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DNA Hybridization for Diagnosis of Enteric Adenovirus Infection from Directly Spotted Human Fecal Specimens

G. HAMMOND,[1,2]* C. HANNAN,[1] T. YEH,[1] K. FISCHER,[1] G. MAUTHE,[1] AND S. E. STRAUS[3]

University of Manitoba[1] and Cadham Provincial Laboratory,[2] Winnipeg, Manitoba R3C 3Y1, Canada, and National Institutes of Health, Bethesda, Maryland 20892[3]

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By using a genomic probe, DNA hybridization for adenovirus type 41 (Ad41) showed equivalent sensitivity with a direct spot method from clinical specimens compared with a more laborious DNA phenol extraction procedure. By using this direct spot preparation method, fecal specimens of 67 patients were examined under code for blind testing for the presence of adenovirus by DNA hybridization by using two Ad41 probes (genomic and cloned *Bgl*II-D) and an adenovirus type 2 genomic probe. Identical results were obtained with both of the Ad41 probes. Of the fecal specimens from 42 children with adenovirus gastroenteritis studied prospectively (16 of whom had enteric adenoviruses), 13 specimens (81%) were detected by DNA hybridization with a cloned Ad41 *Bgl*II-D probe. There were 14 fecal specimens that were positive by electron microscopy (EM) and culture for nonenteric adenovirus, and 2 specimens were positive by DNA hybridization (87% specificity); these 2 specimens may have been from a mixed enteric adenovirus and nonenteric adenovirus infection. None of 26 specimens from age-matched healthy control patients was positive for adenovirus by EM or DNA hybridization. Our data indicated that DNA hybridization gives highly reproducible results. The direct spot technique is the method of choice for specimen preparation in the diagnostic laboratory, since it requires only the simplest manipulations in specimen preparation. By using DNA hybridization with the *Bgl*II D fragment of a cloned enteric Ad41, both adenovirus type 40 and Ad41 were detected directly from fecal specimens, but it was less sensitive than EM following direct ultracentrifugation of specimens. The *Bgl*II-D Ad41 DNA probe was highly specific for enteric adenoviruses, and DNA hybridization with this probe could be a useful diagnostic test for these fastidious adenoviruses.

DNA hybridization offers the potential for the rapid diagnosis of virus infections, but the sensitivity and specificity of this method compared with those of conventional diagnostic methods is not yet fully known.

Enteric adenoviruses (EAds; adenovirus types 40 [Ad40] and 41 [Ad41] of subgenus F) (6, 26) have been implicated as causes of gastroenteritis in infants (3, 5, 7, 12, 24, 25). Because EAds are often difficult to cultivate, alternative specific methods for the detection of these viruses directly in clinical specimens are necessary, and results of several studies with that aim have been reported (13, 16, 19). DNA hybridization with radioactive probes has been evaluated recently for the detection of adenovirus in fecal specimens, with either cloned DNA fragments of Ad40 or Ad41 (14, 20, 22) or genomic probes of adenovirus type 2 (Ad2) (20, 21). A nonradioactive cloned DNA probe has been evaluated recently (17).

We report here an evaluation of DNA hybridization for the detection of adenoviruses in fecal specimens and compare its sensitivity and specificity with those obtained by electron microscopy (EM) and virus isolation in tissue culture. For this evaluation we employed adenovirus DNA probes representing two subgenera (C and F). We also compared genomic DNA and a cloned DNA restriction fragment (*Bgl*II-D) of Ad41 when used as probes (22). The efficacy of a direct spot method that is technically simpler than phenol extraction of fecal specimens was also evaluated. Finally, we applied the direct spot test with the Ad41 *Bgl*II-D probe for the diagnosis of EAds in a prospective study of pediatric gastroenteritis associated with adenovirus shedding.

* Corresponding author.

MATERIALS AND METHODS

Fecal specimens. Two separate groups of fecal specimens were employed in the investigation, as described below.

(i) Group A. Of a collection of fecal specimens (stored at −70°C) that were submitted for diagnosis to the Virus Detection Laboratory, Cadham Provincial Laboratory, in 1983 and 1984 from individual children with sporadic disease (usually gastrointestinal), 67 were selected on the basis of diagnostic results (see below) and coded for blind testing by hybridization with three separate DNA probes (Ad41 genomic DNA, Ad41 *Bgl*II-D DNA, and Ad2 genomic DNA). The selected group included specimens that were found to be, on the basis of prior EM (10) or tissue culture results on HEp-2 and rhesus monkey kidney cells, positive for adenovirus (n = 38), negative for any virus (n = 22), and negative for adenovirus and positive for rotavirus or enterovirus (n = 7). Aqueous fecal suspensions (10%) were clarified by centrifugation and processed further as described below.

(ii) Group B. Initial fecal specimens were obtained from 42 children with acute adenovirus gastroenteritis and stored at −70°C. These children were enrolled in a prospective study between 1982 and 1984 (G. Hammond, L. Thompson, H. Lukes, and G. Mauthe, manuscript in preparation). The fecal specimens were coded prior to blind testing by DNA hybridization with the Ad41 *Bgl*II-D DNA probe only. The diagnosis of adenovirus infection was established by examination of fecal specimens by EM following direct ultracentrifugation of the fecal supernatant (10). Specimens were cultured with a single additional pass on HEp-2 cells, and adenoviruses were identified by neutralization. Specimens that were positive for adenovirus by EM but negative for

adenovirus by culture on HEp-2 cells were cultured on Graham 293 cells (8, 23) with two additional passes. The types of the adenoviruses isolated on Graham 293 cells were determined by restriction endonuclease analysis (11). Fecal specimens from 26 age-matched controls enrolled at the same time as the index patients were also tested by EM, culture, and DNA hybridization.

Virus DNA and DNA probes. Ad2 and Ad41 were purified by centrifugation on a CsCl gradient after propagation in HEp-2 and Graham 293 cells, respectively. DNA was recovered from purified virus by phenol extraction and ethanol precipitation. The Ad41 *Bgl*II D DNA fragment (22) was prepared by subcloning into the *Bam*HI site of the plasmid vector pUC8. Virus DNAs were radioactively labeled in vitro by nick translation with ^{32}P in 50-ng amounts; the average specific activity was 4×10^8 cpm/μg of DNA. Adenovirus type 3 (Ad3) and adenovirus type 8 (Ad8) DNAs, which were used as adenovirus hybridization specificity controls, were prepared from similarly purified virus after propagation in HEp-2 cells.

Specimen preparation. Fecal suspensions were clarified routinely by centrifugation at 1,500 rpm for 15 min or, when fine particulate matter remained in the supernatant, by centrifugation at 15,000 rpm for 5 min in a centrifuge (model 5412; Eppendorf).

The clarified aqueous fecal suspensions were processed by one of two procedures: phenol extraction or direct spot. In the phenol extraction procedure, a solution of phenol-extracted and ethanol-precipitated DNA from an initial volume of 0.5 ml of fecal supernatant was dissolved in 100 μl of buffer. After denaturation and adjustment to 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 20 μl of DNA was deposited on a nitrocellulose membrane in duplicate in a 96-well manifold apparatus. In the direct spot procedure, which was an adaptation of the technique described by Anderson et al. (2), 5 μl of untreated clarified fecal suspension was deposited directly onto the nitrocellulose membrane in duplicate. Disruption of virus and denaturation of DNA was achieved by placing the membrane on filter paper soaked in 0.1 M NaOH with 1 M NaCl for 20 min, followed by neutralization in 0.1 M Tris buffer (pH 7.2) with 1 M NaCl and by washing twice in 2× SSC buffer for 20 min. Positive and negative controls were also deposited onto each membrane.

Hybridization. After baking at 68°C for 6 h, membranes were hybridized for 18 h at 68°C with 10^7 cpm of adenovirus DNA probe labeled as described above. The results were recorded by autoradiography on film (XAR-2; Eastman Kodak Co., Rochester, N.Y.) at −70°C for various periods of time in the presence of an enhancing screen.

RESULTS

Specificity of three probes. Results of preliminary experiments with purified unlabeled adenovirus DNAs from Ad2, Ad3, Ad8, and Ad41 demonstrated that under the hybridization conditions employed in this study, the reaction was essentially subgenus specific and that the probes detected at least 100 pg of unlabeled homologous DNA (Fig. 1 and 2), and occasionally, as little as 5 pg was detected (data not shown).

Comparison of the phenol extraction method with the direct spot method with group A specimens. Group A fecal specimens were employed in the initial evaluation of DNA hybridization and the evaluation of the two procedures for specimen preparation. In separate experiments, phenol-extracted DNA of 57 of the 67 coded fecal specimens was tested, with Ad41 genomic DNA and Ad41 *Bgl*II-D DNA used as probes. The results of one such experiment that demonstrates the sensitivity and specificity of the two Ad41 DNA probes, as well as their reactivity with DNA extracted from 24 of these fecal specimens, are shown in Fig. 1. Both Ad41 probes reacted with the same seven specimens under conditions that allowed the detection of 100 pg of Ad41 DNA, whereas 1-ng amounts of Ad2, Ad3, and Ad8 DNAs were either not detectable (Ad41 *Bgl*II-D probe) or barely detectable (Ad41 genomic probe). Of the 57 specimens tested by phenol extraction, 14 reacted with both of the Ad41 DNA probes. When the tests were repeated, the results were identical to those obtained initially.

To evaluate an easier method of specimen preparation, the direct spot method (2) was compared with the phenol extraction method. The results for 12 specimens showed that the sensitivity of hybridization was equivalent by each procedure; 3 of the 12 fecal specimens reacted positively with an Ad41 genomic DNA probe by both procedures and with comparable intensity, as did various amounts of unlabeled Ad41 DNA, ranging from 5 pg to 1 ng (data not shown). The results of repeat direct spot tests were identical to the original results.

All 67 fecal specimens were tested with both of the Ad41 DNA probes by the phenol extraction method ($n = 20$), the direct spot procedure ($n = 10$), or both ($n = 37$). The Ad41 genomic and cloned *Bgl*II-D probes gave identical results in all tests, and adenovirus DNA was detected by both Ad41 probes in a total of 15 specimens. The 67 fecal specimens were also tested with an Ad2 genomic DNA probe, and 17 reacted positively.

Sensitivity and specificity of probes by culture and EM. Overall, 20 of the 67 group A fecal specimens were positive for hybridization; 12 reacted with both Ad2 and Ad41 probes, 5 reacted only with the Ad2 probe, and 3 reacted only with the Ad41 probes. Evaluation of the hybridization results of the group A fecal specimens by comparison with virus isolation in HEp-2 cell cultures and by comparison with direct electron microscopic examination of ultracentrifuged specimens (10) is given in Table 1. Adenovirus was isolated from 28 specimens, 17 of which were identified by neutralization with antisera to adenovirus type 1 (Ad1), Ad2, Ad3, Ad5, adenovirus type 6 (Ad6), and adenovirus type 7 (Ad7) (7 belonged to subgenus B and 10 belonged to subgenus C). In relation to virus culture, the sensitivity of hybridization was 43% when Ad2 and Ad41 probes were used, and the specificity was 80%. In relation to the EM results, the comparable values were 70 and 91%, respectively. All seven specimens which contained either rotavirus or enterovirus were hybridization negative. However, 8 of 39 specimens which were negative for adenovirus by culture in HEp-2 cells and 4 of 44 specimens which were negative by EM were positive by hybridization (Table 1).

Evaluation of group B fecal specimens by Ad41 *Bgl*II-D probe. Group B fecal specimens from 42 children that had gastroenteritis and that were shedding adenovirus and fecal specimens from 26 age-matched controls were processed by the direct spot procedure and tested by hybridization with the Ad41 *Bgl*II-D probe. Under conditions that detected 10 to 100 pg of Ad41 DNA, 15 of 42 EM-positive specimens reacted with the probe (Fig. 2 and Table 2). Hybridization therefore was less sensitive than EM [sensitivity = (13/16) × 100 = 81%]. The Ad41 *Bgl*II-D probe did not react with the 26 fecal samples from healthy age-matched controls. This probe was also highly specific in excluding non-EAds [spec-

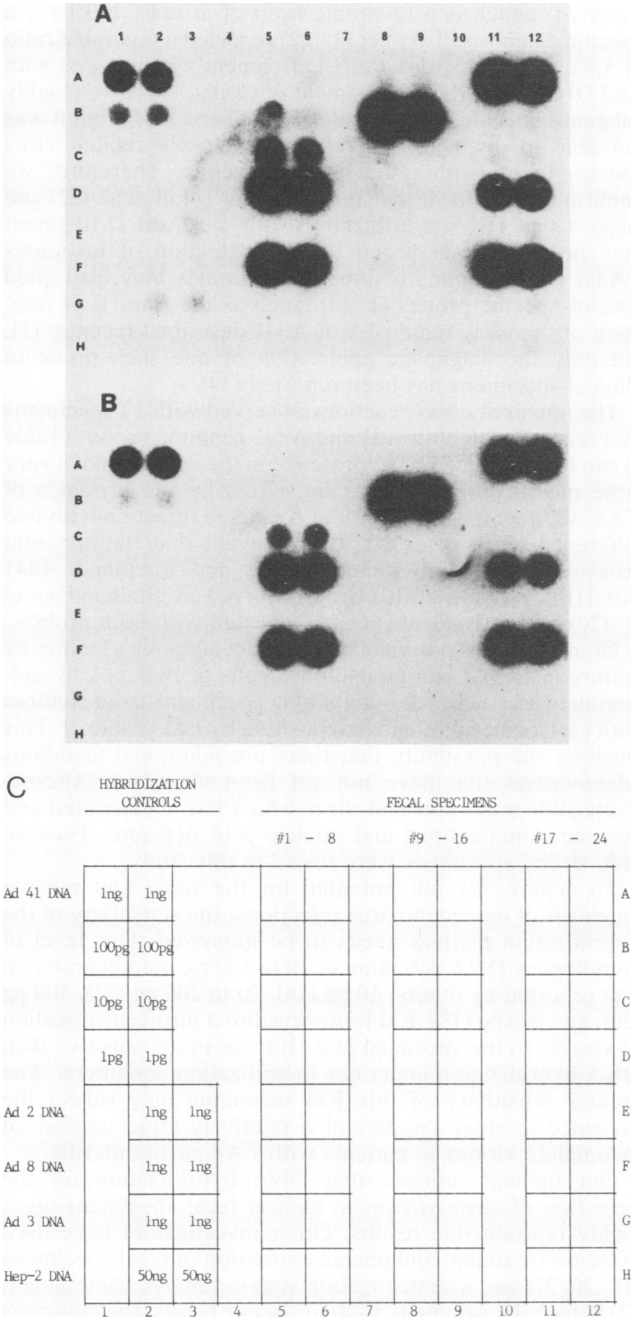

FIG. 1. Grid (C) for comparison of the sensitivity, specificity, and reactivity with fecal specimens of Ad41 genomic DNA (A) and Ad41 *Bgl*II-D DNA (B) as hybridization probes (specific activities, 6.4×10^8 and 1.8×10^8 cpm/μg, respectively). Twenty-four group A fecal specimens were tested as phenol (DNA) extracts. Control DNAs were extracted from purified virions or from uninfected HEp-2 cells. Duplicate fractions of all samples were deposited on separate membranes in an identical arrangement. The membranes were hybridized with 10^7 cpm of the indicated probe for 18 h at 68°C. Autoradiographic exposure time was 24 h. Identical fecal specimens were positive, but the background activity appeared greater with the Ad41 genomic probe.

TABLE 1. Hybridization with genomic probes for detection of adenovirus in group A fecal specimens compared with virus isolation in HEp-2 cell culture and direct EM examination

Reference diagnostic test	No. of specimens	No. of hybridization-positive specimens with:			
		Ad2 probe only	Ad41 probe only	Ad2 and Ad41	Total
CC[a] positive	28	4	1	7	12[b]
CC negative	39	1	2	5	8
EM positive	23	2	3	11	16[c]
EM negative	44	3	0	1	4
Total		5	3	12	

[a] CC, HEp-2 cell culture.

[b] Results of hybridization versus those of HEp-2 cell culture were as follows: sensitivity, 12 of 28 (43%); specificity, 31 of 39 (79.5%); positive predictive value, 12 of 20 (60%); negative predictive value, 31 of 47 (66%).

[c] Results of hybridization versus those of EM were as follows: sensitivity, 16 of 23 (70%); specificity, 40 of 44 (91%); positive predictive value, 16 of 20 (80%); negative predictive value, 40 of 47 (85%).

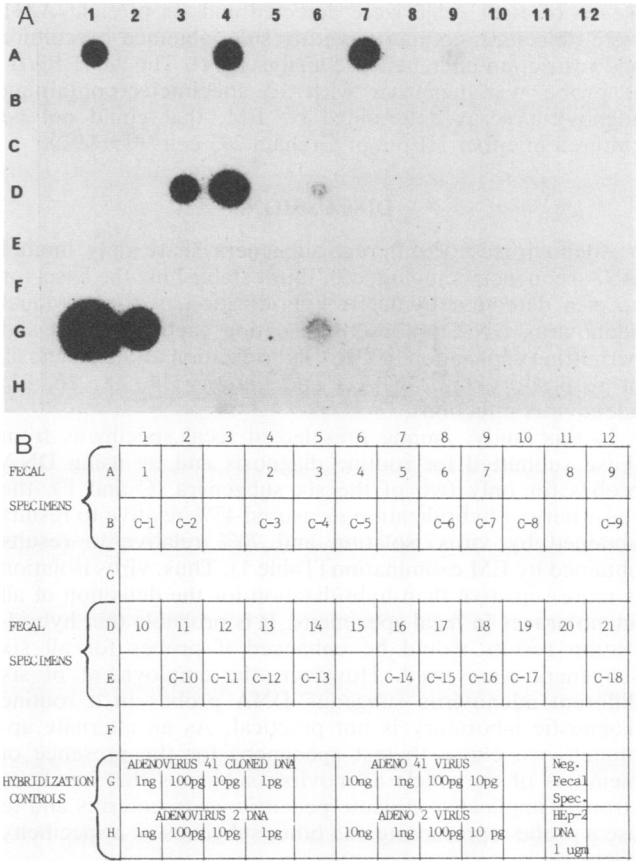

FIG. 2. Hybridization of the Ad41 *Bgl*II-D DNA probe to group B fecal specimens by using the direct spot method for specimen preparation. Hybridization was at 68°C for 18 h. Autoradiographic exposure was for 48 h. Fecal specimens were from children with acute adenovirus gastroenteritis ($n = 42$) and healthy controls ($n = 26$) negative for adenovirus by EM and culture, of which 15 specimens were positive by hybridization (Table 2). (A) Examples of reactions from 7 positive (boxes 1, 2, 5, 7, 12, 13, and 15) and 14 negative fecal specimens from gastroenteritis patients and from 18 control specimens (C-1 to C-18). (B) Arrangement of fecal specimens and controls.

TABLE 2. Sensitivity and specificity of DNA hybridization with the Ad41 *Bgl*II-D probe for the diagnosis of EAd infection (group B fecal specimens)

DNA hybridization	No. examined by EM and culture			
	Patients with adenovirus gastroenteritis positive by EM (n = 42)			Healthy age-matched controls negative by EM and culture (n = 26)
	EAd	Non-EAd	Noncultivable adenovirus	
Positive	13[a]	2[b]	0	0[c]
Negative	3	12	12	26

[a] Sensitivity = (13/16) × 100 = 81%.
[b] Specificity versus non-EAd = (12/14) × 100 = 86%.
[c] Specificity versus other controls = (26/26) × 100 = 100%.

ificity = (12/14) × 100 = 86%]. These two false-positive reactions occurred with fecal specimens from which Ad2 was cultured and may have represented either mixed EAd–non-EAd infections or shedding of a large amount of Ad2, as the Ad41 *Bgl*II-D probe did not cross-react with 1 ng of purified Ad2 DNA (Fig. 1). However, the probe did not distinguish between Ad41 and Ad40 in clinical specimens (seven of eight Ad40 were detected and six of eight Ad41 were detected), compared with results obtained by culture and restriction endonuclease analysis (11). The Ad41 *Bgl*II-D probe was negative with 12 specimens containing adenoviruses, as determined by EM, that could not be cultured in either HEp-2 or Graham 293 cells (Table 2).

DISCUSSION

Adenoviruses of different subgenera share only limited DNA sequence homology (9), thus establishing the basis for species detection by DNA hybridization, with individual adenovirus DNA probes representing each subgenus and permitting application of DNA hybridization to the diagnosis of respiratory (15, 18, 21) and enteric (14, 18, 20, 22) adenovirus infections.

In this study, among preselected fecal specimens from those submitted for routine diagnosis and by using DNA probes for only two of the six subgenera (C and F), the sensitivity of hybridization measured 43% relative to results obtained by virus isolation and 70% relative to results obtained by EM examination (Table 1). Thus, virus isolation is more sensitive than hybridization for the detection of all adenoviruses in fecal specimens. It is probable that hybridization results would be enhanced if probes for all six subgenera were used. However, the employment of six different adenovirus subgenus DNA probes in a routine diagnostic laboratory is not practical. As an alternate approach, we chose to test specimens for the presence of members of the single adenovirus subgenus that has been strongly implicated in acute pediatric gastroenteritis and to use a probe representing one other subgenus as a specificity control.

In previous hybridization studies of fecal specimens, the following DNA probes have been used: a cloned fragment of Ad2 (18), genomic Ad2 and a clone of the *Bam*HI G fragment of Ad41 (20), the *Pst*I H fragment of Ad40 and the *Pst*I B fragment of Ad41 (14), and Ad2 and the Ad41 *Bgl*II D fragment (22). In our study equivalent sensitivity of an Ad41 genomic probe was shown in comparison with that of the *Bgl*II D fragment, and a greater specificity of the latter was shown, because no cross-hybridization was observed with a large excess of DNA derived from subgenera B, C, and D viruses. In our hands, the Ad41 *Bgl*II-D probe did not hybridize with up to 1 ng of heterologous Ad2, Ad3, or Ad8 DNAs, while as little as 10 pg of homologous Ad41 DNA was detected, which is a favorable ratio of at least 100:1 for a specific diagnosis. Takiff et al. (22) reported a favorable ratio of 5,000:1 for the Ad41 *Bgl*II D fragment as compared with Ad2 DNA. The *Bgl*II D fragment of cloned Ad41 was highly subgenus specific for both enteric Ad40 and Ad41, but it was not able to distinguish between the closely related virus species found within the same subgenus. Therefore, we confirm the results of the study done by Takiff et al. (22) and suggest that DNA hybridization with the *Bgl*II D fragment may be a practical probe for the detection of fastidious EAds. Other regions of the EAd genomes may also yield species-specific probes (1, 14), such as the *Bam*HI H fragment of the early region 1A of Ad41 described recently (1), but only the diagnostic application of one such probe to clinical specimens has been reported (14).

The apparent cross-reactions observed with 12 specimens that reacted with both Ad2 and Ad41 genomic probes (Table 2) can be explained by the presence in the specimen of a very large quantity of cross-reacting virus, by the presence of EAd-Ad2 recombinants (22), or by mixed infection with two different adenoviruses (4). It is relevant that the apparent cross-reactivity with genomic Ad2 and a cloned Ad41 *Bam*HI-G probe has also been observed by Stalhandske et al. (20) in 5 of 16 fecal specimens tested with both probes.

Of interest was our inability to detect adenovirus either by culture in HEp-2 and Graham 293 cells or by DNA hybridization of 12 adenovirus-containing specimens from children with gastroenteritis-that were positive by EM (Table 2). This suggests the possibility that there are additional fastidious adenoviruses that have not yet been identified. Alternatively, this may represent virus which has degenerated and become noninfectious and nucleic acid deficient, because only stored specimens were tested in this study.

To achieve its full potential for the rapid and reliable diagnosis of any adenovirus infections, the sensitivity of the hybridization method needs to be improved. Our level of homologous DNA detection of 10 to 100 pg is comparable to that reported by others: 10 pg (14), 20 to 200 pg (22), 100 pg (20), and 200 pg (18). EM following direct ultracentrifugation of viruses to the specimen grid (10) was more sensitive than DNA hybridization under our hybridization conditions. The greater sensitivity of this EM technique may reflect the presence in stool samples of a relatively large number of incomplete virions in patients with EAd gastroenteritis.

Our findings indicate that DNA hybridization for the detection of adenoviruses in clinical fecal specimens gives highly reproducible results. Other investigators have used protease treatment and phenol extraction of fecal specimens (14, 20, 22) and a centrifugation step to remove bacteria (14, 22) before the sample is spotted. In our hands, the laborious phenol extraction method was no more effective than the simple direct spot specimen application, and the technical simplicity of the latter method makes it the method of choice for specimen preparation in diagnostic hybridization. This method would not work, however, for the detection of RNA viruses. This method requires evaluation by other investigators, but its speed, simplicity, and small sample volume has many advantages.

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