SAMPLING, SHIPPING, ORGANISATION AND TESTING OF INACTIVATED SAMPLES TO TAKE ADVANTAGE OF MOLECULAR ANALYSIS METHODS

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ABSTRACT

Introduction
FMD is endemic in many countries and causes significant economic losses in livestock farming despite efforts directed towards control by vaccination. Efficient vaccination for protection against FMD in such settings is often hampered by a limited access to or excessive costs of high quality vaccines and/or the lack of a well planned vaccination strategy based on thorough epidemiological information. Limited access to or excessive costs of high quality vaccines is a matter of economic resources and may be solved by sufficient support from International Organisations. However, in such settings the methodological tools for generating thorough epidemiological information and sufficient and timely characterisation of circulating and epidemiological important strains of FMDV required for setting up a well planned vaccination strategy need a leap forward in regard to the ways epidemiological studies and strain characterisation are organised and performed. Previous experimental studies performed by us and others have looked at new sampling strategies for relatively easy estimation of e.g. prevalence of infected animals by using real-time RT-PCR analysis of swab samples collected directly into a suitable lysis buffer. The next step was to look at this approach under field conditions by using sampling of mouth swabs directly into a suitable lysis buffer for easy shipping and downstream analyses as described here.

Materials and methods
For this study we selected the Landhi Dairy/Cattle Colony (LCC) outside Karachi, the largest dairy colony in Pakistan and the biggest buffalo colony in the world. Due to its size and fragmented vaccination coverage, together with the Pakistani husbandry tradition of frequent transportation of animals to and from the large dairy colonies, FMDV infection is prevalent in LCC and therefore, LCC provided a suitable study population. Moreover, the information generated would also provide epidemiological information that could potentially improve the control of FMD in the colony and thus be of benefit to the many farmers enduring significant economic losses. From April 2006 to April 2007 we collected mouth swab samples of randomly selected, non-clinically affected herds as one group (group A), from non-clinically affected animals in herds with clinical evidence of a prior FMD outbreak (group B, i.e. herds with a few animals with evidence of old and healing lesions consistent with acute FMD for more than 2-3 weeks prior to sampling) and from non-clinically affected animals in a few herds in which acute FMD was obvious in at least one animal (group C). Swabs were immediately placed in Qiagen RLT buffer, which immediately stabilised the viral RNA and rendered the sample non-infectious. In addition, epithelial samples were collected from clinical FMD cases from group C. The results from analysis of these epithelial samples are described in a separate paper together with the detailed sequence and phylogeny studies of selected positive mouth swab samples from all 3 groups. The mouth swab sampling scheme included four major samplings together with smaller monthly samplings. Each sampling was accompanied by a questionnaire to gather general health status and vaccination information and the exact location of the herd was determined by GPS. The laboratory-based analysis employing real-time RT-PCR is described here.
While VP1, capsid and full genome sequences derived from swabs and epithelial samples, together with the questionnaire-based information, are described in an accompanying paper.

Results

Sampling of mouth swabs directly into lysis buffer followed by shipment of the samples either directly to Denmark for real-time RT-PCR analysis at Lindholm, or alternatively via the National Reference Laboratory in Islamabad for RNA extraction followed by cDNA preparation and shipping of prepared cDNAs to Denmark, proved to be a reliable method for detecting FMDV RNA. Sampling in this way preserved the FMDV RNA and such samples contain no infectious FMDV and can therefore relatively easy be shipped as diagnostic samples and clearly provided valuable information on prevalence of FMDV infection in LCC over the one-year study period. Moreover, sequencing of such samples were possible provided that they contained a reasonable amount of FMDV RNA (sequencing results to be described in the accompanying paper) and in addition, preliminary in vitro results (Graham Belsham, personal communication) indicate that it may be possible to re-generate infectious FMDV from such purified RNA samples by transfection into suitable cell cultures.

Discussion

The approach described here has in our opinion clearly shown merit. Stabilised and inactivated swab samples can easily be collected and shipped internationally and may be used to determine the temporal-spatial distribution and prevalence of FMDV infection and further analysis of selected positive samples by sequencing can be used to characterise circulating strains of FMDV. Thus, the described method is a viable and valuable alternative to shipment of epithelial samples, containing infectious virus, for strain characterisation.

1. INTRODUCTION

Pakistan has a number of large dairy buffalo and cattle colonies, FMD is endemic there and it has borders with India, Afghanistan, Iran and China where FMD is also a continued problem. Only fragmented information exists on the prevalence and extent of circulation of FMDV, including characterisation of current strains, in Pakistan. Improvement of this situation requires intensified surveillance and facilities and expertise in molecular epidemiology and may initially best be achieved by international collaboration. The project described here is such a collaboration between experts and staff in Islamabad and Karachi in Pakistan and at Lindholm in Denmark in close coordination with the FAO and the WRL for FMD in Pirbright, UK.

The traditional diet in Pakistan includes buffalo milk or buffalo milk-based products and large dairy colonies are located around the major cities like e.g. Karachi to provide a continued supply of fresh non-pasteurised milk. Buffalo milk is preferred over cattle milk due to the high fat content and many of the large dairy colonies have 90-95% buffalo and only 5-10% cattle. The Landhi Dairy/Cattle Colony (LCC) is located in the eastern suburbs of Karachi, was established in 1959 and is now the largest dairy colony in Pakistan and the biggest buffalo colony worldwide. There is estimated to be a total of around 250-280 000 buffalo and 15-25 000 cattle in the colony. The number of individual farms is not known with certainty, but it has been estimated that there is between 1000-2000 farms each with around 100-200 animals on average. All of this is located on only 750 acres of land (including 250 acres for primitive roads). The farmers mainly purchase animals from the three major breeding regions in Punjab and in upper and lower Sindh and animals are primarily kept for one lactation period. After the lactation period, a few of the buffalos/cattle are used for re-breeding by the owner, some are slaughtered, and an estimated 50-75% of them go back to the regions mentioned above to be used for breeding once more. Such animals will, when they are ready to enter a new lactation period, again be transported across the country and sold to individual farmers. Many goats, sheep, cattle and buffalo from all over Pakistan are once a year transported to large markets just outside the big cities in connection with the celebration of the major Muslim festival Eid-ul-Azha. Animals that are not sold for sacrifice may, after having been mixed with many other animals from different places, then go back to where they came from or perhaps be sold and go somewhere else.

The majority of the commercial farmers in LCC vaccinate their animals, either with a good quality trivalent vaccine (O, A, Asia1), but only given once due to the high cost of the vaccine, or with a monovalent type O vaccine produced in Pakistan which is of unknown quality and efficiency and apparently only contains antigen from a single, very old type O strain. This lack of an efficient and coordinated vaccination policy, together with the large number of animals kept in a relatively small area and the frequent transportation of animals in and out of the colony as well as the general lack of biosecurity procedures and awareness among LCC farmers, creates conditions that favour
continued FMDV circulation. Therefore, the LCC provides a suitable study population for establishing the methods needed for direct inactivation, stabilisation and shipment of field samples containing FMDV RNA and such studies may in turn provide useful epidemiological information to be used to improve FMD control in LCC.

In order to potentially set up a future vaccination strategy that is efficient in protecting the LCC against currently circulating strains of FMDV, it is necessary to get data on the seasonal occurrence and prevalence of FMDV in the colony and to characterise the circulating strains. This may be achieved by a structured sampling strategy based on mouth swab sampling in connection with the relatively easy shipment of inactivated and stabilised samples to a laboratory with the capacity to perform large-scale RT-PCR testing to detect samples positive for FMDV which can then subsequently be further analysed by sequencing as described below and in an accompanying paper.

2. MATERIALS AND METHODS

2.1 Study Design and Sample Collection, Organisation and Shipping.

The study was designed as a longitudinal and cross-sectional survey to provide information on clinical and in particular subclinical FMDV infection prevalence in LCC. We collected samples during 4 trips to Pakistan from April 2006 to April 2007 and local Veterinary Officers collected in addition a smaller number of samples each month during the same period. The monthly smaller samplings included 5 farms selected at random and on each farm 6 animals (5 buffalo and 1 cow if possible) were selected at random, clinically examined and a mouth swab taken by careful swabbing of the tongue with a sterile cotton swab (Libby Sterilin). During our 4 visits, the number of randomly selected farms was increased to 18 and the number of randomly selected animals on each herd increased to 9 buffalo as well as 1 cow if possible. When visiting the farms, the location was logged by handheld GPS equipment and the farmers thoroughly questioned for knowledge that may be of relevance for the analysis, e.g. clinical signs, vaccination status, vaccine used, age of the animal, outbreak history and any other relevant information. The randomly selected animals were checked clinically while taking samples. Farms without any clinical signs of current or relatively recent acute FMD were assigned to study group A (randomly selected, non-clinically affected animals from non-affected herds). Animals from farms with a history and signs of recent clinical FMD were assigned to study group B (samples were taken from non-clinically affected animals in herds where the presence of a few other animals with evidence of old and healing lesions indicated that acute FMD had been present in the herd for more than 2-3 weeks before sampling) and farms with animals with current signs of acute FMD found during the initial examination were assigned to study group C (samples were taken from non-clinically affected animals in herds in which acute FMD was obvious in at least one other animal). The animals in the herds of group B and C with clinical signs of earlier or current FMD were examined in more detail, in particular concerning animals in group C in order to locate suitable vesicular lesion epithelia (lesion epithelia samples were in addition also collected during a pre-study visit in January/February of 2006; the results of the testing of collected epithelial samples are described in an accompanying paper). Mouth swabs were immediately placed, after breaking and removing the plastic handle of the swab, into 1.0 ml of RLT lysis buffer (Qiagen) in 2 ml screw-cap polypropylene tubes (Sarstedt). Such swabs in lysis buffer were often kept at ambient temperature for many hours before being stored at -20°C and then shipped either directly to Denmark, or alternatively via the National Reference Laboratory in Islamabad for RNA extraction followed by cDNA preparation and shipping of prepared cDNAs to Denmark. Swabs in lysis buffer and prepared cDNAs as described here contain no infectious FMDV (only stabilised RNA or cDNA) and can relatively easily be shipped as diagnostic samples both locally and internationally.

2.2 RNA Extraction, cDNA Preparation and Quantitative RT-PCR

Total RNA was extracted from thawed sample aliquots using the Qiagen RNA Blood Kit (Qiagen) according to the manufacturer’s instructions. The extracted RNA samples were then converted into cDNA and analysed by quantitative “real-time” RT-PCR to determine the amount of FMDV RNA present in the mouth swab samples as described in detail elsewhere, with the assumption that the swabs were initially diluted around 10-fold when placed in 1 ml of RLT lysis buffer. Sequencing. Selected samples with a relatively high content of FMDV cDNA based on the RT-PCR result were further analysed by sequencing essentially as described by us previously. The results of the sequencing are described in an accompanying paper.
3. RESULTS

3.1 FMDV RNA Detected in Swab samples.

During the 1 year study we collected 960 mouth swabs from 124 farms in group A (herds with no clinical signs of acute or recent FMD), 45 samples from 4 herds in group B (animals without clinical signs, but from herds where other animals had signs of healing/healed FMD) and 29 samples from 2 herds in group C (animals without clinical signs, but from herds where other animals had acute clinical signs of FMD) giving a total of 1034 swab samples analysed. The number of FMDV RNA positive swabs was 106 in group A (no clinical signs), 22 in group B (herds with relatively recent FMD) and 25 in group C (herds with acute signs of FMD), corresponding to 11%, 49% and 86% of the samples being positive in groups A, B and C, respectively. Of the 106 positive samples collected from subclinically infected animals/herds in group A, 58 contained sufficient FMDV RNA to facilitate subsequent sequencing. This selected group of 58 positive swabs from subclinically infected animals had a mean Ct value in the real-time RT-PCR of 36.0 (range 26 to 39) equivalent to around 10 \(^{4.6} \) (range 10 \(^{3.7-7.5} \)) FMDV RNA target copies per ml of saliva/swab while the other 48 swabs from this group, not selected for sequencing, had a mean Ct of 42.5 (range 40-48) equivalent to around 10 \(^{1.7} \) (range 10 \(^{1.0-3.4} \)) copies per ml of saliva/swab. For comparison, the positive swabs from the farms with recent signs of FMD had a mean Ct of 37.5 (range 25-46) equivalent to around 10 \(^{4.1} \) (range 10 \(^{1.6-7.8} \)) copies per ml of saliva/swab while the positive swabs collected in farms with acute FMD had a mean Ct of 32.0 (range 24-39) equivalent to around 10 \(^{5.8} \) (range 10 \(^{3.7-8.1} \)) copies per ml of saliva/swab. These copy numbers usually correspond to roughly 2 \(^{1.5} \) (range 10 \(^{0-1.5} \)), 1 \(^{1.5} \) (range 10 \(^{0-6} \)) and 10 \(^{1.5} \) (range 10 \(^{1-5.5} \)) tissue culture infectious doses (TCID50) per ml when assayed in bovine thyroid cells. For further details of seasonal prevalence of subclinical infection and sequence analysis etc please see the accompanying paper and our previously published results.

4. DISCUSSION

The data presented point out that carefully planned field epidemiological studies combined with collection of swab samples directly into a suitable lysis buffer for subsequent easy shipping, locally or internationally, to a laboratory for molecular testing by RT-PCR followed by sequencing of selected samples may provide a powerful tool for generating epidemiological information and valuable characterisation of circulating strains of FMDV. More than 10% of samples collected from herds with no signs of FMD turned out to be positive for FMDV RNA, while herds having animals with signs of an earlier outbreak of FMD (healed lesions) had approximately 50% of the samples from apparently uninfected neighbouring animals being positive and more than 80% of unaffected animals were positive in herds where other animals had signs of acute FMD. Taken together, these data indicate that the method described provides an accurate account of the numbers of animals being exposed to FMDV within a relatively short time period prior to sampling and in fact, may indicate that the positive animals are indeed infected. Data from our experimental studies indicate that mouth swabs are usually detected as positive within a time window of up to 2 weeks after exposure and are negative in carrier animals. Consequently, we consider it likely that the positive animals detected here in a field setting were exposed or infected within a 14 days window. Although FMDV RNA positive animals without clinical signs of disease may not for certain be infected, we consider the fact that more than 50% of the positive samples from farms with no clinical signs of infection contained enough RNA sufficient for sequence analysis (a level of 10 \(^{1.5} \) TCID50 per ml of saliva/swab) an indication that these animals may be subclinically infected. This notion is substantiated by the additional facts that the RT-PCR targets the 5’-untranslated region (5’-UTR) and that the sequencing PCRs involved amplification of relatively long targets, which together have been shown to be good indicators of picornavirus infectivity, and that our previous studies have indicated a good correlation between copy numbers and infectivity; i.e. further evidence for these animals indeed being subclinically infected and that FMDV likely replicated in these animals to levels facilitating detailed molecular analysis. This view is further supported by the finding that non-affected animals in farms with other animals having evidence of previous, but healed, FMD lesions had a higher percentage of swabs positive for FMDV RNA, indicating an earlier, but apparently relatively widespread, circulation of virus in this group and potentially still
subclinical infection in some animals. Also, the finding of a very high percentage of positive swabs and a higher RNA content in swabs from non-affected animals from herds with ongoing acute FMD, further substantiates that such animals may truly be subclinically infected or alternatively, be in an early stage of infection before lesions develop. Consequently, although some of the animals detected as FMDV RNA positive by our methods may only have been exposed to FMDV and not necessarily actively infected, we consider it likely that a significant proportion was indeed subclinically infected.

The use of on-site/in-the-field inactivated and stabilised swab samples as described here for easy shipping and subsequent analysis in advanced international laboratories, may also be combined with collection of epithelial samples from clinically affected animals as such epithelial samples also can be placed directly into a suitable solution, e.g. RNAlater (Ambion), which inactivates viral infectivity and stabilises the RNA provided that only small samples are collected and that the tissues are fully immersed in the RNAlater and kept at room temperature for at least 24 hours before shipping (Alexandersen, S. et al. unpublished). Finally, preliminary in vitro results (Graham Belsham, personal communication) indicate that, provided collected samples contain sufficient FMDV RNA, it may likely be possible to re-generate infectious FMDV from such RNA purified from inactivated and stabilised swabs or epithelia by using transfection into suitable cell cultures should the need arise to study the virus further for e.g. virulence or vaccine studies etc.

5. CONCLUSIONS

- Combined field and molecular epidemiological studies using randomly selected mouth swabs collected directly into a suitable lysis buffer provide a powerful tool for generating detailed epidemiological information and molecular characterisation of circulating strains.
- Direct lysis of collected swab samples into a suitable lysis buffer circumvents the need for international shipment of samples as dangerous goods and provides inactivated and stable samples that without any safety problems can be shipped as diagnostic samples.
- Analysis of high numbers of mouth swabs by a highly sensitive, quantitative real-time RT-PCR indicates that this method may provide an efficient way of establishing the extent of virus circulation during periods of low clinical activity and for providing estimates of prevalence of infection at a given point in time. Furthermore, subsequent sequencing of selected samples can be used for detailed analysis of the relationship of circulating strains with strains circulating in other countries or with known vaccine strains in order to provide appropriate vaccine coverage. Extracted RNA may possibly be used for making infectious FMDV by using transfection into suitable cell cultures; however, this aspect needs further study.

6. RECOMMENDATIONS

- Collaborative studies using inactivated and stabilised samples and involving the relevant authorities and laboratories of countries with endemic FMD, together with International Organisations such as FAO, the WRL for FMD and a National Reference Laboratory from a resource-rich country may provide an efficient avenue for strengthening FMD control programs.
- Further studies should be supported to further establish the suitability of the proposed methods, to provide more epidemiological data and knowledge of circulating strains of FMDV in various settings and to establish the potential for using such samples to re-generate infectious FMDV by optimising the methods used for lysis and stabilisation, RNA extraction and in particular the methods for maximising the efficiency of transfecting susceptible cells.

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8. REFERENCES


