

## Diagnostic Evaluation of a Real-Time Reverse Transcriptase PCR Assay for Detection of Classical Swine Fever Virus

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**A fluorogenic-probe hydrolysis (TaqMan)-reverse transcriptase (RT) PCR for classical swine fever virus (CSFV) was evaluated for diagnostic sensitivity and specificity by using clinical samples obtained from the Dominican Republic, where the disease is enzootic. The sensitivity of this test, using nasal swab samples taken from both symptomatic and asymptomatic animals, exceeded the diagnostic sensitivity of virus isolation (100% versus 72.4%, respectively) with little loss of specificity (98.9% versus 100%, respectively). At the herd level, three of four infected farms were identified by virus isolation, while the CSFV real-time RT-PCR assay identified all four infected premises. This simple and accurate test permits rapid detection of CSFV in affected herds.**

Classical swine fever (CSF) is a highly contagious and often fatal disease of swine. *Classical Swine Fever Virus* (CSFV), the causative agent of CSF, is a member of the genus *Pestivirus* in the family *Flaviviridae* (22). CSFV is an enveloped virus with a 12.5-kb single-stranded RNA genome of positive polarity (9). The genome encodes a 4,000-amino-acid polyprotein that is co- and posttranslationally processed by viral and cellular proteases into 12 polypeptides (15). Both ends of the genome contain untranslated regions (UTR), which are highly conserved among virus isolates (3, 13, 14, 20, 24).

Although not present in the United States, CSF is distributed worldwide. In the Western hemisphere the disease occurs in Central and South America, southern Mexico, and the Caribbean basin. Sporadic outbreaks of CSF are frequently recorded in the European Union. Countries affected by the disease experience an international ban on trade of live animals and pork products. As a result, the European Union has had a stamping-out/nonvaccination policy since 1990 (23). The CSF outbreak in The Netherlands in 1997 to 1998 resulted in 11 million pigs being destroyed at a cost of over 2 billion US dollars (17). In large part, the disease status of these animals was unknown (5).

Rapid and precise detection of CSFV is critical for disease containment. Current diagnostic methods, including detection of viral antigens in tonsils by using fluorescent antibody (18), antigen capture enzyme-linked immunosorbent assay (4, 21), and detection of genomic RNA by reverse transcription-PCR (3, 8, 11, 12, 24), are relatively rapid diagnostic tests; however, these techniques require centralized laboratory facilities and clinical specimen submissions, which delay disease diagnosis,

thus affecting the efficiency of emergency disease management measures.

A rapid, presumptive diagnosis at the site of a suspected disease outbreak would be extremely useful for controlling CSF. To address this need, a fluorogenic-probe hydrolysis (TaqMan)-reverse transcriptase (RT) PCR assay for CSFV was evaluated with samples from experimentally infected swine (19). The assay's sensitivity equalled or exceeded the sensitivity of virus isolation (VI). Viral RNA was detected in nasal and tonsil scraping samples 2 to 4 days prior to the onset of clinical disease. In addition, the assay can be performed in 2 h or less, thus providing a rapid method for the diagnosis of CSF.

Here we evaluated the diagnostic sensitivity and specificity of this test using clinical samples collected from swine holdings in the Dominican Republic (DR). In the DR, CSFV is enzootic in the domestic pig population, although actual disease incidence is unknown. A large part of the national herd is vaccinated regularly with live attenuated and, more recently, CSFV E2 subunit vaccines (2).

A total of 449 nasal swabs were collected from nine farms in the central region of the DR (Table 1). Samples were collected with sterile cotton swabs (Fisherbrand, Fisher Scientific, Pittsburgh, Pa.) and placed into 1.5-ml sterile tubes containing 1 ml of Dulbecco's minimal essential medium (Gibco, Grand Island, N.Y.) plus antibiotics and antimycotic (Gibco). Tubes were placed in dry ice, transported to the laboratory, and kept at  $-70^{\circ}\text{C}$  until processed. The CSFV real-time RT-PCR assay was performed as described previously (19). SK6 swine kidney cell monolayers were used for VI. Monolayers were inoculated with 250  $\mu\text{l}$  of filtered (0.22  $\mu\text{m}$ ) nasal swab transport media and then incubated at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . Inoculated cell cultures were passed twice more at 4-day intervals by transferring freeze-thawed cultures onto fresh SK6 cell monolayers before being considered negative for virus. CSFV was detected in cultures by peroxidase staining (1), using the CSFV monoclo-

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TABLE 1. Detection of CSFV in clinical samples by CSFV real-time RT-PCR assay, VI, and nested PCR

Farm	Location	CSF clinical signs	No. of samples	Vaccine <sup>a</sup>	No. of samples			CSFV RNA detected by nested PCR <sup>b</sup>
					VI positive	Real-time PCR positive	VI negative PCR positive	
1	Licey	No	22	Marker/subunit	0	2	2	ND (0/2)
2	Licey	No	19	LAV	0	0		
3	Licey	Yes	57	None	3	5	2	DR02.3 (2/2)
4	Licey	No	18	LAV	0	0		
5	Licey	Yes	112	LAV	45	52	7	DR02.6 (7/7)
6	Licey	No	10	LAV	0	1	1	ND (0/1)
7	La Vega	Yes	102	Marker/subunit	0	1	1	DR02.1 (0/1)
8	La Paloma	No	65	Marker/subunit	0	1	1	ND (1/1)
9	La Paloma	Yes	44	LAV	2	7	5	DR02.6 (1/5); DR02.4 (4/5)
Total			449		50	69	19	15/19

<sup>a</sup> "Marker/subunit" indicates that at the time of sample collection a E2 CSFV vaccine was in use, before that live attenuated vaccine had been used. LAV, live attenuated vaccine.

<sup>b</sup> Values are number of samples with indicated RNA/number of VI-negative PCR-positive samples. ND, not detected.

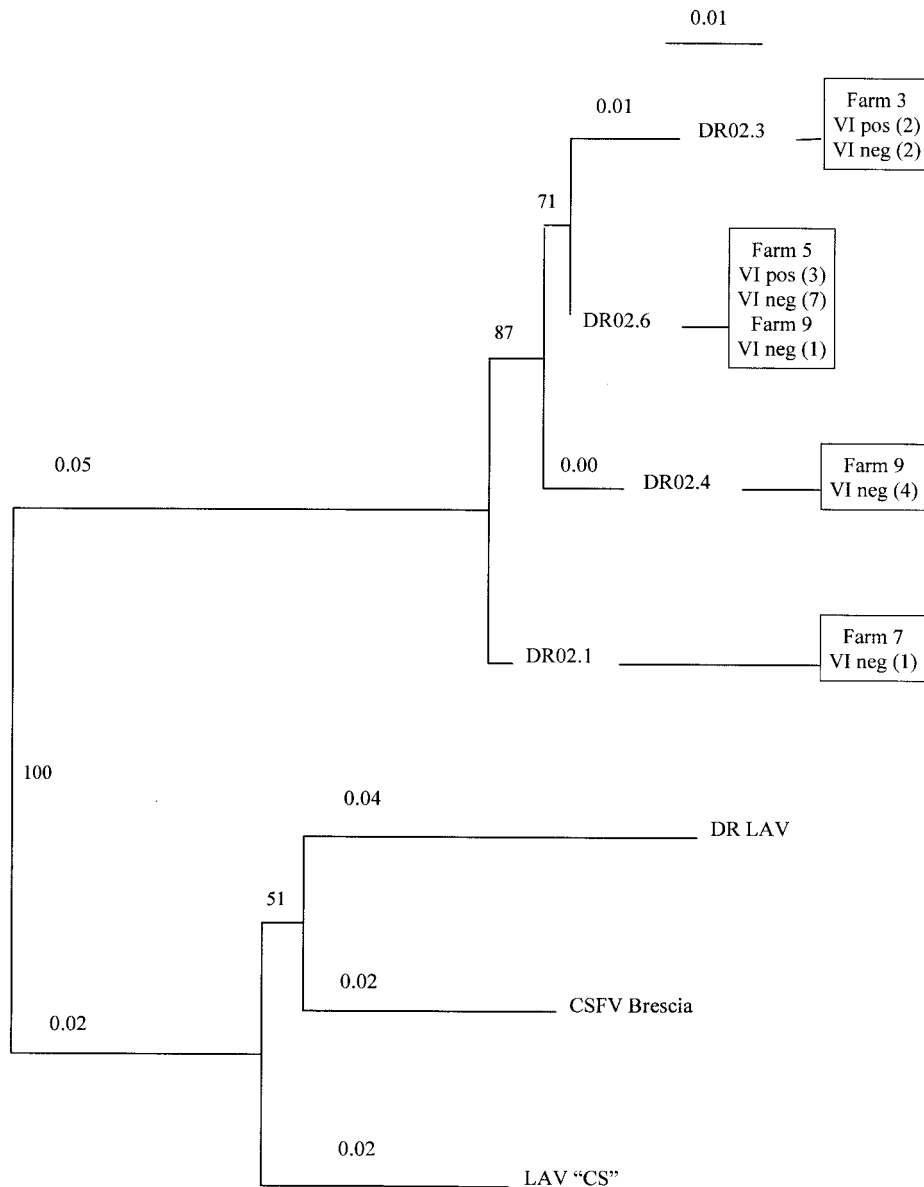


FIG. 1. Comparative sequence analysis of CSFV isolates from the DR. 5' UTR/N<sup>pro</sup> region sequences (see the text) were aligned by using ClustalW. Unrooted trees were generated by using the neighbor-joining algorithm with a Poisson correction for multiple substitutions. DR LAV, CSFV live attenuated vaccine currently used in the DR; LAV "CS", live CSFV attenuated vaccine from Russia; DR02.1, DR02.3, DR02.4, and DR02.6, DR field isolates; pos, positive; neg, negative. The number of isolates sequenced is shown in parentheses.

TABLE 2. Performance of the CSFV real-time RT-PCR assay after evaluation of VI-negative CSFV real-time RT-PCR-positive samples by RT-nested PCR

Test index <sup>a</sup>	%	95% Confidence interval
Sensitivity	100 (65/65)	100–100
Specificity	98.9 (380/384)	97.9–99.9
Positive predictive value	94.3 (65/69)	88.6–99.7
Negative predictive value	100 (380/380)	100–100
False-negative rate	0.00 (0/65)	0.0–0.0
False-positive rate	5.7 (4/69)	1.8–14.9
Overall accuracy	99.1 (445/449)	98.9–99.9

<sup>a</sup> Sensitivity, number of positive samples over number of true-positive samples; specificity, number of negative samples over number of true-negative samples; positive predictive value, number of samples that were positive and had CSFV; negative predictive value, number of samples that were negative and did not have CSFV; false-negative rate, number of negative samples confirmed positive over true-positive samples; false-positive rate, number of positive samples confirmed negative over true-positive samples; overall accuracy, total of true-positive and true-negative samples over the total number of samples.

nal antibody WH303 (7) and a Vectastain ABC kit (Vector Laboratories, Burlingame, Calif.).

Of 449 samples, 69 were positive by the CSFV real-time RT-PCR assay (Table 1); of these, 50 were VI positive. The 19 samples that tested positive by real-time PCR and negative by VI were evaluated further for the presence of CSFV RNA by a nested PCR. The first PCR consisted of the amplification of a 777-bp segment encompassing the 5' UTR and regions of N<sup>pro</sup>, using the primer pair 5'GTCGTCAGTAGTTCGACG 3' and 5'ATGCTCTTTTGGGGCTAT 3'. Nested PCR was performed with the primers 5'TCTCTGCTGTACATGGCA 3' and 5'TATCCTTTTGGTCACCTC 3', which generated a 355-bp amplicon. PCR products obtained were sequenced on a PRISM 3700 automated DNA sequencer (PE Biosystems, Foster City, Calif.). Sequence data were assembled with the Phrap software program, with confirmatory assemblies performed by

using CAP3 (10). Of the 19 VI-negative samples examined, CSFV RNA was detected by nested PCR in 15 (Fig. 1; Table 1). Four samples that were negative by nested PCR were assumed to be CSFV real-time RT-PCR assay false positives (Tables 1 and 2). Notably, these samples were obtained from farms (no. 1, 6, and 8) that lacked evidence of clinical disease and from which virus was not isolated (Table 1; Fig. 1). The real-time PCR and nested PCR assays do not target the same genomic region (5'UTR and N<sup>pro</sup>, respectively). Thus, there is a possibility that the discrepancy between tests results obtained might be due to differences between the sensitivity of real-time PCR and that of the first round of amplification in the nested PCR assay. The DR isolates clustered together and differed from the live attenuated vaccine currently used in the DR (Pestiffa, Merial). Isolates were unique to a particular farm, with the exception of farm 9, where two different viruses were detected (Table 1). Additional samples that were CSFV real-time RT-PCR assay positive and VI positive were also sequenced ( $n = 5$ ). Two VI-positive samples from farm 3 and three VI-positive samples from farm 5 were similar to nested-PCR-positive VI-negative samples obtained from those farms (Fig. 1; Table 1).

Analysis of the cycle threshold ( $C_t$ ) values of the CSFV real-time RT-PCR-positive samples indicated that all samples with  $C_t$  values below 38 (46 of 69) were CSFV positive by either VI or nested PCR. The four false-positive samples were identified beyond cycle 38, one sample was identified between cycles 38 and 40, and three samples were identified between cycles 40 and 45 (Fig. 2). All samples with  $C_t$  values above 45 were CSFV negative by the three tests used here.

Between cycles 40 and 45, a total of eight samples were positive (Fig. 2). Three of these samples were false positives (75% of false-positive results), one sample was VI positive, and four samples were confirmed by nested PCR and sequencing as CSFV positive. Thus, samples testing positive between cycles

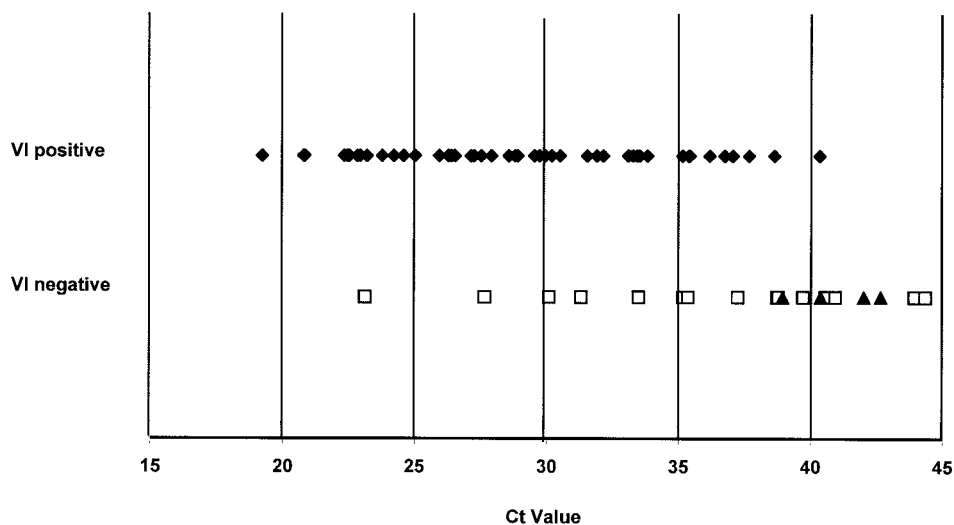


FIG. 2. Relationship between ( $C_t$ ) values of real-time PCR-positive samples and CSFV status of the samples. Filled diamonds represent samples that were positive by both CSFV RT-PCR assay and VI ( $n = 50$ ). Empty squares represent samples that were CSFV RT-PCR assay positive, VI negative, and nested PCR positive ( $n = 15$ ). Filled triangles represent false-positive samples. These samples were CSFV RT-PCR assay positive but negative by both VI and nested PCR ( $n = 4$ ).

TABLE 3. Comparison of performance of VI with CSFV real-time RT-PCR assay

Test index <sup>a</sup>	%	95% confidence interval
Sensitivity	72.4 (50/69)	61.9–83.0
Specificity	100 (380/380)	100–100
Positive predictive value	100 (50/50)	100–100
Negative predictive value	95.2 (380/399)	93.1–97.3
False negative rate	27.5 (19/69)	17.0–31.9
False positive rate	0.0 (0/50)	0.0–0.0
Overall accuracy	95.7 (430/449)	93.9–97.6

<sup>a</sup> Sensitivity, number of positive samples over number of true-positive samples; specificity, number of negative samples over number of true-negative samples; positive predictive value, number of samples that were positive and had CSFV; negative predictive value, number of samples that were negative and did not have CSFV; false-negative rate, number of negative samples confirmed positive over true-positive samples; false-positive rate, number of positive samples confirmed negative over true-positive samples; overall accuracy, total of true-positive and true-negative samples over the total number of samples.

40 and 45 require the use of a second confirmatory test, in this case nested PCR, to determine true sample status.

In this diagnostic evaluation, CSFV real-time RT-PCR assay outperformed VI, which is considered the diagnostic standard for CSF (5, 16, 18). The performances of VI and CSFV real-time RT-PCR were estimated as previously described (University of Oklahoma Health Sciences Center [www.fammed.ouhsc.edu/robhamm/cdmc.htm]). The diagnostic sensitivity of the CSFV real-time RT-PCR assay (100%) was higher than that of VI (72.4%) with little loss of specificity (Tables 2 and 3). The false-negative rate of VI in this evaluation was 27.54%, compared with no false negatives observed with the CSFV real-time RT-PCR assay (Tables 2 and 3). Importantly, the overall accuracy of the CSFV real-time RT-PCR assay (99.14%) was higher than that of VI (95.77%) (Tables 2 and 3).

Analysis of these data at the herd level indicates that CSFV was detected by CSFV real-time RT-PCR assay on four farms (farms 3, 5, 7, and 9) (Table 1), while only three farms were positive by VI (farms 3, 5, and 9) (Table 1). CSFV real-time RT-PCR detected CSFV on farms 1, 3, 5, 6, 7, 8, and 9. Farms 1, 6, and 8 were confirmed as CSFV negative by the confirmatory nested PCR assay. Thus, the CSFV real-time RT-PCR assay allowed the identification of a fourth CSFV-infected farm, farm 7, which was not identified by VI. A low predictive value for VI has been previously noted during a CSF outbreak in The Netherlands where only 4.5% of infected herds were detected by VI (6, 17). Similarly, a lower sensitivity for VI was observed by us previously, using experimentally infected animals (19).

In summary, the CSFV real-time RT-PCR assay had a higher predictive value than the current diagnostic standard, VI. Notably, the high sensitivity of the test is accompanied by little loss of specificity. The assay allowed rapid identification of CSF-affected farms by using a nasal swab sample. This diagnostic tool should prove useful during a CSF outbreak, when decisions regarding infection status need to be made rapidly to curtail disease transmission.

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