Comparison of RNA Extraction Methods to Augment the Sensitivity for the Differentiation of Vesicular Stomatitis Virus Indiana1 and New Jersey

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Two methods for the extraction of RNA of vesicular stomatitis virus (VSV) Indiana1 and New Jersey and their simultaneous amplification by one-step polymerase chain reaction using reverse transcriptase were evaluated. A guanidine-thiocyanate-based RNA extraction (Qiagen RNeasy Mini Kit, Qiagen, Valencia, CA) followed by column-based purification coupled with one-step RT-PCR proved to be a simple, safe, practicable, and reliable tool for rapid, highly sensitive, and specific differential diagnosis of both types of VSV in cell lysate and spiked tissue samples as compared with the tri-phasic extraction method (Tri-reagent method). When RNA was extracted either from VSV cell culture stock or from VSV spiked bovine lymph nodes by using Qiagen RNeasy Mini Kit, the detection limit in the multiplex RT-PCR was as

low as 0.505 to 2.84 TCID₅₀ for VSV-IND and VSV-NJ, respectively. The multiplex RT-PCR consistently detected VSV-IND and NJ RNA in as little as 0.1–1.0 fg of total RNA from spiked BHK-21 cell suspension when Qiagen RNeasy mini kit was used. The multiplex RT-PCR assay was capable of detecting both types of VSV in a one-step reaction tube. The minimum sensitivity of this assay in various experiments was 0.1683 TCID₅₀ (IND), 0.0946 TCID₅₀ (NJ), and 0.057 fg (IND and NJ) per 2 µl PCR sample, which is significantly more sensitive than reported previously (0.28–2.8 TCID₅₀/1 µl). So the present study improved the sensitivity of previously reported multiplex RT-PCR for the detection and differentiation of VSV-IND and VSV-NJ in a single assay. *J. Clin. Lab. Anal.* 25:95–99, 2011. © 2011 Wiley-Liss, Inc.

Key words: guanidine-thiocyanate; polymerase chain reaction; rapid detection; RNA extraction; sensitivity; vesicular stomatitis virus and viral diagnosis

INTRODUCTION

Vesicular stomatitis (VS), caused by vesicular stomatitis virus (VSV) of the Rhabdoviridae family, is an economically important disease of livestock and occurs throughout the Americas (1). There are two major serotypes of VSV, New Jersey (NJ) and Indiana (IND). VSV-NJ is known to be endemic to Ossabaw Island, GA (2), whereas both serotypes have been observed in Central and South America (2,3).

Yearly outbreaks of VS occur in Central America, particularly at the beginning of the dry and rainy seasons. Most clinical cases in endemic areas are caused by the NJ serotype (3,4). Large outbreaks of VSV-NJ and VSV-IND have occurred periodically in the western

United States and Mexico, causing serious losses particularly to dairy herds (5).

Clinical signs include vesicular lesions, ulceration, and erosion of the oral and nasal mucosa and epithelial surface of the tongue, coronary bands, and teats, along with crusting lesions of the muzzle, ventral abdomen, and sheath. Clinical disease has been observed in cattle, horses, and pigs and very rarely in sheep, goats, and

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Received 12 May 2010; Accepted 24 November 2010
DOI 10.1002/jcla.20439

Published online in Wiley Online Library (wileyonlinelibrary.com).

llamas. Serologic evidence of exposure has been found in many species, including cervids, nonhuman primates, rodents, birds, dogs, antelope, and bats. The viruses are potentially zoonotic and may cause influenza-like disease in people working in close contact with the virus (6).

Clinical signs of VS resemble foot-and-mouth disease (FMD), a severe exotic vesicular disease, which makes it essential to diagnose and differentiate VS from FMD rapidly and effectively. Virus isolation and complement fixation test (CFT) are the most commonly used methods for the detection of VSV from clinical samples. CFT, despite being a reliable method, requires the preparation and standardization of reagents on a weekly basis, and requires several grams of tissue. Moreover, in many cases, field samples submitted for diagnosis of VSV are not appropriate for tissue culture due to heavy bacterial contamination or other cytotoxic substances used by the farmers to treat the vesicular lesions (7). Multiplex RT-PCR provides a sensitive and specific diagnostic alternative that circumvents the need for culture. The aim of this study was to improve the sensitivity of previously reported multiplex RT-PCR (8) for the detection and differentiation of VSV-IND and NJ in a single assay.

MATERIAL AND METHODS

Virus Strains

Viral stocks of VSV-IND ($10^{3.625}$ TCID₅₀/25 µl) and VSV-NJ ($10^{4.375}$ TCID₅₀/25 µl) grown on BHK-21 cells were kindly provided by Dr. Hana Van Campen of the Veterinary Diagnostic lab of Colorado State University (CSU), Fort Collins, CO.

Viral RNA

Extracted RNA (10,000 fg/µl) of reference strains of VSV-IND1 and VSV-NJ—Hazelhurst were kindly provided by Roberta J. Magnuson of the Animal Population Health Institute, CSU, Fort Collins, CO.

Experiment 1: RNA Extraction From VSV-Cell Culture Stock

Viral stocks of VSV-IND ($10^{3.625}$ TCID₅₀/25 µl) and VSV-NJ ($10^{4.375}$ TCID₅₀/25 µl) were diluted in molecular grade water to 10^{-5} TCID₅₀/ml in 1.0 log₁₀ intervals. RNA from various dilutions of each VSV type was extracted by using Qiagen RNeasy mini kit as per manufacturer's instructions (Qiagen, Valencia, CA). The eluted RNA (35 µl) from each dilution (100 µl), containing both virus types, was stored at -80°C until further use.

Experiment 2: RNA Extraction From VSV Spiked BHK-Cell Suspensions

Known VSV negative BHK-21 cell suspensions were spiked with various dilutions (1,000, 100, 10, 1, and 0.1 fg) of previously quantified RNA of reference strains of VSV-IND1 and VSV-NJ—Hazelhurst. Each spiked sample contained RNA from both VSV serotypes and each samples had the same amount of each serotype added. Total RNA was extracted from the spiked cell lysate by using Qiagen RNeasy kit as per manufacturer's instructions. The eluted RNA (35 µl) was stored at -80°C until further use.

Experiment 3: RNA Extraction From VSV Spiked Bovine Lymph Nodes

Bovine lymph nodes, known to be negative for VSV, were homogenized by using mini bead-beater mill and spiked with various dilutions (10^{-1} – 10^{-5} TCID₅₀/ml) of VSV-IND ($10^{3.625}$ TCID₅₀/25 µl) and VSV-NJ ($10^{4.375}$ TCID₅₀/25 µl).

RNA was extracted from the spiked samples (approx. 100 mg) by using either Qiagen RNeasy mini kit or Tri-reagent protocol (Molecular Research Center, Inc., Cincinnati, OH). Each spiked sample had the same amount of each serotype added. Total RNA obtained by these extraction methods was used directly in one-tube multiplex RT-PCR. The amount of RNA contained in the 2 µl volume added to the reaction varied from sample to sample (0.0057–57.14 fg or 0.0168 TCID₅₀ to 946.6 TCID₅₀). It represented 4–5.7% of the total volume obtained from each RNA extraction. Each experiment was repeated thrice at least.

Oligonucleotide Primers

Oligonucleotide primers sequences were previously published and their genomic locations are shown in Table 1. P (polymerase-associated phosphoprotein) gene of VSV-IND and L (large polymerase protein) gene of

TABLE 1. Sequence and Genomic Location of the VSV Oligonucleotide Primers

Primer	Sequence 5'–3'	Amplicon length
Forward VSV-IND-P	GCA-GAT-GAT-TCT-GAC-AC	614 bp
Reverse VSV-IND-P	GAC-TCT-CGC-CTG-ATT-GTA	
Forward VSV-NJ-L	TTG-GTT-CGG-AAC-TTG-GAT-TC	301 bp
Reverse VSV-NJ-L	ACT-CAT-GCG-GTA-TTT-ACC-CTT-G	

VSV-NJ were selected on the basis of their known serotype specificity (3,8,9).

One-Step Multiplex RT-PCR

One step RT-PCR kit (Qiagen GmbH, Hilden, Germany) was used in the VSV multiplex hot-start RT-PCR. The reactions were done in a volume of 25 μ l containing: 2 μ l of RNA template, 5 $\times$ Buffer [Tris $\cdot$ Cl, KCl, $(\text{NH}_4)_2\text{SO}_4$, 12.5 mM MgCl_2 , DTT; pH 8.7], 1 $\times$ Q solution, 0.4 mM of each deoxynucleotide triphosphate (dNTP), 1.0 μ M of each VSV primers, 5 U RNasin (Promega, Madison, WI), 1.0 μ l enzyme mix (Omniscript and Sensiscript Reverse Transcriptases, HotStartTaq DNA polymerase).

Thermal cycler (Gene Amp[®] PCR System 9700, Applied Biosystem, Carlsbad, CA) was preheated to 50°C before placing samples in it. Reverse transcription and amplification was accomplished in one-step with the following incubation program: 30 min at 50°C, 15 min at 95°C, 40 cycles at 94°C 1 min, 50°C 1 min, 72°C 3 min, and finally 10 min at 72°C. Reactions were held at 4°C until the amplicons could be analyzed by gel electrophoresis. Amplification products (12 μ l) were mixed with tracking dye (2 μ l) and separated by electrophoresis on a 1.5% agarose gel, containing 0.33 μ g ethidium bromide (Invitrogen, Paisley, UK) per ml, at a constant 220 V for 40 min in TAE buffer. The gel was photographed with a UV transilluminator (Kodak EDAS 290, UVP Inc., San Gabriel, CA) and a digital graphic printer (Sony, UP-D895).

Negative controls consisted of RNA extracted from uninfected culture supernatant and molecular grade water in place of RNA in the RT-PCR. All reactions were set up in a dedicated RT-PCR hood (PCR Work Station[™], Model# P-030, C.B.S scientific Co, Del Mar, CA) with dedicated decontaminated pipettors

(Eppendorf) and in a room separate from that used for RNA extraction and gel electrophoresis of amplified products. Barrier tips and nuclease-free supplies and reagents were used at all times. Sizes of the products were determined by comparison with 100 bp DNA marker. A 614 bp band indicated amplification of the appropriate P gene portion of VS-IND and a 301 bp band indicated amplification of the L gene portion of VS-NJ.

RESULTS

Analytical Sensitivity of the Assay

In experiment-1, the sensitivity was consistently observed to be 0.505 TCID₅₀ and 2.84 TCID₅₀ (0.1683 TCID₅₀ and 0.0946 TCID₅₀) for VSV-IND and VSV-NJ, respectively, by either VSV uniplex or multiplex RT-PCR (Fig. 1).

In experiment-2, the multiplex RT-PCR consistently detected VSV-IND and VSV-NJ RNA in as little as 0.1–1.0 fg (0.057–0.0057 fg) of total RNA from spiked BHK-21 cell suspension when Qiagen RNeasy mini kit was used for extraction of VSV RNA (Fig. 2).

In experiment-3, VSV virus sample mixtures, containing known amounts of both VSV-IND and NJ viruses spiked onto bovine lymph nodes, were analyzed in order to evaluate the efficiency of the extraction methods from tissue samples. Specific amplified products were found in each sample according to the primer used. The lowest detection limit of the multiplex RT-PCR assays for VSV-IND and NJ were 50.5 and 284 TCID₅₀, respectively, when tri-reagent protocol was used for the extraction of total RNA from the spiked tissue samples (Fig. 3), whereas this detection limit was 0.505 TCID₅₀ and 2.84 TCID₅₀ (0.1683 TCID₅₀ and 0.0946 TCID₅₀) VSV-IND and NJ, respectively, when Qiagen RNeasy mini kit was used for the VSV RNA extraction (Fig. 4).

Fig. 1. Multiplex RT-PCR sensitivity and specificity assay for VSV-IND and VSV-NJ detection. RNA extracted from serial dilutions of both types of VSV with titers of $10^{3.625}$ TCID₅₀/25 μ l and $10^{4.375}$ TCID₅₀/25 μ l, for IND and NJ, respectively, using Qiagen RNeasy mini kit. Lanes 2–5: typical positive VSV-IND reactions; lane 6: negative VSV-IND reaction; lane 7–10: positive VSV-NJ reactions; lane 11: negative VSV-NJ reaction; lanes 12–15: VSV-IND and VSV-NJ positive reactions utilizing both primer sets; lane 16: negative for VSV-IND & NJ reaction; CE: negative extraction control; CP: negative PCR control; M: DNA marker.

Fig. 2. Reverse transcription polymerase chain reaction amplification of RNA extracted from spiked BHK-21 cell suspensions by using Qiagen RNeasy mini kit. Lane 1: 100 bp marker; lane 2–6: IND and NJ RNA with IND Primer set; lane 7–11: IND and NJ RNA with NJ Primer set; lane 12: Negative extraction control (IND primer set); lane 13–17: IND and NJ RNA with both IND and NJ primer sets; lane 18: Negative extraction control (NJ primer set); lane 19: Negative extraction control (IND and NJ primer sets); lane 20: Negative PCR control.

Fig. 3. Reverse transcription polymerase chain reaction amplification of RNA extracted from VSV-IND and VSV-NJ spiked bovine lymph nodes by the tri-reagent protocol. Lane 1: 100 bp marker; lane 2–6: IND and NJ RNA with NJ Primer set; lane 7–11: IND and NJ RNA with IND Primer set; lane 12–16: IND and NJ RNA with both IND and NJ primer sets; lane 17: Negative extraction control; lane 18: Negative PCR control; lane 19: 100 bp marker.

Fig. 4. Reverse transcription polymerase chain reaction amplification of RNA extracted from VSV-IND and VSV-NJ spiked bovine lymph nodes by Qiagen RNeasy mini kit. Lane 1: 100 bp marker; lanes 2–6: IND and NJ RNA with NJ Primer set; lanes 7–11: IND and NJ RNA with IND Primer set; lane 12–16: IND and NJ RNA with both IND and NJ primer sets; lane 17: Negative extraction control; lane 18: Negative PCR control; lane 19: 100 bp marker.

Specificity

The specificity of the multiplex RT-PCR was assessed by analysis of viral genomes from VSV-IND and VSV-NJ. Samples were considered positive for VSV-IND and VSV-NJ only if a 614 and 301 bp fragments were clearly

seen in the product analysis gel. The specificity of the assay was demonstrated, as no amplification was detected in the negative extraction and PCR controls. No cross reactions were observed between VSV-IND templates and VSV-NJ primers, and vice versa, when using RNA purified from VSV (IND and NJ) spiked

samples (Figs. 1–4). The RT-PCR assay was capable of detecting both types of VSV from spiked samples in a one-step reaction tube.

DISCUSSION

In this work, a highly sensitive and specific multiplex RT-PCR assay is described and applied for the simultaneous and differential detection of VSV-IND and VSV-NJ. Two different VSV RNA extraction methods were compared to select the fast, simple, and reliable in obtaining high quality RNA from the tissue culture lysates and spiked tissue samples. The use of Qiagen RNeasy mini kit in this regard proved better when compared with acid-guanidine method.

The detection limit in the developed multiplex RT-PCR was as low as 1.0 fg and 2.84 TCID₅₀ when Qiagen RNeasy mini kit was used for extraction of VSV RNA from the samples. However, this limit was significantly higher, 50.5 TCID₅₀ for VSV-IND and 284 TCID₅₀ for VSV-NJ, when tri-reagent method was used for the extraction of total RNA from the samples. These detection limits are superior or comparable to previously reported detection limits of similar assays (10–15). Negative controls confirmed that only RNA was transcribed and amplified, no virus had contaminated the control cultures, and no contaminating RNA or DNA was present in any of the reagents used in this study.

The primers used in this study were specific for the P gene of VSV-IND and L gene of VSV-NJ. Primers for these genes were either used separately or collectively in multiplex RT-PCR to check the possibility of interactions between the primer sets. The primer sets were found highly specific for the amplification of their target genes and no cross reaction occurred.

Occasionally, the multiplex RT-PCR was able to detect VSV-IND as low as 0.1 fg and VSV-NJ as low as 1.0 fg when both primer sets were used in the assay. However, VSV-NJ was detected as low as 0.1 fg of total RNA when only one primer set for the amplification of L gene of VSV-NJ was used in the assay (Fig. 2). The extraction and purification of RNA by using Qiagen RNeasy mini kit is very rapid, less time consuming (less than one hour) and requires less expertise. The use of this extraction method in addition to the multiplex RT-PCR assay, allows a rapid approach for the differential detection of VSV-IND and VSV-NJ a single assay. The test also allowed the specific detection of the two viruses simultaneously mixed in spiked samples, resembling a hypothetical co-infection. In summary, the results of the study showed that the use of Qiagen RNeasy mini kit in addition to the one-step RT-PCR kit (Qiagen, Valencia, CA)

as a simple, practicable, and reliable tool for rapid, highly sensitive, and specific differential diagnosis of VSV-IND and VSV-NJ in cell lysate and spiked samples. Future studies should include testing of clinical samples for the diagnosis and differentiation of VSV types for further validation of this assay.

REFERENCES

1. Hole K, Clavijo A, Pineda LA. Detection and serotype-specific differentiation of vesicular stomatitis virus using multiplex, real time, reverse transcription-PCR assay. *J Vet Diagn Invest* 2006;18:139–146.
2. Rodríguez LL, Fitch WM, Nichol ST. Ecological factors rather than temporal factors dominate the evaluation of vesicular stomatitis virus. *Proc Natl Acad Sci USA* 1996;93:13030–13050.
3. Rodríguez LL, Vernon S, Morales AI, Letchworth GJ. Serological monitoring of vesicular stomatitis virus New Jersey serotype in enzootic regions of Costa Rica. *Am J Trop Med Hyg* 1990;42:272–281.
4. Perez E, Cornelissen B. Retrospective study of the temporal distribution of vesicular stomatitis in cattle in Costa Rica, 1972–1986. *Prev Vet Med* 1988;6:1–8.
5. Goodger WJ, Thurmond M, Nehay J, Mitchell J, Smith P. Economic impact of an epizootic of bovine vesicular stomatitis in California. *J Am Vet Med Assoc* 1985;186:370–373.
6. Quiroz E, Moreno N, Peralta PH, Tesh RB. A human case of encephalitis associated with vesicular stomatitis virus (Indiana serotype) infection. *Am J Trop Med Hyg* 1988;39:312–314.
7. Rodríguez LL, Letchworth GJ, Spiropoulou CF, Nichol ST. Rapid detection of vesicular stomatitis virus New Jersey serotype in clinical samples by using polymerase chain reaction. *J Clin Microbiol* 1993;31:2016–2020.
8. Magnuson RJ, Triantis J, Rodríguez LL, et al. A single-tube multiplex reverse transcription-polymerase chain reaction for detection and differentiation of vesicular stomatitis Indiana1 and New Jersey viruses in insects. *J Vet Diagn Invest* 2003;15:561–567.
9. Nunez JI, Blanco E, Hernandez T, et al. A RT-PCR assay for the differential diagnosis of vesicular viral diseases of swine. *J Virol Methods* 1998;72:227–235.
10. Abravaya K, Esping C, Hoenle R. Performance of a multiplex qualitative PCR LCx assay for detection of human immunodeficiency virus type 1 (HIV-1) group M subtypes, group O, and HIV-2. *J Clin Microbiol* 2000;38:716–723.
11. Echavarría M, Forman M, Ticehurst J. PCR method for detection of adenovirus in urine of healthy and human immunodeficiency virus-infected individuals. *J Clin Microbiol* 1998;36:3323–3326.
12. Fernández J, Agüero M, Romero LJ, et al. Rapid and differential diagnosis of Foot-and-Mouth disease virus (FMDV), swine vesicular disease virus (SVDV) and vesicular stomatitis virus (VSV) by one-step multiplex RT-PCR assay in clinical samples. *J Virol Methods* 2008;147:301–311.
13. Hofner MC, Carpenter WC, Ferris NP, Kitching RP, Botero FA. A hemi-nested PCR assay for the detection and identification of vesicular stomatitis virus nucleic acid. *J Virol Methods* 1994;50:11–20.
14. Johnson G, Nelson S, Petric M. Comprehensive PCR-based assay for detection and species identification of human herpesviruses. *J Clin Microbiol* 2000;38:3274–3279.
15. Knight AP, Messer NT. Vesicular stomatitis. *Compend Contin Edus Pract Vet* 1983;5:S517–S522.