Analysis of the epidemiological dynamics during the 1982–1983 epidemic of foot-and-mouth disease in Denmark based on molecular high-resolution strain identification

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INTRODUCTION

Foot-and-mouth disease virus (FMDV) remains a serious infection in husbandry worldwide, and the devastating effects if introduced to a disease-free area became clear during the epidemic in the UK during 2001. The depopulation of infected premises by incineration of animals in an attempt to control the epidemic in the UK raised considerable economic and ethical concerns, and first-choice alternatives to the non-vaccination policy such as emergency vaccination after which the vaccinated animals are culled or even allowed to live have since then been vigorously debated (Council Directive, 2003). Our knowledge of potential epidemiological scenarios upon introduction of FMDV to a disease-free, non-vaccinated area is limited and in the case of future crises it could be invaluable for decision makers to rapidly understand the initial dynamics of an epidemic.

After absence of the disease in Denmark since April 1970, vaccination against foot-and-mouth disease (FMD) was prohibited from January 1977. However, during 14 March to 4 May 1982, FMD was diagnosed in 20 cattle herds and two pig herds in Denmark (Westergaard, 1982). Twenty-one of the affected farms were located on the island of Funen and one was located on the west coast of the island of Zealand. Still adopting the policies of non-vaccination and depopulation of FMDV-infected premises, a single outbreak of FMD was diagnosed in a cattle herd on the island of Funen on 13 January 1983 (Anonymous, 1983). Thus, this epidemic in Denmark resembles the one in the UK by appearing in an FMDV-free, highly susceptible population of cloven-hoofed animals but differs significantly in terms of dissemination of the infection after introduction.

The present study focusing on the 1982–1983 epidemic of FMD in Denmark was conducted as a contingency exercise in molecular epidemiology with the additional purpose of gaining knowledge of epidemiological scenarios after introduction of FMDV to a disease-free, non-vaccinated area. The study, in particular, was an attempt to exploit the quasi-species nature of FMDV strains for molecular high-resolution strain identification and its use in analysis of epidemiological dynamics.
METHODS

Virus samples and cultivation. Twenty-three outbreaks of FMD were recorded in Denmark in 1982–1983, located as shown in Fig. 1 (Westeraard, 1982; Anonymous, 1983). A total of 21 of the 23 outbreaks were confirmed during the epidemic by virus isolation. The majority of isolates were propagated in primary cultures of calf kidney cells, but primary cultures of pig kidney or calf thyroid cells were also used according to the diagnostic protocol of the institute (including also inoculation of baby mice). Seventeen of these isolates collected from different farms were found as primary passage isolates in the stock collection of our institute and included in the present study. The isolates were named according to serotype followed by geographical location followed by the three-letter country code, followed by chronological outbreak number according to Westergaard (1982), followed by year of isolation. From one case of FMD, stocks of the primary isolate and two successive cell culture passages performed during the epidemic were included and identified as a, b and c, respectively. In other cases, terminal end-point dilutions (twofold) were done in an attempt to reveal the diversity of variants co-existing in an isolate. All isolates were propagated once on monolayers of primary calf kidney cells grown to confluence in Hanks’ balanced salt solution supplemented with 0.5% lactalbumin hydrolysate, vitamins as used in Eagle’s MEM, streptomycin (0.1 mg ml⁻¹), neomycin (0.05 mg ml⁻¹) and 10% calf serum.

RT-PCR and sequencing. Viral RNA was purified from cell culture supernatants using the QiAamp viral RNA kit (Qiagen) and reverse transcription was done using random hexamer primers with Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences) according to the manufacturer’s instructions. Full-length VP1 was amplified by PCR based on the forward primer 5’-CCCGAAAAAGCGGTTATACG-3’ and the reverse primer 5’-GGTTGGACTCCACATCTCC-3’ in 50 μl volumes including standard incubation buffer for the AmpliTaq Gold DNA Polymerase (Applied Biosystems). The cycling conditions were 5 min at 94 °C followed by 45 cycles of 1 min at 94 °C, 30 s at 60 °C and 90 s at 72 °C, and finally 5 min at 72 °C. Amplification products were visualized by electrophoresis in 1.5% agarose gels with ethidium bromide and subsequent UV exposure. The PCR products were excised from the gel, desalted twice with distilled water and purified using 0.45 μm Ultrafree-MC Centrifugal filter units (Millipore). Cycle sequencing was done with the BigDye Terminator v1.1 kits (Applied Biosystems) using the PCR primers and, in addition, an internal forward primer, 5’-TTGAGCTAAGGGTCCCG-3’, and an internal reverse primer, 5’-CCTCTCAAGTTGGACACG-3’. The cycle sequencing reactions were analysed with an ABI310 genetic analyser (Applied Biosystems). Sequences were edited by reading all four reactions, which means that sequence information was available from at least three sequence reactions for every part of the VP1 coding region. All sequences were truncated to the full VP1 coding region for the present study.

Phylogenetic analyses. The VP1 sequences used in these analyses are listed in Table 1. Sequences with highest identity to the VP1 coding regions of the Denmark 1982–1983 epidemic were identified by a BLAST search (Altschul et al., 1997) on sequence data deposited at the GenBank sequence database. Multiple alignments and phylograms of the sequences generated during the present study and sequences retrieved from GenBank were done with CLUSTALX (EMBL, Heidelberg, Germany, May 1994) (Thompson et al., 1997) using the default parameters and 1000 bootstrap replications. The aligned sequences were presented with GeneDoc (Nicholas et al., 1997) and dendograms were visualized with TREEVIEW (Page, 1996), version 1.6.6, consistently rooted with the O1/Kaufbeuren/FRG/66 strain as outgroup.

\[D_{s}/D_{n}\] ratio. The ratio of non-synonymous to synonymous \((D_{s}/D_{n})\) nucleotide differences was calculated according to Nei & Gojobori (1986), ignoring two ambiguous nucleotide positions (A or G) in one isolate in position 34 and A or G in five isolates in position 580; see supplementary Fig. S1 in JGV Online.

Meteorological estimations. In order to assess time periods with meteorological conditions suitable for airborne transmission from the outbreaks at northern Funen and the outbreak on the west coast of Zealand, meteorological data were processed in the Rimpuff dispersion model (Mikkelsen et al., 1997). Data were retrieved from the meteorological recording stations at Sprogø (recordings every 10 min), Beldringe (recordings every hour) and Omo (recordings every 3 h). A source in the outbreak area in northern Funen releasing virus particles at a constant rate (arbitrary units) was assumed and mean virus concentrations (arbitrary units) at ground level were calculated for intervals of 24 h during the period 1 April to 11 May 1982.

![Fig. 1. Location of the outbreaks of FMD in Denmark in 1982–1983. Outbreak zones of each of the three putative strains are indicated by ellipses. Putative primary introductions and the case of airborne transmission from the island of Funen to the island of Zealand are indicated by arrows.](image-url)
RESULTS

Sequences of the VP1 coding region of all 17 isolates collected in Denmark during the 1982–1983 epidemic were aligned with VP1 sequences of representatives of the seven serotypes of FMDV (data not shown) and as shown in Fig. 2 were found to cluster together with vaccine strain O1/Kaufbeuren/FRG/66 used as vaccine in the FRG at that time, as well as with O1/Lausanne/SWI/65 and the field isolates collected from Germany (Beck & Strohmaier, 1987), O/Murchin/GDR/82 and O/Zusmarshausen/FRG/84. Fig. 2 also shows the epidemiological links as revealed by interviews of farmers conducted by the Danish Veterinary Service during the epidemic (Westergaard, 1982; Anonymous, 1983). The three distinct populations representing individual introductions (discussed below) are tentatively indicated by ellipses and designated strains O/DEN.A/82, O/DEN.B/82 and O/DEN/83.

Strain O/DEN.A/82 is apparently subdivided into two groups, the only difference being that the three isolates constituting the minor group have an A in position 545 and two of these three isolates have an ambiguous A or G in position 580 (supplementary Fig. S1 in JGV Online). Terminal end-point dilution of isolate O/Gudbjerg/DEN/1/82, representing the major group, revealed that an A in position 545 is also present in a minority population of this isolate, and the ambiguous A or G in position 580 is a characteristic of O/Kappendrup/DEN/2/82, another representative of the major group. In addition the A in position 545 was found among variants resulting from terminal end-point dilution in some of the isolates belonging to strains O/DEN.B/82 and O/DEN/83, respectively, and the ambiguous A or G in position 580 was revealed in two isolates belonging to O/DEN.B/82.

All isolates of strain O/DEN.B/82 are characterized by a G in position 83 and a C in position 136, none of which is observed in strains O/DEN.A/82 and O/DEN/83. The apparent difference between the two pairs of identical isolates belonging to O/DEN.B/82 is due to ambiguities in nucleotide positions 545 and 580 (plus two singletons). Terminal end-point dilution again reveals that this variability is inherent in isolates from each of the two pairs. The O/Skelskor/DEN/22/82 isolate compared to the other isolates of the O/DEN.B/82 does not deviate in any other marker than the two variable markers. However, cloning by terminal dilution of this isolate did not reveal any inherent diversity in any of the two markers.

The three representatives of strain O/DEN/83 are cell culture passage levels 1, 2 and 3 of the same specimen, generated in an attempt to reveal any inherent diversity of the virus population in the specimen. All three passages share a T in position 3, a C in position 149, a C in position 344, a C in position 444, and an A in position 542, none of which was

Table 1. VP1 sequences involved in the phylogenetic analyses

<table>
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<tr>
<th>Isolate name</th>
<th>Accession no.</th>
<th>Date of symptoms</th>
<th>Reference</th>
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<tr>
<td>O1/Kaufbeuren/FRG/66</td>
<td>X00871</td>
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<td></td>
<td>Beck &amp; Strohmaier (1987)</td>
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<tr>
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<td>M15979</td>
<td></td>
<td>Beck &amp; Strohmaier (1987)</td>
</tr>
<tr>
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<td>M16078</td>
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<td>Beck &amp; Strohmaier (1987)</td>
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<td>–</td>
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observed in the strains O/DEN.A/82 and O/DEN.B/82. Cloning by terminal dilution of the primary isolate does not reveal any inherent variability in these five markers. Neither does cloning reveal any inherent variability in position 580, found to be ambiguous in strains O/DEN.A/82 and O/DEN.B/82.

The $D_{m}/D_{e}$ ratio was calculated as a means to indicate if mutations are neutral or represent selective advantages or, alternatively, if the coding region in question is subject to functional constraints. The ratio was found to be 0.50, which is indicative of neutrality of mutations.

The conclusion that isolate O/Skelskor/DEN/22/82 belongs to strain O/DEN.B/82 (discussed below) led to a hypothesis of airborne transmission as indicated in Fig. 1 from the herds in northern Funen to Skelskor on the western coast of Zealand, where symptoms were recorded 13 days after the culling of the last herds in northern Funen. The meteorological recordings from three stations on the route of the putative transmission were used in the Rimpuff dispersion model for a period of 6 weeks (see supplementary Fig. S2 in JGV Online) to reveal periods with plumes of airborne particles allowing airborne transmission. A number of periods with a substantial risk of airborne transmission between the two locations could be identified, such as 3–4 April, 8–12 April, 16–17 April, 27 April–1 May, and 11 May. Breaking down these periods into 3 h intervals the optimal conditions were found at midnight of 16–17 April as shown in Fig. 3.

**DISCUSSION**

The quasi-species nature of virus strains implies that strains should be regarded as populations of variants and the diversity and topography in sequence space of the population should be described in order to identify a strain. For epidemiological purposes, the objective can be reformulated as to describe the diversity of the transmissible entity resulting in introduction of infection to an individual or a herd. Based on fluctuations in proportions of the various subpopulations this can be attempted by characterization of virus in multiple isolates or samples from the same individual or herd or, in some cases, multiple plaque clones of the same sample. This approach, based on restriction fragment pattern analyses, has been previously exploited in the epidemiological resolution of recurrent introductions of Aujeszky’s disease virus to Danish border areas during the eradication campaign in the late 1980s (Christensen et al., 1990, 1993). It was concluded that herpesvirus strains are also of a quasi-species nature and shown that the genetic diversity of co-existing variants is a highly conserved characteristic during sequential transmissions in the field, in general unaffected by cell culture passage. The term ‘strain’ was tentatively defined as ‘a transmissible entity’ to reflect the fact that such entities could be discriminated with high resolution (Christensen, 1995).

High-resolution strain identification based on the characterization of inherent diversity by sequencing of multiple samples has never been extensively exploited, possibly due to the need to provide and handle very large amounts of data. In attempts to use strain identification as an epidemiological tool, questions to be addressed include the following. (i) Are two populations of isolates, collected at different farms, identical, suggesting transmission between the two farms? (ii) Are two populations of isolates, collected at different farms, different, yet related in sharing a subpopulation of identical variants, suggesting a common source or epidemiologically related sources of the two outbreaks? (iii) Could one population of isolates collected at one farm be a subpopulation of one collected at another farm, suggesting a bottleneck transmission from one farm to
the second? Such an approach obviously has the potential to provide more valid conclusions and better resolution of epidemiological dynamics than characterizing only one isolate from each farm. However, the possibilities of genetic instability of a strain and bottleneck transmissions of unrepresentative subpopulations from one outbreak to another should not be neglected. The actual impact of these limitations has to be assessed during the application of this approach in resolving epidemics.

The present study was conducted in an attempt to assess the potential of exploiting the quasi-species nature of FMDV strains in resolving epidemiological dynamics and to gain knowledge of the 1982–1983 epidemic of FMD in Denmark. The study was based on 17 primary isolates available from that epidemic. Due to the lack of multiple isolates from every farm, populations of variants in the strains in the present study had to be visualized by the analysis of isolates from different farms based on the assumption that these farms were infected by the same strain. However, the sequence information per se and the compliance between sequence information and epidemiological links as revealed by the Danish Veterinary Service during the epidemic (Westergaard, 1982; Anonymous, 1983) make the approach feasible in this case.

The identification of three populations representing three strains introduced to Denmark is in agreement with the appearance in three separate regions shown in Fig. 1 and with epidemiological links as revealed during the epidemic (Westergaard, 1982; Anonymous, 1983) shown in Fig. 2. As regards the apparent two populations of strain O/DEN.A/82, the finding of the two variable markers of the minor population also among terminally diluted variants of the major population renders it most likely that the two subgroups merely represent minor fluctuations in the compositions of the same population of variants. As regards the isolates of strain O/DEN.B/82, it is likewise obvious that the apparent differences found between the two pairs of identical isolates and O/Skelskor/DEN/22/82 represent fluctuations in the composition of the same population of variants. Strain O/DEN/83 was collected from an outbreak 9 months after the last outbreak on Funen in spring 1982. Based on a number of observations and a potential reservoir being sheep introduced to the barn in the winter of 1982–1983 it was anticipated that this outbreak was due to an introduction in spring 1982 (Anonymous, 1983). The farm was located in an area where two outbreaks were diagnosed in April 1982 in pig herds from which no virus was isolated. Whether these three outbreaks represent separate introductions or only one remains an open question.

The question whether any of the three strains could have evolved from each other during a spread in Denmark should be addressed. Mutations emerging in single virus particles and accumulating in a population would expand the diversity of the population unless a bottleneck appeared and provided there was no selective advantage of such mutations. The $D_n/D_s$ ratio of 0.5 of the nucleotide differences between the variants indicates neither any constraints nor any selective advantage of the differences and, if a bottleneck appeared, the manifestation of a mutation in a major subpopulation of a strain therefore would be relatively rare. Given the two unique markers found in all representatives
of strain O/DEN/B/82 and five unique markers found in all representatives of O/DEN/83, successive incidences of bottleneck transmissions resulting in the manifestation of minority variants as a majority population of the resulting strain and mutations would have to take place; this renders a linkage of any of the three strains by mutational evolution most unlikely in such a short time span. The other question is whether any of the three strains could have evolved from any of the others by bottleneck transmission of a pre-existing minority variant. As no such minority transmission is seen in cases where transmission routes were indicated (Fig. 2) this also is considered most unlikely.

Although it is unlikely that the three strains could have evolved from each other, the isolates share an inherent diversity in two nucleotide positions, suggested to be markers, as specific as any other marker. This strongly indicates a common source or epidemiologically related sources, the three strains representing subpopulations of a more heterogeneous population of the source(s). Furthermore, the combination of an apparent strict linkage in isolates of some markers different for each of the three strains and the sharing of hyper-variable markers by the three strains suggests that they share in their evolutionary history common recombination events between variants of different phylogenetic lineages. This is consistent with the possibility that the source(s) of the strains introduced to Denmark was located in an area with endemic FMD, i.e. an area where animals could become co-infected or superinfected with different strains. A series of FMD outbreaks in the GDR beginning on 14 March 1982 were reported to l’Office International des Épizooties as reviewed by Sørensen et al. (2000). In addition, contacts between the national veterinary services at that time revealed that the epidemic had emerged in the north-west coastal area of the GDR approximately 3 weeks before the outbreak in Denmark. This epidemic emerged after the importation of large amounts of meat from Ukraine to the city of Anklam, GDR (Dr E. Stougaard, former Chief Veterinary Officer of Denmark, personal communication). The isolate O/Murchin/GDR/1982 was collected from the epidemic in the GDR in 1982 but due to the differences between this isolate and the isolates collected in Denmark it does not assist in concluding any origin of the epidemic in Denmark.

Vaccines against FMD used in the 1980s were often inadequately inactivated and cases of FMD in Europe were concluded to be serotype O1 vaccine-related (Beck & Strohmaier, 1987), such as O/Zusmarshausen/FRG/84, included in alignments of the present study (Fig. 3). However, the characteristics of the three strains emerging in Denmark and in particular the distinct differences between them do not support a hypothesis of a common vaccine ancestry of these outbreaks.

The significance of adaptive mutations selected during passage in cell cultures is often questioned when sequence information is used for strain identification and should also be addressed here. Except for two singleton markers (the ambiguous A or C in position 34 and the A in position 418) all differences among the Danish isolates can be recognized in multiple isolates, and the multiple differences based on which the isolates are grouped into three populations appear in linkage patterns correlating with the three populations. All isolates except the two passages of O/DEN/83 were primary passages in cell culture and it is very unlikely that any adaptive mechanism could cause such patterns of differences. The $D_{o}$/$D_{s}$ ratio of 0·5 also suggests no selective advantage of mutations. To exclude the possibility of adaptive mutations during cell culture passage, the performance of PCR directly on specimens is preferred but only six such specimens were still available from the epidemic. Sequencing of PCR products from those specimens gave results that agreed with the sequence information obtained from cell culture passages of virus isolates (data not shown).

Long-distance airborne transmission has previously been shown to incidentally play a significant epidemiological role in the introductions of Aujeszky’s disease virus to the island of Funen and to other border areas of Denmark (Christensen et al., 1990, 1993) and it is plausible to suggest that the FMD epidemic in 1982–1983 was also due to airborne transmission. The conditions necessary for long-distance airborne transmission of infections are not fully understood. However, low-turbulence airflow minimizing dilution of infectious material is one prerequisite and is facilitated by a steady, low wind (speed and direction) and transportation over sea or terrains without hills (cf. Richardson, 1920; Businger, 1973). In addition, temperatures of 3–0 °C and a relative humidity of 55–65 % facilitate survival in an airborne condition of the infectious aerosol (Schoenbaum et al., 1990). We have no data to support definitively an explanation of airborne transmission of FMDV to Funen but the conclusion that three strains were introduced almost concurrently renders a number of alternative explanations, such as personal contacts to farms in FMDV-infected areas, importation of live animals from such areas and contaminated migratory birds, less likely. An airborne transmission from northern parts of the GDR would imply transportation over a distance of about 200 km, mostly over the sea, which is not unprecedented (Gloser et al., 1982; Donaldson et al., 1982b), and it was previously shown by use of the Rimpuff dispersion model to be a possibility (Sørensen et al., 2000). The conclusion that the outbreak on the south-west coast of Zealand was caused by airborne transmission from the northern part of Funen is based not only on strain identity but also on meteorological data. The Rimpuff dispersion model was exploited for a period of 3 weeks before and 3 weeks after 21 April 1982, which was the day of the culling of three herds in northern Funen. This time period was chosen to see if there was a significant coincidence between the presence of a strong source of FMDV and the appropriate meteorological conditions. In fact, occurrence of appropriate meteorological conditions – as assessed by the Rimpuff dispersion model – allowing the putative transportation...
from northern Funen to the west coast of Zealand was not a rare incidence. Among other time periods, such conditions were found during the night of 16–17 April. Given that FMDV was introduced to the last three farms with FMD outbreaks on northern Funen by a truck collecting pigs for slaughter (Fig. 2) it is plausible that there was shedding of substantial amounts of FMDV from infected pigs before the appearance of symptoms in cattle. This explanation would also accord with the findings that pigs are the host species excreting the largest amounts of virus (Donaldson et al., 1982a).

The present report basically confirms the transmission routes as suggested by the investigations carried out by the Danish Veterinary Service during the epidemic in Denmark in 1982–1983 (Westergaard, 1982; Anonymous, 1983). Thus, contact transmission by a practising veterinarian during the initial phase and by truck drivers collecting dairy milk or pigs for slaughter could explain all cases – except two – of secondary spread of the infection. The remaining two cases were one case with no indication of a transmission route and one case of putative airborne transmission from an infected neighbouring farm. Strict measures were imposed on veterinarians and truck drivers in Denmark during the epidemic in the UK in 2001 to avoid similar contact transmissions. Had such measures been implemented after the first suspicion of FMD during the epidemic in Denmark in 1982–1983 it is likely, based on the epidemiological dynamics as now revealed, that very limited or no transmission from foci of primary introductions would have taken place.

The significant difference in dissemination between the two epidemics in Denmark 1982–1983 and UK 2001 stresses that high-resolution molecular analysis of the initial epidemiological dynamics performed within 1–2 days could play an important role in deciding the most cost-effective strategy of control. The approach of the present study was only an example to show that high-resolution studies based on the quasi-species nature of FMDV strains is possible – yet the material for the present study was not optimal and raises many questions. The validity of this approach has to be assessed in studies in the future where sequence information used for strain identification is compared to other epidemiological information. Ideally, such molecular epidemiological analyses should be performed by sequencing of PCR products from 5–10 specimens collected as diagnostic samples from affected animals in each infected herd.

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