Short communication

Diagnostic specificity of a real-time RT-PCR in cattle for foot-and-mouth disease and swine for foot-and-mouth disease and classical swine fever based on non-invasive specimen collection


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Abstract

Foot-and-mouth disease virus (FMDV) and classical swine fever virus (CSFV) are highly contagious and can cause great economic losses when introduced into disease-free regions. Accurate estimates of diagnostic specificity (Sp) are important when considering the implementation of surveillance for these agents. The purpose of this study was to estimate diagnostic Sp of a real-time reverse-transcriptase PCR assay developed for detection of FMDV in cattle and domestic swine and CSFV in domestic swine based on non-invasive specimen collection. One thousand and eighty-eight range beef cattle were sampled from thirteen geographic locations throughout Texas. One thousand and one hundred market hogs and cull sows were sampled. Results for both FMDV and CSFV were considered positive if amplification occurred at or before 40 PCR cycles, inconclusive between 40 and 45 cycles and negative otherwise. Ten cattle had nonspecific PCR amplifications for FMDV, but none were classified as positive and only one as inconclusive. Specificity (95% confidence interval) was estimated as 100% (99.7, 100). There were 19
nonspecific PCR amplifications for FMDV in sampled swine with 1 classified as positive, 6 as inconclusive, and 12 as negative. Specificity (95% confidence interval) was estimated as 99.9% (99.5, 100). There were 21 nonspecific PCR amplifications for CSFV, and 1 was classified as positive. Specificity (95% confidence interval) was estimated as 99.9% (99.5, 100). These assays have high Sp, but nonspecific PCR amplifications can occur.

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### 1. Introduction

Foot-and-mouth disease virus (FMDV) belongs to the Picornaviridae family within the genus *Aphthovirus* (Lubroth, 2002). The viral genome is comprised of a single molecule of positive-sense single-stranded RNA (Alexandersen et al., 2003), and seven serotypes (O, A, C SAT 1–3, and Asia 1) have been identified (Thomson and Bastos, 2004). Classical swine fever virus (CSFV) belongs to the family Flaviviridae within the genus *Pestivirus* (Kleiboeker, 2002). The CSFV genome is a single molecule of positive-sense single-stranded RNA, and only a single serotype has been recognized (Paton and Greiser-Wilke, 2003).

PCR-based diagnostic assays have been developed to identify FMDV and CSFV (Harding et al., 1994; McGoldrick et al., 1998; Reid et al., 2000; Callahan et al., 2002; Hearps et al., 2002; Aguero et al., 2004; Hoffmann et al., 2005; Oem et al., 2005; Risatti et al., 2005; Depner et al., 2006; Ferris et al., 2006; Ophuis et al., 2006). FMDV can be present in most physiologic fluids and isolated several days prior to development of clinical lesions (Alexandersen et al., 2003; Thomson and Bastos, 2004). Oral cavity swabs can be used to identify the virus in animals prior to development of these lesions. Tonsils are often the first tissue where CSFV can be identified after oral exposure (van Oirschot, 2004), and oral and nasal swabs can be used as specimens for diagnostic detection of the virus in infected swine.

Validation of a diagnostic test is the process of evaluating the effectiveness for a particular use (Jacobson, 1998). A validated assay identifies the presence of a particular analyte (e.g. RNA sequence) that allows predictions to be made concerning the true disease status of the animal. Accuracy is measured as sensitivity (Se) and specificity (Sp), which are the probability of correctly identifying diseased and non-diseased animals, respectively. Field validation of a diagnostic assay is primarily concerned with measuring the effects due to factors that affect the concentration and composition of the analyte in the collected specimen to be tested (host factors). Host factors can be classified as intrinsic (e.g. age, sex, breed, genetic resistance or susceptibility) or acquired (e.g. actively or passively acquired immunity). The strain of the disease agent might also affect the assay through mechanisms associated with the host (tests based on immunity) and the agent itself (tests based on agent detection). Non-host factors in the field, such as contamination or deterioration of the sample, might also affect analyte quality and quantity and subsequently overall diagnostic accuracy.

Accurate estimates of Sp are important when considering surveillance for foreign animal diseases such as FMD and CSF. Sensitivity is important for early recognition of disease to reduce spread, but it must be balanced against required high Sp because false-positive results need complete epidemiologic and diagnostic workups to eliminate the possibility that these viruses were present. The objective of this study was to estimate diagnostic Sp of a real-time reverse-transcriptase PCR assay developed for the identification of FMDV infection in cattle and domestic swine and CSFV in domestic swine based on non-invasive specimen collection.

### 2. Materials and methods

Animal protocols were approved by the Clinical Research Review Committee at the College of Veterinary Medicine and Biomedical Sciences, Texas A&M University.
2.1. Animal sampling

Texas was categorized into 15 ecological zones based on the Texas Agriculture Statistical Districts (USDA, 2003). The districts were Northern High Plains, Southern High Plains, Northern Low Plains, Southern Low Plains, Cross Timbers, Blacklands, North East Texas, South East Texas, Trans-Pecos, Edwards Plateau, South Central, Coastal Bend, Upper Coast, South Texas, and Lower Valley. The sample size was estimated based on an assumed Sp of 0.99 and the desire to estimate this value with a precision of 0.009 and 99% confidence. The sample size was estimated as 1096 animals using exact binomial methods (Fosgate, 2005). The sample size per ecological zone was selected proportional to the total number of cattle in the zone based on the 2002 census (USDA, 2002). Adjacent ecological regions were combined when the number of cattle to sample was less than 40. Cattle were selected from Texas Agricultural Experiment Research & Extension Centers (TAEREC) in each ecological zone. Private herds were enrolled for regions in which a TAEREC was not present or when cattle were not available for sampling. Cattle were sampled from January to December 2004 and age, breed, sex, and pregnancy status were recorded. Sterile Dacron® tipped swabs were introduced into the oral cavity, and a circular motion was used to swab the mandibular gingival surface while avoiding contamination with feed and other particulate matter. Swabs were inserted into 2 ml cryovials that contained 1.5 ml of chilled Dulbecco’s Modified Eagle Medium, the handles broken off, and the vials sealed. Swabs were transported on dry ice to the laboratory and stored at −70 °C until testing.

A single commercial swine slaughter plant was selected in the Blacklands ecological zone in Texas. This plant received market hogs and cull sows from large production units predominantly located in Texas, Iowa, and Oklahoma. All swine presented for slaughter at the times of sampling were collected until the total sample size of 1100 was obtained. Sampling occurred during June and July 2004. Sterile Dacron® tipped swabs were introduced into the oral cavity, and a circular motion was used to swab the mandibular gingival surface. Sterile cotton tipped swabs were inserted 2.5 cm into both nostrils making circular motions wiping the surface of each cavity.

Samples were collected after swine were stunned by a high-voltage electrical charge and prior to exsanguination. Swabs were inserted into 2 ml cryovials that contained 1.5 ml of chilled Dulbecco’s Modified Eagle Medium, the handles broken off, and the vials sealed. Swabs were transported on dry ice to the laboratory and stored at −70 °C until testing. Oral and nasal swabs were collected for FMDV and CSFV testing, respectively.

2.2. Diagnostic testing

Total RNA was extracted using a commercially available kit (RNeasy Mini Kit, Qiagen, Valencia, CA, USA) according to manufacturer’s instructions. In brief, swabs were thawed and fluid aseptically squeezed from the tips. Tips were discarded, and 140 μl of the remaining viral transport media was vortexed and added into a tube with extraction buffer. Seventy percent ethanol was added to the lysate and mixed by pipetting up and down. Sample solution was added to the mini spin column provided with the kit and centrifuged for 30 s at 13,000 rpm. The spin column was washed three times and then dried by a 1 min centrifugation at 10,000 rpm. RNA was eluted by adding 40 μl RNase free water onto the spin column and centrifuging for 1 min at 13,000 rpm. Eluate containing the extracted RNA was stored at −70 °C until testing.

The FMDV and CSFV real-time RT-PCR assays were performed as a single-tube hydrolysis reaction using commercially available reagents (PerkinElmer, EZ-RT-PCR, PE Biosystems, Foster City, CA, USA) based on a diagnostic platform developed at the Foreign Animal Disease Diagnostic Laboratory (FADDL) at Plum Island, New York, USA (Callahan et al., 2002). Reactions were designed for a final volume of 25 μl using 2.5 μl of template. The RT-PCR reaction mixture was composed of the 5× buffer solution with the addition of Mn(OAc)₂ (5 mM), primers (0.3 mM), probe (0.3 mM), dATP/CTP/GTP (0.1 mM), dUTP (0.2 mM), rTth DNA polymerase (0.1 U/ml), bovine serum albumin (0.1 μg/μl), and trehalose (0.5 M) which was provided dried in the kit reaction tubes. Two different kits were used, each developed specifically for FMDV or CSFV RNA detection. Specimens were tested by adding sample and diluent to the
dried reaction mixture and performing a 10-min reverse-transcriptase (RT) step at 60 °C linked to a 55 cycle PCR with each cycle composed of 2 s at 95 °C and 30 s at 60 °C. Reactions were performed on a portable 10-kg real-time thermocycler (The SmartCycler, Cepheid Inc., Sunnyvale, CA, USA) that was operated via a computer. A fluorogenic signal is produced when a PCR product develops and a positive test result for both FMDV and CSFV was defined as an identified signal at or before 40 PCR cycles. Samples in which a signal developed between 40 and 45 cycles were classified as inconclusive, and all other results (no signal or signal at >45 cycles) were considered negative. All specimens developing fluorogenic signal were sent to the FADDL to confirm that reactions were false-positives and not due to true presence of FMDV or CSFV. At FADDL, the real-time RT-PCR was repeated and sequencing of the amplicon was attempted if a signal developed.

Twenty controls were randomly allocated to receive different concentrations of inactivated FMDV, CSFV, and vesicular stomatitis virus (VSV) and created to be indistinguishable from field samples. They were randomly interspersed among field samples and testing was performed without knowledge of sample origin (field vs. control).

2.3. Statistical analysis

Specificity was estimated for each assay (FMDV and CSFV) as the simple proportion of negative test results divided by the total number of field samples tested. The design effect was estimated to quantify the effect of clustered sample collection on estimated variance of the proportion. Fleiss quadratic confidence intervals incorporating the design effect to account for clustering and mid-$P$ exact intervals were calculated for cattle and swine estimates, respectively. Descriptive statistics for the cycle counts (Ct) were determined for all nonspecific PCR amplifications. Age, breed, sex, and pregnancy status were evaluated for their association with nonspecific amplifications by estimating prevalence ratios and calculating Fisher exact $P$-values. All statistical procedures were performed in available software (Epi Info, version 6.04, CDC, Atlanta, GA, USA), and results were interpreted at the 5% level of significance.

3. Results

One thousand and eighty-eight cattle were sampled from 13 geographic locations within 10 ecological zones of Texas. The mean (S.D.) age of sampled cattle was 4.4 (3.8) years. Breeds of sampled cattle included Brahman, Black Angus, Charolais, Hereford, Simmental, Bonsmara, Beefmaster, and their crosses. Forty-four percent (482/1088) of sampled cattle were Brahman or Brahman cross cattle. Three percent of sampled cattle were bulls (32/1088) and the remainder heifers or adult cows. Of the 1056 female cattle, 46% were not pregnant, 32% in early pregnancy (<4 months), and 22% late pregnant (≥4 months) at the time of sampling.

Ten sampled cattle were identified as having a fluorogenic signal for FMDV with one classified as inconclusive and nine classified as negative. Median (minimum, maximum) Ct for these reactions was 51.3 (41.9, 54.9). The design effect was estimated as 1.27 for these nonspecific amplifications demonstrating a tendency for clustering within herds. Specificity (95% confidence interval) was estimated as 100% (99.7, 100) including the inconclusive sample as negative (Table 1). Age, breed, sex, and pregnancy status were not significantly associated with development of a fluorogenic signal. Forty-six percent (6/13) of sampled locations had at least one animal with a fluorogenic signal and the number per location ranged from one to three (Fig. 1).

One thousand and one hundred market hogs and cull sows were sampled for evaluation of the FMDV and CSFV PCR assays. There were a total of 19 specimens that developed a fluorogenic signal with one being classified as positive and six as inconclusive for FMDV in the sampled swine. The median (minimum, maximum) Ct for these reactions was 51.3 (41.9, 54.9). Specificity (95% confidence interval) was estimated as 100% (99.7, 100) including the inconclusive sample as negative (Table 1). Age, breed, sex, and pregnancy status were not significantly associated with development of a fluorogenic signal. Forty-six percent (6/13) of sampled locations had at least one animal with a fluorogenic signal and the number per location ranged from one to three (Fig. 1).

All specimens that developed a fluorogenic signal on either assay were confirmed negative for FMDV
and CSFV at FADDL and classified as nonspecific PCR amplifications. Reliable sequence data were not obtainable from amplicons. All blinded samples spiked with inactivated FMDV, CSFV, and VSV were correctly classified.

4. Discussion

Validation of diagnostic tests should be performed using specimens or animals that accurately represent the target population of the assay before it is deployed.
for use. There are many animal- and environmental-related factors that can affect accuracy of a test (Jacobson, 1998; Greiner and Gardner, 2000). Variability associated with these factors typically cannot be accounted for when the assay is evaluated in the laboratory using well-characterized specimens from animals of known disease status. Accurate estimates of diagnostic Sp are especially important when an assay will be used to screen apparently healthy animals for disease. Traditional screening programs for regulated diseases including brucellosis and tuberculosis frequently employ a serial testing protocol to increase Sp and limit the number of false-positive classifications of animals. Similar serial testing protocols are in place for CSF and likely would also be used for FMD. Specificity is the most important test characteristic to increase the positive predictive value when the prevalence of the disease of interest is low. The prevalence of foreign animal diseases is zero up until the time one is accidentally or intentionally introduced. The United States is considered free of both CSF and FMD and it is important that screening procedures for these diseases have high Sp to limit false-positive disease investigations.

Animal-related data collected in this study did not appear to be associated with nonspecific FMDV PCR amplifications in cattle. However, there did appear to be a tendency for nonspecific FMD reactions to be present more in east and north Texas. The reasons for this observation are unknown, but it might be related to the presence of organisms in the environment that caused nonspecific amplifications. It is also possible that such organisms are present throughout the environment but would only be recognized under certain management conditions. Handling facilities were different at all locations, and some cattle when presented for testing contained feed material in their oral cavity. Other cattle presented with oral cavities containing feces, and it was not always possible to avoid contamination of swabs. Data were not recorded concerning which animals had feed or feces contamination at the time of sampling.

One each of the sampled swine was classified as positive in the assays for CSFV and FMDV. Both of these samples and all other fluorogenic signals were confirmed virus negative at FADDL, and the origins of false-positive amplifications are unknown. The Ct for the false-positive CSFV sample was 20.7 and less than the mean of the extraction positive controls included in each run of the assay, which was 30.6 (range; 28.4–33.3). The rapid amplification in this sample suggests strong primer binding affinity and relatively high concentration of the nucleic acid in the sample. At the time of this report the source of this reaction is unknown, and the reaction was not replicated when evaluated at FADDL.

This study provides estimates of diagnostic Sp for a real-time RT-PCR platform that could be used for FMDV and CSFV surveillance in domestic cattle and swine. Despite the recognized limitations, these results could be used to determine feasibility of implementing healthy animal surveillance for these foreign animal disease viruses. These assays have been documented here to have high Sp. However, difficulties might arise if all samples that develop a fluorogenic signal require investigations at the animal or herd level (rather than sample level). Surveillance targeting animals at highest risk of infection with confirmatory testing at the sample level would improve the positive predictive value of screening methods for FMDV and CSFV.

5. Conclusions

PCR-based diagnostic assays for detection of FMDV and CSFV in cattle and swine have high Sp and will be valuable tools in the early detection of these viruses in populations at highest risk of exposure.

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References


