

## 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  $\mu$ l of a 10% stool suspension and 75  $\mu$ l 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<sub>2</sub>SO<sub>4</sub>. The result was determined by both visual reading of the color change and measurement of the A<sub>490</sub> 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  $\mu$ l of sample (RNA extract) mixed with 10  $\mu$ l 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 <sup>a</sup>	No. of specimens (%)
<b>Identical ELISA and PAGE results</b>	
ELISA+, PAGE+ .....	325 (24.9)
ELISA-, PAGE- .....	936 (71.8)
<b>Conflicting ELISA and PAGE results</b>	
ELISA+, PAGE- .....	26 (2.0)
ELISA-, PAGE+ .....	17 (1.3)

<sup>a</sup> +, 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).

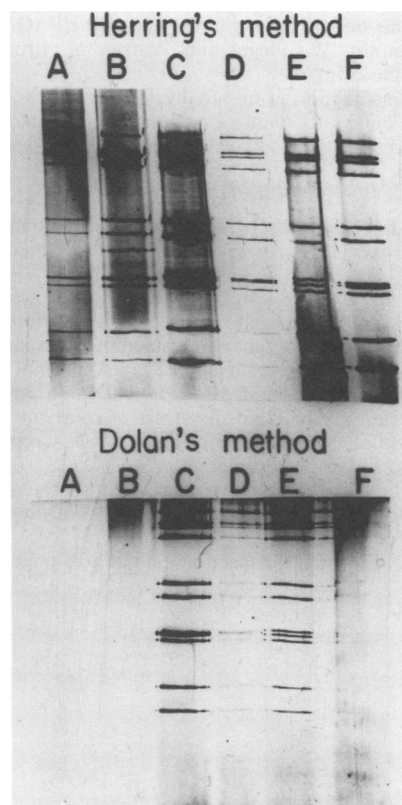


FIG. 1. Comparison of PAGE diagnostic results of the techniques of Herring et al. (3) and Dolan et al. (2) with 6 of the 53 rotavirus-positive fecal samples randomly selected for this purpose. Although the same amounts of fecal samples were used in each technique, only the method of Herring et al. demonstrated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

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