



Diagnostic comparison of serum and EDTA-stabilized blood samples for the detection of foot-and-mouth disease virus RNA by RT-qPCR



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ABSTRACT

Foot-and-mouth disease (FMD) remains a globally important disease but there have only been occasional recent outbreaks in Europe, e.g. in the U.K. in 2001, U.K. 2007 and Bulgaria 2010/2011. However, this infection still poses a threat to Europe as the disease occurs close to its borders and incursions can occur through importation of contaminated animal products and through the air. To deal with a suspected outbreak, fast sampling, transportation and accurate laboratory diagnosis are critical; testing for FMDV is normally performed on epithelium samples or serum. Assessment of the use of stabilized blood in assays for FMDV RNA is useful as this sample material can be prepared on site for safe transportation and rapid analysis at the laboratory. Such samples are also collected for diagnosis of other diseases giving similar clinical signs. Testing serum and EDTA-stabilized blood samples from FMDV-infected cattle and pigs, using real time quantitative RT-PCR assays, yielded similar results. However, detection of FMDV RNA was less sensitive (about 10-fold) when using EDTA-stabilized blood compared to serum. Thus, diagnosis of FMD can be achieved using EDTA-stabilized blood samples in an outbreak situation on a herd basis, but serum is preferred at the single animal level for optimal sensitivity.

1. Introduction

Foot-and-mouth disease (FMD) is one of the most contagious diseases seen in domestic and wild cloven-hoofed animals. The causative agent of FMD is foot-and-mouth disease virus (FMDV), a small RNA virus within the *Aphthovirus* genus of the family *Picornaviridae*. FMDV particles comprise a single-stranded positive-sense RNA genome enclosed within a near spherical protein capsid including 60 copies of 4 different proteins, VP1-VP4 (Belsham, 2005). A total of 7 distinct serotypes of FMDV have been found around the world, these are: O, A, C, Asia1 and Southern African Territories (SAT) 1, SAT 2, SAT 3. Serotype O FMDV is the most widely distributed (responsible for about 70% of outbreaks globally) whereas serotype C virus has not been observed anywhere since 2004 (Jamal and Belsham, 2013).

The virus can be transmitted by direct or indirect contact between animals, by aerosol spread and in contaminated food waste (Alexandersen and Mowat, 2005). The main virus entry site in the host is the pharyngeal region but infection can also occur through abrasions of the skin or mucous membranes (Alexandersen et al., 2001; Arzt et al., 2010; Murphy et al., 2010). After virus introduction, the infection follows a rapid time course and infected animals develop clinical signs demonstrated by fever and slight to moderate depression, quickly

followed by the appearance of vesicular lesions in and around the oral cavity and on the feet. Excessive salivation in cattle and lameness in pigs are also clinical signs consistent with FMD. In contrast, small ruminants, i.e. sheep and goats, are often less clinically affected. The viremic period usually lasts for up to 4–5 days, and is co-incident with the appearance of clinical signs. Circulation of the virus in the blood gives rise to secondary amplification of the virus in the keratinized stratified squamous epithelia of the skin and mouth and also in the myocardium of young animals (Alexandersen and Mowat, 2005). The latter event may lead to myocarditis with a fatal outcome in young ruminants and pigs. Following viremia, the appearance of circulating antibodies from 4 to 7 days post-infection is accompanied by a rapid reduction in the level of virus in the blood. The infection then declines and healing of the lesions will begin. After this acute infection phase, many infected cattle (up to 50%, Alexandersen et al., 2003), will carry low levels of infectious virus in the oropharynx for a prolonged period of time without any clinical symptoms. This is called the ‘carrier-state’ and can be maintained for months or even years (this does not occur in pigs). However, it is unusual, for these ‘carrier’ animals to transmit the virus to other animals (Tenzin et al., 2008) but direct transfer of oropharyngeal fluid from carrier cattle to naïve animals can efficiently transmit the infection (Arzt et al., 2018), thus this oropharyngeal fluid

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does constitute a risk.

As outbreaks of FMD immediately result in a ban on international trade of livestock animal products, a positive laboratory diagnosis of FMD has important socio-economic consequences for the affected country. It is, therefore, of the highest importance to diagnose this infection as fast as possible in order to prevent further virus spread and to get the disease under control.

The outbreak of FMD in the U.K. in 2001 affected about 2000 premises and resulted in economic losses of around US\$ 8 billion including direct and indirect costs (Knight-Jones and Rushton, 2013). In Denmark, Boklund et al. (2013) have estimated, using modelling, that a “typical” outbreak of FMD would cost the Danish economy between € 410–665 million. If an outbreak should occur in an area with a high density of susceptible livestock or if the diagnosis is delayed, then the economic impact could be even higher. Furthermore, the present recommendations for controlling FMD outbreaks, using stamping-out procedures, inevitably give rise to major public ethical concerns.

For these reasons, in European countries, clinical signs indicative of FMD, immediately lead to collection of blood (to obtain serum) and relevant tissue samples for laboratory analysis. Currently, the first line system for the detection of FMDV from the sample materials is real time quantitative RT-PCR (RT-qPCR) as described previously (Reid et al., 2003; Callahan et al., 2002). These assays are serotype independent since they target highly conserved sequences within the FMDV genome. The primary aim of this study was to investigate the diagnostic utility of EDTA-stabilized blood samples for the detection of FMDV RNA using this assay system. Stabilized blood may be a relevant alternative to serum because this material can be prepared, without centrifugation, and mixed with lysis buffer on the farm when the blood samples are collected. This method has been used for Ebola virus for safety reasons in a diagnostic context, when ready-to-use inactivation collection tubes were prepared (Rosenstjerne et al., 2016). A similar system could prove to be relevant for easy pen-side collection of low-risk blood samples for FMDV testing. The lysis buffer chosen should essentially inactivate the virus (since the capsid structure will be disrupted by chaotropic lysis buffers) and the sample will contain free viral RNA. This will reduce the risk of virus transmission during transportation from farm to diagnostic laboratory and upon arrival may speed up the laboratory procedures for sample examination. In the event of an outbreak, use of such pre-treated samples may facilitate analysis of large numbers of samples. Furthermore, EDTA-stabilized blood is collected for the diagnosis of other animal diseases, e.g. bluetongue, for which FMD could be a potential differential diagnosis.

2. Materials and methods

2.1. Animal experiments and blood samples

This study is based mainly on samples collected from 2 animal experiments, performed in cattle and in pigs, respectively. An initial pilot study was based on samples from a third study. All three animal experiments were conducted in accordance with EU legislation on animal experimentation (EU Directive 2010/63/EU) and received ethical approval from the Danish Animal Experiments Inspectorate (License 2012-15-2934-00182). The work was performed in the experimental stables and laboratories under high containment at DTU-Vet, Lindholm.

The pilot study was conducted for comparison of the sensitivity of both EDTA- and heparin-stabilized blood samples versus serum for the detection of FMDV by RT-qPCR (see below). Blood samples collected from 4 Jersey bull calves (2-6-months old) undergoing a vaccination study at the Institute were used. The animals were challenged with a serotype O virus strain (FMDV O/UKG 34/2001).

The main cattle experiment included samples from 13 Jersey bull calves (2-6-months old). The main aim of the experiment was to evaluate a recombinant FMDV vaccine candidate and this has been described in detail elsewhere (experiment 2 within Gullberg et al., 2016).

Five of the calves (id nos. C1, C2, C5, C6, C7) were challenged by sub-epithelial inoculation into the tongue with a serotype O virus strain (FMDV O/UKG 34/2001) on post infection day 0 (PID 0), while the rest of the animals (id nos. C3, C4, C8, C9, C10, C11, C12, C13) remained as contacts. The animals were observed, on a daily basis, for the appearance of clinical signs and rectal temperatures were recorded. The experiment was terminated 14 days post challenge. Unstabilized and EDTA-stabilized blood samples collected at pre-selected days throughout the entire infection period were analysed.

The pig experiment included 9 Landrace crossbreed pigs (7-weeks old). These animals were a part of a study focusing on a comparison of the pathogenicity of modified FMDVs (essentially as described in Lohse et al., 2012). Four pigs (id nos. 17, 19, 27 and 28) were challenged by sub-epidermal inoculation into the heel bulb with a serotype A virus (FMDV A/TUR/2/2006) on PID 0. The rest of the pigs (id nos. 16, 18, 20, 23, and 25) remained as un-inoculated contacts. The animals were observed, on a daily basis for clinical signs and rectal temperatures were recorded. Five of the pigs (id nos. 17, 18, 19, 27 and 28) were euthanized on PID 9. Another 3 pigs were euthanized on PID 11 (id nos. 16, 20, 25) and the remaining pig (id no. 23) was euthanized when the experiment was terminated on PID 15 (note: this pig was housed with other pigs included in the original experimental study and therefore was not alone after PID 11). Unstabilized and EDTA-stabilized blood samples were collected, on pre-selected days, from the 9 pigs throughout the experimental period.

2.2. Sample management

Unstabilized and stabilized blood samples were collected from the jugular vein from each animal on the following days:

Pilot study (in cattle): PID 0 (before virus challenge) and also on PID 1, 2, 3, 4, 6, 7 and 9.

Main cattle experiment: PID 0 (before virus challenge) and also on PID 1, 2, 3, 4, 6, 7, 9, 11 and 14.

Pig experiment: PID 0 (before virus challenge) and also on PID 1, 2, 3, 4, 6, 7, 9, 11 and 15 (when possible).

Venosafe[®] plastic tubes for serum (clot activator), EDTA-stabilized blood (8.4 mg EDTA-K3) and with heparin (135 USP. U. sodium heparin) were used for the blood collection (Terumo).

Transportation of blood samples, from the stables to the laboratory, took approximately 15 min due to the disinfection steps required for biosafety.

On arrival at the laboratory, the unstabilized blood samples were stored at 4 °C until the next day when they were centrifuged at 1775 × g for 8 min at 5 °C and the serum fraction was transferred into 2 ml microtubes and stored at –80 °C until further use. EDTA- and heparin-stabilized blood samples were prepared on the same day as sampling; 100 µl whole blood was added to 300 µl MagNA Pure lysis buffer from the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche), in 2 ml microtubes, and incubated for 10 min (to achieve lysis of blood cells, denaturation of proteins and disruption of the virus). The samples were then stored at –80 °C until further use.

2.3. Detection of FMDV RNA in serum and blood samples using quantitative real-time RT-PCR assays

The presence of FMDV RNA in serum and in lysed EDTA- and heparin-stabilized blood samples was determined using RT-qPCR assays (essentially as described by Reid et al., 2003); this assay is targeted to the 5'-untranslated region of the viral RNA. For RNA extraction, 200 µl of each serum sample was mixed with 300 µl MagNA Pure lysis buffer while the lysed EDTA- and heparin-stabilized blood samples (as described above) were added to 100 µl phosphate buffered saline (PBS) (Gibco[®] Life Technologies) to make a total volume of 500 µl, in each case, for use in the MagNA Pure LC extraction robot. Total RNA was isolated from these samples according to the manufacturer's

instructions using this automated RNA purification system and eluted in 50 μ l Elution Buffer. An aliquot of each RNA (6 μ l) was used to prepare cDNA in a total volume of 15 μ l, using a Taq-Man RT mastermix kit with random hexamer primers (Applied Biosystems); samples were incubated at 48 °C for 45 min and then 95 °C for 5 min. A portion of the cDNA (7 μ l) was mixed with 18 μ l of 2 \times TaqMan universal PCR mastermix (Applied Biosystems) containing 22.5 pmol of each primer and 7.5 pmol of FAM-labeled probe. PCR amplification was performed in a Thermal cycler (Mx3005P, Stratagene); reaction procedure: 50 °C for 2 min, 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min. The data were analysed using MxPro qPCR software (Stratagene), samples giving Ct values < 40 were considered positive.

3. Results

3.1. Pilot study

Serum, EDTA-stabilized blood and heparinized blood samples from 4 FMDV-infected cattle were analysed in parallel. In total, FMDV RNA was detected in 14/22 serum samples, 13/22 EDTA-stabilized blood samples, and in 10/22 samples of heparinized blood. The profile of detection of positive samples for the three sample materials was very similar in each case (see Fig. 1). However, when compared to the serum samples (which showed the highest levels of RNA), the heparinized blood showed RNA levels which were detected, typically, with Ct values about 10 higher, i.e. the use of this material was about 1000 \times less sensitive. The EDTA-blood samples appeared intermediate in sensitivity for FMDV RNA detection in this system; the EDTA-treated blood samples yielded a slightly lower level of FMDV RNA than the serum samples from each of the 4 cattle analysed (Fig. 1). On this basis, the remaining study was focused on comparison of serum and EDTA-stabilized blood.

3.2. Progression of clinical infection and viremia

Cattle experiment: all animals (5 inoculated and 8 contacts), developed clinical signs indicative of FMD.

Pig experiment: all 9 pigs (4 inoculated and 5 contacts) developed clinical signs indicative of FMD.

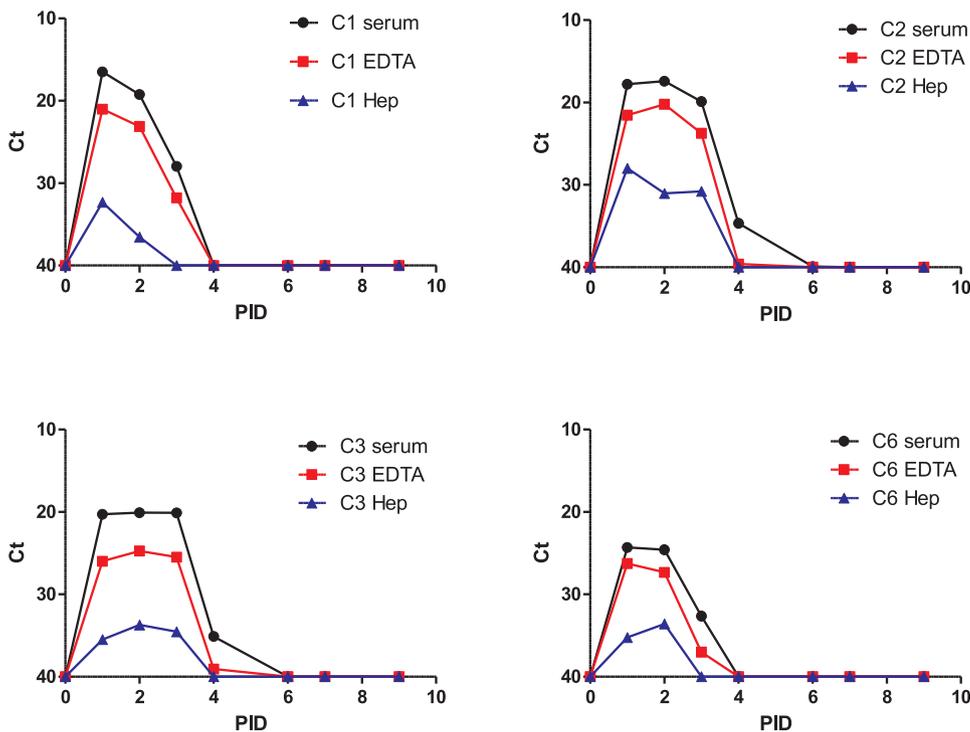


Fig. 1. Pilot study for the detection of FMDV RNA in cattle serum and stabilized blood samples during acute phase of infection. FMDV RNA was detected, by RT-qPCR, in serum, EDTA- and heparin-stabilized blood samples collected from 4 calves (id nos. C1, C2, C3, C6) on the indicated days. Black line = serum, red line = EDTA-stabilized blood, blue line = Heparin-stabilized blood. The four cattle were inoculated, into the tongue, with FMDV O/UKG 34/2001 on PID 0.

3.3. Detection of FMDV RNA in serum and EDTA-stabilized blood samples

The presence of FMDV RNA was determined using RT-qPCR (as above) in serum and EDTA-stabilized blood samples collected throughout the time course of the acute infection. The results are presented in Fig. 2 (for cattle samples) and Fig. 3 (for pig samples).

Samples from each calf were found to be FMDV RNA positive at some stage during the course of the experiment (Fig. 2) in both the serum and the EDTA-treated blood samples. The samples from the five inoculated calves tested positive for FMDV RNA in serum, as well as in EDTA-stabilized blood, as early as the first day after challenge (PID 1). The contact animals became FMDV RNA positive some 2–3 days later (PID 3–PID 4). From among all the animals, 3 calves (ID nos. C8, C9 and C10) first scored positive for FMDV RNA one day earlier, when assessed using the serum samples than could be observed using the EDTA-stabilized blood samples. Note, for the calves C8 and C9 earlier detection of FMDV RNA in serum is clear from Fig. 2 while for C10 only a very low positive reading (Ct = 39.85) was observed on PID4 which is not apparent from the graph. The other 10 animals first tested positive in both blood sample preparations on the same day. In general, all calves had 2–4 days of viremia. After the acute infection period, FMDV RNA disappeared from the blood and concurrently, clinical lesions started healing. Nine animals became FMDV RNA negative in both serum and EDTA-stabilized blood samples on the same day, while the remaining 4 animals (ID nos. C2, C5, C6 and C7) showed one day's difference (see Fig. 2); the EDTA-stabilized blood samples scored negative one day before the corresponding serum samples (in each case the serum samples yielded Ct values > 35 on the last day, thus the level of FMDV RNA was very low).

The time course of FMDV RNA detection in each of the calves is shown in Fig. 2 and represents the entire infection period in the experiment. It is clear that the detection of FMDV RNA from the two types of blood sample followed the same pattern. However, the EDTA-stabilized blood samples generally produced slightly higher Ct-values (mean difference 2.8 Ct-values), indicating a lower concentration of FMDV RNA in this sample type which, thereby, produced an overall lower sensitivity in the RT-qPCR assays. In total, FMDV RNA was detected in 42 serum samples from the 13 infected cattle while positive results were

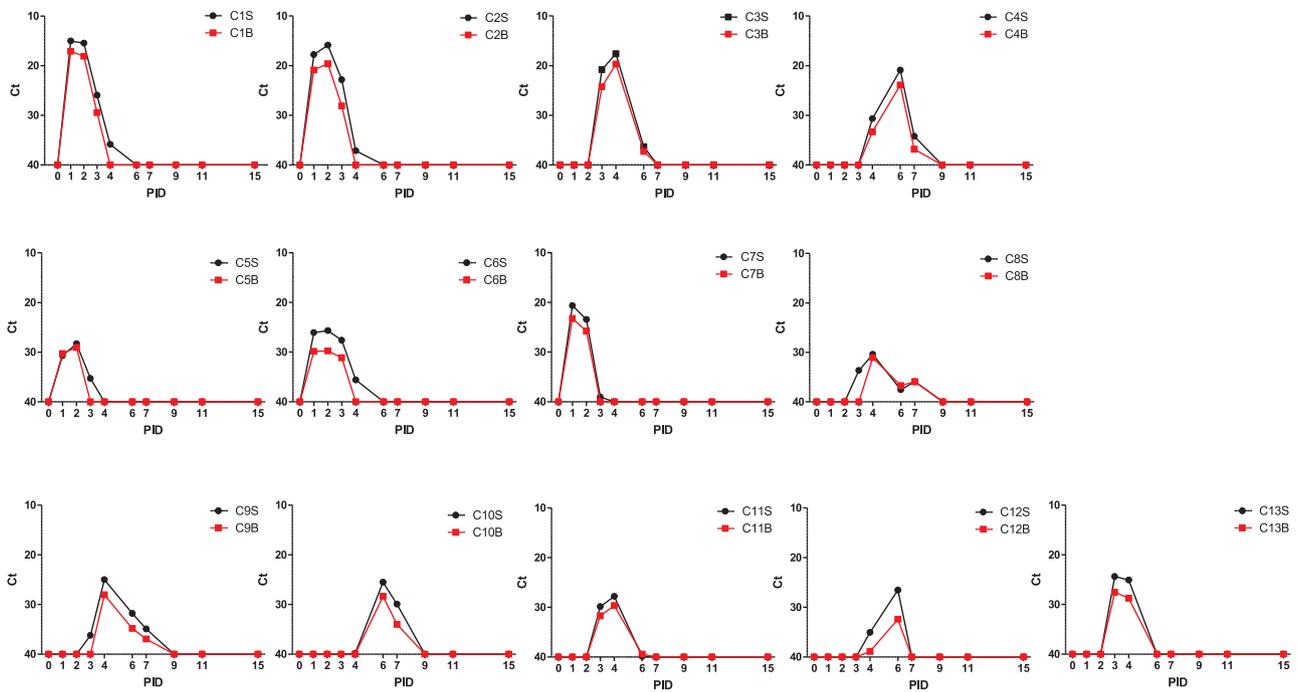


Fig. 2. Detection of FMDV RNA in serum and EDTA-stabilized blood samples from cattle. Five calves (id nos. C1, C2, C5, C6, C7) were inoculated into the tongue with a serotype O virus strain (FMDV O/UKG 34/2001) on PID 0. The other 8 calves (id nos. C3, C4, C8, C9, C10, C11, C12, C13) remained as contacts. All 13 cattle developed clinical disease. Serum samples and EDTA-stabilized blood samples were collected on the indicated days and the presence of FMDV RNA in these samples was determined by RT-qPCR. Black line = serum, red line = EDTA-stabilized blood.

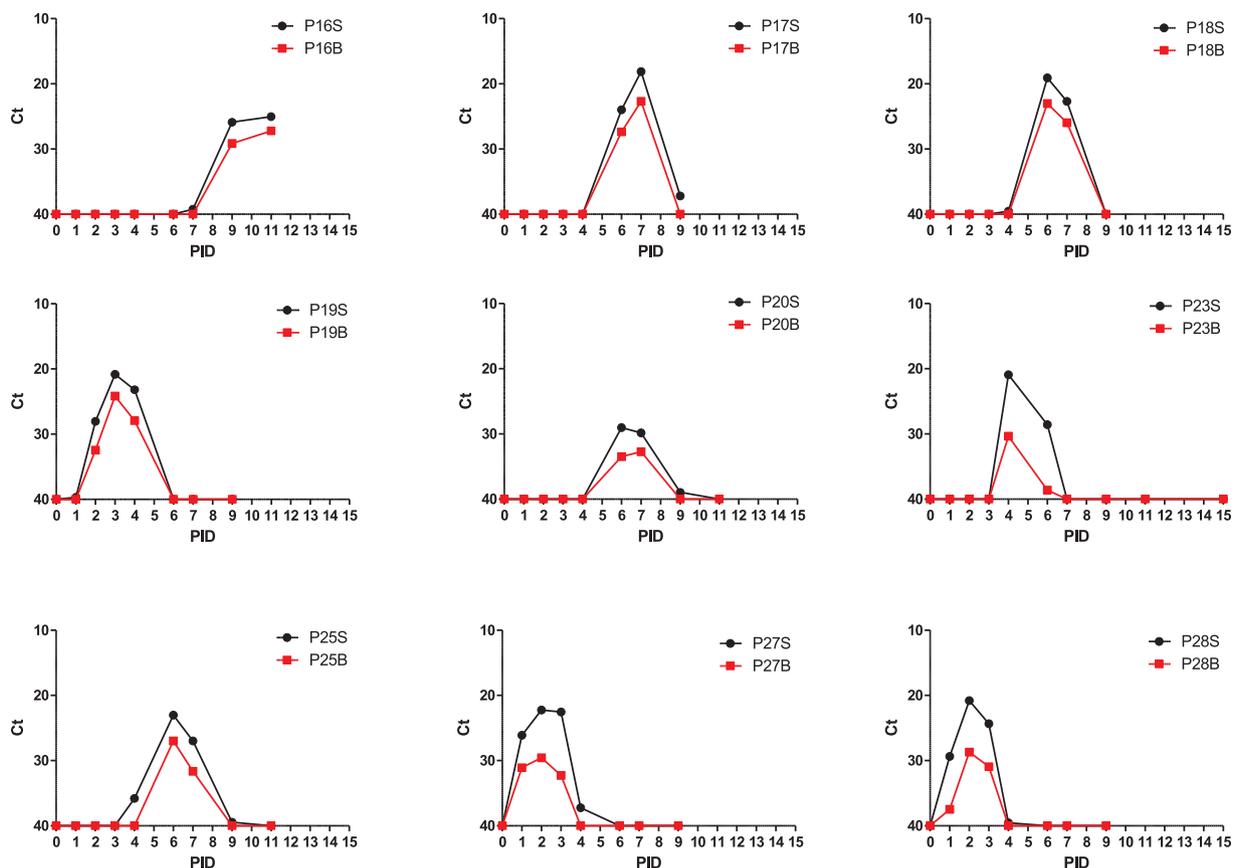


Fig. 3. Detection of FMDV RNA in serum and EDTA-stabilized blood samples from pigs. Four pigs (id nos. 17, 19, 27 and 28) were inoculated into the heel bulb with a serotype A virus (FMDV A/TUR/2/2006) on PID 0. The other 5 pigs (id nos. 16, 18, 20, 23, and 25) remained as un-inoculated contacts. All 9 pigs developed clinical disease. Serum samples and EDTA-stabilized blood samples were collected on the indicated days and the presence of FMDV RNA in these samples was determined by RT-qPCR. Black line = serum, red line = EDTA-stabilized blood. Note some of the pigs were euthanized during the course of the infection for animal welfare reasons and thus samples could not be collected for the full 15 days.

only observed in 35 of the corresponding EDTA-stabilized blood samples; however, it was only the samples containing very low levels of viral RNA (Ct > 33) that gave a different result. Such samples were also responsible for the stated differences in duration of viremia (see above).

All 9 pigs were found to become FMDV RNA positive in both blood sample types (defined as a Ct-value < 40) during the course of the experiment. Three of the 4 inoculated pigs (id nos. 19, 27, 28) were FMDV RNA-positive on the day after inoculation (PID 1) in serum (albeit pig 19 had a very low positive reading (Ct = 39.72) at this time) and two of these (pigs 27 and 28) were also scored positive on this day using the EDTA-stabilized blood samples. The third pig (pig 19) was scored positive in the stabilized blood sample from PID 2. The last inoculated pig (pig 17) remained negative for several days and followed the infection time course profile of the contact challenged pigs. The contact pigs (and pig 17) became FMDV RNA-positive 3–6 days later than most of the inoculated animals (that is on PID 4–PID 7). Three pigs (pig id nos. 17, 20, 23) became FMDV RNA-positive in both serum and EDTA-stabilized blood samples on the same day, while the remaining 3 pigs (pig id nos. 16, 18, 25) demonstrated FMDV RNA-positivity in serum before EDTA-stabilized blood samples (although only at low levels, as above). Some of the pigs were euthanized, for animal welfare reasons, before the end of the experiment so the entire infection period is not shown for all animals. It was apparent that, 5 out of 9 (56%) pigs had FMDV RNA-positive serum for longer than the corresponding EDTA-stabilized blood samples. As in the cattle experiment, both sample preparations produced the same time course profile during the infection, thus the EDTA-stabilized blood samples again showed lower test sensitivity. Of 30 pig serum samples that were scored positive, the corresponding EDTA-stabilized blood samples were also scored positive in 21 cases and the only discrepancies occurred when the level of FMDV RNA detected in serum was very low (Ct > 35).

4. Discussion

The aim of this study was to investigate the utility of stabilized blood for the detection of FMDV RNA by real-time RT-PCR. The performance of this diagnostic material was compared to corresponding results for serum samples. Material from cattle and pigs, livestock species that are highly susceptible to FMDV, was selected for the analysis.

A pilot study included both EDTA-stabilized blood and heparinized blood for comparison with serum samples, however, heparinized blood samples gave a much lower diagnostic sensitivity and were therefore excluded from the main study. Similar issues with low sensitivity using heparinized blood samples have been observed previously for other viruses (see Ding et al., 2011).

Analysis of the data from both the pilot study and the main study showed that FMDV RNA could be efficiently detected in both serum and EDTA-stabilized blood, however, for the EDTA-stabilized blood samples the detection occurred with a slightly lower sensitivity. This is clearly demonstrated by the graphical presentation of the Ct-values for the two sample types in Figs. 1–3. In some cases, the lower sensitivity with the EDTA-stabilized samples influenced the length of the diagnostic window for detection of viremia in this material. Looking at the course of infection, it was evident that serum samples provided a longer time window for detection of FMDV RNA as 54% and 56% of the cattle and pigs, respectively, had fewer FMDV RNA-positive EDTA-stabilized blood samples than serum sample positives. Furthermore, on sample days with FMDV RNA-positivity in both materials, the Ct-values were higher in the EDTA-stabilized blood samples with a mean difference of 2.8 ± 1.5 Cts and 4.2 ± 2.8 Cts, for cattle and pig sera, respectively.

Thus, the sensitivity of FMDV RNA detection using EDTA-stabilized blood samples may be better for the calf samples than for the pig samples. However, as the calves and the pigs were challenged with

different strains of FMDV (serotype O and A, respectively), this could also influence the results in the individual experiments.

The laboratory handling of the different sample materials was not identical. The unstabilized blood samples had to be centrifuged, and the serum separated, prior to RNA extraction while the EDTA-stabilized blood samples were immediately put into lysis buffer and then stored until extraction. Both samples types were handled promptly after collection. For extraction of RNA, different volumes of sample material were used. Serum samples of 200 µl were used (as in accredited tests at the DTU Vet's national reference laboratory) while only 100 µl of EDTA-stabilized blood was used. This can account for a 1 Ct value difference in performance of the assays but does not explain the entire discrepancy (ca. 3–4 Cts). The smaller volume of EDTA-stabilized blood was used in order to avoid clotting, as described by Reid et al. (2009). In the present study, clotting of the EDTA-blood in the presence of the MagNA Pure lysis buffer suspension was not apparent.

The results presented in Fig. 1 (pilot study) indicated that the use of heparinized blood was much less satisfactory than using serum or EDTA-stabilized blood. However, different kits should be evaluated for FMDV RNA extraction from both EDTA-stabilized blood and heparin-treated blood since such samples have been successfully used for the detection of other viruses (see Ding et al., 2011). Before final kit selection, the relevant lysis buffer should also be tested for FMDV inactivation competence.

Diagnosis of FMD using EDTA-stabilized blood samples can be reliably performed on a large sample size from an outbreak setting. During an acute outbreak, infected animals, at different stages of infection, are expected to be present and one positive sample is sufficient to classify the farm as infected. From “in contact” herds, several blood samples are usually collected, thus increasing the overall sensitivity for a batch of samples provided from a given herd. The EDTA-stabilized blood samples can be mixed with lysis buffer on the farm and the samples can leave the premises classified as low risk samples. Although, logistically, on farm inactivation may not be ideal, it could be a preferred option, during an outbreak, when space for storage and handling of samples in a high containment laboratory is tightly constrained. This treatment will also allow simpler and more cost efficient transportation of the samples to the diagnostic laboratory for processing; the pre-treatment can save time as well since the samples will be ready for RNA extraction and further analysis on arrival. Also, in an outbreak situation, inactivated samples could be handled at a lower biocontainment level, thus increasing the laboratory capacity for handling and analysing numerous field samples.

With the present system, it cannot be advised to use EDTA-stabilized blood for individual animal monitoring. This material will not be the sample of choice for a suspect case in a FMDV-free region, however, if EDTA-stabilized blood samples have been collected for other diagnostic assays (e.g. for bluetongue virus) then it is apparent that such samples can give a clear positive result for the presence of FMDV.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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