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The aim of this study was to evaluate a number of foot-and-mouth disease (FMD) test methods for use in red deer. Ten animals were intranasally inoculated with the FMD virus (FMDV) O UKG 11/2001, monitored for clinical signs, and samples taken regularly (blood, serum, oral swabs, nasal swabs, probang samples and lesion swabs, if present) over a 4-week period. Only one animal, deer 1103, developed clinical signs (lesions under the tongue and at the coronary band of the right hind hoof). It tested positive by 3D and IRES real-time reverse transcription polymerase chain reaction (rRT-PCR) in various swabs, lesion materials and serum. In a non-structural protein (NSP) in-house ELISA (NSP-ELISA-IH), one commercial ELISA (NSP-ELISA-PR) and a commercial antibody NSP pen side test, only deer 1103 showed positive results from day post-inoculation (dpi) 14 onwards. Two other NSP-ELISAs detected anti-NSP serum antibodies with lower sensitivity. It also showed rising antibody levels in the virus neutralization test (VNT), the in-house SPO-ELISA-IH and the commercial SPO-ELISA-PR at dpi 9, and in another two commercial SPO-ELISAs at dpi 12 (SPO-ELISA-IV) and dpi 19 (SPO-ELISA-IZ), respectively. Six of the red deer that had been rRT-PCR and antibody negative were re-inoculated intramuscularly with the same O-serotype FMDV at dpi 14. None of these animals became rRT-PCR or NSP-ELISA positive, but all six animals became positive in the VNT, the in-house SPO-ELISA-IH and the commercial SPO-ELISA-PR. Two other commercial SPO-ELISAs were less sensitive or failed to detect animals as positive. The rRT-PCRs and the four most sensitive commercial ELISAs that had been used for the experimentally inoculated deer were further evaluated for diagnostic specificity (DSP) using 950 serum samples and 200 nasal swabs from non-infected animals. DSPs were 100% for the rRT-PCRs and between 99.8 and 100% for the ELISAs.
Foot-and-Mouth Disease in Red Deer

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1974; Forman et al., 1974; Gibbs et al., 1975; McVicar et al., 1974). The lack of significant clinical signs in deer makes FMD laboratory testing a crucial tool for the identification of infection in farmed deer.

Even though FMD test methods are well evaluated for cattle, and to a satisfactory extent for sheep, goats and pigs (Alexandersen et al., 2003a,b, Brocchi et al., 2006), no test method evaluation data are available for red deer and only very limited data have been published for other deer species.

The aim of this study was therefore to evaluate FMD test methods for use in red deer, which is the predominant deer species farmed in New Zealand. These tests will improve diagnostic capability for this high impact disease during investigations and responses, if the need ever arises. Clinical samples (positive control reagents) collected from red deer are required for proper validation of any diagnostic assay for the detection of FMDV and antibodies to the virus in these animals.

Materials and Methods

Animal experiments

The animal experiments were performed within a high containment facility at the National Centres for Animal Disease (NCAD) – Winnipeg Laboratory [National Centre for Foreign Animal Disease (NCFAD)], Winnipeg, Manitoba, Canada, with the approval of the institutional Animal Care Committee under the close supervision of a veterinarian. Ten red deer had been purchased from a red deer farm in Ontario and transported to the NCFAD, Winnipeg, Manitoba. They were five male and five female red deer, aged between 8 and 18 months and weighing between 35 and 80 kg. Six red deer were housed in two groups of three animals per cubicle. The remaining four were housed as two groups of two in a double sized cubicle divided by puck board and black mesh extending from floor to ceiling.

The inoculum (FMDV strain O UKG 11/2001) was produced from a seed stock originating from the Pirbright Institute, UK, by passaging the virus twice in lamb kidney (LK) cells. The P1 region of progeny virus was sequenced and compared to an original field isolate GenBank Accession number DQ404180 (Cottam et al., 2006) to confirm the absence of sequence changes in the P1 region, specifically where the determinant of the receptor cell tropism of FMDV is contained, particularly VP2 and VP3. After presampling, every animal received an intranasal inoculation of 10⁶ 50% tissue culture infective dose (TCID₅₀) of the FMD virus strain O UKG 11/2001 and diluted in a total volume of 5 ml per animal (2.5 ml per nostril) in alpha minimal essential medium (α-MEM). Following inoculation, deer were monitored for clinical signs of disease and their rectal temperatures and progression of disease recorded regularly.

Animals were anaesthetized with 0.5–1.0 mg/kg of xylazine intramuscular (via dart gun) and were then given 1–2 mg/kg ketamine intravenous as needed to prolong sedation. Sedation was reversed with yohimbine at 0.1–0.2 mg/kg half the volume given intravenous and half given intramuscular. To minimize the amount of anaesthetics received by individual animals, close examination and sampling were scheduled on alternate days, giving each animal at least 2 days between manipulations. Deep rectal temperatures (both baseline and post-infection) were taken using a probe and recorded while the deer were recumbent under sedation. At the end of the experimental infection period, animals were euthanized using sodium pentobarbital.

It became apparent, following a preliminary analysis of the virus and antibody detection data, that re-inoculation of a subset of animals was necessary to generate sufficient clinical material and positive serum samples. At 14 dpi, 6 of 9 red deer were therefore re-inoculated with 10⁶ TCID₅₀ of FMDV strain O UKG 11/2001 in a total volume of 1 ml per animal, this time by intramuscular injection. Animals that had no detectable virus in swabs or blood and no antibody response at the time were selected for re-inoculation.

Sample collection from experimental deer

Heparinised (whole) blood and clotted blood (for serum), oesophageal–pharyngeal (probang) samples and swabs (oral and nasal) were collected from all animals just before inoculation and every 2 to 3 dpi per animal according to a predetermined schedule.

Probang samples were diluted in an equal volume of Eagle’s–HEPES medium (pH 7.2) containing 5% foetal calf serum and stored at −70°C until required.

One deer, animal 1097, exhibiting mild signs consistent with FMD was euthanized at 5 dpi and various tissue samples collected for virus detection and/or histopathology. Tissues collected for histopathology included lymph nodes (cervical, submandibular), lung, liver, spleen, tonsil, coronary band, tongue, heart and nasopharynx/soft palate. Tissues were immediately fixed in 10% formalin (formal-FIXX™, Thermo Fisher Scientific Inc., Waltham, MA, USA) for at least 24 h or separately placed in transport media.

At 14 dpi and following re-inoculation, blood, probang and swabs were collected (as described earlier) from all animals every 2–3 days until 26/12 dpi (12 days post-re-inoculation) and on the day of post-mortem examination. Three deer were then euthanized at 28/14 dpi, 2 at 29/15 dpi, the final 4 at 30/16 dpi and tissue samples collected and preserved as described earlier.

For tissue homogenate preparation, samples were rinsed with Dulbecco’s PBS (D-PBS) to obtain rid of residual transport medium, weighed and emulsified to obtain a
10% weight/volume suspension in D-PBS. The suspension was then clarified by centrifugation at 2000 g for 20 min at 4°C and the supernatant treated with antibiotics for 30 min at room temperature.

Sample collection from non-infected red deer

Collection of samples from non-infected New Zealand red deer was performed at a number of slaughter plants throughout New Zealand by Ministry for Primary Industries (MPI) meat inspectors. FMD has never occurred in New Zealand. A total of 950 blood samples were collected into 10-ml blood tubes with clotting agent. Another 200 blood samples and matching 200 nasal swabs were also collected. Bloods and swabs were couriered chilled to the Animal Health Laboratory (AHL), Upper Hutt, New Zealand. Bloods were spun at the laboratory and serum collected, aliquoted and stored frozen. The 950 serum samples were used for testing in various ELISAs; the 200 extra serum samples and 200 nasal swab samples were used for rRT-PCR testing.

Virus isolation

Isolation of FMDV from blood, swabs, probang samples, vesicular fluid and 10% tissue homogenates was carried out in primary LK cells and performed according to a published protocol (Moniwa et al., 2012).

FMDV RNA detection

RNA extraction from blood, serum, nasal swabs, oral swabs, probang samples and tissue homogenates for viral RNA detection was performed using a MagMax-96 Viral RNA Isolation Kit, AM1836 (Ambion, Life Technologies) following manufacturer's protocol. The MagMAX™ Express-96 Instrument was used for purification using a Deep Well Magnetic Particle Processor (Life Technologies). Fifty five μl of each sample was used for extraction and the RNA eluted into 90 μl of Elution buffer.

Detection of viral RNA using FMDV 3D TaqMan rRT-PCR was carried out according to published protocols (Moniwa et al., 2007). This rRT-PCR used primers and probe that specifically target a conserved region of the FMDV 3D gene resulting in the amplification of an 88 bp product. The forward primer FMDV 1186F (5’-ACCTGGTTTATAAAACCTGTGATG3’) and reverse primer FMDV 1237R (5’TCAACCTCTCTCAGTGACCTCCAC3’) were custom made by Invitrogen (Life Technologies Inc., Burlington, ON, Canada). The FMDV TaqMan probe (5’ATCCCTCTGTTGACGC3’) labelled on the 5’ end with 6FAM and the 3’ end with minor groove binder (MGB) was synthesized by Applied Biosystems Inc. (ABI, Life Technologies). AgPath-ID™ One-Step RT-PCR reagents (Life Technologies) were used for all tests. The test was performed in 96-well plates on ABI SDS7900ht with a final volume of 25 μl per well containing 1× RT-PCR buffer, 0.5 μmol forward primer, 0.5 μmol reverse primer, 0.2 μmol probe, 1× RT enzyme mix and 5 μl RNA template. The thermal cycler was programmed for 50°C for 30 min, 95°C for 15 min for the RT step and 95°C for 10 s, 60°C for 1 min for the amplification step which was repeated 45 times. The threshold cycle (Ct) was determined for each sample and positive controls. Ct values <35.99 were considered positive. Amplification with Ct value between 35.99 and 40.00 and or low curve to the threshold sample was considered suspicious or doubtful, while Ct values >40 were considered negative.

Detection of viral RNA using FMDV IRES TaqMan rRT-PCR was carried out according to published protocols (Reid et al., 2002; Anonymous, 2012) with some modifications. The forward primer FMDV SA-IR-219-246F: 5’-CACTTTAGGGTGCAATGCTGAC-3’ and reverse primer FMDV SA-IR-315-293R: 5’-CAGATYCCRAGTGWCICITGTTA-3’ were custom made by Invitrogen. The FMDV TaqMan probe (FMDV SAmulti2-P-IR-292-269R: 5’-CCTGCGGGGTACCTGAAGGGCATCC-3’) labelled on the 5’ end with 6FAM and the 3’ end with MGB was synthesized by Applied Biosystems Inc. (ABI, Life Technologies). AgPath-ID™ One-Step RT-PCR reagents (Life Technologies) were used for all tests. The test was performed in 96-well plates on ABI SDS7500 with a final volume of 25 μl per well containing 1× RT-PCR buffer, 1.25 μl of 18 μmol forward primer, 1.25 μl of 18 μmol reverse primer, 1 μl of 5 μmol probe, 1× RT enzyme mix and 5 μl RNA template. The thermal cycler was programmed for 45°C for 10 min, 95°C for 10 min for the RT step and 95°C for 15 s, 60°C for 45 s for the amplification step which was repeated 45 times. The Ct value was determined for each sample and positive controls. Ct values <35.99 were considered positive. Amplification with Ct value between 35.99 and 40.00 and or low curve to the threshold sample was considered suspicious or doubtful, while Ct values >40 were considered negative.

Viral antigen detection

A double antibody sandwich (DAS) ELISA was used to detect FMDV present in samples according to a previously described protocol (Moniwa et al., 2012). Average OD values >0.1 were considered positive.

Detection of FMDV antigen using a lateral flow device (LFD), the SVANODIP® FMDV-Ag (Boehringer Ingelheim Svanova, Uppsala, Sweden) was carried out according to the manufacturer’s instructions.

Detection of FMDV antigen using the in-house FMDV serotype O specific LFD was carried out as described in Yang et al. (2013).
Virus neutralization test

Detection of neutralizing antibodies for serotype O was performed on IBRS 2 cells according to a protocol developed at the NCFAD. For screening purposes, 1/4 dilution of serum in diluent (MEM-α containing 50 µg/ml gentamicin and 2 mM l-glutamine) was added to three wells of a tissue culture microtitre plate. Two wells were used for the test, and the third well was used as a serum toxicity control. For the determination of virus neutralization test (VNT) titre, three columns of eight 2-fold dilutions of serum starting from 1/4 were needed. Similarly, two wells of each dilution were used for the test and the third well used as a serum toxicity control. To each test well containing 25 µl of diluted serum, an equal volume of culture medium (diluent with 10% foetal bovine serum) containing challenge virus (100 TCID₅₀) was added. Culture medium alone was added to serum toxicity control wells. Positive and negative control sera were included for each assay.

A back titration of the challenge virus was also performed to confirm the titre used. Plates were incubated for 60 ± 5 min. at 37 °C in a humidified 5% CO₂ incubator. IBRS2 cells (50 µl at 1 x 10⁶ cells/ml) were then added to all wells and the plates incubated at 37 ± 2°C in a humidified 5% CO₂ incubator for 2–3 days. Serum samples inhibiting cytopathic effect (CPE) in at least 50% of virus-infected wells were considered positive. The VNT titre was determined as the reciprocal of the serum dilution inhibiting CPE in at least 50% of virus-infected wells.

Detection of serum antibodies against FMDV structural proteins

Antibodies against FMDV serotype O structural proteins (SP) were detected using four assays: the in-house solid phase competitive FMDV serotype O antibody ELISA (SPO-ELISA-IH) from the NCFAD, Winnipeg, Canada, and the commercially available ELISAs PrioCHECK® FMDV-O antibody detection ELISA (SPO-ELISA-PR) from Life Technologies (formerly Prionics), Lelystad, The Netherlands, the ID SCREEN® FMD type O competition ELISA (SPO-ELISA-IV) from ID-Vet, Montpellier, France, and the FMDV serotype O Solid Phase Competitive ELISA (SPO-ELISA-I2Z) from IZSLER, Brescia, Italy.

The SPO-ELISA-IH detects anti-serotype O FMDV antibodies in test sera. Briefly, 96-well Maxisorp microplates were coated overnight at 4°C with 100 µl per well of an optimal dilution of rabbit anti-FMDV serotype O serum in coating buffer (0.06 M carbonate buffer, pH 9.6 ± 0.05). Next, the plates were blocked with 100 µl of blocking/dilution buffer [5% normal rabbit serum, 10% normal bovine serum in 0.01 M phosphate-buffered saline containing 0.05% Tween, pH 7.2 ± 0.1 (PBST)] at 37°C on an orbital incubator shaker. After 1 h blocking, plates were washed five times with PBST followed by the addition of 100 µl per well of FMDV serotype O antigen diluted to optimal concentration in blocking/dilution buffer. Plates were incubated for 1 h at 37°C on an orbital incubator shaker.

After washing, 50 µl of 1/5 dilution of test serum and controls was added to each well, immediately followed by 50 µl of diluted guinea pig anti-FMDV serotype O serum. The plates were again incubated for 1 h at 37°C on an orbital incubator shaker. Plates were washed, 100 µl per well of polyclonal donkey anti-guinea pig IgG (H&L) conjugated to horseradish peroxidase diluted in blocking/diluent buffer added and incubated for 1 h at 37°C on an orbital incubator shaker.

After washing, 100 µl of o-phenylenediamine dihydrochloride (OPD) substrate was dispensed into each well and incubated for 10 min at room temperature. The OPD colour change reaction was stopped with 50 µl of 2 M sulphuric acid. The optical density (OD) of each well of the plate was determined on a microplate reader at 490 nm. Assay ODs were corrected for background by subtracting average OD of eight wells containing substrate and stop solution alone. Results for test sera were expressed as a per cent inhibition (PI) of signal strength. The PI value for each sample was calculated by the following formula: PI = (100 – mean OD of 2 replicate test samples/(mean OD of the 4 replicate target standards) × 100. Based on the testing of over 900 cattle field sera and over 600 porcine field sera from an FMDV-free country (Canada) and the frequency distribution of their PI values, samples were considered positive if PI ≥ 50%.

The SPO-ELISA-PR, a competitive ELISA, was performed according to the manufacturer’s instructions. Test results were derived by the following formula: PI = 100 – (test sample OD/negative reference serum OD) × 100. According to the manufacturer’s evaluation data, samples were considered positive if the PI value was ≥50%.

The SPO-ELISA-IV, a competitive ELISA, was performed according to the manufacturer’s instructions. Test results were calculated as follows: S/N% = 100 × (test sample OD – positive control OD)/(negative control OD – positive control OD). According to the manufacturer’s evaluation data, samples with S/N% ≤35% were considered positive, >45% negative and between 35% and 45% doubtful. In order to make these competitive ELISA results comparable to other competitive ELISA results, per cent inhibition (PI) was calculated: PI% = 100 – S/N%, resulting in samples with PI% ≥65% being positive, <55% being negative and between 55% and 65% doubtful. For simplicity reasons, we used a 55% cut-off and no doubtful range.
The SPO-ELISA-I2Z, a competitive ELISA, was performed according to the manufacturer’s instructions. Test results were derived by the following formula: PI = 100 \( - \) (test sample OD/negative reference serum OD) \( \times \) 100. According to the manufacturer’s evaluation data, samples were considered positive if the PI value was \( \geq 70\% \).

Detection of serum antibodies against FMDV non-structural proteins

Antibodies against NSPs were detected using four NSP-ELISAs and a LFD.

The in-house 3ABC-ELISA (NSP-ELISA-IH) is a competitive ELISA directed towards antibodies against the FMDV 3ABC polypeptide, which is a NSP. It uses a recombinant 3ABC protein with a 5-histidine fusion tag as antigen (Moniwa et al., 2012). The assay was performed as previously described (Moniwa et al., 2012). Test results were derived by the following formula: PI = [(negative reference serum OD) \( - \) test sample OD]/(negative reference serum OD \( - \) positive reference serum OD)] \( \times \) 100. Based on the testing of over 500 bovine, ovine and porcine field sera from an FMDV-free country (Canada) and the frequency of distribution of their PI values, samples were considered positive if the PI value was \( \geq 50\% \).

The commercially available PrioCHECK® FMDV NS ELISA (NSP-ELISA-PR), a blocking ELISA, was performed according to the manufacturer’s instructions (Life Technologies, Lelystad, The Netherlands). Test results were derived by the following formula: PI = 100 \( - \) (test sample OD/negative reference serum OD) \( \times \) 100. According to the manufacturer’s evaluation data, samples were considered positive if the PI value was \( \geq 70\% \).

The commercially available Anigen FMD NSP Ab ELISA (NSP-ELISA-BN), a competitive ELISA, was performed according to the manufacturer’s instructions (BioNote, Gyeonggi-do, Korea). Test results were derived by the following formula: PI = [(1 \( - \) (test sample OD/negative reference serum OD))] \( \times \) 100. According to the manufacturer’s evaluation data, samples were considered positive if the PI value was \( \geq 50\% \).

The commercial IDEXX FMD 3ABC Ab Test (NSP-ELISA-ID), an indirect ELISA, was performed according to the manufacturer’s instructions (IDEXX, Westbrook, ME, USA). Test results were derived by the following formula: % S/P = 100 \( \times \) [(sample OD \( - \) negative control OD)/(positive control OD \( - \) negative control OD)]. According to the manufacturer’s evaluation data, samples were considered positive if the S/P value was \( \geq 30\% \), negative if the S/P value \( < 20\% \), and suspect if the S/P value was between 20% and 30%.

The Anigen Rapid FMD NSP Ab Test Kit (NSP-penside test) was used according to manufacturer’s instructions (BioNote, Inc, Gyeonggi-do, Korea).

Histopathology and immunohistochemistry

Fixed tissues were routinely processed, cut and stained with haematoxylin and eosin (H&E) for histopathologic examination. For immunohistochemistry, 5 \( \mu \)m sections were cut, air dried overnight and placed into a 60°C oven for 1 hour. The deparaffinized and rehydrated sections were quenched for 10 min in aqueous 3% hydrogen peroxide, rinsed in MilliQ water and placed into Tris-buffered saline plus Tween (TBST) for 5 min. For the monoclonal antibody, epitopes were retrieved using low pH Glyca (Biogenex, Freemont, CA, USA) in a decloaking chamber (Biocare Medical, Concord, CA, USA). Sections were loaded from a TBST bath onto the DakoCytomation autostainer and incubated with mAb F8-3Bp (NSP) or mAb F14-12SA (structural protein) at a dilution of 1 : 100 or 1 : 800, respectively, for 1 h. The sections were incubated for 30 min using the Envision + anti-mouse-HRP polymer kit (Dako, Carpinteria, CA, USA) followed by a TBST rinse. Diaminobenzidine was used as the substrate chromogen, and the slides were counterstained with Gill’s haematoxylin.

Statistical methods

Diagnostic specificities (DSP) and 95% confidence intervals (CI) were calculated using the software Epitool ‘test evaluation against a gold standard’ (Sergeant ESG: 2009, Epitools epidemiological calculators. AusVet Animal Health Services and Australian Biosecurity Cooperative Research Centre for Emerging Infectious Disease. Available at: http://epitools.ausvet.com.au).

Results

Clinical observations

Rectal temperatures did not show any significant changes in the red deer following primary FMDV inoculation. The average rectal temperature for all deer before inoculation was 38.8°C \pm 0.3°C, and 39.0°C \pm 0.6°C after inoculation. Likewise, no significant temperature changes were observed in red deer re-inoculated at dpi 14 (data not shown).

Only one of the 10 red deer, animal 1103, showed obvious clinical signs of FMD. This deer had mild signs of lameness beginning on dpi 6. In addition, there were two erosions (about 2 mm wide) on the underside of the tongue apparently due to ruptured vesicles (Fig. 1a). By dpi 12, the tongue lesions had almost healed (Fig. 1b) and healing was complete at dpi 16 (Fig. 1c).

The development of foot lesions in deer 1103 started at dpi 6 with a softening and apparently fluid-filled coronary band on the right hind limb (Fig. 1d). By dpi 9, the
The coronary band lesion was blanched, soft and fluid filled in one area while in another area the vesicle had ruptured leaving a brownish open wound (Fig. 1e). At dpi 12, the coronary band lesions became more extensive (Fig. 1f). By dpi 16, the foot lesion had begun to heal (Fig. 1g). However, at dpi 21 the lesion apparently got worse possibly due to secondary bacterial infection (Fig. 1h). At dpi 26, mild lameness was observed and the lateral claw of the right hind limb was apparently retracting beginning at the caudal portion (Fig. 1i). Nevertheless, at dpi 29 when this deer was euthanized, the foot lesions had almost healed leaving behind dry wart-like crusts (Fig. 1j).

Mild clinical signs were also observed in most of the deer starting at dpi 6, but these were inconclusive. Such signs included mild lameness and apparent swelling of the coronary band, but these changes never progressed to identifiable lesions. However, deer 1105 had a 2 mm wide vesicle on the underside of the tongue on dpi 5 (Fig. 1k) and rapid respiration on dpi 6 which returned to normal on dpi 7. By dpi 9–14, eight deer except 1103 had returned to normal. Re-inoculation by intramuscular injection did not induce any disease in the six deer that were re-inoculated. However, at dpi 21 deer 1101 had erosion on the inside of the cheek which could have been due to a ruptured vesicle (Fig. 1l).

Pathology

Necropsy findings were unremarkable in all animals except for the foot lesion described above in deer 1103 and multifocal coalescing haemorrhages of the soft palate in deer 1100. Microscopic examination was performed on tissues from deer 1097, 1100 and 1103. For deer 1097 euthanized at dpi 5, only non-specific changes including mild haemorrhage and neutrophil infiltration of the cervical lymph node were observed. In deer 1100, there was mild haemorrhage and perivascular accumulation of mononuclear inflammatory cells in the subepithelial connective tissue of the palate...
which may have been related to the probang procedure. For deer 1103, no significant microscopic findings were observed. Immunohistochemistry was performed on tissues from deer 1100 and 1103 using antibody F14-12SA, and FMDV antigen could not be detected. Additionally, tissues from animal 1103 were run using antibody F8-3Bp and FMDV antigen was not detected.

Detection of viral RNA by rRT-PCR and of virus by FMDV isolation

The presence of FMDV in samples from infected deer was tested by both 3D and IRES rRT-PCR and by virus isolation. Except for a few samples, data from both rRT-PCRs were almost identical (Table 1).

Most positive results in all three tests were from nasal swabs, such as deer 1100 on dpi 1 and 4, deer 1103 on dpi 3 and 6, deer 1104 on dpi 2 and 5, and deer 1107 on dpi 3. Deer 1103 was also positive in both rRT-PCRs but negative by virus isolation in serum on dpi 3 and in oral swab dpi 16. The oral swab samples from deer 1102 at dpi 12 and probang sample from deer 1103 at dpi 6 had detectable FMDV RNA by 3D rRT-PCR but were negative by IRES rRT-PCR. The reverse was true for the serum samples from deer 1104 at dpi 2 with FMDV RNA detection by IRES but not by 3D rRT-PCR.

Only two samples (oral swab form deer 1104 at dpi 2 and nasal swabs from deer 1102 at dpi 3) with $C_{\text{t}} \geq 36$ contained infectious virus. Interestingly, virus could be isolated from three samples negative for FMDV genome (probang samples from deer 1100 at dpi 4 and 1104 at dpi 2, blood from deer 1104 at dpi 5). Most of the samples required two passages on LK cells before infectious virus could be detected.

Vesicular fluid and all swabs of lesions (tongue at dpi 6 and coronary band at dpi 12 and 16) from deer 1103 were positive for FMDV genome with comparable results for the 3D and IRES rRT-PCR (Table 2). However, infectious virus could only be isolated from vesicular fluid and tongue lesion swabs (Table 2).

Tissues collected at necropsy, including lymph nodes, soft palate and tonsils, from all but one animal were negative for FMDV genome by rRT-PCR. Tonsil from deer 1103, euthanized at dpi 29, tested positive for FMDV genome by rRT-PCR with $C_{\text{t}} = 30.7$ by 3D and 33.7 by IRES rRT-PCR. However, infectious virus or viral antigen could not be isolated or detected by immunohistochemistry, respectively.

Detection of viral antigen

The in-house DAS ELISA was used to confirm the presence of FMDV in supernatants from LK cell cultures for virus isolation. As shown in Table 3, all but one of the supernatants from cell cultures showing CPE either after the 1st or 2nd passage, were positive for FMDV antigen by the DAS-ELISA. Although the vesicular fluid isolate tested negative by DAS-ELISA twice, virus presence was confirmed by rRT-PCR.

Rapid antigen detection tests were performed using an in-house LFD test for FMDV serotype O and the commercially available Svanodip FMDV-Ag test. Testing was limited to samples that were positive or suspicious for the presence of FMDV genome by rRT-PCR.

Both tests detected cell culture-derived FMDV serotype O UKG 11/2001 and O SKR 4/2010 used as strong positive controls. However, from the deer samples, only vesicular fluid obtained at dpi 9 from deer 1103 produced a very faint band at the test line with the in-house LFD test and was negative in the commercial test.

Detection of FMDV antibodies after initial inoculation

Of the 10 experimentally inoculated red deer, only animal 1103 showed an antibody response after the initial inoculation (Fig. 2a,b). Neutralizing antibodies were detected from dpi 9 onwards with titres in the VNT peaking at 1/256 at dpi 12 (Fig. 2b). Thereafter, titres dropped but remained low positive over the remainder of the trial period with a slight peak at dpi 23.

The antibody response in the four NSP-ELISAs was variable (Fig. 2a). The NSP-ELISA-IH and the NSP-ELISA-PR performed equally well with antibody detection from dpi 14 onwards, but the NSP-ELISA-PR reaction was already just below the cut-off at dpi 12. Antibody levels peaked at dpi 16 and slowly declined over the course of infection. The NSP-ELISA-BN and the NSP-ELISA-ID performed relatively poorly. NSP-ELISA-BN detected samples as positive at one time point only (dpi 16) and NSP-ELISA-ID at two time points (dpi 19 and 21).

The NSP antibody LFD pen side test detected antibodies earlier in whole blood as revealed by a faint band on dpi 14, while the earliest detection of antibodies in serum was at dpi 16 (Fig. 3). Antibodies could be detected up until dpi 29 when the animal was euthanized.

Serotype specific antibodies were detected by the SPO-ELISA-IH and the SPO-ELISA-PR from dpi 9 and 12, respectively (Fig. 2b). Data from both tests were almost identical, except that the SPO-ELISA-PR had not yet crossed the cut-off level at dpi 9, even though the PI was on the increase and close to the cut-off. SPO-ELISA-IV showed a similar increase of sero-positivity to the previous assays but with lower PI values. The SPO-ELISA-IZ lagged behind and only showed positive results from dpi 19 onwards.
Detection of FMDV antibodies after re-inoculation

For the reason that only deer 1103 had shown an antibody response from dpi 9 onwards, six of the red deer were re-inoculated intramuscularly with the same FMDV strain that had been used initially but at a higher dose at dpi 14. Results are shown in Fig. 2c–h for the six animals. None of the re-inoculated animals staged an antibody response to NSP. None of these animals became positive in rRT-PCR or by virus isolation after re-inoculation. Nevertheless, all six animals became positive for SP serotype O antibodies (Fig. 2c–h) and in the VNT.

Five of the animals became positive in the VNT at dpi19/5 and one animal, deer 1106, became positive at dpi 21/7. In the SPO-ELISA-IV and SPO-ELISA-PR, the strongest SPO antibody responses over the course of re-inoculation were observed for each animal. Antibody activities were comparable for both ELISAs, showing similar patterns over the experimental period. Each of the six animals became positive in both ELISAs from dpi 19/5 onwards. SPO-ELISA-PR was slightly weaker than SPO-ELISA-IH in deer 1101 (Fig. 2d) but otherwise comparable.

The SPO-ELISA-IV showed comparable pattern to the previous SPO-ELISAs in deer 1098, 1101 and 1102 (Fig. 2c–e) with slightly lower PI values. Nevertheless, it failed to detect deer 1105, 1106 and 1107 (Fig. 2f–h) as positive, even though the PI values were increasing over time. The SPO-ELISA-IZ generally did not identify samples as positive over the course of inoculation in any of the six re-inoculated deer. Generally, the PI levels during the phase prior to rising PI values were rather high at around 40%. Even if there was a rise of PI values in the SPO-ELISA-IZ, it was not much and did not rise above the relatively high cut-off value of 70%.

Diagnostic specificities

The diagnostic specificities (DSP) of most of the commercial test methods used were determined at the AHL, New Zealand, using samples from non-infected deer from local slaughter houses (Table 4). Generally, DSP values were...
high for every test method, well above 99% or above 88% for the lower 95% CI.

Discussion

Foot-and-mouth disease virus has been shown to infect cervids such as reindeer, moose and deer, including white tailed deer (McVicar et al., 1974; Haigh et al., 2002; Dunbar et al., 2009; Arzt et al., 2011; Moniwa et al., 2012). FMDV is transmitted mainly by direct contact between an infected and a susceptible animal and most often entry is through the pharyngeal region and upper respiratory tract but can also occur through cuts and abrasions in skin and mucosa (Alexandersen et al., 2003b). Clinical signs of FMD in deer are varied, with red deer and fallow deer showing milder signs compared to white tailed deer, roe deer and muntjac deer (Forman and Gibbs, 1974; Gibbs et al., 1975; Moniwa et al., 2012). In the current study, red deer were inoculated intranasal, as close as possible to natural infection. Only one of ten red deer developed the typical foot lesions of FMD, which were confined to just one foot. In addition, fever and tongue lesions in this animal were mild.

Even though few other animals had the virus in oral and nasal swabs at dpi 1–5 by both rRT-PCR and virus isolation, only the animal with clinical disease had FMDV RNA levels considered positive by rRT-PCR in serum. However, transient viraemia based on infectious virus isolation was also detected in another deer, but this was not matched by clinical disease. This could mean that the genomes were not detected in the other deer due to bad timing (animals could not be sampled daily), low sensitivity or low viral load. In contrast, in a previous study with white tailed deer at the NCFAD, most of the animals inoculated by the intranasal route developed viraemia at dpi 1–4 and severe disease even at lower doses of the challenge virus (Moniwa et al., 2012). Based on the current study’s data, FMD in red deer was mild and only one animal truly became infected. Despite failure to isolate infectious virus, the apparent persistence of FMDV RNA (up to dpi 29) in the tonsils of the red deer that developed clinical disease is indicative of a possible carrier state in red deer. In experimentally infected white tailed deer, virus was not detected beyond dpi 28 (Moniwa et al., 2012) but in other reports virus detection in white tailed deer lasted for up to 11 weeks after infection (McVicar et al., 1974).

The detection of antibodies to FMDV NSP is often used to confirm a history of active infection with replication of virus and systemic spread. This also serves as a means of distinguishing vaccinated from infected animals (DIVA) as vaccinated animals lack antibodies to FMDV NSP (Clavijo et al., 2004). In the current experiment, only the one deer that had transient viraemia and developed clinical FMD lesions (1103) was positive for antibodies to FMDV NSP. Furthermore, using serotype O ELISA, antibodies to whole virus antigen were detected in the same animal following the primary inoculation. In addition, the VNT, which is a functional assay, detected neutralizing antibodies only in the one deer following the primary inoculation. These results confirmed that just one deer had a systemic FMDV
Foot-and-Mouth Disease in Red Deer

(a) Deer 1103

(b) Deer 1103

(c) Deer 1098

(d) Deer 1101

(e) Deer 1102

(f) Deer 1105

(g) Deer 1106

(h) Deer 1107

- SPO-ELISA-IH
- SPO-ELISA-PR
- SPO-ELISA-IV
- SPO-ELISA-IZ

- NSP-ELISA-IH
- NSP-ELISA-PR
- NSP-ELISA-BN
- NSP-ELISA-ID

% Inhibition

VNT titre

dpi
infection sufficient to induce an immune response. Intramuscular re-inoculation of the deer that had no clinical signs or no detectable antibodies at dpi 14 induced neither clinical disease nor antibodies to FMDV NSP by the time the experiment was terminated.

Intramuscular inoculation can lead to infection in susceptible species as the virus spreads to the blood stream and also reaches epithelial cells (Alexandersen et al., 2003b). Such cells are most abundant in the stratified squamous epithelium of the tongue, in and round the mouth, and in the skin (Zhang and Alexandersen, 2004). With the high dose of the inocula used for re-inoculation of six animals, it was expected that some virus would get into the blood and spread to more susceptible sites. Therefore, the absence of disease following re-inoculation either means low susceptibility of red deer to FMDV or suggests that the initial inoculation might have induced specific immunity, including cell mediated immunity, despite the absence of clinical disease. Such an immune response would delay and/or prevent virus replication following a repeat infection. This hypothesis is consistent with the antibody reaction observed after the second, but not the first, inoculation as discussed.

Contrary to the FMDV NSP response, intramuscular re-inoculation with FMDV induced a rapid rise in neutralizing antibodies and serotype O specific antibody response to whole virus antigen, with all 6 re-inoculated deer testing positive at day 5 after re-inoculation. This suggests that the intramuscular inoculum, instead of leading to infection, possibly served mainly as a source of antigen for the immune system. The rapid response at dpi 5 compared to the one animal 1103 that sero-converted following the initial inoculation could be a consequence of the route of inoculation and the high inoculum (10⁸ TCID₅₀ per animal). Furthermore, there is a high possibility that the immune system had been primed by the initial inoculation.

**Fig. 2.** Antibody detection in seven red deer after experimental inoculation (a and b clinically infected deer 1103) and re-inoculation (c–h). (a) Deer 1103. Antibody reaction in four non structural protein (NSP) ELISAs during the course of experimental infection. NSP-ELISA-IH = in house blocking ELISA; NSP-ELISA-BN = commercial competitive ELISA from BioNote; NSP-ELISA-PR = commercial blocking ELISA from Life Technologies; NSP-ELISA-ID = commercial indirect ELISA from IDEXX. % S/P = ratio of optical density of sample/optical density of positive control in percent. (b–h) Antibody reactions in the virus neutralization test (VNT) and four FMDV structural protein serotype O (SPO)-specific ELISAs: SPO-ELISA-IH = in-house competitive ELISA, SPO-ELISA-PR = commercial blocking ELISA (Life Technologies), SPO-ELISA-IV = commercial competitive ELISA (ID-VET), SPO-ELISA-IZ = commercial competitive ELISA (IZSLER) for the detection of serotype O specific antibodies. (a, b) Deer 1103, experimentally infected. (d–h) Deer 1098, 1101, 1102, 1105, 1106 and 1107. These deer were re-inoculated with a FMD serotype O virus. Dpi, day post-inoculation.

**Fig. 3.** Detection of antibodies by a lateral flow device (pen-side test) against non-structural viral proteins in serum and whole blood during the course of experimental infection in deer 1103. DPI, days post-inoculation.

**Table 4.** Diagnostic specificities of a number of commercial FMDV screening tests

<table>
<thead>
<tr>
<th>Test Method</th>
<th>Number of deer tested</th>
<th>Sample type</th>
<th>Samples positive</th>
<th>DSP %</th>
<th>DSP % Lower CI 95%</th>
<th>DSP % Upper CI 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D rRT-PCR</td>
<td>200</td>
<td>Nasal swab</td>
<td>0</td>
<td>100.00</td>
<td>98.17</td>
<td>100.00</td>
</tr>
<tr>
<td>3D rRT-PCR</td>
<td>200</td>
<td>Serum</td>
<td>0</td>
<td>100.00</td>
<td>98.17</td>
<td>100.00</td>
</tr>
<tr>
<td>IRES-HRT-PCR</td>
<td>200</td>
<td>Nasal swab</td>
<td>0</td>
<td>100.00</td>
<td>98.17</td>
<td>100.00</td>
</tr>
<tr>
<td>IRES-HRT-PCR</td>
<td>200</td>
<td>Serum</td>
<td>0</td>
<td>100.00</td>
<td>98.17</td>
<td>100.00</td>
</tr>
<tr>
<td>NSP-ELISA-PR</td>
<td>950</td>
<td>Serum</td>
<td>1</td>
<td>99.89</td>
<td>99.41</td>
<td>100.00</td>
</tr>
<tr>
<td>SPO-ELISA-PR</td>
<td>950</td>
<td>Serum</td>
<td>2</td>
<td>99.79</td>
<td>99.24</td>
<td>99.97</td>
</tr>
<tr>
<td>SPO-ELISA-IZ</td>
<td>950</td>
<td>Serum</td>
<td>2</td>
<td>99.79</td>
<td>99.24</td>
<td>99.97</td>
</tr>
<tr>
<td>SPO-ELISA-IV</td>
<td>950</td>
<td>Serum</td>
<td>0</td>
<td>100.00</td>
<td>99.61</td>
<td>100.00</td>
</tr>
<tr>
<td>NSP-LFD Bionote</td>
<td>950</td>
<td>Serum</td>
<td>0</td>
<td>100.00</td>
<td>99.61</td>
<td>100.00</td>
</tr>
</tbody>
</table>

DSP, diagnostic specificity; CI, confidence interval.
While the FMDV IRES TaqMan rRT-PCR was carried out by following the method described in the OIE manual (Anonymous, 2012), the FMDV 3D TaqMan rRT-PCR was carried out according to a published protocol (Moniwa et al., 2007). Nevertheless, the primers and probe for the 3D gene as described in this study have been validated for use in FMD diagnosis at the NCFAD (Moniwa et al., 2007) and have been used with external proficiency panels from the World Reference Laboratory for FMD, Pirbright, with no discrepancy to the OIE rRT-PCR assays over the years.

Regardless of the comparison of test methods, both the FMDV 3D and IRES rRT-PCR were to a large extent equally good for the detection of FMDV RNA. In addition, most of the samples positive by rRT-PCR were also positive by virus isolation. However, most of the lesion swabs had \( C_t \leq 35.99 \), but no infectious virus was recovered from them. On the other hand, some samples with \( C_t \geq 56 \) by rRT-PCR were positive by virus isolation. Virus isolation is considered the gold standard for detecting the presence of FMDV in clinical material (Moniwa et al., 2012). Based on the data reported here, more samples from infected red deer tested positive by rRT-PCR than by virus isolation. It is not unusual, for whatever reason, to see discrepancies in some samples. This has been observed before (Reid et al., 2002; Alexandersen et al., 2003a,b; Zhang and Alexandersen, 2004). With virus isolation being laborious and time consuming, requiring 2–4 days to obtain results, and with the data available, rRT-PCR assays would play a central role during an FMD incursion as screening methods for red deer.

For rapid antigen detection, only the NCFAD in-house LFD and not the commercial pan-reactive LFD (Svanodip) detected FMDV antigen in vesicular fluid from the infected red deer. However, the band was very faint and in the field, such a result would probably be considered inconclusive, necessitating further testing in the laboratory. It is worth noting that, because of the small volume recovered from the vesicles, vesicular fluid was diluted in PBS. Therefore, an undiluted sample would have likely come up positive on, at least, the NCFAD in-house LFD.

Sufficient samples were obtained from the one infected animal and the six re-inoculated animals to compare the performance of the SPO-ELISAs to each other and to the VNT. The SPO-ELISA-IH and the commercial SPO-ELISA-PR detected antibody responses with confidence and comparable to the VNT. Unexpectedly, two commercial SPO-ELISAs failed to detect some or most animals as positive. Therefore, the SPO-ELISA-PR will be the choice of screening ELISA for FMD preparedness for red deer.

There were limited positive samples for a comparison of FMDV NSP-ELISAs obtained from one animal only. However, results from this one deer showed that the NSP-ELISA-IH and the NSP-ELISA-PR detected anti-NSP serum antibodies during the course of infection. The other two ELISAs, NSP-ELISA-BN and NSP-ELISA-ID identified most samples as negative that came up positive in the other NSP-ELISAs. The NSP-ELISA-BN is a competitive ELISA and was expected to perform at a similar level to other NSP competitive/blocking ELISAs. A possible explanation for this is the monoclonal antibody used, which has specificity for one epitope of the NSP antigen. If this one deer failed to make sufficient serum antibodies against the specific epitope, the ELISA could fail to detect this animal as positive, even though it showed some antibody reactivity below the cut-off level. The NSP-ELISA-ID is an indirect ELISA and uses an anti-ruminant IgG-HRP conjugate. It may well be that this conjugate does not work as well with deer antibodies as it does with cattle or sheep IgGs.

Again, we were able to identify an ELISA, the NSP-ELISA-PR, which can be used for FMD preparedness in laboratories where the in-house ELISA is not readily available. As in other animal species, the antibody response to NSPs appeared later than the antibody response to SPs (Ryan et al., 2008).

The NSP antibody LFD test was comparable to the NSP-ELISA-PR in terms of initial detection of serum antibodies to FMDV NSP and duration of antibody detection. Considering that results can be acquired by this penside test within minutes of obtaining samples, this test might be ideal for rapid detection of antibodies in FMDV-infected red deer. However, the sample size is too small to allow for any firm conclusions.

In summary, the main aims of this study have been achieved, such as providing sample material that could be used for comparing FMD test method performance in red deer, and in determining which readily available test methods could be used and should be maintained at laboratories for FMD preparedness. The two rRT-PCR methods detected animals harbouring the virus comparably to virus isolation. The SPO-ELISA-PR and the NSP-ELISA-PR performed comparably well to their in-house ELISA equivalents and to the VNT. The NSP antibody detection LFD showed potential for easy and early detection of FMDV infection in the field using blood or serum. Finally, red deer seem to be relatively resistant to FMDV infection.

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References


