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Rapid Detection of Vesicular Stomatitis Virus New Jersey Serotype in Clinical Samples by Using Polymerase Chain Reaction

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Vesicular stomatitis virus of the New Jersey serotype (VSV-NJ) causes vesicular disease in cattle, pigs, and horses throughout the Americas. Vesicular disease is clinically indistinguishable from foot-and-mouth disease (FMD). Therefore, outbreaks of vesicular disease in FMD-free areas must be rapidly diagnosed by laboratory methods and affected farms must be quarantined until laboratory results confirm the absence of FMD. Diagnosis is currently performed in high-containment (biosafety level 3) laboratories by using complement fixation and virus isolation in tissue culture. We describe here an alternative method for the detection of VSV-NJ RNA in clinical samples. This method includes a rapid acid guanidine-phenol RNA extraction procedure coupled with a one-tube polymerase chain reaction (PCR) using reverse transcriptase. By using this test, we were able to detect the largest number of positive samples (53 of 58), followed by complement (48 of 58) and isolation in tissue culture (43 of 58). The primers chosen for this assay amplify a 642-nucleotide region of the phosphoprotein gene of VSV-NJ but not of VSV-IN. Sequencing of the PCR product enables genetic typing of virus isolates and epidemiological studies. Since no infectious materials are necessary to perform this test and any infectious virus in clinical samples is destroyed by acid guanidine-phenol treatment, diagnosis can be safely performed in regular diagnostic laboratories.

Vesicular stomatitis (VS) is caused by viruses of the genus *Vesiculovirus* of the *Rhabdoviridae* family. There are two major serotypes of VS virus (VSV), New Jersey (NJ) and Indiana (IN). Enzootic VSV activity occurs in the southeastern part of the United States, Mexico, Central America, Panama, and northern South America (Colombia, Ecuador, Peru, and Venezuela). Three subtypes of the IN serotype have been found: IN_1 (found in enzootic areas), IN_2 (isolated sporadically in Trinidad, Brazil, and Argentina), and IN_3 (reported in Brazil and Colombia) (2). In Central America, outbreaks of VS occur every year, particularly at the beginning of the dry and rainy seasons (9, 12, 14).

Most clinical cases in enzootic areas are caused by the NJ serotype (9, 12). However, both serotypes cause identical vesicular lesions on the epithelia of the mouths and tongues, and coronary bands of hooves of cattle, pigs, and horses. In cattle, the two serotypes also affect the epithelia of the teats, making milking difficult and causing secondary subclinical and clinical mastitis (9, 14, 21). In addition, VSVs infect humans in enzootic areas in North America, Central America, and South America (16, 17). Clinical symptoms in humans range from a self-limited flu-like illness to severe encephalitis (13, 16). Periodically, large epizootics of VS NJ and IN_1 have occurred in western United States and Mexico, causing serious losses particularly to dairy herds (8, 10).

Initial clinical symptoms of VS resemble foot-and-mouth disease (FMD), a severe vesicular disease which has been eradicated from Central America and North America. Therefore, cases of vesicular disease must be rapidly diagnosed by laboratory methods and affected farms must be quarantined until laboratory results confirm the absence of FMD. In Central America, samples of epithelium from the lesion are usually collected from clinically affected animals and sent in transport medium to a high-containment regional diagnostic laboratory for vesicular diseases (Laboratorio de Diagnóstico de Enfermedades Vesiculares, Panama City, Panama). There, samples are processed by the complement fixation (CF) test for detection of FMD virus (FMDV) and VSV antigens. Delays of up to 5 days may occur between the collection of samples and reports of test results. Samples are often insufficient or inadequate for testing or unreactive to all antibodies tested (7). Because of this delay and the problems associated with quarantining their herds, many producers choose not to report clinical cases and therefore increase the risk of an FMD outbreak going unreported until it is too late to be contained. This problem could be overcome, at least in part, by a rapid method to detect VSV directly from tissue samples which could be performed in regular diagnostic facilities throughout the region.

We have used the polymerase chain reaction (PCR) to amplify a 642-nucleotide fragment of the gene encoding the polymerase-associated phosphoprotein (P) of VSV-NJ in RNA extracted from epithelial samples from clinically affected animals. This method not only allowed highly sensitive detection, rapid diagnosis, and highly specific identifi-

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TABLE 1. Description of 58 epithelium samples tested by viral isolation, CF, and PCR

Province, breed, or lesion site	No. of samples
Province in Costa Rica	
Alajuela	41
Cartago	3
Guanacaste	5
Limón	3
Puntarenas	4
San José	1
Unknown	1
Cattle breed	
Dairy (holstein, jersey)	30
Beef (Brahman, others)	17
Mixed	11
Unknown	0
Site of lesion	
Mouth	30
Teat	22
Hoof	1
Unknown	5

cation of VS-NJ but also provided enough DNA for direct sequencing and genetic typing of the isolates.

MATERIALS AND METHODS

Sample collection and description. All samples were obtained from clinically ill cattle between December 1986 and March 1992. Most of the 58 samples were from mouth and teat lesions (30 and 22, respectively) and only 1 was from a foot lesion (Table 1). Samples originated from different animals located in geographic regions throughout Costa Rica, representing all the ecological zones of Costa Rica where VSV is found (3) (Table 1). Epithelium samples, collected from animals clinically affected with vesicular disease, were placed in transport medium (50% phosphate-buffered glycerol, pH 7.2) and sent to LADIVES. A small portion of the epithelium was given to us and kept frozen at −70°C in our laboratory (19). Once CF results were obtained, samples were processed by both virus isolation in tissue culture and enzymatic amplification using reverse transcriptase and PCR (RT-PCR). Most samples were collected within 24 h of clinical appearance of disease. However, some samples were dry scabs obtained several days after clinical disease, and in some cases the animals had been treated with local antiseptics prior to sample collection.

Isolation in tissue culture. Approximately 100 to 500 mg of epithelium was homogenized in 5 ml of minimum essential medium containing 5% fetal bovine serum and penicillin-streptomycin with a sterile mortar and pestle. After centrifugation for 5 min at 12,000 × *g*, each homogenate was inoculated onto Vero cells. Cultures were checked every 24 h for cytopathic effect for 3 days. If cytopathic effect was not observed, cells were frozen and thawed twice and a second passage in Vero cells was done; if after 3 days, no cytopathic effect was observed, the sample was considered negative. All viruses isolated were identified by virus neutralization assay with VSV-NJ- and VSV-IN-specific reference antisera (kindly supplied by J. Pearson, National Veterinary Services Laboratory, Ames, Iowa).

CF tests. CF results were obtained through the Costa Rican Ministry of Agriculture as part of a collaborative study. All CF tests were done at the LADIVES laboratory in Panama by standard procedures (11).

RNA extraction and analysis. RNA extraction procedures were done with RNase-free conditions and diethylpyrocarbonate (DEPC)-treated reagents as described previously (15). RNA was extracted from samples of epithelia from the tongues, mouths, snouts, teats, or feet of clinically affected cattle and healthy control animals by the acid phenol procedure (5). Briefly, approximately 100 mg of tissue was homogenized in 500 μl of acid guanidine isothiocyanate (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% sodium sarcosyl, 100 mM beta-mercaptoethanol, 200 mM sodium acetate [pH 4.0]) in a 1.7-ml microcentrifuge tube with a plastic pestle (Kontes, Inc.). Once completely homogenized, 500 μl of water-equilibrated phenol and 150 μl of chloroform-isoamyl alcohol (49:1) were added and mixed by vortexing. After a 20-min incubation on ice, samples were centrifuged at 14,000 × *g* at 4°C for 15 min and the aqueous phase was removed to a different tube. RNA was purified with an RNA purification kit (RNaid; Bio Labs) following the instructions of the manufacturer. RNA was diluted in 50 μl of sterile DEPC-treated water. The integrity of the RNA was corroborated by size fractionation in 1.5% agarose gels containing 2.2 M formaldehyde (6). Gels were stained for 5 min in a solution containing 0.5 μg of ethidium bromide per ml, destained for 1 h, and photographed with Polaroid film.

One-tube RT-PCR. Reaction conditions were developed to allow both the synthesis of the first strand of cDNA of the virus RNA template by using RT and the subsequent amplification of DNA by using the *Taq* polymerase in a single-tube reaction. This simplifies the procedure for routine purposes and minimizes the opportunity for cross contamination between samples. A premix containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.01% gelatin (wt/vol) 0.1% Triton X-100 (vol/vol) 1.6 mM beta-mercaptoethanol, 0.24 mM dGTP, 0.24 mM dATP, 0.24 mM dCTP, and 0.2 mM dTTP, 200 ng of positive-sense primer, 200 ng of negative-sense primer, 3 U of RT (avian myeloblastosis virus; Promega, Madison, Wisconsin), and 1.5 U of *Taq* polymerase (Promega) in a total volume of 95 μl was prepared for each sample to be tested and dispensed into 0.5-ml tubes. Five microliters of each sample was added to each tube. This reaction mixture (final volume, 100 μl) was overlaid with 50 μl of mineral oil, incubated at 41°C for 1 h and then subjected to 40 cycles of the following profile: 93°C for 90 s, 50°C for 2 min, and 72°C for 4 min. All incubations were done in an automatic temperature cycler (Coy Labs [Grass Lake, Mich.] or Ericomp [San Diego, Calif.]).

Primers used for both RT-PCRs were specific for the phosphoprotein gene of VSV-NJ or -IN. These primers were as follows: NJ-P 102, 5′ GAGAGGATAAATATCTCC 3′; NJ-P 744(−), 5′ GGGCATACTGAAGAATA 3′; IN-P 179, 5′ GCAGATGATTCTGACAC 3′; and IN-P 793(−) 5′ GAC TCT(C/T)GCCTG(A/G)TTGTA 3′. Primers were synthesized on an automated synthesizer (Applied Biosystems model 380A or Dupont, NEN Research Products Coder 300).

VSV-NJ primers were chosen because their viral P-gene target sequences were known to be serotype specific and conserved among VSV-NJ isolates from throughout North America and Central America (4). VSV-NJ primers were used to provide the PCR-based virus detection system with the maximum potential to identify VSV-NJ in infected material from anywhere throughout the geographic range of the disease. In addition, the viral genome sequence flanked by these primers is a hypervariable region located in the

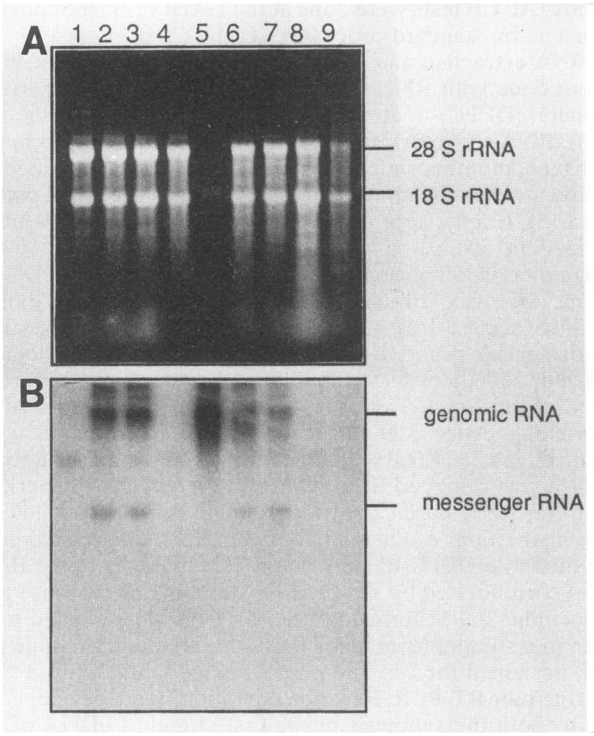

FIG. 1. (A) Agarose-formaldehyde gel showing total RNA purified from Vero cells infected with VSV-NJ (lanes 1) or mock infected (lane 2), and purified VSV-NJ genomic RNA (lane 3). (B) Autoradiograph of the same gel probed with a ^{32}P-labelled probe specific for VSV-NJ G-protein gene.

phosphoprotein gene of VSV-NJ (4). The extent of nucleotide sequence variability in this region is such that direct sequence analysis of the PCR products generated may allow precise genotyping and provide insight into the epizootiology of the disease.

Products of PCRs (10 μl of each reaction mixture) were analyzed in a 10-cm-long, 1.5% agarose minigel that was run for 20 min at 75 V in TAE buffer (15) containing 0.5 μg of ethidium bromide per ml and photographed with a UV transilluminator, a camera (Fotodyne), and Polaroid film. The sizes of the products were determined by using ϕX174 replicative-form DNA digested with *Hae*III as size markers (Bethesda Research Laboratories, Gaithersburg, Md.). The identity of the 642-bp fragment obtained with VSV-NJ primers was confirmed in some samples by DNA sequencing, using the primer extension dideoxy-chain termination method described by Winship (22).

Hybridization. In order to show the presence of intact viral RNA in the total RNA extractions, 4 μg of total RNA from Vero cells infected with different isolates of VSV-NJ were separated in formaldehyde gels as described above. Destained gels were dried, and viral RNA was detected directly by in situ hybridization of the gel using a ^{32}P-labelled probe specific for VSV-NJ G-protein gene as described previously (1, 18, 20). Briefly, VSV-NJ G-protein DNA was excised with *Pst*I from a pGem plasmid containing the VSV-NJ G-gene sequence (kindly supplied by S. Vernon). Purified DNA (25 ng) was labelled with ^{32}P, using a random primer labelling kit (Bethesda Research Laboratories) and Klenow DNA polymerase (15), and the specific activity of the probe was 3×10^5 cpm/ng. Hybridization was done with 10 ng of labelled probe in a solution consisting of 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 0.1% sodium dodecyl sulfate, and 50% formamide and incubating the solution at 42°C for 12 h (15).

RESULTS

RNA extraction. Up to 25 tissue samples could be extracted simultaneously in approximately 2 h by using this procedure. Intact cell RNA could be obtained from tongue epithelia or Vero cells by using the acid guanidine-phenol extraction method (Fig. 1A). Intact viral RNA, both genomic RNA (11 kb) and G-protein mRNA (1.72 to 1.77 kb), was also obtained by this method, as shown by hybridization to a virus-specific probe (Fig. 1B). Total RNA obtained by this extraction method was used directly in the one-tube PCR. The amount of RNA contained in the 5-μl volume added to the reaction varied widely from sample to sample (0.001 to 1 μg). In order to avoid an additional RNA quantification step and maintain the simplicity of this diagnostic procedure, we tested several volumes of template RNA in the RT-PCR, using five PCR-positive and five PCR-negative samples and obtained the same results with 1, 5, or 10 μl of template (data not shown). On the basis of these results, we continued using 5 μl of RNA template, which represents 10% of the total volume obtained from each RNA extraction.

Specificity. Samples were considered positive for VSV-NJ only if a 642-bp fragment was clearly seen in the product analysis gel (Fig. 2). The identity of the 642-bp band was confirmed in a few cases by direct sequencing of the product band. The information provided by sequencing the 642-bp fragment allowed phylogenetic and epidemiologic studies of VSV-NJ in Costa Rica (10a). However, for diagnostic purposes, it is not necessary to sequence the PCR product. The specificity of the assay was demonstrated, since noninfected tissue and water controls included in all tests always tested

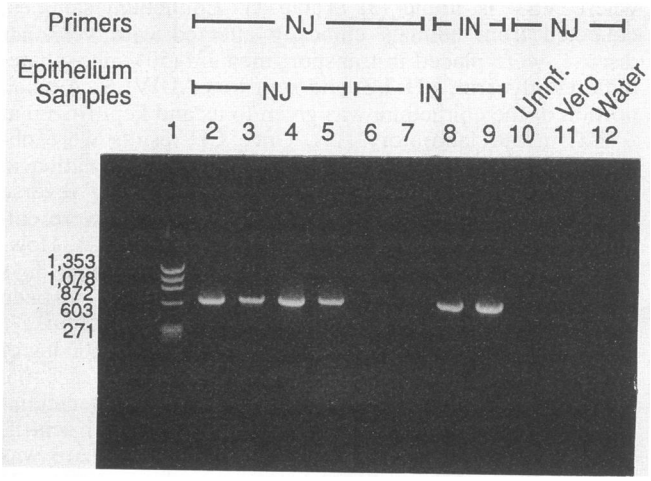

FIG. 2. Agarose gel of products obtained by RT-PCR of epithelium samples from clinical vesicular cases from three provinces of Costa Rica. The samples in lanes 2 to 5 showed typical positive VSV-NJ reactions, showing a 642-bp product band. The samples in lanes 6 to 9 were VSV-IN tested with NJ primers (lanes 6 and 7) or IN primers (lanes 8 and 9). The sample in lane 10 was epithelium from an uninfected (uninf.) cow, and lanes 11 and 12 contain uninfected Vero cells and water (control), respectively. Markers in lane 1 were ϕX174 replicative-form DNA digested with *Hae*III. The numbers to the left of the gel are the sizes (in base pairs) of size markers.

TABLE 2. Comparison of VSV-NJ detection in clinical samples by virus isolation, CF, and RT-PCR

Method	No. of positive samples (n = 58)	Detection rate (%)
Virus isolation	43	74
CF	48	83
RT-PCR	53	91

negative (Fig. 2). In addition, we failed to amplify a 642-bp fragment from epithelium samples from teats, noses, and coronary bands of 10 seronegative cows from VSV-free areas of Costa Rica and the United States (data not shown). Furthermore, the test was serotype specific for VSV-NJ, since the 642-bp fragment was absent from epithelium samples from VSV-IN-infected animals. These samples tested positive, giving a 614-bp product band when VSV-IN-specific primers were used (Fig. 2). The RT-PCR assay was shown to be capable of detecting VSV-NJ from infected tissues from different regions of Costa Rica and amplified products of the same size (642 bp) in each case.

Comparison with currently used diagnostic methods. The largest number of positive samples was obtained by RT-PCR, followed by CF and virus isolation in tissue culture (Table 2). There was a 76% (44 of 58) agreement among all three tests (Table 3). CF had a higher agreement with PCR (88% [51 of 58]) than did virus isolation (83% [48 of 58]). Of 15 samples that gave negative results by virus isolation, 10 gave positive results by PCR, whereas only 1 was positive by CF test. Only one sample was positive by CF and negative by PCR, and none of the samples that were positive by virus isolation were negative by PCR (Table 3).

DISCUSSION

A rapid diagnostic test for vesicular disease using PCR requires a fast and simple procedure to obtain RNA from epithelium samples taken under field conditions. Many times these samples are contaminated with dirt, manure, or substances used to treat the vesicular lesions. The acid guanidine-phenol extraction procedure proved to be fast, simple, and reliable in obtaining high-quality RNA from a variety of clinical samples. Some of these samples were of very poor quality for isolation in tissue culture or CF assay.

The primers used in this study were specific for the viral P gene. On the basis of previous VSV-NJ P-gene sequence analyses of isolates from throughout North and Central America, RT-PCR will be capable of detecting virus in tissue from throughout the geographic range of the disease. The primers were however, specific for the VSV-NJ serotype. Several VSV-IN-infected tissues were tested and gave negative results. These samples were positive however, when VSV-IN-specific primers were used (Fig. 2). The 642-bp product band could be found only in VSV-NJ infected tissue but not in VSV-IN-infected or uninfected tissue (Fig. 2). A weak nonspecific 50- to 100-bp band was observed occasionally in some noninfected Vero cell controls. The identity of this minor band was not determined, since it did not interfere with the diagnostic 642-bp band. Spurious bands in this small size range have frequently been reported in PCR literature and have been considered concatenated primer bands (15).

TABLE 3. Agreement of virus isolation, CF, and RT-PCR test results in detecting VSV-NJ[a]

VSV-NJ detection by the following test:			No. of samples	Agreement
Virus isolation	CF	RT-PCR		
+	+	+	40	69.0
+	+	−	0	0.0
+	−	+	3	5.2
+	−	−	0	0.0
−	+	+	7	12.0
−	+	−	1	1.7
−	−	+	3	5.2
−	−	−	4	6.9

[a] A total of 58 samples were tested.

The RT-PCR procedure detected the largest number of positive samples, despite the fact that numerous samples were retroactively processed by RT-PCR, often several months after CF and tissue culture isolation were performed. Therefore, samples for RT-PCR were frequently smaller, and in some cases very little epithelium was left. One such sample was positive by CF and negative by RT-PCR. This sample did not contain enough epithelium to allow repeating the RNA extraction and RT-PCR test. The small amount of epithelium in this sample may provide an explanation for the negative PCR result.

The one-tube RT-PCR procedure described in this study performed very well when used with field samples submitted for diagnosis. These samples in many cases were not appropriate for tissue culture because of heavy bacterial contamination or because they were cytotoxic or contained antiseptics or other cytotoxic substances (such as gasoline and paint remover) used by the farmers to treat the vesicular lesions. The CF test, despite being a reliable method, requires the preparation and standardization of reagents on a weekly basis, and several grams of tissue are necessary to perform the CF test. In contrast, RT-PCR reagents are stable for months at −20°C, and the RT-PCR test was not affected by bacterial contamination or cytotoxic substances and required only 100 mg of tissue. Both CF and RT-PCR procedures could be done the same day, whereas tissue culture isolation required at least 2 days and in many cases 4 days and two passages in Vero cells to detect viral growth.

Since the procedure described here does not involve the use of live agents, it is possible to establish this method in regular diagnostic facilities.

Finally, by using the basic procedure described here, it should be possible to mix different sets of primers for VSV-NJ, VSV-IN, and FMDV in the same reaction tube. If these primers are designed to produce amplification products easily discerned by size in agarose gels, then the identification of the causal agent could be determined. An internal control using primers specific for a common cellular RNA species (e.g., actin) could be included in order to confirm that intact RNA was isolated and the PCR occurred. Positive RNA controls, including a small cloned fragment of FMDV could be safely used without the necessity of biosafety level 3 containment. Such an assay could have significant impact by providing a highly sensitive, rapid differential diagnosis of vesicular disease in livestock throughout the Americas.

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