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Development of a reverse transcription loop-mediated isothermal amplification assay for the detection of vesicular stomatitis New Jersey virus: Use of rapid molecular assays to differentiate between vesicular disease viruses

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A B S T R A C T

Vesicular stomatitis (VS) is endemic in Central America and northern regions of South America, where sporadic outbreaks in cattle and pigs can cause clinical signs that are similar to foot-and-mouth disease (FMD). There is therefore a pressing need for rapid, sensitive and specific differential diagnostic assays that are suitable for decision making in the field. RT-LAMP assays have been developed for vesicular diseases such as FMD and swine vesicular disease (SVD) but there is currently no RT-LAMP assay that can detect VS virus (VSV), nor are there any multiplex RT-LAMP assays which permit rapid discrimination between these 'look-a-like' diseases in situ. This study describes the development of a novel RT-LAMP assay for the detection of VSV focusing on the New Jersey (VSNJ) serotype, which has caused most of the recent VS cases in the Americas. This RT-LAMP assay was combined in a multiplex format combining molecular lateral-flow devices for the discrimination between FMD and VS. This assay was able to detect representative VSNJV's and the limit of detection of the singleplex and multiplex VSNJV RT-LAMP assays were equivalent to laboratory based real-time RT-PCR assays. A similar multiplex RT-LAMP assay was developed to discriminate between FMDV and SVDV, showing that FMDV, SVDV and VSNJV could be reliably detected within epithelial suspensions without the need for prior RNA extraction, providing an approach that could be used as the basis for a rapid and low cost assay for differentiation of FMD from other vesicular diseases in the field.

1. Introduction

Vesicular stomatitis New Jersey virus (VSNJV), in the family *Rhabdoviridae*, genus *Vesiculovirus*, is endemic in southern Mexico, Central America and northern regions of South America (Colombia, Venezuela, Ecuador and Peru). VSNJV, an arbovirus, can affect humans and causes vesicular stomatitis (VS) in horses, cattle and pigs (Velazquez-Salinas et al., 2014). There have been a number of multiyear outbreaks of VSNJV in the United States (Letchworth et al., 1999; Rainwater-Lovett et al., 2007) which can cause heightened concern when the disease is observed in cattle or pigs due to the similarity in clinical presentation to foot-and-mouth disease (FMD) which is an OIE-listed notifiable disease caused by an RNA virus (FMDV) belonging to the family *Picornaviridae,* genus *Aphthovirus*. Difficulties in distinguishing the agents responsible for the clinical signs of vesicular disease are not restricted to VSV and FMDV. In pigs, swine vesicular disease (SVD), caused by SVD virus (SVDV: genus *Enterovirus,* family *Picornaviridae*) can also cause characteristic vesicular lesions. Therefore, there is a pressing need for rapid, sensitive and specific differential diagnostic assays that are suitable for decision making in the field within Europe (FMD differentiation from SVD) and the Americas (FMD differentiation from VS) when screening animals displaying clinical signs consistent with vesicular disease.

Simple pen-side diagnostics which can be deployed on farm to rapidly detect viral antigen have been developed in the form of lateral flow devices (LFD's) for detection of FMDV (Ferris et al., 2009,2010a; Oem et al., 2009; Yang et al., 2013; Morioka et al., 2015), SVDV (Ferris et al., 2010b) and VSV (Ferris et al., 2012). However the analytical sensitivity of these antibody-based tests is lower than those of molecular based methods and the sample type applicable for use is normally restricted to epithelial tissue. As

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such, there has been an impetus to develop and transfer molecular assays for detection of these diseases into field settings. Initially research was focused on solutions to enable real-time RT-PCR (rRT-PCR) to be performed in the field (Callahan et al., 2002; King et al., 2008; Wilson et al., 2009; Madi et al., 2012; Howson et al., 2015), but since most of these platforms are expensive and need to be decontaminated when moved between farms, emphasis has shifted to alternative molecular assays such as loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000).

LAMP is an isothermal, autocyling, strand-displacement DNA amplification technique which can be performed at a single temperature in a water bath and can be combined with simple, disposable visualisation using molecular LFD's (James et al., 2010). Singleplex reverse transcription (RT)-LAMP assays have been previously designed and evaluated within laboratory settings for detection of FMDV (Dukes et al., 2006; Shao et al., 2010; Chen et al., 2011a,b; Yamazaki et al., 2013; Ding et al., 2014; Waters et al., 2014) and SVDV (Blomström et al., 2008) and in the case of FMDV these assays have been adapted for field use and validated within endemic settings (Howson et al., 2015). However, currently there is no equivalent LAMP assay for the detection of VSV, nor are there multiplex assay formats available for rapid differentiation between 'look-a-like' diseases within a single reaction.

This manuscript describes the development of the first RT-LAMP assay for the detection of VSNJV which is the serotype responsible for the majority of the recent clinical cases in North America and Central America (Velazquez-Salinas et al., 2014). This new test was combined in a multiplex (LFD) format to allow differential detection of VSNJV and FMDV. In parallel to this work, the performance of a second multiplex RT-LAMP assay that can detect FMDV and SVDV was also evaluated, providing two assays that can be used for the rapid detection and discrimination of viruses causing vesicular disease of livestock.

2. Methods

2.1. Ethics

All animal samples utilised in this project were archival samples from previous experimental studies approved by The Pirbright Institute ethical review committee under the auspices of the Animal Scientific Procedures Act (ASPA) 1986 (as amended), or comprised samples collected and submitted by endemic country authorities or NCFAD-Canadian Food Inspection Agency (Winnipeg, Canada) to The World Reference Laboratory for FMD (WRLFMD) at The Pirbright Laboratory.

2.2. Virus isolates

Archival epithelial suspensions (10% (w/v) in M25 phosphate buffer: 35 mM Na_2HPO_4 , 5.7 mM KH_2PO_4, pH 7.6) for FMDV (n = 5), SVDV (n = 5), VSNJV (n = 6) and VS Indiana virus (VSIV) (n = 2) used within this study were obtained from the WRLFMD archive at The Pirbright Institute (Table 1). Archival VSNJV cell culture supernatants from clade I (n = 3), clade II (n = 1), clade III (n = 4) and clade IV (n = 1) were obtained from the WRLFMD, in addition to cell culture supernatant for FMDV O UAE 2/2003 (n = 1). Archival RNA extracted from VSNJV clade IV (n = 1) and clade V (n = 1) isolates were supplied by Central Veterinary Institute (CVI), Netherlands (Table 1). Four RNA samples were supplied by Agence nationale de sécurité sanitaire de l'alimentation (ANSES, France) comprising VSNJV clade I Hazlehurst (n = 2; 10^{-1} and 10^{-3} from a decimal dilution series) and Indiana 1 Mudd-Summers (n = 2; 10^{-2} and 10^{-4} from a decimal dilution series).

2.3. RNA extraction

Total RNA was extracted by an automated procedure on a MagNA Pure LC robot using total nucleic acid kit reagents following manufacturer's guidelines or extracted using QIAcube and the QIAamp viral extraction kit (Qiagen).

2.4. RT-LAMP and RT-LAMP-LFD

For detection of VSNJV, a new singleplex RT-LAMP assay was designed. Sixteen VSNJV full-length genomes (Accession numbers: KU296051-KU296057, JX122220, JX12112, JX121111, JX121109, JX121108, JX121104, JX121105, JX121106, JX121107) representing the six clades of VSNJV were obtained from GenBank. Sequences were aligned in BioEdit (Version 7.0.5.3) from which a region spanning nucleotides 1376–1598 (junction between nucleocapsid (N) and phopshoprotein (P)) was selected for LAMP primer design (Table 2) using Primer Explorer V4. The RT-LAMP mastermix and cycling parameters for the VSNJV RT-LAMP assay were as per the wet reagents described by Howson et al. (2015) and the primer ratio in a 25 μl reaction as reported by Blomström et al. (2008); F3/B3 (External primers: EP's): 5 pmol, FIP/BIP (Internal primers: IP's): 40 pmol and Floop/Bloop (loop primers: Loops): 20 pmol. The singleplex FMDV and SVDV RT-LAMP assays used primers and primer concentrations from published assays (Dukes et al., 2006 and Blomström et al., 2008; respectively), the RT-LAMP mastermix for both assays were as per the wet reagents described by Howson et al. (2015). In all cases, 5 μl of template (RNA or clinical sample) was added to the assay formats. For clinical samples epithelial suspensions were diluted 1:5 in nuclease free water as described previously (Waters et al., 2014; Howson et al., 2015). RT-LAMP reactions were performed using the Genie® II (OptiGene Ltd.) or a standard laboratory based real-time PCR machine (Stratagene, Mx5000p). Visualisation of RT-LAMP products was achieved by real-time fluorescence (T_P: Time to positivity, rounded to the nearest minute) and/or end point molecular LFD's. In all cases, assays were run at 65 °C for 60 min followed by 85 °C for 5 min. To confirm that amplicons were virus specific, annealing analysis was performed at the end of the RT-LAMP reaction on the RT-LAMP products using the Genie® II (OptiGene Ltd.). LAMP products were heated to 98 °C, then cooled to 80 °C ramping at 0.05 °C per second. Anneal temperature (T_a) calculations were automated using Genie® Explorer v0.2.1.1 software (OptiGene Ltd). Samples were defined as positive if amplification had occurred and the LAMP product annealed in the virus amplicon specific temperature range: FMDV: 87.5–89.5 °C; VSNJV: 83.5–84.5 °C; SVDV: 86.0–87.0 °C. In all cases, samples were tested in duplicate. To enable visualisation on molecular LFDs the singleplex RT-LAMP reactions for detection of VSNJV combined internal FIP and BIP primers which were labelled at the 5′ termini with Flc: fluorescein (FIP) and Btn: biotin (BIP) and visualised using an immuno-chromatographic LFD (Forsite Diagnostics, York, UK). In this case, a positive was concluded if two bands were present, the T: Test line and C: Control line. To create the multiplex format, the primers were added at half the volume but twice as concentrated as the singleplex assay to account for the dilution effect of the second set of primers. In addition the internal FIP and BIP primers for the VSNJV, FMDV and SVDV assays were labelled at the 5′ termini as per Table 3 to permit spatial separation on multiplex molecular LFD's (AMODIA MultiFlow®, Germany). The multiplex format combined a total of two disease target reagents, discerning either FMDV and SVDV, or FMDV and VSNJV (Fig. 1). Multiplex LFD's were considered positive if there was a presence of two out of a possible three bands which should include one T: Test line and one C: Control line. The first T line (anti-Flc) was always the indicator for FMDV positive samples, whilst the second T line (anti-DIG) was either an indicator for VSNJV or SVDV depending