Furthermore, both previous studies recommended using rectal swabs for detection of rotaviruses as an alternate to the bulk stool.

Previous studies comparing performance of these two specimen types had however some weaknesses such as an inadequate number of paired samples tested, detection of other gastroenteritis agents than rotavirus or testing of only RS specimens without comparing results

to BS [14]. Studies focusing on bacterial gastroenteritis agents also demonstrated a lower sensitivity and specificity of RS in comparison to BS [10, 11].

In our study, however, specimens were collected prospectively from a large cohort of children with paired samples obtained simultaneously from each enrolled patient. This made it possible to compare two types of clinical specimens using different diagnostic methods.

In conclusion, we found a high correlation between bulk stool and rectal swab specimens for diagnosis of rotavirus by EIA and PCR. Some pediatric patients suffering from rotavirus infection are hospitalized for a short time period, making it difficult to obtain a bulk stool sample before discharge from the hospital. Therefore, RS specimens can be used as an alternative specimen type especially in situations where immediate and logistically easy sampling is required.

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Ethical approval

The Regional Committees for Medical and Health Research Ethics (REK) approved this study. Written informed consent was obtained from parents or legal guardians of all included children.

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Conflict of interest

The authors declare no competing interests or conflict of interest.

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