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

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A novel highly sensitive and specific gel-based one-step hot-start multiplex RT-PCR has been developed and standardized for rapid diagnosis of OIE List A vesicular diseases in clinical samples. The test allows a simultaneous and differential detection of Foot-and-mouth disease virus (FMDV, the seven serotypes), Swine vesicular disease (SVDV) and Vesicular stomatitis virus (VSV, the two main serotypes) which may cause undistinguished clinical symptoms and lesions in infected pigs.

The method uses three primer sets, one specific for each virus (FMDV, SVDV or VSV), amplifying DNA fragments different in length, that allows a gel-based differential detection. Primer sets were selected in highly conserved viral genome regions to be compatible in a multiplex RT-PCR assay. Specific primer set for FMDV amplifying a fragment of 275 bp in all the seven FMD viral serotypes A, O, C; Sat1, Sat2, Sat3 and Asia1, was selected in the 3D gene coding region: FMD-B (Saiz et al, 2003) and a newly designed FMD-C. For SVDV, a previously described primer set SS4/SA2 (Nuñez et al., 1998) was selected, defining an amplicon of 154 bp. A new VSV-1/VSV-2 primer pair specific for VSV was designed in the L gene region that amplifies both Indiana-1 and New Jersey serotypes, delimiting an amplicon of 110 bp.

Total RNA was extracted from samples using a unique extraction method, the commercial Tripure Isolation Reagent (Roche Molecular Biochemicals), following manufacturer’s instructions. For the specificity assays using DNA viruses, viral genome was purified using the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals).

Optimal conditions for the vesicular multiplex RT-PCR assay were established and a hot start amplification was performed using the three specific primer sets (FMD-B/C, SS4/SA2, VSV-1/2) in a multiplex format in a single step, single tube reaction. Reverse transcription-amplification was carried out using MuMLV reverse transcriptase and Taq Gold polymerase (Applied Biosystems).
In order to determine the detection limit of the test, ten-fold serial dilutions, in serum, of infected cell cultures with each of the following viruses were used in the multiplex RT-PCR assay: FMDV A (A22 Iraq), O (O1 Manissa/Turkey) and C (C1 Noville) and VSV Indiana-1 and New Jersey serotypes grown in BHK-21, as well as SVDV (UK-72) grown in IBRS-2. A sensitivity of 0.2 TCID$_{50}$, 0.02 TCID$_{50}$ and 0.07 TCID$_{50}$ were reached for each of the FMDV A, O and C serotypes respectively in the multiplex RT-PCR. Moreover, 0.126 TCID$_{50}$ was the detection limit for SVDV and a sensitivity of 6.4 TCID$_{50}$ was achieved after analysis of VSV Indiana-1 serotype as well as 0.7 TCID$_{50}$ was the corresponding detection limit in the case of New Jersey serotype using the multiplex format.

The specificity of the multiplex RT-PCR reaction was tested using seventeen FMDV isolates representatives of the seven serotypes A, O, C, Sat 1, Sat 2, Sat 3 and Asia 1, eleven SVDV strains and VSV Indiana-1 and New Jersey reference strains, as well as Coxsackie virus B5 and other domestic animal related viruses (CSFV, BDV, PCV-I, PCV-II, BVDV, PRRSV, ASFV, ADV, MCFV, BTV). In addition, nucleic acids from porcine whole blood, serum, swabs and tissue samples from healthy pigs, and several non-infected cell lines were employed in the specificity test. Multiplex RT-PCR was observed to be positive for all the FMDV, SVDV and VSV isolates obtaining the expected specific amplified product in each case (fig.1 and 2), while no positive products were found in the RT-PCR when other related viruses, cell lines or non-infected porcine tissues samples were assayed (data not shown).

Specificity of the amplicons was confirmed by restriction endonuclease digestion using AhdI (Saiz et al., 2003) and/or BsrFI for FMDV, ClaI for SVDV and BsmAI and BsaJI for VSV.

In order to assess the validity of the method in clinical specimens, samples from pigs experimentally inoculated with FMDV, SVDV or VSV were used. All the in vivo experimentations were performed in the BSL-3 animal facilities at CISA, Valdeolmos. Pigs were inoculated with one of the following viruses: A22 Iraq, O UK-2001 or C1 Noville FMDV isolates; UK-72 SVDV strain; and Indiana-1 and New Jersey VSV serotypes. EDTA-blood, serum, nasal and pharyngeal swabs, faecal faeces (in the case of SVDV), vesicular epithelium and tissues samples were collected at different days and analysed by multiplex RT-PCR. Specific positive amplification product was observed in each case according to the inoculated virus.
**Fig 1.** Detection of FMDV strains by Multiplex RT-PCR assay: A: 1: A22 Iraq (IRQ24/64); 2: A Pehuajo, Argentina/92; 3: A24 Cruzeiro, Brazil 3/55; 4: A5 Allier, France/60; O: 5: O1 Manisa/Turkey/69; 6: O Algeria 1/99; 7: O1 BFS 1860/UK/67; 8: O1 Kaufbern, Germany/67; 9: O1 Campos, Brazil 1/58; 10: O1 Caseros, Argentina 2/67; C: 11: C1 Spa70 (CS-8 Sta Pau); 12: C1 Noville, Switzerland/65; 13: C3 Argentina/85; SAT1: 14: RV 11/37 Rhodesia; SAT2: 15: RHO 1/48, Rhodesia; SAT3: 16: RV 7/34, Rhodesia; Asia1: 17: LEB/88, Lebanon. C+: Multiplex RT-PCR positive control. M: Molecular Weight Marker V. *(Roche Molecular Biochemicals)*.

**Fig 2.** Simultaneous and differential detection of FMDV (O serotype), SVDV (UK-72 strain) and VSV (Indiana and New Jersey serotypes) in multiplex RT-PCR assay. CoxB: Coxsackie B5 virus; C-: Negative multiplex RT-PCR control. M: Molecular weight marker V *(Roche Mol. Biochem.)*
The multiplex test showed to be a simple, economical and reliable tool for rapid diagnosis of vesicular diseases in clinical samples, spending less than six hours in obtaining the results and it can be used even in the cases of an hypothetical co-infection.

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**References**
