FOIA request to CDC re: purification of any "virus" on "immunization" schedules, by any method

Christine Massey <cmssyc@gmail.com> Tue, Nov 23, 2021 at 1:28 PM
To: "FOIA Requests (CDC)" <FOIARequests@cdc.gov>

November 23, 2021

To:
Roger Andoh
Freedom of Information Officer
1600 Clifton Rd NE MS T-01
Atlanta, Georgia 30333
Email: FOIARequests@cdc.gov
Phone: 770-488-6277
Fax: 770-488-6200

Dear Freedom of Information Officer,

This is a formal request for access to general records, made under the *Freedom of Information Act.*

Description of Requested Records:

***Please note**: this request is very similar to another request that I submitted on March 1, 2021 (**21-00792-FOIA**) where I had **specified** purification via maceration, filtration and use of an ultracentrifuge and received a "no records" response. The difference with this new request is that it does **not** specify maceration, filtration and use of an ultracentrifuge; it mentions filtration, ultracentrifugation and chromatography and only by way of an example.*

All studies and/or reports in the possession, custody or control of the Centers for Disease Control and Prevention (CDC) and/or the Agency for Toxic Substances and Disease Registry (ATSDR) describing the **purification** (i.e. via filtration, ultracentrifugation and chromatography) of any "virus" addressed by any "vaccine" on either the childhood or adult U.S. "immunization" schedule, directly from a sample taken from a diseased host, where the sample was not first combined with any other source of **genetic** material (i.e. a cell line; fetal bovine serum).

Please note that I am not requesting studies/reports where researchers failed to **purify** the suspected "virus" and instead:

- cultured something, and/or
- performed an amplification test (i.e. PCR), and/or
- fabricated a "genome", and/or
- produced electron microscopy images of unpurified things.

I am already aware that according to virus theory a "virus" requires host cells in order to replicate. I am **not** requesting records describing the **replication** of a "virus" without host cells, or records that describe a suspected "virus" floating in a vacuum, or a strict fulfillment of Koch's Postulates, or private patient records.

I simply request records that describe "its" **purification** (**separation** from everything else in the patient sample, as per standard laboratory practices for the purification of other very small things).

Please note that my request includes any study/report matching the above description, **authored by anyone, anywhere.**

If any records match the above description of requested records and are currently available to the public elsewhere, please provide enough information about each record so that I may identify and access each one with certainty (i.e. title, author(s), date, journal, where the public may access it). Please provide URLs where possible.

Format:
Pdf documents sent to me via email; I do not wish for anything to be shipped to me.

Contact Information:
Last name: Massey
First name: Christine
Address: [redacted]Peterborough, ON, Canada
Phone: [redacted]
Email: cmssyc@gmail.com

Thank you in advance and best wishes,
Christine Massey, M.Sc.

Gmail Christine Massey <cmssyc@gmail.com>

Your CDC FOIA Request #22-00402-FOIA

MNHarper@cdc.gov <MNHarper@cdc.gov> Mon, Nov 29, 2021 at 9:28 AM
To: cmssyc@gmail.com

November 29, 2021

Request Number: 22-00402-FOIA

Dear Ms. Massey:

This is regarding your Freedom of Information Act (FOIA) request of November 23, 2021, for request for all studies and/or reports in the possession, custody or control of the Centers for Disease Control and Prevention (CDC) and/or the Agency for Toxic Substances and Disease Registry (ATSDR) describing the purification (i.e. via filtration, ultracentrifugation and chromatography) of any "virus" addressed by any "vaccine" on either the childhood or adult U.S. "immunization" schedule, directly from a sample taken from a diseased host, where the sample was not first combined with any other source of genetic material (i.e. a cell line; fetal bovine serum). Please note that I am not requesting studies/reports where researchers failed to purify the suspected "virus" and instead: • cultured something, and/or • performed an amplification test (i.e. PCR), and/or • fabricated a "genome", and/or • produced electron microscopy images of unpurified things..

Please see the attached letter.

Sincerely,
CDC/ATSDR FOIA Office
770-488-6399

2 attachments

22-00402 Acknowledgement (Complex) 30 Days (003).pdf
120K

22-00402-FOIA.msg
104K

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Centers for Disease Control
and Prevention (CDC)
Atlanta GA 30333

November 29, 2021

Christine Massey
[redacted]
Peterborough, ON, Canada
Via email: cmssyc@gmail.com

Dear Ms. Massey:

The Centers for Disease Control and Prevention and Agency for Toxic Substances and Disease Registry (CDC/ATSDR) received your attached Freedom of Information Act (FOIA) request dated November 23, 2021. Your request assigned number is 22-00402-FOIA, and it has been placed in our complex processing queue.

In unusual circumstances, an agency can extend the twenty-working-day limit to respond to a FOIA request.

We will require more than thirty working days to respond to your request because we reasonably expect that two or more CDC centers, institutes, and offices (C/I/Os) may have responsive records.

To process your request promptly, please consider narrowing the scope of your request to limit the number of responsive records. If you have any questions or wish to discuss reformulation or an alternative time frame for the processing of your request, you may contact the analyst handling your request Mark Harper at 770-488-8154 or our FOIA Public Liaison, Roger Andoh at 770-488-6277. Additionally, you may contact the Office of Government Services (OGIS) to inquire about the FOIA mediation services they offer. The contact information for OGIS is as follows: Office of Government Information Services; National Archives and Records Administration; 8601 Adelphi Road-OGIS; College Park, Maryland 20740-6001; e-mail at ogis@nara.gov; telephone at 202-741-5770; toll free at 1-877-684-6448; or facsimile at 202-741-5769.

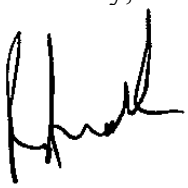
Because you are considered an "Other requester" you are entitled to two hours of free search time, and up to 100 pages of duplication (or the cost equivalent of other media) without charge, and you will not be charged for review time. We may charge for search time beyond the first two hours and for duplication beyond the first 100 pages. (10 cents/page).

If you don't provide us with a date range for your request, the cut-off date for your request will be the date the search for responsive records starts.

You may check on the status of your case on our FOIA webpage https://foia.cdc.gov/app/Home.aspx and entering your assigned request number. If you have any questions regarding your request,

please contact me at 770-488-8154 or via email at wzj6@cdc.gov.

Sincerely,



Roger Andoh
CDC/ATSDR FOIA Officer
Office of the Chief Operating Officer
(770) 488-6399
Fax: (404) 235-1852

Enclosure

22-00402-FOIA

Gmail Christine Massey <cmssyc@gmail.com>

Your CDC FOIA Request #22-00402-FOIA

MNHarper@cdc.gov <MNHarper@cdc.gov> Fri, Mar 11, 2022 at 12:03 PM
To: cmssyc@gmail.com

March 11, 2022

Request Number: 22-00402-FOIA

Dear Ms. Massey:

This is regarding your Freedom of Information Act (FOIA) request of November 23, 2021, for request for all studies and/or reports in the possession, custody or control of the Centers for Disease Control and Prevention (CDC) and/or the Agency for Toxic Substances and Disease Registry (ATSDR) describing the purification (i.e. via filtration, ultracentrifugation and chromatography) of any "virus" addressed by any "vaccine" on either the childhood or adult U.S. "immunization" schedule, directly from a sample taken from a diseased host, where the sample was not first combined with any other source of genetic material (i.e. a cell line; fetal bovine serum). Please note that I am not requesting studies/reports where researchers failed to purify the suspected "virus" and instead: • cultured something, and/or • performed an amplification test (i.e. PCR), and/or • fabricated a "genome", and/or • produced electron microscopy images of unpurified things..

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Sincerely,
CDC/ATSDR FOIA Office
770-488-6399

6 attachments

KASEMPIMOLPORN et al 1988.pdf
593K

MOOSAI 1984.pdf
1015K

Potgieter et al 2009.pdf
230K

HERRING et al 1982.pdf
893K

Dubal et al 2015.pdf
695K

Final Response Full Grant.pdf
147K

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

Centers for Disease Control
and Prevention (CDC)
Atlanta GA 30333

March 11, 2022

Christine Massey
[redacted]
Peterborough, ON, Canada
Via email: cmssyc@gmail.com

Dear Ms. Massey:

This letter is our final response to your Centers for Disease Control and Prevention and Agency for Toxic Substances and Disease Registry (CDC/ATSDR) Freedom of Information Act (FOIA) request of November 23, 2021, assigned #22-00402-FOIA, for:

Description of Requested Records:

Please note: *this request is very similar to another request that I submitted on March 1, 2021 (**21-00792-FOIA**) where I had **specified** purification via maceration, filtration and use of an ultracentrifuge and received a "no records" response. The difference with this new request is that it does **not** specify maceration, filtration and use of an ultracentrifuge; it mentions filtration, ultracentrifugation and chromatography and only by way of an example.*

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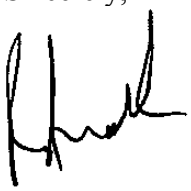
We located the attached 5 journal publications. After a careful review of these pages, no information was withheld from release.

If you need any further assistance or would like to discuss any aspect of the records provided please contact either our FOIA Requester Service Center at 770-488-6399 or our FOIA Public Liaison at 770-488-6246.

Sincerely,



Roger Andoh
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Office of the Chief Operating Officer
(770) 488-6399
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22-00402-FOIA

JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1982, p. 473–477
0095-1137/82/090473-05$02.00/0

Vol. 16, No. 3

Rapid Diagnosis of Rotavirus Infection by Direct Detection of Viral Nucleic Acid in Silver-Stained Polyacrylamide Gels

ALAN J. HERRING,* NEIL F. INGLIS, CLEMENT K. OJEH, DAVID R. SNODGRASS, AND JAMES D. MENZIES

Moredun Research Institute, Edinburgh, EH17 7JH, Scotland

Received 29 March 1982/Accepted 7 June 1982

A rapid simple technique for the diagnosis of rotavirus has been developed based on the sensitive detection of rotavirus double-stranded RNA genome segments separated in polyacrylamide gels. The method utilizes a recently described ultrasensitive silver stain for polypeptides, which can also detect subnanogram amounts of nucleic acid. The sensitivity of the technique is comparable with that of electron microscopy or enzyme-linked immunosorbent assay.

Rotaviruses cause enteritis in a wide variety of species and have been intensively investigated for more than a decade (reviewed 5, 14). As members of the *Reoviridae* (13), they have a double-stranded RNA (dsRNA) genome consisting of 11 segments ranging in molecular weight from approximately 2.0×10^6 to 0.2×10^6 (9). Electrophoretic analysis has revealed major differences in the mobility of the genome segments between virus isolates from different host species and minor differences between individual isolates from the same species. Thus, genome electropherotyping has been the most common method for both taxonomic and epidemiological studies (9, 12, 18).

Recently, several methods have been reported which utilize silver staining for the ultrasensitive detection of polypeptides resolved by polyacrylamide gel electrophoresis (PAGE) (16, 17, 20). We used one of these methods (20) to monitor the purification of rotavirus from feces and found that the dsRNA bands were also stained with high sensitivity. This result was consistent with the well-described property of silver ions to form a stable complex with nucleic acids (7). Similar nucleic acid staining with another silver staining method has recently been reported by Somerville and Wang (22).

In this communication we describe a diagnostic test for rotavirus in feces based on this ultrasensitive detection of viral dsRNA, which has the advantages of simplicity, economy, and speed, and which simultaneously identifies the electropherotype.

MATERIALS AND METHODS

Fecal specimens. Infected and control fecal specimens were obtained from cattle and human sources. The bovine samples and two of the human samples were examined for rotavirus by electron microscopy (EM) and by enzyme-linked immunosorbent assay (ELISA). The ELISA was performed essentially by the method of Yolken et al. (25), using a hyperimmune rabbit serum raised against tissue culture-grown bovine rotavirus, with a neutralization titer of 1:10,240 both to coat the wells and as a conjugate to detect antigen. Unconcentrated samples were examined by EM as described by Snodgrass et al. (21). The samples in the dilution experiment were coded and scored blind. The majority of the human specimens were kindly provided by the Edinburgh Regional Virus Laboratory. Rotavirus diagnosis had been carried out on these specimens by the cell culture method of Bryden et al. (2).

Nucleic acid extraction. Fecal samples were diluted 1:4 by weight with 0.1 M sodium acetate buffer (pH 5.0) containing 1% (wt/vol) sodium dodecyl sulfate; the normal sample size used was 0.25 g of feces, which provided enough extract for at least 10 separate analyses. An equal volume of a 3:2 (vol/vol) 'phenol'-chloroform mixture was added to the fecal suspension, and the sample was mixed for 1 min. ('Phenol' consisted of a mixture of 500 g of phenol, 70 g of *m*-cresol, and 200 g of water containing 0.5 g of 8-hydroxyquinoline.) The emulsified mixture was then centrifuged for 10 min at 1,200 × *g*, and the resulting clear upper aqueous layer was removed. A sample was then prepared for electrophoresis by the addition of 10 μl of 25% (wt/vol) sucrose containing 0.2% bromophenol blue to 40 μl of the aqueous layer.

Occasional samples failed to yield sufficient clear aqueous layer, but it was found that either further centrifugation for 3 min at 16,000 × *g* in a microcentrifuge (Mechanika Precyzyjna, type 320a) or the addition of 0.5 ml of buffer followed by remixing and centrifugation at 1200 × *g* gave an ample clear layer.

PAGE. The 50-μl samples were loaded onto 5% polyacrylamide slab gels (acrylamide-to-bis-acrylamide ratio of 37.5:1) which were polymerized with 0.01% (vol/vol) *N,N,N',N'*-tetramethylethylenediamine and 0.05% (wt/vol) ammonium persulphate. The gel and electrode buffer was 0.036 M Tris–0.03 M

sodium dihydrogen phosphate–0.001 M EDTA (pH 7.8). Gel dimensions were 14-cm wide by 19-cm long and 0.15-cm thick. It should be noted that the gel thickness is critical with the silver staining technique (20). Deep sample wells (0.6 by 2.0 cm) facilitated loading without the transfer of sample to neighboring wells. Electrophoresis was performed at room temperature for 16 h at 20 mA and 70 V. In most experiments, one of the glass plates used to form the gel mold was treated with a 1% solution of Silane 174A in ethanol (Union Carbide Corp.) for 10 min, dried in air, rinsed in distilled water, and redried. This treatment caused the gel to adhere strongly to the plate and greatly simplified its handling during staining.

Silver staining. The gels were stained by using a slight modification of the method of Sammons et al. (20). The initial fixation steps described for protein staining were omitted, and the gels were washed with 10% ethanol–0.5% acetic acid for 30 min and then soaked in 0.011 M silver nitrate for 2 h. The gel was then rinsed briefly in distilled water, and the reduction step was performed with a solution of 0.75 M sodium hydroxide containing 0.1 M formaldehyde and 0.0023 M sodium borohydride. The bands appeared at this stage, and the reduction was continued until the bands were clearly visible for a maximum of 10 min. In our early experiments the gels were then placed in 0.07 M sodium carbonate, and the intensity of staining of both the bands and the background increased slightly in the 20 min or so after transfer. After 30 min, the gels were placed in fresh carbonate solution. However, it was found that, when using gels which were stuck to a glass plate with Silane 174A, an unacceptable degree of background staining sometimes developed when the gel was placed in the carbonate solution. This could be prevented by treating the gel with a 5% acetic acid solution for 30 min after the reduction and then transferring the gel to carbonate solution for storage. Gels have been successfully stored, sealed in polythene bags, for up to 6 months.

All of the solutions for the staining were made from single distilled water and, with the exception of the initial fixation solution, were degassed before use. The solutions were used in 200-ml volumes in a single plastic staining dish, and care was taken to avoid touching the gel surface with ungloved hands. Constant agitation of the solutions throughout the procedure was achieved with a rocking bed destainer. The gels were photographed by transmitted light, using a Wratten 85B filter.

Purification of virion dsRNA. Virus was purified from infected bovine feces essentially by the method described by Todd and McNulty (24), and the dsRNA was extracted with phenol and further purified by one cycle of CF11 cellulose chromatography (6) performed as described by Bevan et al. (1). The resulting dsRNA was quantified spectrophotometrically.

RESULTS

The results obtained by direct extraction of feces with phenol followed by gel analysis of the extract are shown in Fig. 1. The first nine samples were all from a herd affected with enteritis, and eight may be clearly seen to contain the characteristic dsRNA segments of bovine rotavirus; the other three control samples were negative. As would be expected, the samples from a single outbreak all showed the same electropherotype. Other bands were seen on the gels, especially near the origin where DNA forms a diffuse band, and occasional samples produced a continuous smear of stained material; but neither of these effects interfered with the detection of the dsRNA. The rotaviral genome segments could be identified by the characteristic sharpness and unique pattern of the dsRNA bands.

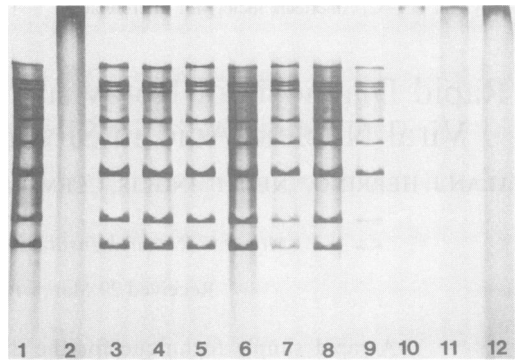

FIG. 1. Gel electrophoresis of fecal nucleic acid extracts. Tracks 1 through 9, extracts of fecal samples from diarrheic calves; tracks 10 through 12, extracts of feces from uninfected calves.

The results of a comparison between PAGE, ELISA, and EM are summarized in Table 1, together with the results of PAGE analysis on 24 human specimens which had been tested for rotavirus by cell culture. There was complete concordance between PAGE and ELISA results and only a single conflicting result in the PAGE and EM results. The level of virus in this one sample was clearly low, as the dsRNA bands were faint. The results with the human samples similarly showed just one conflicting result, which was positive by PAGE; this sample was obtained from a patient who also yielded other samples which were positive by cell culture.

The sensitivity of PAGE was investigated by dilution experiments. A positive sample judged to contain an average level of viral dsRNA was serially diluted with a negative sample to give a range of viral concentrations from 12.5 to 0.2% of that in the original sample, but with approximately the normal amount of contaminating nonviral material in each dilution. Extracts of these samples were analyzed by PAGE, and the results are shown in Fig. 2. Rotavirus dsRNA segments 1 through 4 were detected in dilutions down to 0.39%, but the lower-molecular-weight bands were not apparent at the higher dilutions. Figure 2 also shows the result of diluting the positive sample extract with electrophoresis

TABLE 1. Comparison of PAGE with other methods of rotavirus diagnosis

Specimen	No. of samples	Diagnostic method[a]				No. of samples in category
		PAGE	ELISA	EM	Cell culture	
Bovine	68	+	+	+	ND	41/68
		−	−	−	ND	26/68
		+	+	−	ND	1/68
Bovine	13	+	+	ND	ND	5/13
		−	−	ND	ND	8/13
Human	2	+	+	+	ND	1/2
		−	−	−	ND	1/2
Human	24	+	ND	ND	+	18/24
		−	ND	ND	−	5/24
		+	ND	ND	−	1/24

[a] +, Positive; −, negative; ND, test not performed.

buffer. All of the dsRNA segments were detected at a level of 0.39% of the original positive material.

A similar dilution series, using the same positive and negative feces and the appropriate buffers, was constructed and tested by ELISA and by EM (Table 2). EM was found to detect virus to a level of 1.56% of the original sample. The ELISA results are expressed as ratios of the positive and negative optical densities (P/N) as suggested by Yolken et al (25), who considered any value in excess of 2.1 to be rotavirus positive. Table 2 shows two values of P/N for each dilution. The first, and higher value, was based on the optical density given by the particular negative feces used for dilution, and the second is based on the optical density given by our standard uninfected feces. These data show the dilution series positive to the 0.39% and 1.56% levels, respectively. Thus, with the methods used, the sensitivities of PAGE, EM, and ELISA were approximately equal.

One further dilution series was investigated to determine the sensitivity of the silver staining method in absolute terms. Examination of gels loaded with a dilution series of purified dsRNA showed that the detection limit of the silver stain for a single band was 300 to 400 pg.

DISCUSSION

The diagnosis of rotavirus infection has been achieved by a variety of methods (reviewed in 10) based on either the direct visualization of the virion by EM or the detection of viral antigens by a wide diversity of immunological techniques, including the highly advanced and sensitive enzyme-linked fluorescence assay (26). The test described above is based on the direct extraction and detection of viral dsRNA. Two direct extraction methods have been reported previously, but both have been designed for epidemiological studies and genome analysis rather than for diagnosis. Clarke and McCrae (3) described a method based on end labeling of total fecal nucleic acid followed by CF11 cellulose purification of the dsRNA and analysis by PAGE and autoradiography. This procedure could detect rotavirus dsRNA with very high sensitivity, but is too protracted and expensive to use as a routine diagnostic test. Theil et al. (23) recently reported a method for the bulk extraction of dsRNA from large fecal samples (6 ml), using CF11 cellulose as a batch procedure. Their results confirmed that directly extracted dsRNA was identical to virion dsRNA. It is an indication of the increased sensitivity of the silver staining method relative to ethidium bromide fluorescence that Thiel et al. (23) used the dsRNA from 0.4 ml of feces for a single analysis, whereas we routinely use the nucleic acid from only 0.01 ml of feces.

The greatest advantages of the PAGE and silver stain method are its lack of ambiguity and

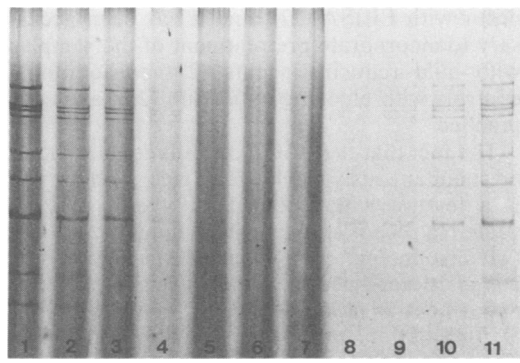

FIG. 2. Gel electrophoresis of fecal nucleic acid extracts. Tracks 1 through 7, extracts of a dilution series of bovine feces containing 12.5, 3.13, 1.56, 0.78, 0.39, 0.2, and 0% of positive sample, respectively; tracks 8 through 11, four dilutions in buffer of the nucleic acid extract of the positive feces containing 0.39, 0.78, 1.56, and 3.13% of the positive extract, respectively.

TABLE 2. EM and ELISA results on the dilution series of positive feces[a]

Positive feces in dilution (%)	Test		
		ELISA	
	EM	P/N [1]	P/N [2]
12.5	+ [1]	9.12 (+)	4.8 (+)
6.25	+ [16]	5.55 (+)	2.92 (+)
3.13	+ [1]	4.79 (+)	2.52 (+)
1.56	+ [2]	4.25 (+)	2.23 (+)
0.78	−	3.53 (+)	1.85 (−)
0.39	−	2.28 (+)	1.20 (−)
0.20	−	1.86 (−)	0.98 (−)
0	−	1.0 (−)	0.53 (−)

[a] The figures in brackets indicate the number of viral particles found in a standard 10-min search. Positive-to-negative (P/N) ratios are explained in the text. The test results (+, −) are shown in parentheses.

the fact that it provides information about viral electropherotype. Since the test detects the viral genome which has a unique number and pattern of dsRNA segments the results are unequivocal. None of the samples we have examined to date has given any spurious bands which could be confused with viral dsRNA. The only problem encountered in our early trials of the technique was the accidental transfer of sample to a neighboring well in the gel at the time of loading; certainly care is required at this stage, and accurate loading is facilitated by the use of deep sample wells. If confirmation of a weak positive result is required, the sample may be concentrated very simply by ethanol precipitation.

The unambiguous nature of a positive PAGE test contrasts with the difficulties in the interpretation of low-positive values in the ELISA. False-positive results have been reported to occur with ELISA (27), and it has been necessary to incorporate pretreatment of the samples with mild reducing agents (27) or additional controls with blocking antiserum (25), as is our practice.

The fact that the PAGE and silver stain method simultaneously produces an electropherotype is a feature which considerably enhances its value. In recent studies with a human virus the two distinct patterns which are seen for segments 10 and 11 appear to correlate with two major neutralization subgroups of the virus (4, 8). In addition, other major surveys of human viral genome electropherotypes have revealed considerable minor variations (12, 18), and one study has suggested that isolates from neonates may be distinct (18). Lourenco et al. (12) noted the limitation that their clinical samples were too small to allow multiple electrophoretic analyses. Silver staining should permit far more economical use of the dsRNA and has the added advantage that the low loadings required enhance the resolution obtained. The adoption of the gel method for diagnosis should lead to a rapid increase in our understanding of rotavirus epidemiology. The method avoids the problems posed by the recent discoveries of rotaviruses without the group antigen (15, 19) and may also reveal whether the virus can cross species barriers as was recently suggested by McNulty et al. (15).

Most of the samples we have studied to date would be suitable for electropherotype analysis without further purification, but those which give high backgrounds could be conveniently purified by CF11 chromatography (1, 6). The gel system we describe above was selected to allow rapid staining of the gel after electrophoresis and not for maximum resolution of the dsRNA. Discontinuous buffer system gels (11) give the best resolution (18), but such gels require the full fixation and washing procedure described by Sammons et al. (20) to remove sodium dodecyl sulfate. Gels containing agarose cannot be stained by this method.

The method has several other minor advantages. The initial phenol extraction is both virucidal and bacteriocidal and thus eliminates the biohazard and much of the unpleasantness associated with fecal samples. The apparatus and chemicals employed are relatively inexpensive, and there is no dependance on immunological reagents which are variable and expensive to purchase or prepare.

The use of silver staining to detect nucleic acids in such low amounts should have considerable application in the study and diagnosis of other viruses, but it is particularly applicable to dsRNA because of the very sharp bands formed by this species in PAGE and because of the ease with which it may be purified from complex mixtures by CF11 cellulose chromatography (6). We have already found the method most useful for the detection of DNA in velocity sedimentation experiments and for the analysis of small restriction enzyme fragments.

ACKNOWLEDGMENTS

We thank J. M. Inglis and M. F. Jamieson of the Edinburgh Regional Virus Laboratory for supplying the human fecal specimens, Union Carbide Corp. for their kind gift of Silane 174A, P. J. Richardson for his capable technical assistance, and B. J. Easter for photographing the gels.

LITERATURE CITED

1. **Bevan, E. A., A. J. Herring, and D. J. Mitchell.** 1973. Preliminary characterisation of two species of dsRNA in yeast and their relationship to the killer character. Nature (London) **245**:81–86.
2. **Bryden, A. S., H. A. Davies, M. E. Thouless, and T. H. Flewett.** 1977. Diagnosis of rotavirus infection by cell culture. J. Gen. Microbiol. **10**:121–125.
3. **Clarke, I. N., and M. A. McCrae.** 1981. A rapid and sensitive method for analysing the genome prolifes of field isolates of rotavirus. J. Virol. Methods **2**:203–209.

4. **Espejo, R. T., O. Munoz, F. Serafi, and P. Romero.** 1980. Shift in the prevalent human rotavirus detected by ribonucleic acid segment differences. Infect. Immun. **27**:351–354.
5. **Flewett, T. H., and G. N. Woode.** 1978. The rotaviruses. Arch. Virol. **57**:1–23.
6. **Franklin, R. M.** 1966. Purification and properties of the replicative intermediate of the RNA bacteriophage R.17. Proc. Natl. Acad. Sci. U.S.A. **55**:1504–1511.
7. **Jenson, R. J., and N. Davidson.** 1966. Spectrophotometric, potentiometric and density gradient ultrascentrifugation studies of the binding of silver ion by DNA. Biopolymers **4**:17–32.
8. **Kalica, A. R., H. B. Greenberg, R. T. Espejo, J. Flores, R. G. Wyatt, A. Z. Kapikian, and R. M. Chanock.** 1981. Distinctive ribonucleic acid patterns of human rotavirus subgroups 1 and 2. Infect. Immun. **33**:958–961.
9. **Kalica, A. R., R. G. Wyatt, and A. Z. Kapikian.** 1978. Detection of differences among human and animal rotaviruses using analysis of viral RNA. J. Am. Vet. Med. Assoc. **173**:531–537.
10. **Kurstak, E., C. Kurstak, J. van den Hurk, and R. Morisset.** 1981. Animal rotavirus, p. 105–141. *In* E. Kurstak and C. Kurstak (ed.), Comparative diagnosis of viral disease. IV. Vertebrate animal and related viruses part B—RNA viruses. Academic Press, Inc., New York.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
12. **Laurenco, M. H., J. C. Nicolas, J. Cohen, R. Scherrer, and F. Bricout.** 1981. Study of human rotavirus genome by electrophoresis: attempt of classification among strains isolated in France. Ann. Virol. (Paris) **132**:161–173.
13. **Matthews, R. E. F.** 1979. The classification and nomenclature of viruses. Intervirology **11**:133–135.
14. **McNulty, M. S.** 1979. Morphology and chemical composition of rotaviruses. Colloq. Inserm. **90**:111–140.
15. **McNulty, M. S., G. M. Allan, D. Todd, J. B. McFerran, and R. M. McCraken.** 1981. Isolation from chickens of a rotavirus lacking the rotavirus group antigen. J. Gen. Virol. **55**:405–413.
16. **Merril, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert.** 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science **211**:1437–1438.
17. **Oakley, B. R., D. R. Kirsch, and N. R. Morris.** 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. **105**:361–363.
18. **Rodger, S. M., R. F. Bishop, C. Birch, B. McLean, and I. H. Holmes.** 1981. Molecular epidemiology of human rotaviruses in Melbourne, Australia, from 1973 to 1979, as determined by electrophoresis of genome ribonucleic acid. J. Clin. Microbiol. **13**:272–278.
19. **Saif, L. J., E. H. Bohl, K. W. Theil, R. F. Cross, and J. A. House.** 1980. Rotavirus-like, calicivirus-like and 23-nm virus-like particles associated with diarrhea in young pigs. J. Clin. Microbiol. **12**:105–111.
20. **Sammons, D. W., L. D. Adams, and E. E. Nishizawa.** 1981. Ultrasensitive silver-based color staining of polypeptides in polyacrylamide gels. Electrophoresis **2**:135–141.
21. **Snodgrass, D. R., W. Smith, E. W. Gray, and J. A. Herring.** 1976. A rotavirus in lambs with diarrhea. Res. Vet. Sci. **20**:113–114.
22. **Somerville, L. L., and K. Wang.** 1981. The ultrasensitive silver 'protein' stain also detects nanograms of nucleic acids. Biochem. Biophys. Res. Commun. **102**:53–58.
23. **Theil, K. W., C. M. McCloskey, L. J. Saif, D. R. Redman, E. H. Bohl, D. D. Hancock, E. M. Kohler, and P. D. Moorhead.** 1981. Rapid, simple method of preparing rotaviral double-stranded ribonucleic acid for analysis by polyacrylamide gel electrophoresis. J. Clin. Microbiol. **14**:273–280.
24. **Todd, D., and M. S. McNulty.** 1976. Characterisation of pig rotavirus RNA. J. Gen. Virol. **33**:147–150.
25. **Yolken, R. H., H. W. Kim, T. Clem, R. G. Wyatt, A. R. Kalica, R. M. Chanock, and A. Z. Kapikian.** 1977. Enzyme-linked immunosorbent assay (ELISA) for detection of human reovirus-like agent of infantile gastroenteritis. Lancet **ii**:263–267.
26. **Yolken, R. H., and P. J. Stopa.** 1979. Enzyme-linked fluorescence assay: ultrasensitive solid-phase assay for detection of human rotavirus. J. Clin. Microbiol. **10**:317–321.
27. **Yolken, R. H., and P. J. Stopa.** 1979. Analysis of nonspecific reactions in enzyme-linked immunosorbent assay testing for human rotavirus. J. Clin. Microbiol. **10**:703–707.

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Rapid detection of enteric adenovirus and rotavirus: a simple method using polyacrylamide gel electrophoresis

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SUMMARY A simple and rapid procedure for identifying adenovirus and rotavirus in stool extracts has been developed. The technique is based on polyacrylamide gel electrophoresis of the virus nucleic acid, but sample preparation is straightforward and does not entail phenol extraction or the use of a radioactive label. Furthermore, processing is not influenced by the amount of specimen obtained and is thus suitable for application as a batch testing method. This approach removes the need for specific antisera, which are not readily available since most of these viruses cannot be grown using routine tissue culture procedures. Trials in this laboratory have indicated that the technique is of comparable sensitivity to electron microscopy.

Several groups of viruses have been implicated in the production of diarrhoeal disease in humans.[1] Of these, the rotaviruses and adenoviruses are probably the most common, although the involvement of small round viruses, both structured and featureless, should not be underestimated.[2] Consequently, there is a need to characterise as far as possible the agents responsible for a particular outbreak both for public health reasons and also to gather epidemiological data with which to assess the importance of each type of virus. These viruses were originally discovered in stools by electron microscopy, but they are difficult to isolate in tissue culture and this has led to a search for alternative methods for their detection. Recently, several diagnostic kits have been produced for the detection of rotaviruses. These are based on immunological recognition and are thus limited to the specificity of the antibodies provided. In view of the difficulties experienced in the growth of these viruses in vitro antisera are not readily available and no kit has yet been marketed which can be used to identify enteric adenoviruses. In addition, the application of these procedures is often not straightforward and may be influenced by the amount of specimen or the titre of virus it contains (Moosai *et al*, unpublished observations). In the absence of sera adequate in quality or quantity, electron microscopy has remained the only method capable of recognising all these virus groups in a single test. The method is simple and rapid, but it is insensitive and confined to viruses which have well defined morphology.

We have recently described the use of polyacrylamide gel electrophoresis (PAGE) in the differentiation of several rotavirus strains, and we have introduced a nomenclature (Rotacode) for the profiles of genomic RNA thus produced.[3] This approach has now been developed further in order to distinguish the genome of rotavirus (segmented, double stranded RNA) from that of adenovirus (linear, double stranded DNA) and thus provide a diagnostic tool for the identification of these viruses. This technique is simple, convenient, and similar in sensitivity to diagnosis by electron microscopy.

Material and methods

TREATMENT OF SPECIMENS FOR PAGE AND ELECTRON MICROSCOPY

All stool samples used in this study had been sent to the routine diagnostic laboratory (Royal Victoria Infirmary) and were obtained from children and infants admitted to hospitals in north east England. The stool samples were suspended in Hank's balanced salt solution at a concentration of 10%. This could then be analysed directly by PAGE (see below), but had to be processed further for examination in the electron microscope. Large particles were removed by low speed sedimentation (2000 rpm in a

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MSE Mistral centrifuge, 4°C) and the virus was concentrated by high speed sedimentation (45 000 rpm for 1 hour in a Beckman Sw 55 rotor at 4°C). Pellets containing virus were resuspended in electron microscopy diluent (distilled water containing 0·1% bacitracin) and applied to electron microscope grids. The preparations were negatively stained by floating the grids on 2% potassium phosphotungstate (pH 7·0) for 15 s. The surplus fluid was removed with filter paper and the grids were allowed to dry.

PAGE

Electrophoresis was performed using the discontinuous gel system described by Laemmli.[4] Resolving gels (7·5% polyacrylamide) were cast between 20 × 40 cm glass plates separated by 1·5 mm spacers, and a stacking gel (3% polyacrylamide) was then poured on top. Resolving gel mixtures contained: 20 ml of 1·5 M TRIS-HCl (pH 8·8)/4% sodium dodecyl sulphate (SDS), 20 ml of 30% acrylamide/0·8% bis-acrylamide, 38·7 ml of water. After degassing gels were polymerised with 1·2 ml of 20% ammonium persulphate and 30 μl of N,N,N′,N′-tetramethylethylenediamine (temed). The stacking gel contained: 6·25 ml of 1 M TRIS-HCl (pH 6·8)/0·8% SDS, 5 ml of 30% acrylamide/0·8% bis-acrylamide, 31·3 ml of water. Stacking gels were polymerised with 0·75 ml of 20% ammonium persulphate and 50 μl of temed.

Stool suspensions and cell lysates were prepared for electrophoresis in the same way. Each was made 5% with respect to SDS by the addition of an equal volume of 10% SDS in distilled water[6] and incubated for 30 min at 37°C to release nucleic acids and dissociate protein complexes. SDS also acts as a powerful inhibitor of nuclease activity. Then 6·25 μl of the treated sample was mixed with an equal volume of sample buffer (62 mM TRIS-HCl pH 6·8, 3% SDS, 5% 2-mercaptoethanol, and 40% glycerol) and applied to the gel (0·5 cm slot). Gels were electrophoresed for 16 h at 30 mA using a "Biorad Protean" cell operated with tap water coolant.

Nucleic acid bands were visualised using the silver staining procedure described by Herring *et al*,[5] with slight modifications. Gels were removed from the glass plates and fixed for 60 min in a solution of 50% ethanol and 10% acetic acid. Rehydration was performed in two stages, each of 60 min duration, by transfer to a solution containing 25% ethanol, 10% acetic acid, and thence to 10% ethanol, 1% acetic acid. The rehydrated gels were stained in silver nitrate (0·19% in water) for a further 60 min, rinsed in distilled water for 10 min, and immersed in a reducing solution (3% NaOH, 0·37–0·41% formaldehyde, 0·05% $NaBH_4$) for 10 min to initiate colour development. Optimum staining was achieved by transferring the gels to sodium carbonate solution (0·75%) for 5 min. This procedure stained nucleic acids black in a light brown gel matrix. Wet gels could be stored in fixing solution and kept in the dark until photographed.

DNA molecular weight markers, adenovirus 2, and bacteriophage lambda DNA, were purchased from Sigma Ltd.

GROWTH OF ADENOVIRUS TYPE 2

Adenovirus type 2, isolated from a nasopharyngeal secretion, was grown at 37°C in HeLa cells under Eagle's minimum essential medium containing 10% fetal calf serum. Sixteen hours after infection the medium was replaced with Eagle's minimum essential medium containing 2% serum and incubation was continued. The cells were harvested when the cytopathic effect was maximum (usually about four days after infection) and disrupted by three cycles of freezing and thawing. The resulting cell lysates were clarified by centrifugation (1500 rpm for 10 min) in a MSE Mistral centrifuge at 4°C and stored frozen. Uninfected HeLa cells were treated in a similar manner to provide negative controls. All these lysates were processed for electrophoresis exactly as the 10% stool suspensions described above.

NUCLEASE DIGESTION

Samples for nuclease digestion were precipitated from the SDS treated preparations with 2·5 volumes of ethanol and collected by centrifugation (10 000 *g* for 10 min). Nucleic acids were resuspended in 1·5 M NaCl, 0·15 M sodium citrate, for treatment with RNAse A (Sigma, 100 μg/ml) and T1 (Sigma, 100 μg/ml), or in 0·1 M TRIS-HCl (pH 7·5), 0·1 M NaCl, 15 mM $MgCl_2$, for treatment with DNAse 1 (Sigma, 50 μg/ml). Reaction mixtures were incubated at 37°C for 30 min (RNAse) or 28°C for 2 h (DNAse). Treated samples were mixed with an equal volume of sample buffer and analysed by electrophoresis.

Results

The protocol described above could be applied routinely to all samples; concentration or dilution according to virus titre was not required. With this procedure, samples containing rotaviruses displayed all the double stranded RNA segments of the virus genome as previously reported.[3] Stool samples which were known from electron microscopy to contain adenoviruses gave a pattern which was clearly distinguishable from that produced by samples containing rotaviruses, reoviruses, or phage lambda (Fig. 1). Gels run in this manner sometimes showed a diffuse band about halfway down; this was appar-

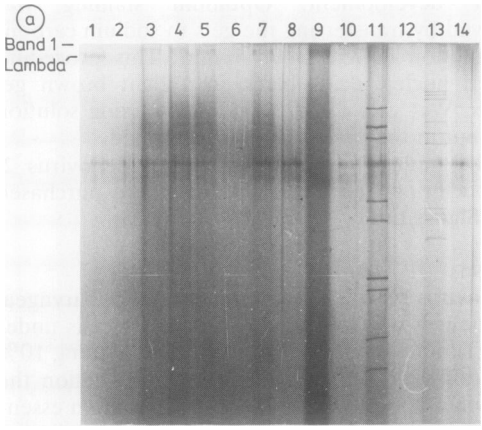

Fig. 1a *Analysis of stools containing rotavirus. Stool samples containing rotavirus (tracks 11 and 14), adenovirus (tracks 3–5, 7, 9), or those in which no virus could be detected by electron microscopy (2, 6, 8, 10, 12) were electrophoresed in a 7·5% polyacrylamide gel as described in the text. Nucleic acids prepared from viruses grown in vitro were used as markers: reovirus type 1 (track 13), phage lambda (track 1). The uppermost band (band 1) produced by samples containing adenoviruses is marked. The diffuse band present in all tracks is an artefact of electrophoresis.*

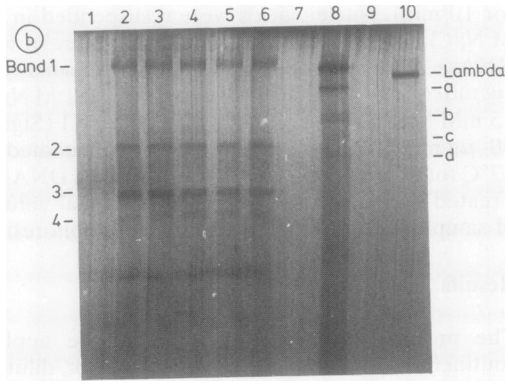

Fig. 1b *Comparison of bands produced by stools containing adenovirus (tracks 2–6) with those obtained using adenovirus 2 grown in vitro (track 8) and stools in which no virus was detected by electron microscopy (tracks 1 and 7). Small molecular weight species seen in adenovirus positive stools (bands 2–4) and the different species detected in adenovirus preparations grown in vitro (a–d) are indicated. The diffuse band present in all tracks is an artefact of electrophoresis.*

ent even when no sample was applied and represented an artefact of electrophoresis. Adenovirus positive stool preparations produced a prominent band (band 1 in Fig. 1a, b) near the top of the gel and several others (bands 2–4) of lower molecular weight (Fig. 1b). Virus grown in tissue culture also produced additional bands (a–d) which were different from those seen in the stool samples (Fig. 1b). The high molecular weight band (band 1) had an almost identical mobility to that observed when adenovirus infected cell lysates or commercially available preparations of adenovirus 2 DNA were analysed on the same gel (Fig. 2). The silver staining procedure is able to detect both protein and nucleic acid, but the high molecular weight band 1, observed in these experiments, was also found to fluoresce when stained with ethidium bromide (not shown), thus confirming that it consisted of nucleic acid. Furthermore, this band was not seen when samples derived from uninfected cells or stools in which no adenovirus had been detected were used (Fig. 1a, b). Consequently, it was likely that the high molecular weight band seen in samples containing adenovirus represented the virus genomic DNA. This was confirmed by testing specimens for DNA content using specific nucleases. The high molecular

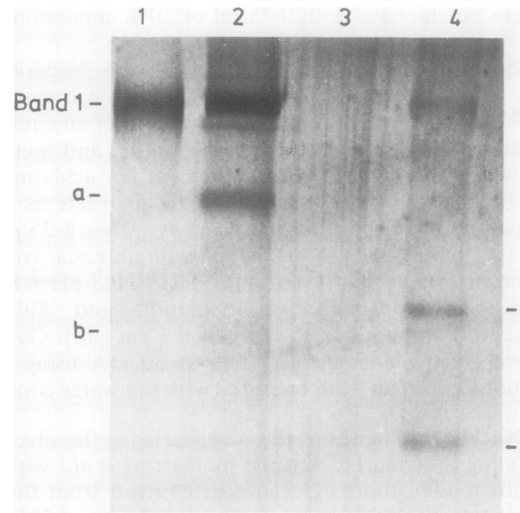

Fig. 2 *Identification of adenovirus DNA. Adenovirus DNA samples were electrophoresed on a 7·5% polyacrylamide gel: track 1, purified adenovirus 2 DNA (Sigma Ltd); track 2, adenovirus 2 grown in tissue culture in this laboratory; track 3, stool sample containing no detectable adenovirus; track 4, stool sample containing enteric adenovirus. Additional DNA bands are visible in both the tissue culture grown virus (a, b) and the enteric virus sample (2, 3).*

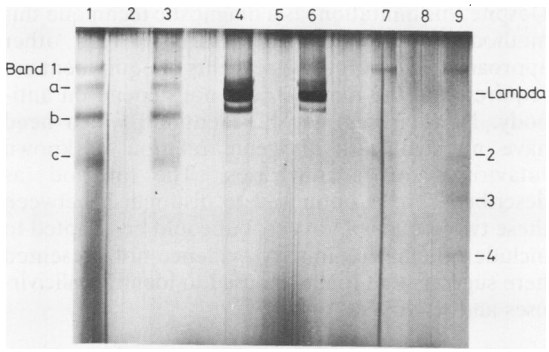

Fig. 3 *Nuclease treatment. Effect of nuclease treatment on bands produced by adenovirus or lambda DNA. Tissue culture grown adenovirus, tracks 1–3; bacteriophage lambda DNA, tracks 4–6; enteric adenovirus, tracks 7–9. Samples applied to tracks 2, 5, and 8 have been treated with DNAse 1. Samples applied to tracks 3, 6, and 9 have been treated with RNAses A and T1. Additional bands present in the tissue culture grown adenovirus (a–c) and the enteric virus (2–4) are also marked.*

weight band was resistant to ribonucleases A and T1 but sensitive to deoxyribonuclease. A purified preparation of bacteriophage lambda DNA (MW 31 × 10^6) was used as a positive control in these experiments and showed an identical pattern of sensitivity to digestion by these enzymes (Fig. 3). We therefore concluded that band 1 consisted of DNA which displayed electrophoretic migration properties almost identical to those of adenovirus genomic DNA and which could be used as a diagnostic feature for the detection of this virus group. The lower molecular weight bands were also stained by ethidium bromide and digested by DNAse (Fig. 3), thus showing that they were also DNA. Although found only in association with band 1 DNA, they varied widely in amount, and thus were not considered suitable for use in virus identification. Consequently, these bands have not been investigated further. The lambda DNA migrated with an anomalously high mobility in this SDS-PAGE system and formed a band below the adenovirus DNA, which is known to be smaller (MW 25 × 10^6). This was found to be an artefact of PAGE. Using the same samples the adenovirus DNA migrated more rapidly than the lambda DNA in 0·5% agarose gels, as expected.

The efficiency of diagnosis by PAGE was compared with that of diagnosis using the electron microscope by conducting a "blind" trial. Sixty stool samples known to be either positive (44) or negative (16) for adenovirus by electron microscopy were coded by one worker and then tested by PAGE. In all cases samples found to contain adenoviruses by microscopy were correctly identified by PAGE. In one case a sample which was negative for virus by electron microscopy was positive for adenovirus by PAGE. This sample was then re-examined in the microscope and adenovirus particles were found, although in lower numbers than in the other samples. Consequently, both methods agreed in all cases examined.

Discussion

We have described a method which we believe could provide a rapid and reliable means of detecting adenoviruses and rotaviruses in stool suspensions. PAGE has been applied to this problem previously, but has suffered from the necessity to prepare pure nucleic acid which could then be radiolabelled in vitro. This was a cumbersome technique which required expensive reagents.[6] The use of the silver staining procedure has removed this limitation and permitted the use of simple nucleic acid extraction procedures. Deproteinisation is not required; after combination with SDS proteins acquire a large negative charge and presumably migrate through the gel more rapidly than the nucleic acids. Stool samples are also rich in protease[7] so that any high molecular weight proteins are probably degraded in these specimens. Protease activity would also help inactivate nucleases in the stools. These factors may explain the surprising effectiveness of this approach and the clarity of the results.

The finding of DNA species of lower molecular weight is as yet unexplained. These molecules, found in stool samples containing adenovirus, were of similar size, although they often varied in amount. They have never been detected in specimens containing rotavirus or in samples negative for both viruses. Consequently, they do not seem likely to cause any confusion in the identification of stool viruses. The stools used in these experiments in which no virus could be detected (negative stools) were obtained from patients with gastrointestinal symptoms. This makes it unlikely that such additional bands have arisen as a byproduct of the illness. Their association with enteric adenovirus specimens suggests that they may represent incomplete adenovirus or adeno-associated virus genomes. The extra bands noted in preparations of adenovirus grown in tissue culture may also represent incomplete genomes. These species of lower molecular weight were not considered in the diagnosis of adenovirus. Band 1 DNA was sufficient for this purpose and the method was of equal sensitivity to electron microscopy when applied to clinical specimens. This technique should be suitable for routine specimen testing since samples are all treated in the same manner. The virus specific DNA bands were

identified by the use of specific nucleases, but this is not necessary on a routine basis. Provided marker nucleic acids are used, we have found that the identification of adenovirus DNA may be made on the basis of comigration alone. Each gel should therefore include a sample of commercially obtained adenovirus DNA as well as stool samples known to contain adenovirus and rotavirus as markers. Any artefactual effect on the migration of adenovirus DNA in PAGE is fully reproducible, and it is not therefore necessary to use agarose gels as well. Furthermore, the silver staining method is not applicable to agarose gels and it is more complicated to ensure sufficient material is loaded for clear detection by ethidium bromide staining.

The findings presented here suggest that the PAGE test could be used instead of electron microscopy for routine diagnosis. However, at least in the early stages, it is anticipated that both techniques would be used in parallel. Later on, only samples showing unusual PAGE patterns—for example, the atypical rotaviruses—need be rechecked in the electron microscope.

In the case of the rotaviruses, different virus strains may be identified by the particular pattern the segmented genome produces on electrophoresis (electrophoretype). This may be useful in assessing the epidemiological importance of individual strains. A similar analysis is not possible for adenoviruses because their genome is not segmented and differences in mobility between strains are small or absent. Consequently, the further investigation of adenovirus epidemiology will depend on the identification of individual virus strains, and we are currently extending these experiments to permit such identification using restriction enzymes. Despite this limitation, as a diagnostic technique this method has major advantages over other approaches: no expensive reagents or equipment are required and the method does not depend on antibody. New viruses may be identified which need have no significant antigenic relation to known rotaviruses or adenoviruses. The method as described here is optimised to distinguish between these two groups of viruses, but could be adapted to include others. Preliminary evidence not presented here suggests that it may be used to identify caliciviruses and reoviruses.

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References

[1] Holmes IH. Viral gastroenteritis. *Prog Med Virol* 1979;**25**:1–36.
[2] Madeley CR. Viruses in the stool. *J Clin Pathol* 1979;**32**:1–10.
[3] Moosai RB, Alcock R, Madeley CR. A cryptogram for recording rotavirus strains: the rotacode. *J Hyg (Camb)* (in press).
[4] Laemmli UK. Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature* 1970;**227**:680–5.
[5] Herring AJ, Inglis NF, Ojeh CK, Snodgrass DR, Menzies JD. Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver stained polyacrylamide gels. *J Clin Microbiol* 1982;**16**:473–7.
[6] Clarke IN, McCrae MA. A rapid and sensitive method for analysing the genome profiles of field isolates of rotavirus. *J Virol Meth* 1981;**2**:203–9.
[7] Moosai RB, Gardner PS, Almeida JD, Greenaway MA. A simple immunofluourescent technique for the detection of human rotavirus. *J Med Virol* 1979;**3**:189–94.

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Polyacrylamide Gel Electrophoresis and Silver Staining for Detection of Rotavirus in Stools from Diarrheic Patients in Thailand

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Detection of rotavirus by polyacrylamide gel electrophoresis in combination with silver staining and by enzyme-linked immunosorbent assay showed 96.7% identical results in tests with 1,304 stool specimens from diarrheic patients. The polyacrylamide gel electrophoresis method can be modified to reduce cost and working time. Phenol extraction of stools, however, is essential in maintaining the sensitivity of the method.

Diarrheal diseases have been recognized as one of the major health problems in Thailand, and rotavirus is responsible for most diarrheal episodes in infants and young children (9). Laboratory diagnosis of rotaviral infection in Thailand depends mainly on enzyme-linked immunosorbent assay (ELISA) and occasionally on electron microscopy. Both methods require expensive instruments, materials purchased from abroad, and highly skilled technicians. Imported commercial diagnostic kits are simple but too expensive for routine diagnosis. For such reasons, only a few laboratories in Thailand are able to diagnose rotaviral infections, and this limited number is insufficient to serve the demand from hospitals and clinics all over the country.

We considered that the technique for detection of the rotaviral double-stranded RNA genome in stool specimens by polyacrylamide gel electrophoresis (PAGE) in combination with silver staining as developed by Herring et al. (3) is simple and inexpensive and can be established in small diagnostic laboratories. The method was tested for its suitability in routine detection of rotavirus in stool samples collected from diarrheic patients who visited hospitals in Bangkok and Nondhaburi from June 1982 to July 1986. The diagnostic potential of PAGE with silver staining was evaluated by comparison with the ELISA method adapted from that previously described by Yolken et al. (11).

In the ELISA, 25 μl of a 10% stool suspension and 75 μl of phosphate-buffered saline–Tween containing 0.01 M disodium EDTA were added in duplicate to each well of a polystyrene microtiter plate (Nunc Immunoplate IF with certificate) precoated with rabbit anti-human rotavirus serum (Dakopatts a/s-Denmark), and the plate was incubated overnight at 4°C. After the plate was washed with phosphate-buffered saline–Tween, rabbit anti-human rotavirus serum conjugated with horseradish peroxidase (Dakopatts a/s-Denmark) was added, and the mixture was incubated for 90 min at 37°C. Each well was washed before the addition of *o*-phenylenediamine substrate (Sigma), and the plate was incubated for 30 min at room temperature before the addition of 2 N H_2SO_4. The result was determined by both visual reading of the color change and measurement of the A_{490} of the well contents with a Titertek Multiskan photometer.

The PAGE and silver staining techniques used were based on those of Herring et al. (3) and Rodger and Holmes (7). A 0.5-ml amount of a 0.1 M sodium acetate solution containing 1% sodium dodecyl sulfate and a 0.5-ml amount of a phenol-chloroform mixture were added to 50 to 100 mg of a fecal specimen in a 1.5-ml microcentrifuge tube. The mixture was shaken vigorously on a Vortex mixer and centrifuged in a microcentrifuge at 6,000 to 7,000 rpm for 2 min. The clear upper aqueous layer containing viral double-stranded RNA was removed for electrophoresis on a polyacrylamide slab gel. The discontinuous system of Laemmli (4) was used, and sodium dodecyl sulfate was omitted from all of the buffers. A 10% polyacrylamide separating gel with a 3% stacking gel was used. The gel was formed between two glass plates approximately 16 cm long and 14 cm wide. Each gel was 0.75 mm thick and contained 15 sample wells. Each well was carefully loaded with 20 μl of sample (RNA extract) mixed with 10 μl of sample buffer (0.5 M Tris base [pH 6.8], 20% glycerol, 0.1% bromophenol blue). Electrophoresis was carried out at room temperature for 3 to 5 h at a constant current of 20 to 25 mA per gel. Finally, the separated double-stranded RNAs in the slab gel were visualized by silver staining.

Each of the 1,304 stool specimens was coded and separately diagnosed by ELISA and PAGE, which were performed with blinds by different investigators. A complete concordance of the ELISA and PAGE results was found in 1,261 (96.7%) of the tested specimens (Table 1). The remaining 43 samples (3.3%) showed opposite results. Seventeen ELISA-negative samples (1.3%) were clearly shown to be rotavirus positive by a single PAGE test. This demonstrated the sensitivity of PAGE over the ELISA method with some fecal samples. The failure to detect rotaviral antigen despite a high viral RNA content might have been due to interference caused by blocking factor(s) present in some stool specimens (10), to the loss of ELISA activity after long-term storage, or to freezing and thawing of fecal samples before the ELISA (1, 6). However, 5 of these 17 fecal samples contained non-group A rotaviruses, as demonstrated by their genomic profiles. These viruses could not be detected, of course, by immunological methods designed for only group A rotaviruses. The role of non-group A rotaviruses in causing gastroenteritis in Thailand has not been studied. The PAGE method is used now as a tool to monitor the emergence of pararotaviral diarrhea, which might become as important in Thailand as it did in Guangxi, China (8).

Twenty-six samples (2.0%) with positive ELISA results were shown to be PAGE negative even after concentration of viral nucleic acid in the samples by alcohol precipitation.

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TABLE 1. Comparison of ELISA and PAGE for detection of rotavirus in 1,304 fecal samples

Specimen group[a]	No. of specimens (%)
Identical ELISA and PAGE results	
ELISA+, PAGE+	325 (24.9)
ELISA−, PAGE−	936 (71.8)
Conflicting ELISA and PAGE results	
ELISA+, PAGE−	26 (2.0)
ELISA−, PAGE+	17 (1.3)

[a] +, Positive; −, negative.

Examination of 11 of these specimens by electron microscopy rarely showed rotaviral particles; this reflected the low content of viral RNA in fecal samples which PAGE could not detect.

The PAGE system was found to be simple enough to establish in small laboratories, in which facilities and budgets are limited. The requirements are merely a vertical slab-gel electrophoretic system and a supply of power which can be produced locally at low cost. It does not depend on immunological reagents, which are expensive or difficult to prepare and store, to maintain quality. For PAGE analysis of one fecal specimen, the expense for chemicals used in preparing the gel, buffer solution, and silver staining was approximately US $0.20. This cost decreased proportionally when the slab-gel size was reduced or when the reservoir buffer solution was used repeatedly. A mini-slab gel 8 by 10 cm in size containing 12 to 15 sample wells showed the same diagnostic results. Reduction of slab-gel and buffer chamber size reduced both the amount of buffer solution needed and the running time (2.5 h at 15 mA).

Phenol is the only expensive reagent used and it is essential for maintaining the sensitivity of the PAGE test. Dolan et al. (2) reported that stool suspensions without phenol-chloroform preextraction could be examined for rotavirus by PAGE analysis and that PAGE analysis yielded diagnostic results comparable to those of electron microscopy and ELISA (Rotazyme; Abbott Laboratories) methods. To evaluate the sensitivity of this simplified method, 53 rotavirus-positive fecal specimens were examined by the techniques of Herring et al. (3) and Dolan et al. (2). As recommended in the procedure of Dolan et al., a 20% suspension of stool was made in reducing buffer containing 2% sodium dodecyl sulfate, 5 M urea, 20% glycerol, and 4% 2-mercaptoethanol in Tris hydrochloride buffer. This mixture was vortexed, heated at 68°C for 5 min, and vortexed again. The gel was run in the discontinuous system of Laemmli (4) at a 35-mA constant current for 5.5 h. Only 48 of the tested samples (90.6%) were positive by the method of Dolan et al., while all 53 samples were clearly positive by the method of Herring et al. In addition, some stool samples, even though positive by the method of Dolan et al., exhibited weaker staining of RNA bands than by the method of Herring et al. (lanes D and F, Fig. 1). The intensity of silver staining did not increase even when the stool suspension heating time was extended to 10 or 20 min before loading onto the gel. The results show that although rotavirus in most stool samples can be detected by the method of Dolan et al., phenol-chloroform extraction is still essential for some specimens for a sufficient amount of rotaviral RNA to be released and detected. In addition, a thorough deproteinization with phenol might also prevent the RNA from sticking at the top of the gel (5).

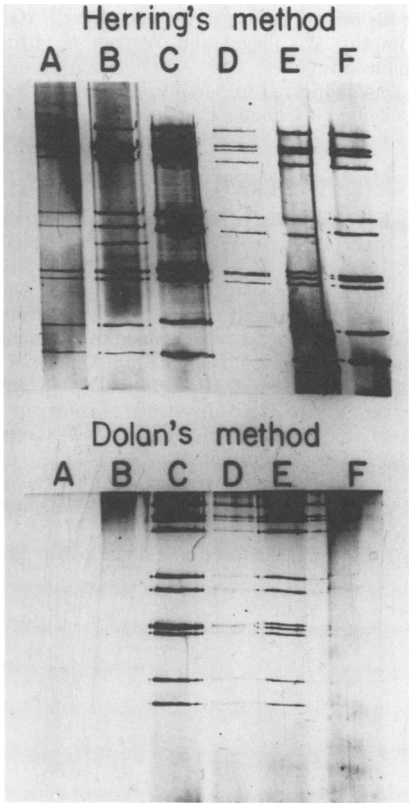

FIG. 1. Comparison of PAGE diagnostic results of the techniques of Herring et al. (3) and Dolan et al. (2) with 6 of the 53 rotavirus-positive fecal samples randomly selected for this purpose. Although the same amounts of fecal samples were used in each technique, only the method of Herring et al. demonstrated RNA in the samples in lanes A and B.

Another drawback of the method of Dolan et al. is the high electrical resistance in the electrophoretic system owing to the presence of 0.1% sodium dodecyl sulfate in both the polyacrylamide and the reservoir buffer. Thus, the separation of RNA segments required more running time and a higher electrical current than was the case in the system of Herring et al.

High-speed centrifugation (7,000 rpm, 2 min) of stool suspensions after phenol-chloroform extraction has been used to accelerate the separation of phenol-chloroform from the aqueous fraction containing viral RNA. Such separation occurred spontaneously without loss of viral RNA content when the mixture was left at room temperature for 5 to 10 min. This separation eliminated the need for a centrifuge, which might not always be available in small laboratories.

Aside from the simplicity, economy, and efficacy of rotaviral and pararotaviral diagnosis, PAGE also provided additional information, such as the detection of rotaviral double infection in three tested specimens and the detection of reinfection with two different strains of rotavirus in a patient. The PAGE method is now being used in Thailand as a tool for rapid identification of rotaviral strains while the serotyping technique is being established.

We acknowledge the staffs of the Siriraj Hospital, The Children's Hospital, and the Bamrasnaradura Infectious Diseases Hospital for their generous help in providing us with stool specimens. We are grateful to Suranga Saguanwongse for her electron microscopy diagnosis and to Ian H. Holmes (University of Melbourne, Victoria,

Australia) for his helpful advice in the use of the PAGE technique. We thank Timothy W. Flegel and Warren Y. Brockelman for reading the manuscript.

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LITERATURE CITED

1. **Bastardo, J. W., and I. H. Holmes.** 1980. Attachment of SA-11 rotavirus to erythrocyte receptors. Infect. Immun. **29**:1134–1140.
2. **Dolan, K. T., E. M. Twist, P. Horton-Slight, C. Forrer, L. M. Bell, Jr., S. A. Plotkin, and H. F. Clark.** 1985. Epidemiology of rotavirus electropherotypes determined by a simplified diagnostic technique with RNA analysis. J. Clin. Microbiol. **21**:753–758.
3. **Herring, A. J., N. F. Inglis, C. K. Ojeh, D. R. Snodgrass, and J. D. Menzies.** 1982. Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. J. Clin. Microbiol. **16**:473–477.
4. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
5. **Loening, U. E.** 1967. The fractionation of high-molecular-weight ribonucleic acid by polyacrylamide-gel electrophoresis. Biochem. J. **102**:251–259.
6. **Middleton, P. J., M. D. Holdaway, M. Petric, M. T. Szymanski, and J. S. Tam.** 1977. Solid-phase radioimmunoassay for the detection of rotavirus. Infect. Immun. **16**:439–444.
7. **Rodger, S. M., and I. H. Holmes.** 1979. Comparison of the genomes of simian, bovine, and human rotaviruses by gel electrophoresis and detection of genomic variation among bovine isolates. J. Virol. **30**:839–846.
8. **Shusheng, W., C. Rifen, C. Jie, L. Rongjian, and J. Rensheng.** 1985. Etiologic studies of the 1983 and 1984 outbreaks of epidemic diarrhea in Guangxi. Intervirology **24**:140–146.
9. **Wasi, C., S. Louisirirotchanakul, K. Thakerngpol, S. Satrasook, M. Surakhaka, W. Varavithya, and P. Thongcharoen.** 1984. The epidemiological study on viral diarrhoea in Thailand. J. Med. Assoc. Thail. **67**:369–376.
10. **Watanabe, H., I. D. Gust, and I. H. Holmes.** 1978. Human rotavirus and its antibody: their coexistence in feces of infants. J. Clin. Microbiol. **7**:405–409.
11. **Yolken, R. H., H. W. Kim, T. Clem, R. G. Wyatt, A. R. Kalica, R. M. Chanock, and A. Z. Kapikian.** 1977. Enzyme-linked immunosorbent assay (ELISA) for detection of human reovirus-like agent of infantile gastroenteritis. Lancet **ii**:263–267.

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Improved strategies for sequence-independent amplification and sequencing of viral double-stranded RNA genomes

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This paper reports significant improvements in the efficacy of sequence-independent amplification and quality of sequencing of viruses with segmented double-stranded RNA (dsRNA) genomes. We demonstrate that most remaining bottlenecks in dsRNA virus genome characterization have now been eliminated. Both the amplification and sequencing technologies used require no previous sequence knowledge of the viral dsRNA, there is no longer a need to separate genome segments or amplicons and the sequence-determined bias observed in cloning has been overcome. Combining very efficient genome amplification with pyrophosphate-based 454 (GS20/FLX) sequencing enabled sequencing of complete segmented dsRNA genomes and accelerated the sequence analysis of the amplified viral genomes. We report the complete consensus sequence of seven viruses from four different dsRNA virus groups, which include the first complete sequence of the genome of equine encephalosis virus (EEV), the first complete sequence of an African horsesickness virus (AHSV) genome determined directly from a blood sample and a complete human rotavirus genome determined from faeces. We also present the first comparison between the complete consensus sequence of a virulent and an attenuated strain of AHSV1. Ultra-deep sequencing (>400-fold coverage) of the AHSV1 reference and attenuated strains revealed different ratios of reassortants in the reference strain and allowed quasispecies detection in the plaque-purified attenuated strain of AHSV1. This approach amounts to a paradigm shift in dsRNA virus research, since it is sensitive and specific enough for comprehensive investigations of the evolution and genetic diversity in dsRNA virus populations.

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INTRODUCTION

The double-stranded RNA (dsRNA) viruses are a large group of viruses that are classified in seven virus families: *Birnaviridae*, *Chrysoviridae*, *Cystoviridae*, *Hypoviridae*, *Partitiviridae*, *Reoviridae* and *Totiviridae*. These viruses infect a large variety of hosts including humans, animals, plants, fungi and bacteria (Mertens *et al.*, 2005). Infection

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AM883164–AM883173, FJ011107–FJ011116, FJ196584–FJ196593 and FJ183353–FJ183393 (given in Table 2).

A supplementary figure and the full technical protocol for sequence-independent amplification of viral dsRNA genomes are available with the online version of this paper.

with pathogenic dsRNA viruses can lead to human and animal deaths and often cause great financial losses. The viral dsRNA genomes are composed of 1–12 segments that range in size from about 200 to 6800 bp. The size of their genomes generally varies between 5000 and 20 000 bp (Mertens, 2004; Mertens *et al.*, 2005; Roy *et al.*, 1994).

Over the past 25 years there have been steady advances in techniques for the cloning, amplification and sequencing of the genomes of dsRNA viruses (Attoui *et al.*, 2000; Bigot *et al.*, 1995; Cashdollar *et al.*, 1982; Lambden *et al.*, 1992; Maan *et al.*, 2007; Potgieter *et al.*, 2002; Rao *et al.*, 1983; Vreede *et al.*, 1998). The most recent improvements achieved cDNA synthesis of the large (>2000 bp) dsRNA genome segments, the preparation of cDNA and cloning of genome sets using single one-tube reactions for oligo-ligation, cDNA synthesis and PCR (Potgieter *et al.*, 2002), as well as the increase of specificity by the introduction of 'anchor primers' which prime themselves for cDNA synthesis (Maan *et al.*, 2007). To date, sequencing of amplified genomes and genome segments has only been achieved by sequencing either individual cloned single genome segments (Attoui *et al.*, 2000; Lambden *et al.*, 1992; Potgieter *et al.*, 2002; Vreede *et al.*, 1998) or purified amplicons of individual genome segments (Maan *et al.*, 2007). Both approaches require the separation, purification and 'primer walking' of clones, amplicons or individual genome segments and primers specific for known sequences or conserved terminal ends (Maan *et al.*, 2007).

This paper reports the first experimental evidence that complete sets of cDNA amplicons and the full-length sequence of viral dsRNA genomes (approx. 20 000 bp) can be obtained directly from field and clinical samples (organs, blood and faeces) without any prior virus propagation, knowledge of sequence information, cloning or separation of amplified cDNA. This is achieved by virtue of significant improvement in the specificity and sensitivity of cDNA amplification of dsRNA viral genomes combined with sequencing in microfabricated high-density picolitre reactors on a massive parallel scale (Margulies *et al.*, 2005), by using GS20/FLX technology (Roche Applied Science). Massive parallel sequencing (more than 400-fold coverage) of the African horsesickness virus (AHSV)-1 reference and attenuated strains demonstrated that these technologies are sensitive and specific enough to reveal sequences and ratios of mixtures of reassortants containing different segments in viral populations. It was also possible to detect viral quasispecies. Finally, we discuss the use of consensus and quasispecies sequence information from virulent and attenuated AHSV populations to assess which factors are involved in viral tropism and virulence of AHSV. This is the first truly robust, generally applicable approach which is sensitive and specific enough to start comprehensive investigations into the genetic diversity in dsRNA virus populations.

METHODS

Viruses. The viruses used in this study and their passage history are given in Table 1. The AHSV, bluetongue virus (BTV), equine encephalosis virus (EEV) and epizootic hemorrhagic disease virus (EHDV) strains used in this study were propagated in baby hamster kidney (BHK)-21 cells grown in Eagle's minimal essential medium (BioWhittaker) in 75 cm^2 flasks, unless indicated otherwise in Table 1. The AHSV and EEV strains, as well as the organs and blood from horses that died of AHSV and the faeces of a calf suffering from rotavirus diarrhoea, were obtained from the OIE World Reference Centre for AHSV and BTV at the Agricultural Research Council–Onderstepoort Veterinary Institute, South Africa. The human rotavirus G9P[6] strain used in this study was kindly provided by Mrs Ina Peenze from the virus collection of the Diarrhoeal Pathogens

Table 1. Origin, passage history and amount of sequence data generated for the viruses used in this study

Eq Spln, Equine spleen; A, adult mice; S, suckling mice; LP, large plaque isolation; KC, KC cells. The raw sequence data are available on request.

Virus	Country of origin	Passage history	Genome sequencer	Company	Area on large PTP	Sequence generated (bp)	Average read length (bp)
AHSV1 reference strain [HS29/62]	South Africa	Eq Spln, 3 S, 2 BHK	GS20	Roche	2 × 1/4	10 024 679	102
AHSV1 attenuated strain	South Africa	Eq Spln, 3 S, 10 BHK, 3 LP, 5 Vero, 1 BHK	GS20	Roche	2 × 1/4	9 448 801	102
Low passage of AHSV1 reference strain [HS29/62]	South Africa	Eq Spl, 1 A, 1 S	GSFLX	Inqaba Biotec	1 × 1/16th	2 514 441	201
AHSV2 Lagos	Nigeria	None, from blood	GSFLX	Inqaba Biotec	1 × 1/16th	2 989 035	232
BTV8 Netherlands	Netherlands	1 KC, 3 BHK	GS20	DYN	1 × 1/16th	901 109	101
EEV Bryanston (HS103/06)	South Africa	1 Vero, 1 BHK	GS20	Inqaba Biotec	1 × 1/8th	2 182 244	101
Human rotavirus G9P[6]	South Africa	None, from faeces	GS20	Inqaba Biotec	2 × 1/16th	951 275	105

Research unit, University of Limpopo, Medunsa, South Africa. The BTV strain used in this study was kindly provided by Professor Peter Mertens from the OIE Reference Centre for BTV, Pirbright, UK.

dsRNA preparation. Total RNA was extracted from cell culture, lung, spleen, faeces or blood using the commercial guanidinium isothiocyanate reagent TRI-REAGENT–LS (Molecular Research Centre), according to the manufacturer's protocol. Single stranded RNA (ssRNA) was removed by precipitation with 2 M LiCl (Sigma) at 4 °C for 16 h followed by centrifugation at 16 000 ***g*** for 30 min. dsRNA was purified from the resulting supernatant using a MinElute gel extraction kit (Qiagen). Where visible, the integrity of dsRNA was evaluated after separation on a 1 % agarose gel (TBE) stained with ethidium bromide. dsRNA of the bovine rotavirus was subjected to SDS-PAGE using 9 % acrylamide for separation (Laemmli, 1970) and silver stained.

Oligo design. An 'anchor primer', PC3-T7 loop, similar to that described by Maan *et al.* (2007), was used in ligation. PC3-T7 loop (5′-p–GGATCCCGGGAATTCGGTAATACGACTCACTATATTTTTAT-AGTGAGTCGTATTA–OH-3′) was synthesized by Tib Molbiol.

Oligo-ligation. PC3-T7 loop (200 ng) was ligated to dsRNA (0.4–200 ng) in 50 mM HEPES/NaOH, pH 8.0 (Sigma), 18 mM $MgCl_2$ (Separations), 0.01 % BSA (TaKaRa), 1 mM ATP (Roche), 3 mM DTT (Roche), 10 % DMSO (Sigma), 20 % polyethyleneglycol $(PEG)_{6000}$ (BDH) and 30 U T4 RNA ligase (TaKaRa) in a final volume of 30 µl. Ligation was performed at 37 °C for 16 h. Ligated dsRNA was purified using MinElute Gel extraction columns following the manufacturer's recommendations (Qiagen).

Sequence-independent cDNA synthesis and PCR amplification. Purified ligated dsRNA was denatured by the addition of 300 mM methyl mercury hydroxide (MMOH; Alfa Aesar) to a final concentration of 30 mM. Alternatively, dsRNA was denatured by the addition of DMSO to a final concentration of 15 % (v/v), heating in a thermal cycler at 95 °C for 2 min and snap-freezing in an ice-water slurry. However, denaturation with MMOH is a lot more efficient than with DMSO and heat, so it is, therefore, the method of choice when only very small amounts of starting material are available. cDNA was reverse transcribed in a cDNA reaction containing 50 mM Tris/HCl, pH 8.3 (Sigma), 10 mM $MgCl_2$ (Separations), 70 mM KCl (Sigma), 30 mM β-mercaptoethanol (Sigma), 1 mM dNTPs (TaKaRa) and 15 U cloned AMV reverse transcriptase (Invitrogen). The reaction was incubated in a thermal cycler at 42 °C for 45 min followed by 55 °C for 15 min. After cDNA synthesis, the excess RNA was removed by adding NaOH (Sigma) to a final concentration of 0.1 M and incubation in a thermal cycler at 65 °C for 30 min. Before cDNA annealing, Tris/HCl, pH 7.5 (Sigma), was added to a final concentration of 0.1 M followed by the addition of HCl (Sigma) to a final concentration of 0.1 M. The cDNA was annealed at 65 °C for at least 1 h.

Amplification of cDNA was performed using primer PC2 (5′-p–CCGAATTCCCGGGATCC-3′) which contains the restriction enzyme sites for *Eco*RI, *Sma*I/*Xma*I and *Bam*HI to facilitate cloning and subcloning of amplified cDNA. The PCR mixture contained 1 × Ex Taq buffer, 0.2 mM dNTPs (TaKaRa), 5 µl cDNA and 2.5 U TaKaRa Ex Taq. The first step during cycling was 72 °C for 1 min to fill incomplete cDNA ends to produce intact DNA. This was followed by an initial denaturation step of 94 °C for 2 min followed by 15–25 cycles of 94 °C for 30 s, 67 °C for 30 s and 72 °C for 4 min (or 1 min per kb of the largest segment). A final extension step of 72 °C for 5 min was included. For increased fidelity, Phusion polymerase (Finnzymes) was used instead of TaKaRa Ex Taq. Amplified cDNA products were viewed after separation on 1 % agarose gels (TBE) containing ethidium bromide. The complete technical protocol for the sequence-independent amplification of viral dsRNA genomes and a schematic representation of this (Supplementary Fig. S1) are available in JGV Online.

Sequencing using GS20/FLX technology. Prior to sequencing using GS20 or GSFLX technology, the amplified cDNA was purified using a QIAquick PCR purification kit (Qiagen). The preparation of DNA libraries, titrations, emPCR and sequencing on the GS20/FLX sequencers, were performed by various companies that provide commercial sequencing services using the GS20/FLX sequencers, with the exception of RochePenzberg, where proof of principle studies were conducted on the AHSV1 genomes. The regions on a large Pico Titre Plate (PTP) used for sequencing each of the genomes and the company that performed the sequencing are listed in Table 1.

Sequence analysis. The initial assembly of sequences using GS20 software (Roche) was insufficient for our purposes. Therefore, the raw sequence data of the genomes were assembled *de novo* in GAP4 (Bonfield *et al.*, 1995) using the normal shotgun assembly. The assembly was confirmed by aligning it with known sequences (where available) and each segment was manually checked and edited. Subsequently, Lasergene7 software from DNASTAR was used for *de novo* assembly of the contigs. Files containing the sequence information, quality values and flowgrams (sff files) were loaded into the Seqman 7 programme of the Lasergene software. Default assembly parameters were used except for the minimum read length which was set to 30 bp for GS20 reads and 50 bp for GSFLX reads. Contigs resulting from the assembly were checked manually and their consensus sequences were exported as FASTA files. Consensus sequences were aligned to known sequences using MEGALIGN (Lasergene7). Finally, sequences were subjected to BLASTN analysis using the National Center for Biotechnology Information website. The consensus sequence of the seven complete dsRNA virus genome sets that were generated during this investigation have been deposited in GenBank under the accession numbers listed in Table 2.

RESULTS AND DISCUSSION

Improving oligo-ligation efficiency by macromolecular crowding

Since it has been reported that the efficacy of enzymes used for ligation of RNA and DNA segments can be improved substantially using macromolecular crowding with high molecular mass chemicals such as PEG (Harrison & Zimmerman, 1984), we investigated whether oligo-ligation with T4 RNA ligase could also be improved by the inclusion of PEG and hexamine cobalt chloride (HCC) in our reactions. Four ligation reactions were performed, as described above, each with 100 ng AHSV4 dsRNA. One ligation reaction was carried out using HEPES ligation buffer (Potgieter *et al.*, 2002) without PEG_{6000}, a second was carried out in the presence of 20 % (w/v) PEG_{6000}, a third contained 1 mM HCC and the fourth reaction contained both 20 % (w/v) PEG_{6000} and 1 mM HCC. cDNA synthesis was carried out as described previously (Potgieter *et al.*, 2002). The cDNA was amplified in 15 PCR cycles and the final amplification products were separated on 1 % TBE agarose gels. The presence of 20 % PEG_{6000} in the oligo-ligation reaction significantly increased the efficiency (Fig. 1b, lane 3). Analysis and quantification of the electrophoresis data using Vision Works Image

Table 2. Gene assignments and GenBank accession numbers of the complete consensus nucleic acid genome sequences of seven dsRNA viruses sequenced using Roche's 454 pyrosequencing GS technology

The raw sequence data are available on request.

Virus	Genome segment number*										
	1	2	3	4	5	6	7	8	9	10	11
AHSV1 reference strain	VP1	VP2	VP3	VP4	NS1	VP5	VP6	VP7	NS2	NS3	–
	AM883164	AM883165	AM883166	AM883167	AM883168	AM883169	AM883170	AM883171	AM883172	AM883173	
AHSV1 attenuated strain	VP1	VP2	VP3	VP4	NS1	VP5	VP6	VP7	NS2	NS3	–
	FJ183364	FJ183365	FJ183366	FJ183367	FJ183368	FJ183369	FJ183370	FJ183371	FJ183372	FJ183373	
AHSV1, 1 A, 1 S†	VP1	VP2	VP3	VP4	NS1	VP5	VP6	VP7	NS2	NS3	–
	FJ011107	FJ011108	FJ011109	FJ011110	FJ011111	FJ011112	FJ011113	FJ011114	FJ011115	FJ011116	
AHSV2 Lagos	VP1	VP2	VP3	VP4	NS1	VP5	VP6	VP7	NS2	NS3	–
	FJ196584	FJ196585	FJ196586	FJ196587	FJ196588	FJ196589	FJ196590	FJ196591	FJ196592	FJ196593	
BTV8 Netherlands	VP1	VP2	VP3	VP4	NS1	VP5	VP7	NS2	VP6	NS3	–
	FJ183374	FJ183375	FJ183376	FJ183377	FJ183378	FJ183379	FJ183380	FJ183381	FJ183382	FJ183383	
EEV Bryanston	VP1	VP2	VP3	VP4	NS1	VP5	NS2	VP7	VP6	NS3	–
	FJ183384	FJ183385	FJ183386	FJ183387	FJ183388	FJ183389	FJ183390	FJ183391	FJ183392	FJ183393	
Human rotavirus G9P[6]	VP1	VP2	VP3	VP4	NSP1	VP6	NSP3	VP7	NSP2	NSP5	NSP4
	FJ183353	FJ183354	FJ183355	FJ183356	FJ183357	FJ183358	FJ183359	FJ183360	FJ183361	FJ183362	FJ183363

*Genome segment number is solely based on the size of the segment and not on SDS-PAGE profiles.
†A, Adult mice; S, suckling mice.

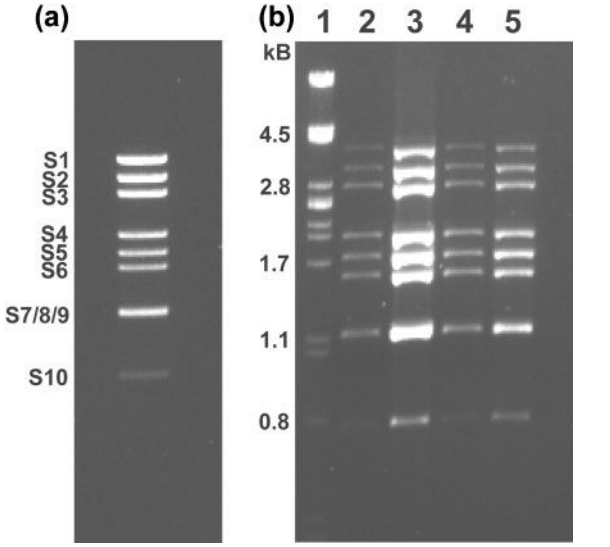

Fig. 1. Ethidium bromide-stained agarose gel (1 %) showing the effect of PEG_{6000} and HCC on oligo-ligation. (a) Purified dsRNA of the AHSV4 reference strain. Amplicons are numbered according to the cognate genome segment. (b) cDNA amplification products after oligo-ligation with different buffers, cDNA synthesis and 15 cycles of cDNA amplification. Lanes: 1, *Pst*I-digested phage λ DNA size marker; 2, cDNA amplified after ligation in normal HEPES-buffered oligo-ligation reaction mixture; 3, cDNA after ligation in HEPES-buffered oligo-ligation reaction mixture containing 20 % PEG_{6000}; 4, cDNA after ligation in HEPES-buffered oligo-ligation reaction mixture containing 1 mM HCC; 5, cDNA after ligation in HEPES-buffered oligo-ligation reaction mixture containing both 20 % PEG_{6000} and 1 mM HCC.

Analysis software (UVP), indicated that the addition of PEG_{6000} to the ligation reaction increased the yield of amplicons approximately fivefold from 100 ng of starting dsRNA and 15 cycles of PCR. The addition of HCC to ligation reactions did not improve the ligation; in fact, addition of HCC to ligation buffer containing PEG_{6000} reduced the efficiency (Fig. 1b, compare lanes 3 and 5).

Determining the amplification sensitivity

To determine the impact of the increased efficiency of ligation on the sensitivity of cDNA synthesis, we compared the sensitivity to that of our previous method (Potgieter *et al.* 2002) using dsRNA purified from the faeces of a calf suffering from diarrhoea caused by a group A rotavirus as template. The dsRNA was diluted 1 : 3 with 10 mM Tris, pH 8.0, from a concentration of 10 ng μl^{-1} down to 40 pg μl^{-1}. Aliquots of 10 µl from each dilution containing approximately 100, 33.3, 11.1, 3.7, 1.2 and 0.4 ng were separated by SDS-PAGE and the dsRNA was visualized after silver staining (Fig. 2a). Another 10 µl aliquot from each dilution was subjected to the improved oligo-ligation and cDNA amplification in the presence of 20 % PEG_{6000}, as described above. The cDNA amplification was performed from 10 µl cDNA in a 30 µl PCR with TaKaRa Ex Taq. After 15, 20 and 25 PCR cycles, a 5 µl aliquot from each amplification reaction was separated on a 1 % agarose

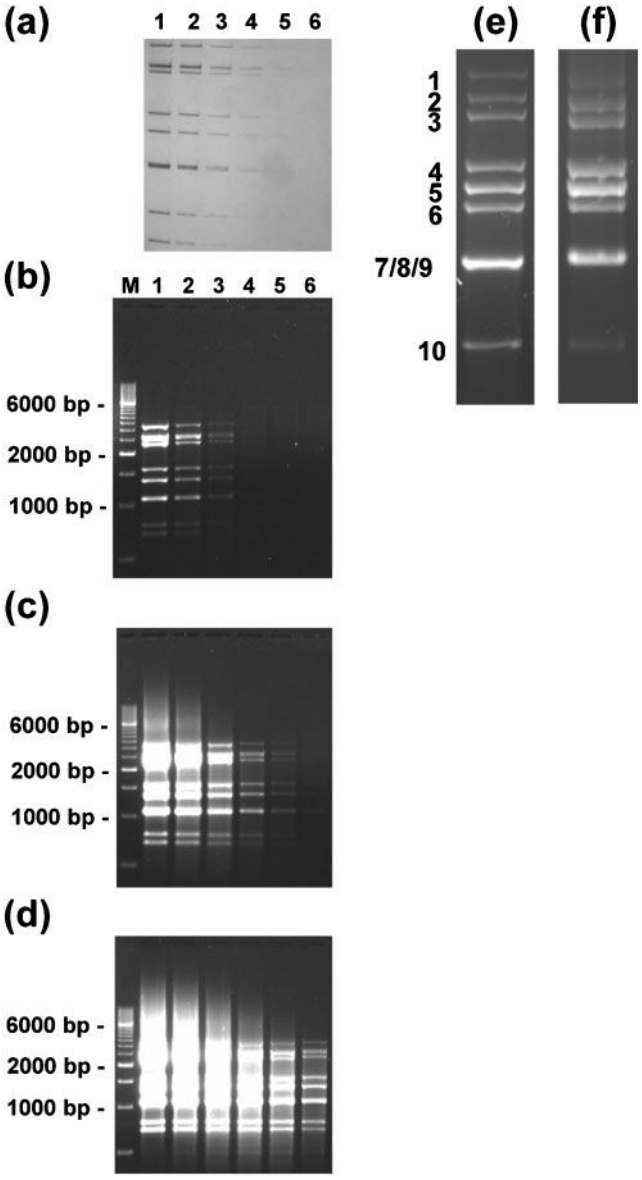

Fig. 2. Determining the sensitivity of the improved complete genome amplification procedure. (a) Silver-stained SDS-PAGE gel of a dilution series of calf rotavirus dsRNA. (b–d) Ethidium bromide-stained 1 % agarose gel of cDNA amplification products from 100, 33, 11, 3.7, 1.2 and 0.4 ng (lanes 1–6, respectively) dsRNA after 15 (b), 20 (c) and 25 (d) PCR cycles. Lane M, 500 bp ladder (Fermentas SM0643). (e, f) Agarose gel analysis of amplicons generated from AHSV dsRNA extracted directly from clinical samples. Genome amplified from dsRNA extracted from the spleen of a horse that died after infection with AHSV9 (e) and a blood clot from a horse that died after infection with AHSV2 (Lagos) (f). Amplicons are numbered according to the cognate genome segment.

gel (TBE) and visualized with ethidium bromide staining. The genomes amplified from different amounts of bovine rotavirus dsRNA and different numbers of PCR cycles are shown in Fig. 2. The cDNA of the complete genome of a bovine rotavirus could be amplified from approximately 400 pg starting material in 25 PCR cycles (Fig. 2d, lane 6). Previously, the amplification of a similar rotavirus genome was reported from 1 ng dsRNA and needed 30 PCR cycles (Potgieter *et al.*, 2002). Although about the same total amount of amplification product from ligation reactions of 100 and 0.4 ng dsRNA could be obtained by increasing the number of PCR cycles, the yields of the larger segments were not equal (compare Fig. 2b, lane 1, after 15 PCR cycles and Fig. 2d, lanes 4–6, after 25 PCR cycles). Therefore, the cDNA of the large genome segments of dsRNA viruses still does not amplify as well as that of the medium and small genome segments.

Encouraged by these results, we attempted to amplify the genomes of AHSV with dsRNA purified from field samples (spleen and blood). The successful amplification of AHSV cDNA from RNA extracted from the spleen and a blood clot from separate field cases are shown in Fig. 2 (e, f). The total amount of dsRNA from these samples is not known, since the yield of purified dsRNA was too low to be visible on ethidium bromide-stained agarose gels. However, the yield of the amplicons of the genome amplified from the blood sample was sufficient to determine the complete genome sequence using GSFLX sequencing (Table 2). This is the first report of sequence-independent genome amplification and sequencing of the genome of an orbivirus directly from a field sample. In other cases, we were able to sequence the terminal ends of genome segment S2 using 'phased primers' similar to those described by Maan *et al.* (2007) and determine the genotype of the viruses (Fasina *et al.*, 2008). In certain cases, the methods described here yielded only partial sequences, representing only some of the ten segments (results not shown). Therefore, the sensitivity of the methods depends on the level of viraemia and the amount of dsRNA extracted from the sample.

Sequencing amplified genomes using GS20/FLX technology

So far, we have amplified and sequenced 52 dsRNA virus genomes of seven different dsRNA viruses (AHSV, BTV, *Cryptosporidium* virus, EEV, EHDV, picobirnavirus and rotavirus) using pyrophosphate-based 454 sequencing technology. Here, as proof of principle of the improved amplification protocol, we report the consensus sequences of seven virus genome sets of four high profile dsRNA viruses (AHSV, BTV, EEV and human rotavirus). The results are summarized in Tables 1 and 2.

The average length of reads generated with the GS20, as expected, was 100 bp. The total sequence data generated per genome on 1/16th region of large PTP on the GS20 (sequenced by DYN) varied between 0.26 and 1.08 Mb (results not shown) and that on 1/8th regions on the GS20 (sequenced at Inqaba Biotec) between 1 and 3 Mb. On the GSFLX, reads were twice as long as on the GS20 by virtue of improvements in the sequencing technology. Total sequence data that were obtained on 1/16th regions on the GSFLX varied between 1 and 3 Mb. Overall, the coverage that was achieved varied between 12- and 150-fold. In our experience, a 40-fold coverage of dsRNA genomes amplified as described here, allows the determination of the complete consensus sequences of the 18–20 kb viral dsRNA genomes (Table 2). A lower coverage does not allow full genome sequence determination, since the larger segments amplify less efficiently and are present in smaller molar amounts. The larger the amount of dsRNA used for amplification, the better the amplification of the large segments (Fig. 2), as measured by the sequence coverage. An inherent technical problem of pyrophosphate-based GS20 sequencing appeared to be the inability to resolve multiple homopolymer base pair repeats accurately (Fig. 3). Manual checking of alignments of homopolymer regions resolves this problem quite easily, as extra base pairs or deletions are usually present in smaller amounts than the correct consensus sequences. The original flowgrams of a particular region can be viewed in Seqman (Lasergene7), which allows visual confirmation of the number of bases that actually occur in a specific homopolymer region. Although the resolution has been improved for the GSFLX, it is still difficult to detect deletion mutations correctly.

Despite these challenges, the experimental approach and methods used here for dsRNA sequencing are significantly more efficient than Sanger cycle sequencing, less expensive, less time consuming and less labour intensive overall. Amplicons of single genome segments do not need to be separated by gel electrophoresis and purified. No sequence information on the terminal ends is needed. These properties make the methods described here suitable for sequencing of known as well as new dsRNA viruses and even those without conserved terminal ends. Roche recently improved the system, allowing several different genomes to be sequenced in a single lane on PTPs (Meyer *et al.*, 2007) and to increase the read lengths to 400 bp and more, resulting in a decrease in the costs for complete viral genome sequencing.

Ultra-deep sequencing AHSV1 reference and attenuated strains, detecting quasispecies and evidence of reassortment with the AHSV3 reference strain

In contrast with the relatively low genome coverage obtained on 1/8th and 1/16th lanes, sequencing of the AHSV1 reference and attenuated strains on two regions each of a four region PTP on the GS20 (Roche, Penzberg) yielded approximately 10 Mb raw data per genome, corresponding to a coverage of >400-fold of the 20 kb genomes (Table 1). The average length of reads was 102 bp. Curiously, assembly of these sequences with GS20 software

did not yield the complete consensus sequences of each virus. When the alignments were repeated using manual GAP4 assembly, 10 large contigs were obtained per genome. Each of the contigs contained the complete consensus sequence of one of the genome segments of the two AHSV1 strains. The accession numbers of the consensus sequences are listed in Table 2.

Further analysis of alignments from the assembly of the AHSV1 reference and attenuated viruses revealed random mutations at various sites in each of the 10 genome segments of both viruses. These differences and deletions were observed in all genome segments. Whether these are true quasispecies or errors introduced due to the low fidelity of the reverse transcriptase and/or DNA polymerases used for cDNA amplification is not known.

Close scrutiny of sequence alignments from the AHSV1 reference strain also revealed mutations with distinct repetitive patterns at the same sites within four of the 10 genome segments, namely S5 (NS1), S8 (VP7), S7 (VP6) and S10 (NS3). An example of this from the alignments of the AHSV1 reference strain S7 (VP6) alignment is shown in Fig. 3. We refer to these repetitive changes as subpopulations. The subpopulations in the AHSV1 reference strain occurred at different frequencies in each of the four genome segments. At first it was thought that these were naturally occurring quasispecies. However, close scrutiny of the sequences revealed that these were in fact a mixture of AHSV1 reference strain and AHSV3 reference strain sequences (data not shown). The ratios of AHSV1 sequences to AHSV3 sequences were as follows: S5 (NS1),

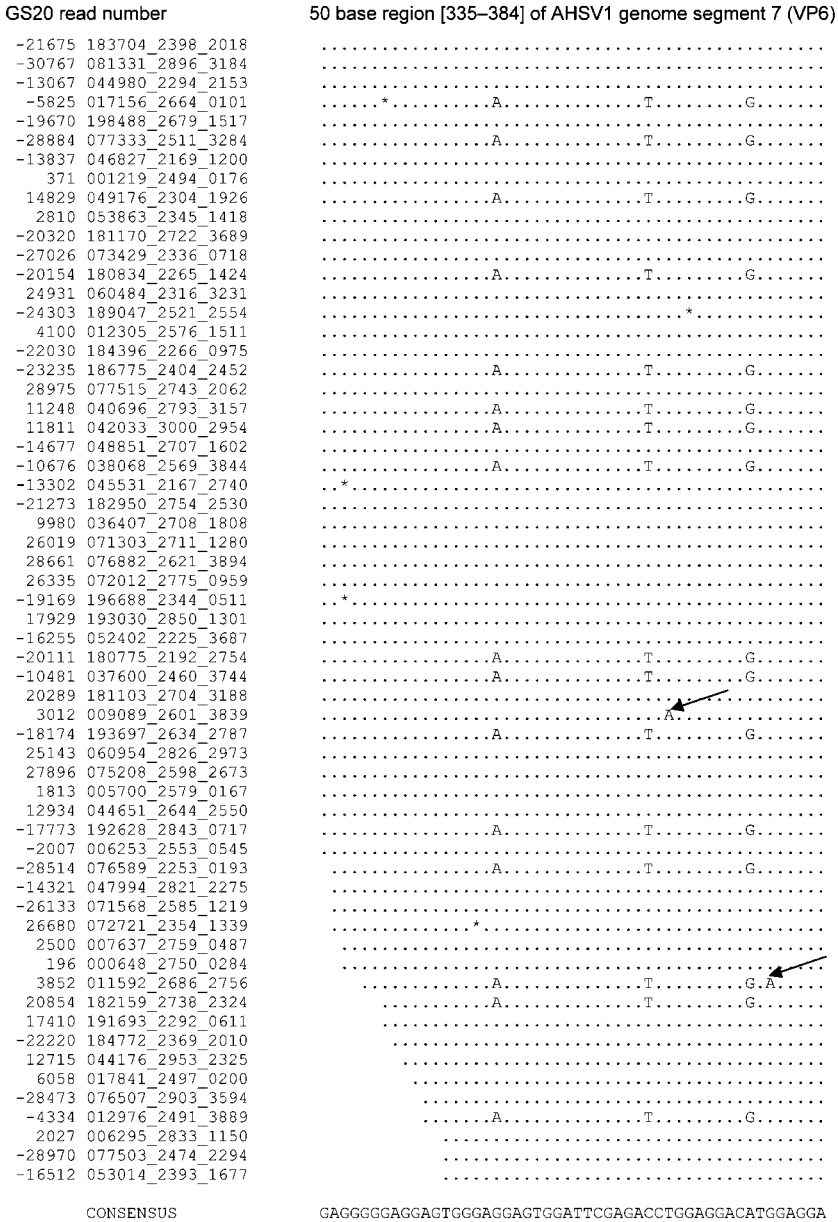

Fig. 3. Detecting reassortment between genome segment 7 (VP6) from AHSV1 and AHSV3. The aligned sequences were generated from the amplified genome of the AHSV1 reference strain on the GS20 sequencer. All sequences identical to the consensus sequence are indicated with a dot (.); sequences that differ from the consensus sequence are indicated with the letter corresponding to the base pair (A, T or G); random deletion mutations are indicated with an asterisk (*); other random mutations are indicated with arrows.

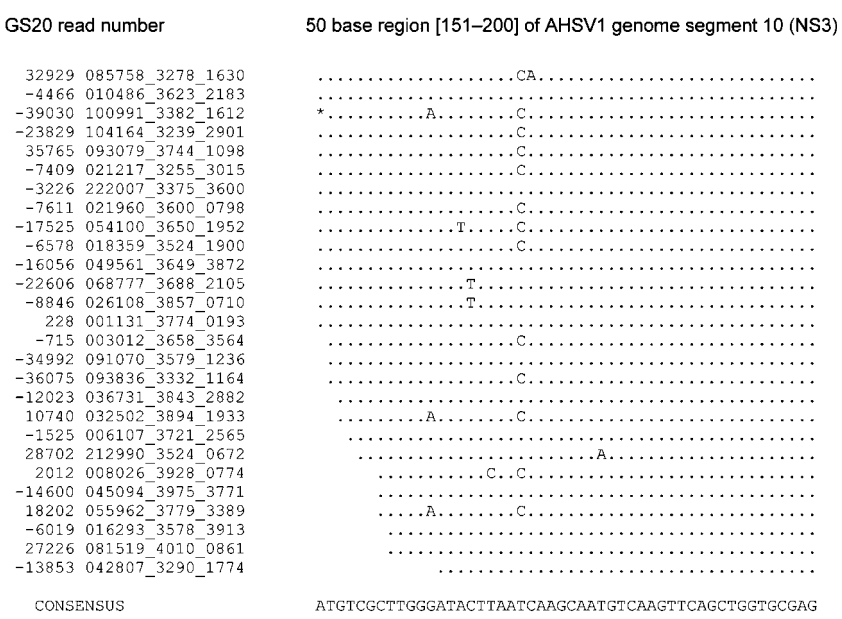

Fig. 4. Detection of quasispecies in genome segment 10 (NS3) of the AHSV1 attenuated (plaque-purified) strain. Sequences and random deletion mutation are labelled with dots, letters or asterisks as in Fig. 3.

15 : 85, S8 (VP7), 95 : 5; S7 (VP6), 70 : 30; and S10 (NS3), 85 : 15. Therefore, it was speculated that the AHSV1 reference strain used in this study (AHSV1 Equine spleen, 3 S, 2 BHK; Table 1) is in the process of reassortment with the AHSV3 reference strain. To prove this, we sequenced the lowest passage of the AHSV 1 reference strain that we have in our virus bank (AHSV1 Equine spleen, 1 A, 1 S; Table 1) from which this reference strain was derived. The consensus sequences of nine of the 10 genome segments of the AHSV1 reference strain and the low passage strain from which it was derived were identical. Only the sequence of genome segment 5, encoding NS1, of the reference strain was found to be almost identical to the AHSV3 reference strain.

It was thus possible to conclude that the AHSV1 reference strain must have been in contact with the AHSV3 reference strain during passage on cell culture and that the two viruses started to reassort. Reassortment was, however, not complete, since the virus population of the AHSV1 reference strain contained a mixture of AHSV1 and AHSV3 segments, indicating the presence of different ratios of reassorted segments. The plaque-purified, attenuated strain of AHSV1 also contains the NS1 protein of AHSV3. Our results indicate that the technologies described here allowed us to follow reassortment events as they take place.

We did not detect any significant repeated quasispecies in nine of the 10 genome segments (S1–S9) of the plaque-purified, attenuated AHSV1 virus population. In genome segment S10 (NS3) we did detect quasispecies that were acquired during the passage of the virus on Vero and BHK cells after it was plaque-purified three times (Fig. 4). These changes were not detected in either of the AHSV1 reference strains. We speculate that the mutation 171T(U)→C, which is shown in Fig. 4 and present in almost 50 % of the reads, occurred first and was followed by the mutation 162G→A, which is present in a much smaller frequency and only in reads where the first mutation is present. However, we do not have sequence information from earlier passages that could confirm this. Nevertheless, in this small section of sequence, we could detect three different varieties of NS3 which were acquired during only six passages in cell culture (5 Vero, 1 BHK). In our view, the other single random changes are not significant and may represent errors due to the low fidelity of the enzymes used.

These results are in agreement with those reported for BTV that was limited to genome segments S2 (VP2) and S10 (NS3) upon passage between sheep, cattle and the insect vector, *Culicoides sonorensis* (Bonneau *et al.*, 2001). In contrast with the latter investigation in which sequence data had to be generated from a limited number of genome segments using genome segment-specific primers or cloning, with our new approach we could screen the complete genome for quasispecies without the need for any genome segment-specific primers or cloning.

We anticipate that the combination of this improved sequence-independent dsRNA genome amplification and ultra-deep pyrosequencing will become an extremely useful tool to study the evolution in dsRNA viruses during either natural passages during virus outbreaks in the field, attenuation or adaptation to cell culture. It should also greatly speed up the development and general implementation of genome-based classification systems for dsRNA viruses for surveillance and epidemiological purposes (Matthijnssens *et al.*, 2008).

Comparing consensus genome sequences from a virulent and an attenuated AHSV1 strain

To initiate investigations aimed at identifying factors that determine virulence in AHSV, we compared the consensus

sequences of the genome of the attenuated strain of AHSV1 and its parental strain. Although the parental strain of the AHSV1 attenuated strain contained a mixture of AHSV1 and AHSV3 sequences, as shown above, AHSV1 low and high passage strains share an identical consensus, except for genome segment S5 (NS1), reflecting true changes in the consensus sequence of the AHSV1 attenuated strain.

The consensus sequence of three of the genome segments, S1 (VP1), S5 (NS1) and S9 (NS2), were identical between the virulent and attenuated strains of AHSV1 (Table 3). Overall, there were 16 nucleotide differences in the consensus sequences of the seven genome segments in which variations occurred, of which seven resulted in amino acid changes. Only two nucleotide changes occurred in the non-coding regions, one in S8 (VP7) and the other in S10 (NS3). Most nucleotide changes were transitions (12 of 16). Since the proportion of the non-coding regions in the genome is small, two of 16 nucleotide differences in this region seems high. However, when the differences were quantified, the proportion of changes is actually much less in the non-coding regions and is, therefore, not more significant. This is generally in agreement with several sequencing studies on other orbiviruses (A. C. Potgieter, unpublished data). We hypothesize that these are the most frequent errors that the viral RNA-dependent RNA polymerase makes during replication.

In our view, the most significant amino acid changes related to virulence might be those in the outer capsid proteins, namely VP2 and VP5. The reason for this hypothesis stems from extensive studies of AHSV3 and AHSV4 in horses, from which it was concluded that virulence of AHSV is related to the affinity of the virus for certain tissues (tissue tropism) and that attenuation of the virus is simply the selection of viruses that do not have selective affinity for vital organs such as the lung (Erasmus, 1973). This also correlates with findings that the outer capsid proteins of orbviruses are involved in cell entry and triggering of apoptosis by the virus (Bhattacharya *et al.*, 2007; Mortola *et al.*, 2004). However, it is also possible that any of the other changes in the genome sequence may be associated with the attenuation. For example, in this specific case, the reassortment of AHSV1 S5 (NS1) with AHSV3 S5 (NS1) may have caused the attenuation. To pinpoint which changes are responsible for attenuation, a reverse genetics system for AHSV would be required. The fact that the AHSV1 sequence data presented here are in agreement with the early AHSV3 and AHSV4 virological and clinical results (Erasmus, 1973) is exciting, since they demonstrate how the different disciplinary approaches support each other by (re)confirming, complementing and extending the findings. It is anticipated that the work started here will give an indication of which factors are involved in tissue tropism and virulence of AHSV.

In conclusion, this report on the significant improvement in the efficiency of generating cDNA from dsRNA viral genomes, combined with the high throughput and coverage of pyrosequencing, introduces a paradigm shift for dsRNA virus research. Viral dsRNA genomes can now be completely sequenced, analysed and cloned directly from field and clinical samples without requiring any prior sequence information. The technology also allows the detection of quasispecies in complete genome sets as opposed to single genome segments. It is envisaged that this progress will facilitate a host of qualitative and quantitative investigations of dsRNA virus population dynamics and viral evolution. It should now be possible to begin to rationally and systematically identify and investigate the factors that are involved in tissue tropism, virulence and virus/host and virus/vector interactions of many dsRNA viruses.

Table 3. Comparison of consensus sequences of the genome segments of the AHSV1 reference and attenuated strains

The consensus nucleic acid sequences of AHSV1 (3 S, 2 BHK) is identical to that of AHSV1 (1 A, 1 S) with the exception of genome segment 5, encoding NS1, which reassorted with AHSV3.

Genome segment	Nucleic acid change	Amino acid change
1 (VP1)	–	–
2 (VP2)	1083 U→A	–
	1365 G→A	357 N→K
	2814 A→G	–
3 (VP3)	720 U→C	232 Y→H
	1589 A→G	–
	1832 G→A	–
4 (VP4)	1748 A→G	–
5 (NS1)	–	–
6 (VP5)	724 C→U	
	1284 G→A	422 S→N
	1320 C→U	434 T→I
7 (VP6)	259 C→U	81 A→V
	523 G→A	69 R→Q
8 (VP7)	3 U→A	–
9 (NS2)	–	–
10 (NS3)	4 U→A	–
	170 C→U	–
	619 U→A	201 M→K

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REFERENCES

Attoui, H., Billoir, F., Cantaloube, J. F., Biagini, P., de Micco, P. & de Lamballerie, X. (2000). Strategies for the sequence determination of viral dsRNA genomes. *J Virol Methods* **89**, 147–158.

Bhattacharya, B., Noad, R. & Roy, P. (2007). Interaction between Bluetongue virus outer capsid protein VP2 and vimentin is necessary for virus egress. *Virol J* **4**, 7.

Bigot, Y., Drezen, J. M., Sizaret, P. Y., Rabouille, A., Hamelin, M. H. & Periquet, G. (1995). The genome segments of DpRV, a commensal reovirus of the wasp *Diadromus pulchellus* (*Hymenoptera*). *Virology* **210**, 109–119.

Bonfield, J. K., Smith, K. F. & Staden, R. (1995). A new DNA sequence assembly program. *Nucleic Acids Res* **23**, 4992–4999.

Bonneau, K. R., Mullens, B. A. & MacLachlan, N. J. (2001). Occurrence of genetic drift and founder effect during quasispecies evolution of the VP2 and NS3/NS3A genes of Bluetongue virus upon passage between sheep, cattle, and *Culicoides sonorensis*. *J Virol* **75**, 8298–8305.

Cashdollar, L. W., Esparza, J., Hudson, G. R., Chmelo, R., Lee, P. W. & Joklik, W. K. (1982). Cloning the double-stranded RNA genes of reovirus: sequence of the cloned S2 gene. *Proc Natl Acad Sci U S A* **79**, 7644–7648.

Erasmus, B. J. (1973). The pathogenesis of African horsesickness. In *Proceedings of the Third International Conference on Equine Infectious Diseases*. Basel, Switzerland: Karger.

Fasina, F., Potgieter, A. C., Ibironke, A., Bakod, B., Bwala, D. & Kumbish, P. (2008). First report of an outbreak of African horsesickness virus serotype 2 in the Northern hemisphere. *J Equine Vet Sci* **28**, 167–170.

Harrison, B. & Zimmerman, S. B. (1984). Polymer-stimulated ligation: enhanced ligation of oligo- and polynucleotides by T4 RNA ligase in polymer solutions. *Nucleic Acids Res* **12**, 8235–8251.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lambden, P. R., Cooke, S. J., Caul, E. O. & Clarke, I. N. (1992). Cloning of noncultivatable human rotavirus by single primer amplification. *J Virol* **66**, 1817–1822.

Maan, S., Rao, S., Maan, N. S., Anthony, S. J., Attoui, H., Samuel, A. R. & Mertens, P. P. (2007). Rapid cDNA synthesis and sequencing techniques for the genetic study of bluetongue and other dsRNA viruses. *J Virol Methods* **143**, 132–139.

Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., Berka, J., Braverman, M. S., Chen, Y. J. & other authors (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**, 376–380.

Matthijnssens, J., Ciarlet, M., Heiman, E., Arijs, I., Delbeke, T., McDonald, S. M., Palombo, E. A., Iturriza-Gómara, M., Maes, P. & other authors (2008). Full genome-based classification of rotaviruses reveals a common origin between human Wa-like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. *J Virol* **82**, 3204–3219.

Mertens, P. (2004). The dsRNA viruses. *Virus Res* **101**, 3–13.

Mertens, P. P. C., Duncan, R., Attoui, H. & Dermody, T. S. (2005). Reoviridae. In *Virus Taxonomy, VIIIth Report of the ICTV*, pp. 447–454. Edited by C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger & L. A. Ball. London: Elsevier.

Meyer, M., Stenzel, U., Myles, S., Prufer, K. & Hofreiter, M. (2007). Targeted high-throughput sequencing of tagged nucleic acid samples. *Nucleic Acids Res* **35**, e97.

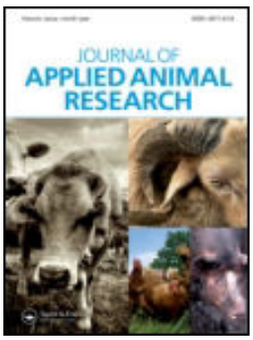
Mortola, E., Noad, R. & Roy, P. (2004). Bluetongue virus outer capsid proteins are sufficient to trigger apoptosis in mammalian cells. *J Virol* **78**, 2875–2883.

Potgieter, A. C., Steele, A. D. & van Dijk, A. A. (2002). Cloning of complete genome sets of six dsRNA viruses using an improved cloning method for large dsRNA genes. *J Gen Virol* **83**, 2215–2223.

Rao, C. D., Kiuchi, A. & Roy, P. (1983). Homologous terminal sequences of the genome double-stranded RNAs of bluetongue virus. *J Virol* **46**, 378–383.

Roy, P., Mertens, P. P. & Casal, I. (1994). African horse sickness virus structure. *Comp Immunol Microbiol Infect Dis* **17**, 243–273.

Vreede, F. T., Cloete, M., Napier, G. B., van Dijk, A. A. & Viljoen, G. J. (1998). Sequence-independent amplification and cloning of large dsRNA virus genome segments by poly(dA)-oligonucleotide ligation. *J Virol Methods* **72**, 243–247.



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Comparison of agarose gel electrophoresis and RNA-PAGE for rapid detection of rotavirus from faecal samples

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Rapid and correct diagnosis of rotavirus infection is very important to offer immediate treatment. In the present study, ribonucleic acid-polyacrylamide gel electrophoresis (RNA-PAGE) and agarose gel electrophoresis (AGE) methods were standardised and compared for the detection of rotaviruses. Double-stranded RNA (dsRNA) of rotavirus was extracted from five positive faecal samples by QIAamp Viral RNA Mini Kit and subjected to RNA-PAGE where the concentration of separating gel was varying, i.e. 7.0%, 8.5% and 12.5%. The bands of the RNA segments were clearly separated on 7.0% and 8.5% separating gel. Extracted dsRNA was also subjected to AGE where the concentration of gel was varying at 0.8%, 1.2%, 1.6% and 2.0% containing ethidium bromide. All the 11 bands of 11 segments of dsRNA were clearly visualised in all the concentrations of gel while 1.2% gel showed clear and separated bands of ladder as well as samples. Different concentrations of rotavirus, i.e. 100–1000 ng, were also loaded on standardised RNA-PAGE and AGE, separately. However, both the methods showed same detection level up to 100 ng particles of dsRNA. Further, the time required for extraction of dsRNA from five samples, their PAGE running and staining by silver stain took around 6–7 h which was comparatively more than the AGE separation, took only 2–3 h for complete procedure. Extracted dsRNA of rotavirus from 100 stool samples of children and 42 faecal samples of piglets were subjected to standardised RNA-PAGE and AGE separately for detection of rotavirus. Interestingly, 38 (38%) samples from children and 3 (7.14%) from piglet were found to be positive for rotavirus by AGE. However, RNA-PAGE could detect only 34 (34%) samples from children and 3 (7.14%) from piglets. Out of 38 positive samples from children, 32 showed long electrophoretic patterns and remaining 6 showed short electrophoretic pattern while that for 3 piglet samples, all showed long electrophoretic pattern. Thus, from our results, it can be concluded that the AGE is highly sensitive (91.17%), marginally less specific (90.90%), reproducible, superior, efficient, less laborious, cost-effective and time-saving than the RNA-PAGE for rapid diagnosis of rotavirus from faecal samples of humans as well as animals.

Keywords: agarose gel electrophoresis; comparison; rotavirus; RNA-PAGE

1. Introduction

Rotavirus infections have been recognised as a common cause of acute gastroenteritis in humans and animals and are the most important cause of severe dehydrating diarrhoea in young children in both developed and developing countries (Kapikian et al. 2001; Temu et al. 2012). Rotaviruses cause an estimated 130 million cases of gastroenteritis and 800,000 deaths in children between the ages of 6 month and 2 year in developing countries (Das et al. 2003). Rotaviruses have a genome composed of 11 segments of dsRNA that encodes 6 structural (VP) and 5 or 6 non-structural proteins (NSPs; Estes & Kapikian 2006). Rotaviruses are classified into seven different groups (A–G) based on the antigenic specificity of the VP6 capsid proteins. Thus, groups A–G can be differentiated with polyclonal and monoclonal antibodies by immunofluorescence, ELISA and immunoelectron microscopy. However, group A rotaviruses are considered as an important viral diarrhoeal agents which can be typed as G (*VP7* gene) and P (*VP4* gene) genotypes based on sequence comparison or G serotypes and P serotypes based on the proteins of the outer capsid that elicit neutralising antibodies. To date, 23 distinct G genotypes and 32 P genotypes have been identified (Mukherjee et al. 2010; Adlhoch et al. 2011).

Owing to the poor adaptability of rotaviruses to serial propagation in cell cultures, laborious and sophisticated procedures are necessary and therefore rapid methods of direct and indirect detection and identification have been introduced into laboratory diagnostics. Initially, direct visualisation of stool material by electron microscopy was employed for rotavirus detection. However, availability of an electron microscope is a constraint in most of the laboratories. Many methods like reverse passive haemagglutination assay, enzyme immunoassay, complement fixation test and immunoelectrophoresis have been

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developed but are less sensitive. The most widely used methods for detection of rotavirus include latex agglutination, enzyme-linked immunosorbent assay (ELISA), lateral flow test and RNA-PAGE (de Góes et al. 2008). Reverse transcriptase-polymerase chain reaction (RT-PCR) is now being extensively used as a confirmatory method for detection of rotavirus (Dewar et al. 2005; Roman & Martinez 2005). RNA-PAGE following silver staining was found to be highly specific technique for detection of rotavirus from faecal samples, but it is tedious and time-consuming.

In India, one of every 250 children or about 90,000–153,000 children die of rotavirus diarrhoea each year (Jain et al. 2001; Broor et al. 2003; Parashar et al. 2009; Tate et al. 2009). Considering the background of morbidity and mortality caused by human rotavirus, detection methods have been employed routinely in clinical diagnostic and epidemiological studies. Therefore, the purpose of the present study was to find out the efficient method for detection of rotavirus from faecal samples at a lowest possible time and at a very low concentration of the virus. The two methods, RNA-PAGE and agarose gel electrophoresis (AGE), were standardised and compared for the evaluation of best suitable rapid method for detection of rotavirus from faecal samples.

2. Materials and methods

2.1. Extraction and purification of dsRNA

Five stool samples which were positive for rotavirus by RT-PCR in earlier studies carried out in 2010 in the Division of Veterinary Public Health, Indian Veterinary Research Institute, Izatnagar, Bareilly, India were used for standardisation of RNA-PAGE and AGE methods. Viral total RNA was extracted by using a QIAamp Viral RNA Mini Kit, Cat. No. 52904 (QIAGEN, Leusden, The Netherlands) containing carrier RNA, extraction buffer AW1 and AW2 and elution buffer as AVE. The extraction was carried out according to the manufacturer's instructions from 150 µl of each stool suspension in phosphate buffer solution (0.01 M phosphate buffer pH 7.2). The RNA pellet was suspended in 20 µl of nuclease-free water and stored at −20°C for further use. Similar protocol was also utilised for the extraction of dsRNA of rotavirus from children stool samples collected from hospitals as well as for piglet faecal samples.

2.2. RNA-PAGE

RNA-PAGE was performed as per the procedure described by Laemmli (1970) and Herring et al. (1982) with little modifications followed by silver staining to determine the presence of rotavirus. Briefly, the reagents of resolving gel [containing Tris HCL (pH 8.8), acry/bisacrylamide solution, 10% sodium dodecyl sulfate (SDS), ammonium persulphate and tetramethylene diamine] were mixed sequentially, and the solution was poured in the gel casting plates fixed in the gel casting apparatus. One ml of distilled water was overlaid on the upper surface of the gel and allowed the gel for polymerization. The reagents of stacking gel [5%; containing Tris HCL (pH 6.8), acrylamide/bisacrylamide solution, 10% SDS, ammonium persulphate and tetramethylene diamine] were mixed sequentially and carefully overlaid on the top of resolving gel after removing the water layer. Subsequently, the comb was put in the stacking gel solution and allowed to polymerize, which took approximately 30 min. After complete polymerization of the gel, the comb was removed, and the electrophoresis buffer (containing Tris base, glycine and distilled water) was loaded in upper and lower containers. The microfuge tubes containing the RNA samples were heated at 56°C for 5–10 min to dissolve the pellet. Subsequently, 10 µl of the sample was mixed with 5 µl of 6× loading dye on paraffin wax paper and loaded into the wells. The gel was run at constant voltage of 100 V till the dye reached the bottom of the gel (normally 1–1.5 h). The staining of the PAGE gel was carried out as per the protocol standardised by Kumar (2006) where the gels were kept in a fixative solution (0.5% glacial acetic acid in 10% ethanol) for 30 min followed by staining in silver stain (0.185 g silver nitrate in 100 ml distilled water) for 30 min. After this, the gels were kept in a developer solution (3 g NaOH pellets in 100 ml distilled water and 0.75 ml formaldehyde) for 10–15 min followed by stop solution (5% glacial acetic acid).

The extracted dsRNA from five positive faecal samples was subjected to RNA-PAGE where the concentration of separating gel was varying, i.e. 7.0%, 8.5% and 12.5%. However, the stacking gel concentration was same as 5.0% in all the gels. The time required for the extraction of dsRNA, PAGE and staining was noted.

2.3. AGE

Electrophoresis was performed as per the procedure described by Chudzio et al. (1989) to determine the presence of rotavirus. Ready-to-use ethidium bromide (2–3 µl) for staining was added directly into the agarose (Sigma) during its preparation. The agarose gel was prepared in 0.09-M Tris-borate buffer, pH 8.2. Approximately, 35 ml of the melted gel was poured into a gel casting tray and comb was placed. After solidification, the comb was removed, the lid was put into an electrophoretic vessel and approximately 300 ml of Tris-borate buffer was poured into it. The extracted dsRNA from five positive faecal samples was subjected to AGE where the concentration of gel was varying at 0.8%, 1.2%, 1.6% and 2.0% containing ethidium

bromide for visualisation in gel documentation system (Kodak 100, Model UXT 20 m 8e). The time required for extraction of dsRNA and AGE was noted.

2.4. Detection of different concentration of dsRNA of rotavirus

The extracted concentrations of dsRNA from positive faecal samples were determined by nano-drop method. Thus, the different concentrations of rotavirus, i.e. 100 ng, 200 ng, 300 ng, 400 ng, 500 ng, 600 ng, 700 ng, 800 ng, 900 ng and 1000 ng, were loaded on standardised RNA-PAGE (7% separating gel) and AGE (1.2%) separately to find out the lowest detection limit by both the methods.

2.5. Screening of the samples by RNA-PAGE and AGE method

A total of 100 diarrhoeal stool samples of children were collected from one private hospital and Nazareth Hospital, Shillong, Meghalaya, India during 2011–2012. Additionally, 42 faecal samples from piglet diarrhoeal cases were also collected from pig farms located in and around, Shillong. All the samples were collected randomly in sterile containers and kept at −70°C till further processing. All these samples were processed for the extraction of dsRNA of rotavirus and subjected to RNA-PAGE and AGE separately for detection of rotavirus. The samples have been collected from same geographical area of Meghalaya (North Eastern Region), India. The climate of the region is tropical wet and humid conditions prevailing for most of the year. The monthly maximum temperature varies between 15 and 30°C (mean, 22°C).

3. Results and discussions

3.1. RNA-PAGE

The extracted dsRNA of rotavirus from five positive faecal samples was subjected to RNA-PAGE with varying concentrations of separating gel as 7.0%, 8.5% and 12.5%; the bands of the RNA segments were clearly separated on 7.0% and 8.5% separating gel while that for 12.5% gel did not show the proper separation of the bands. However, the first four to six segments were not properly separated and stained which resulted into disappearance of these bands from all the concentrations of PAGE gels. This might be due to faulty electrophoresis and/or unstained bands and/or non-separation of the segments. It was also observed that the intensity of the bands showed increasing trend with the increase in concentration of the dsRNA in PAGE.

3.2. AGE

The extracted dsRNA of the five samples was loaded on 0.8%, 1.2%, 1.6% and 2.0% agarose gel, separately. All the 11 bands of 11 segments of dsRNA were clearly visualised in all the concentrations of gel (Figure 1a–d). Similar results were also obtained by Chudzio et al. (1989). In contrast, Pšikal et al. (1991) obtained only eight and nine bands in 1.5% agarose gel. However, ladder and some of the bands were not clearly visualised in 1.6% and 2.0% agarose gel, while 1.2% gel showed clear and separated bands of ladder and samples. As ethidium bromide was added during the preparation of the agarose gel, there was no separate staining procedure required. Thus, total procedure from dsRNA of rotavirus extraction till the band visualisation took 2–3 h for five samples. Further, the method was easy to perform, economical, reproducible and quick. The major advantages of AGE are the straightforward procedure, involving no complicated sample processing and preparation of specific immune sera, and its speed, both making this method usable in routine investigations (Pšikal et al. 1991). It was also observed that 100-ng concentration of dsRNA was sufficient to clearly visualise the samples in agarose gel (1.2%). This indicated that the samples of low virus content could also be detected by AGE method. The clarity and intensity of bands were increasing with the increase in concentration of rotaviral RNA from 100 ng to 1000 ng (Figure 2).

3.3. Screening of the samples

A total of 100 diarrhoeal stool samples of children and 42 of piglet diarrhoeal faecal samples were processed for extraction of genomic RNA. All the extracted dsRNA samples were loaded on 1.2% agarose gel and 7.0% RNA-PAGE for detection of rotavirus. Interestingly, 38 (38%) samples from children (Figure 3) and 3 (7.14%) from piglet were found to be positive for rotavirus by AGE method. However, RNA-PAGE could detect only 34 (34%) samples from children (Figure 4) and 3 (7.14%) from piglets. This indicated that the agarose gel method was more sensitive (91.17%) than the RNA-PAGE (81.57%). The results are in accordance with the results of Pšikal et al. (1991) where it was observed that the reliable results were obtained by electrophoresis of the extracted RNA in agarose allowing a direct identification of rotaviruses, based on the characteristic arrangement of the RNA segments in the gel and on evaluation of respective electrophoretogrammes. Out of 94 samples, 43 were positive for rotavirus after examination by this method and suggested that the method was equally sensitive to ELISA (Matsui et al. 1990). Group A human and animal rotaviruses also displayed two electropherotypes: long and short (Matsui et al. 1990). Short electrophoretic patterns exhibited a larger segment 11 (encoding NSP5) that migrated more slowly and was located between gene segments 9 and 10 (Matsui et al. 1990). Although most group A rotaviruses

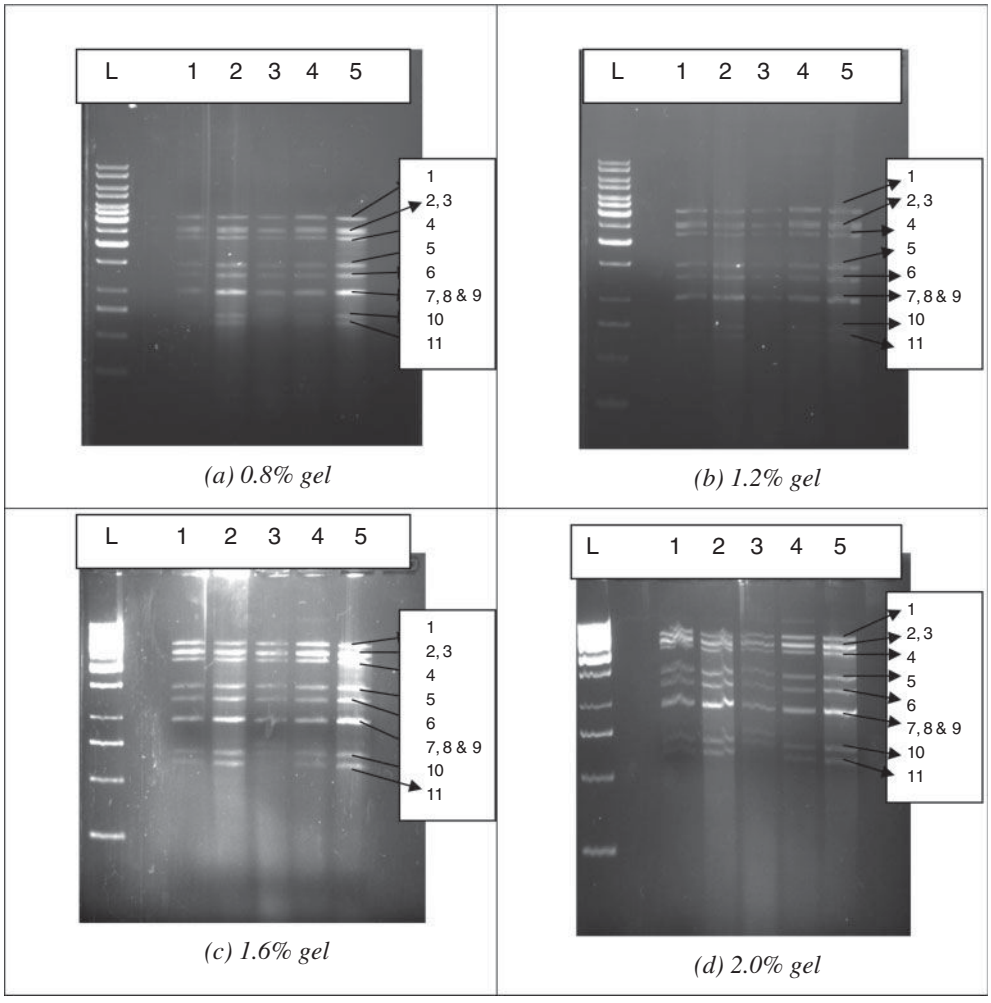

Figure 1. AGE showing segments of rotavirus from five positive samples (a) 0.8% gel, (b) 1.2% gel, (c) 1.6% gel and (d) 2.0% gel.

have either a short or a long pattern, super-short electropherotypes have been documented. These correlations between RNA patterns and serotypes have been maintained and have become a useful epidemiologic tool (Matsui et al. 1990). Detailed descriptions of the correlations between electropherotype and viral antigenic and genetic properties have been reported (Gentsch et al. 1996; Desselberger et al. 2001). Differentiation of 'long' and 'short' electrophoretic pattern is quite difficult in case of AGE than that for RNA-PAGE. It was observed

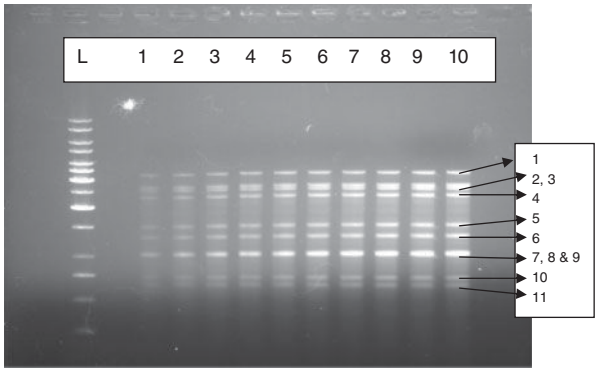

Figure 2. AGE showing segments of rotavirus in 1.2% gel. Note: Lanes 1–10: 100 ng, 200 ng, 300 ng, 400 ng, 500 ng, 600 ng, 700 ng, 800 ng, 900 ng and 1000 ng of RNA of rotavirus.

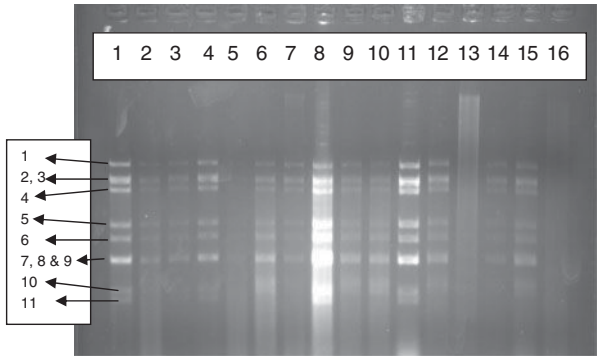

Figure 3. AGE showing segments of rotavirus extracted from stool samples of children.

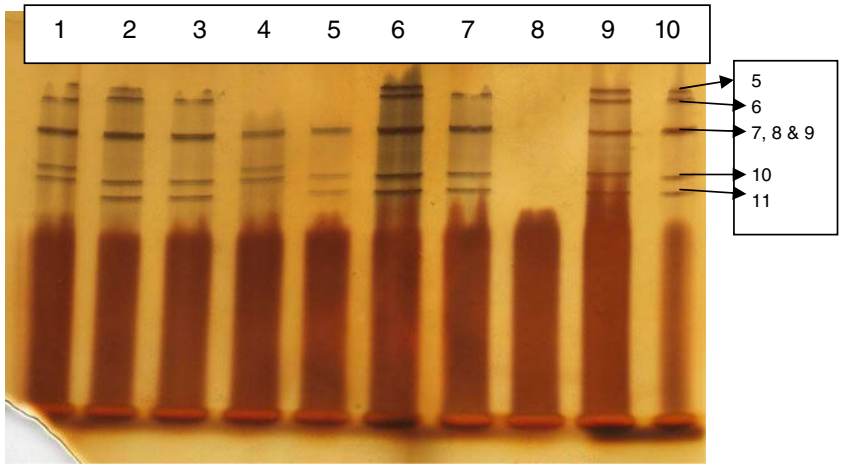

Figure 4. RNA-PAGE showing segments of rotavirus extracted from stool samples of children.

that out of 38 positive samples from children, 32 showed long electrophoretic patterns and remaining 6 showed short electrophoretic pattern while that for 3 piglet samples, all showed long electrophoretic pattern by both the methods, i.e. RNA-PAGE and AGE. In majority of the earlier studies, long pattern seems to be dominant (Brown et al. 1988; Broor et al. 1993; Dubal et al. 2013). The RNA patterns of the 38 animal strains were analysed (Pongsuwanne et al. 1989) using PAGE and showed long RNA profiles, except for two porcine and two bovine specimens.

3.4. Comparison between RNA-PAGE and AGE

Although both the methods detected the rotavirus, they differ in the number of positive samples. The reliable results were obtained by electrophoresis of the extracted RNA in agarose gel allowing a direct identification of rotaviruses based on the characteristic arrangement of the RNA segments in the gel and on evaluation of respective electrophoretogrammes. In case of RNA-PAGE, first four to six segments of dsRNA of rotavirus were not properly separated and stained. However, such problem was not detected in the AGE separation method. All the 11 bands of rotavirus were clearly visualised in AGE. Though all the 11 bands of rotavirus were not visualised, the clarity of the bands was superior in PAGE as compared to agarose gel (1.2%). Further, the time required for extraction of dsRNA from five samples, their PAGE running and staining by silver stain took around 6–7 h which was comparatively more than the AGE separation, took only 2–3 h for complete procedure. According to the WHO (2009) manual, the PAGE method had sometimes been used to diagnose group A rotavirus infections for surveillance studies. However, this method is very labour intensive and time-consuming. Both the methods showed detection level up to 100 ng particles of dsRNA. However, while screening the stool samples from children, only 34 could be detected by RNA-PAGE and 38 by the agarose gel method. This is indicative of superiority of the agarose gel method over the RNA-PAGE method (Pšikal et al. 1991). Moreover, the agarose gel method was easy to perform, less laborious, economical and time-saving than the RNA-PAGE method, as it is complicated, laborious and needs expert to perform. Similarly as PAGE, AGE can be used for the differentiation of the rotavirus RNA not only within a single animal species but also between various species (Rodger & Holmes 1979). Thus, from our results, it can be concluded that the AGE is superior, efficient, less laborious, economical and time-saving than the RNA-PAGE method for rapid diagnosis of rotavirus from faecal samples of humans as well as animals.

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References

Adlhoch C, Kaiser M, Hoehne M, Mas Marques,Stefas I , Veas F and Ellerbrok H. 2011. Highly sensitive detection of the group A rotavirus using Apolipoprotein H-coated ELISA plates compared to quantitative real-time PCR. Virol J. 8: 63.

Broor S, Ghosh D, Mathur P. 2003. Molecular epidemiology of rotaviruses in India. Indian J Med Res. 118: 59–67.

Broor S, Husain M, Chatterjee B, Chakraborty A, Seth P. 1993. Temporal variation in the distribution of rotavirus electropherotypes in Delhi, India. J Diarrhoeal Dis Res. 11: 14–18.

Brown DWG, Mathan MM, Mathew M, Martin R, Beards GM, Mathan VI. 1988. Rotavirus epidemiology in Vellore,

South India. Group, subgroup, serotype, and electropherotype. J Clin Microbiol. 26: 2410–2414.

Chudzio T, Kasatma S, Irvine N, Sankar-Mistry P. 1989. Rapid screening test for the diagnosis of rotavirus infection. J Clin Microbiol. 27: 2394–2396.

Das S, Varghese V, Chaudhury S, Barman P, Mahapatra S, Kojima K, Bhattacharya SK, Krishnan T, Rathod RK, Chhotray GP, et al. 2003. Emergence of novel human group A rotavirus G12 strains in India. J Clin Microbiol. 41: 2760–2762.

de Góes AC, de Moraes MT, de Castro, SW, Araújo IT, de H'alluin JC, da Silva Souza W, da Silva Junior JG, Leite JP. 2008. Development of a rapid and sensitive latex agglutination-based method for detection of group A rotavirus. J Virol Methods. 148: 211–217.

Desselberger U, Iturriza-Gomara M, Gray JJ. 2001. Rotavirus epidemiology and surveillance. Novartis Found Symp. 238: 125–152.

Dewar J, de Beer M, Elliott E, Monaisa P, Semenva D, Steele A. 2005. Rapid detection of rotaviruses – are laboratories underestimating infection in infants? S Afr Med J. 95: 494–495.

Dubal Z B, Bhilegaonkar KN, Barbuddhe SB, Kolhe R P, Simranpreet Kaur, Shriya Rawat, Prejit Nambier, Karunakaran M. 2013. Prevalence and genotypic (G and P) determination of porcine group A rotaviruses from different regions of India. Trop Anim Health Prod. 45: 609–615.

Estes MK, Kapikian AZ. 2006. Rotaviruses. In: Knipe DM, Howley PM, editors. Fields virology. 5th ed., Vol. 1. Philadelphia (PA): Lippincott Williams & Wilkins; p. 1917.

Gentsch JR, Woods, PA, Ramachandran M, Das BK, Leite JP, Alfieri A, Kumar R, Bhan MK, Glass RI. 1996. Review of G and P typing results from a global collection of strains: implications for vaccine development. J Infect Dis. 174: S30–S36.

Herring AJ, Inglis NF, Ojeh CK, Snodgrass DR, Menzies JD. 1982. Rapid diagnosis of rotavirus infection by direct infection of viral nucleic acid in silver stained polyacrylamide gels. J Clin Microbiol. 16: 473–477.

Jain V, Parashar UD, Glass RI, Bhan MK. 2001. Epidemiology of rotavirus in India. The Indian J Pediatr. 68: 855–862.

Kapikian AZ, Hoshino Y, Chanock RM. 2001. Rotaviruses. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE, editors. Fields virology. 4th ed. Philadelphia (PA): Lippincott Williams & Wilkins; p. 1787–1833.

Kumar M. 2006. Polymerase chain reaction for rapid detection of important zoonotic diarrhoeal pathogens [M.V.Sc. Thesis]. Bareilly: IVRI.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage. Nature. 227: 680–685.

Matsui M, Mackow ER, Matsuno S, Paul PS, Greenberg HB. 1990. Sequence analysis of gene 11 equivalents from "short" and "super short" strains of rotavirus. J Virol. 64: 120–124.

Mukherjee A, Chattopadhyay S, Bagchi P, Dutta D, Singh NB, Arora R, Parashar UD, Gentsch JR, Chawla-Sarkar M. 2010. Surveillance and molecular characterization of rotavirus strains circulating in Manipur, North-Eastern India: Increasing prevalence of emerging G12 strains. Infect Genet Evol. 10: 311–320.

Parashar UD, Burton A, Lanata C, Boschi-Pinto C, Shibuya K, Steele D, Birmingham M, Glass RI. 2009. Global mortality associated with rotavirus disease among children in 2004. The J Infect Dis. 200: S9–S15.

Pongsuwanne Y, Taniguchi L K, Choonthanom M, Chiwakul M, Susansook T, Saguanwongse S, Jayavasu C, Urasawa S. 1989. Subgroup and serotype distributions of human, bovine, and porcine rotavirus in Thailand. J Clin Microbiol. 27: 1956–1960.

Pšikal I., Dvorák R., Franz J., Stepánek J. 1991. Rapid identification of bovine rotavirus by electrophoresis in agarose gel. Acta Vet Brno. 60(3): 253–261.

Rodger SM, Holmes IH. 1979. Comparison of the genomes simian, bovine and human rotaviruses by gel electrophoresis and detection of genomic variation among bovine isolates. J Virol. 30: 839–846.

Roman E, Martinez I. 2005. Detection of rotavirus in stool samples of gastroenteritis patients. P R Health Sci J. 24: 179–184.

Tate JE, Chitambar S, Esposito DH, Sarkar R, Gladstone B, Ramani S, Raghava SV, Sowmyanarayanan TV, Gandhe S, Arora R, Parashar UD, Kang G. 2009. Disease and economic burden of rotavirus diarrhoea in India. Vaccine. 27: F18–F24.

Temu A, Kamugisha E, Mwizamholya DL, Hokororo A, Seni J, Mshana SE. 2012. Prevalence and factors associated with Group A rotavirus infection among children with acute diarrhoea in Mwanza, Tanzania. J Infect Dev Countries. 6: 508–515.

World Health Organisation (WHO). 2009. Manual of rotavirus detection and characterization methods. p. 3. www.who.int/vacc.