Genetic analysis of type O viruses responsible for epidemics of foot-and-mouth disease in North Africa

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(Accepted 22 December 1998)

SUMMARY

The nucleotide sequences of the 3′ end of the capsid-coding region were determined for 30 serotype O foot-and-mouth disease (FMD) viruses isolated between 1987 and 1994 from outbreaks in North Africa and the Middle East. These sequences were compared with the previously published sequences of 9 field virus isolates from the Middle East and 5 vaccine virus strains, 3 of which originated from the Middle East (O₁/Turkey/Manisa/69, O₁/Sharquia/Egypt/72 and O₁/Israel/2/85) and 2 from Europe (O₁/Lausanne/Switzerland/65 and O₂/Brescia/Italy/47). Cluster analysis of these sequences using the unweighted pair group mean average (UPGMA) method showed: (i) that the FMD viruses isolated from North Africa and the Middle East were very different from the classical European vaccine strains; (ii) that all the viruses isolated during the 1989–92 North African epidemic formed a cluster differing by no more than 6% from each other; (iii) a virus isolated in Libya in 1988 was unrelated to the aforementioned epidemic; and (iv) viruses from a second, less extensive epidemic, occurring in 1994, fell into yet another cluster.

INTRODUCTION

Foot-and-mouth disease (FMD) is an economically important disease which affects cloven-hoofed animals. Foot-and-mouth disease virus (FMDV) is serologically heterogeneous and seven serotypes are recognized worldwide, namely serotypes O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1. Of these, serotype O is the most prevalent and is considered endemic in parts of South America, the Middle East, Africa and Asia. In the countries of North Africa, serotype O has been reported periodically for more than 25 years (Table 1). The disease is probably the most contagious viral disease of animals and its epidemiology has been

In 1989, a devastating epidemic due to FMDV type O swept across North Africa from East to West and affected Libya, Tunisia, Algeria and Morocco. This paper reports the application of nucleotide sequencing to study isolates of FMDV type O from these

well studied by a variety of techniques [1]. It is now possible to study the relationship between strains of FMDV by comparing the nucleotide sequence of part of the genome coding for the viral capsid [2]. Such studies provide more accurate information than has previously been obtained by examination of the physical nature [3] or antigenicity [1] of the viral proteins. Nucleotide sequencing has been used to study the epidemiology of FMD caused by viruses belonging to serotype O [2, 4–15], serotype A [2, 9, 16–19], serotype C [20–24], SAT 2 [25] and Asia 1 [26, 27].

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Year	Morocco	Algeria	Tunisia	Libya	Egypt
1971	O	O, A, C	?	?	O
1972	O	O, A, C	O	O	O, A†
1973	O	_	_	O	O, A
1974	O	_	_	O	O†, A
1975	_		O†	O	O, A
1976	_	_	_	O	O, A
1977	Αţ	Αţ	_	O	O, A
1978	_	A	_	O	O, A
1979	_	A	Αţ	Α†	O, A
1980	_	_	A	_	O, A
1981	_	_	_	O†	_
1982	_	_	A†	O†	O
1983	Α†	_	_	O†	O
1984		_	_	+	_
1985		_	_	_	_
1986		_	_	_	_
1987	_	_	_	_	O†
1988		_	_	O†	O†‡
1989	_	_	O†	O†§	_
1990	O†	O†	O†	O§	O
1991	O†	O	O§	_	O
1992	O†	O	_	_	_
1993	O	O	_	_	O†
1994	O	_	O†	O†	_
1995	_	_	_	_	_
1996	_	_	_		_
1997	_	_	_	_	_

Table 1. The reported occurrence of foot-and-mouth disease types O, A and C in North Africa between 1971 and 1997*

countries and isolates from the Middle East between 1987 and 1994. The sequences of these isolates are compared with other selected field strains and with vaccine strains. The epidemic had a number of interesting epidemiological features which are discussed, together with their implications for the prospects of FMD control in this region.

MATERIALS AND METHODS

Viruses and cells

The designation and origin of viruses examined in the study are listed in Table 2. Most were isolated on primary bovine thyroid (BTy) cells and subsequently adapted to IB-RS-2 cells for sequence analysis.

Nucleotide sequence analysis

The sequence of approximately 171 nucleotides at the 3' end of the 1D (VP1) gene was determined by directly sequencing the RNA obtained from semi-purified virus harvests using the dideoxy-primer extension sequencing method [28–30].

Computer analysis

Nucleotide sequences were analysed on an IBM compatible personal computer using programs written by one of the authors (N.J.K.). All pairwise comparisons were performed by giving each base substitution equal statistical weight (ambiguities were ignored). A binary tree was constructed according to sequence relatedness across the interval of nucleotides 469–639 of the 1D gene using the unweighted pair

 $^{^{\}ast}$ Compiled from the FAO-WHO-OIE Animal Health Yearbooks and WRL-FMD records.

[†] Confirmed by virus isolation at the WRL-FMD.

[‡] Only in buffalo.

[§] Only in sheep and goats.

^{+,} FMD present but serotype unknown.

Table 2. Foot-and-mouth disease virus type O reference strains and field isolates used in this study

Designation*	Geographical location	Date collected	Animal	Accession number†	Reference
O ₁ /Lausanne	Lausanne, Switzerland	1965	Cattle	M15974	[2]
O ₁ /Sharquia	Sharquia, Egypt	1972	Cattle	AJ004655	[11]
O ₁ /Manisa	Manisa, Turkey	1.iv.69	Cattle	AJ004658	[11]
O ₂ /Brescia	Brescia, Italy	1947	Not known	M55287	[39]
$O_{1}^{27}/ISR/2/85$	Galand, Israel	v.85	Cattle		[15]
O/ALG/3/90	Algeria	15.v.90	Cattle		This work
O/ALG/5/90	Algeria	15.v.90	Sheep		This work
O/ALG/6/90	Algeria	15.v.90	Sheep		This work
O/EGY/4/87	Sharquia, Egypt	iv.87	Sheep		This work
O/EGY/2/89	Ismailia, Egypt	30.i.89	Buffalo		This work
O/EGY/1/93	Dakahlia, Egypt	28.vi.93	Cattle		This work
O/EGY/2/93	Giza, Egypt	10.v.93	Cattle		This work
O/EGY/3/93	Sharquia, Egypt	5.vi.93	Cattle		This work
O/EGY/4/93	Ismailia, Egypt	9.viii.93	Buffalo		This work
O/ISR/1/88	Dalton, Zefat, Israel	24.vi.1988	Cattle		[15]
O/ISR/6/89	Amazya, Israel	22.xii.1989	Cattle		[15]
O/ISR/1/91	Neot-Golan, Israel	5.iii.91	Cattle		This work
O/LEB/A/88	Al-Q-leah, Lebanon	26.iv.88	Cattle		This work
O/LIB/6/88	Bin Walid, Libya	23.viii.88	Not known		This work
O/LIB/10/89	Beer Alganam, Libya	6.xii.89	Sheep or goats		This work
O/LIB/3/94	Benghazi-Alabiar, Libya	8.i.94	Sheep		This work
O/MOR/1/91	Nr. Algerian border, Morocco	xii.90	Sheep		This work
O/MOR/5/91	Nr. Algerian border, Morocco	v.91	Sheep		This work
O/MOR/6/91	Nr. Algerian border, Morocco	v.91	Sheep		This work
O/MOR/8/91	Ben Slimane, Morocco	2.ix.91	Sheep		This work
O/MOR/9/91	Ben Slimane, Morocco	2.ix.91	Sheep		This work
O/MOR/10/91	Ben Slimane, Morocco	2.ix.91	Sheep		This work
O/MOR/10/31 O/MOR/1/92	Ifrane, Morocco	i.92	Not known		This work
O/MOR/1/92 O/MOR/2/92	Ifrane, Morocco	i.92	Not known		This work
O/MOR/2/92 O/MOR/6/92	Tangier, Morocco	vi.92	Cattle		This work
O/SAU/30/88	Riyadh, Saudi Arabia	10.x.88	Cattle		[15]
O/SAU/54/89	Todhia, Saudi Arabia	25.viii.89	Cattle		[15]
	Al Jouf, Saudi Arabia	5.vi.90‡	Sheep		
O/SAU/17/90	Al-Medyan, Saudi Arabia		-		[15]
O/SAU/3/91	· · · · · · · · · · · · · · · · · · ·	19/ii.91	Cattle	A 1004657	[15]
O/SYR/1/87	Duma, Syria	1.iii.87	Cattle	AJ004657	[11]
O/SYR/1/89	Dar'a, Syria	20.ii.89	Cattle		[11]
O/SYR/1/91	Mallaga, Almaara, Edled, Syria	10.v.91	Sheep		[11]
O/TUN/3/89	Mateur, Bizerte, Tunisia	7.xii.89	Cattle		This work
O/TUN/6/89	Haniam, Tunisia	8/xii.89	Not known		This work
O/TUN/8/89	Bélier, Ariana, Tunisia	8.xii.89	Not known		This work
O/TUN/9/89	Brebizi, Ariana, Tunisia	8.xii.89	Not known		This work
O/TUN/10/89	Chevre Leve, Tunisia	8.xii.89	Not known		This work
O/TUN/1/90	Du Fahs, Tunisia	20.iv.90	Cattle		This work
O/TUN/100/94	Tataouine, Tunisia	22.vi.94‡	Goat		This work

^{*} WRL-FMD reference number.

group mean average (UPGMA) method as implemented in the computer program NEIGHBOR and dendrograms plotted using the program DRAWGRAM both from the PHYLIP 3.5c phylogeny package [31]. The UPGMA method constructs a tree by successive

(agglomerative) clustering using an average-linkage

Bootstrap resampling analysis [32] was performed and a neighbour-joining tree constructed using the program Clustal X [33].

[†] EMBL/GenBank/DBJ accession number.

[‡] Date sample received at the WRL-FMD, Pirbright.

RESULTS AND DISCUSSION

Foot-and-mouth disease in North Africa – historical perspective

The frequency with which FMD has been reported from the countries of North Africa over the last 25 years varies from East to West (Table 1). Towards the East the disease is considered endemic whereas the West suffers only from periodic incursions, interspersed with episodes of apparent freedom from the disease. In Egypt, the disease is essentially endemic affecting buffalo, cattle and small ruminants, recent outbreaks being due to FMDV type O. In Libya, periodic outbreaks of FMDV type O have been reported since 1959 and this serotype was recorded annually from 1972 to 1978. In 1979 Libya and Tunisia suffered an apparent extension of an epidemic of FMDV type A which had occurred in Morocco and Algeria during 1977 and 1978. Since 1982 only FMDV type O has been reported from Libya. In Tunisia FMD occurs sporadically; prior to the 1989-92 epidemic types O (1972 and 1975), A (1979–82) and C (1965, 1967 and 1969) had been reported. The situation in Algeria is less clear; prior to the 1977 epidemic of FMD type A, type O had been reported in 1966 and there were unconfirmed reports of type O, A and C in 1971 and 1972. Similarly, in Morocco, prior to 1977, there had been periodic epidemics of FMD of varying severity interspersed with episodes of apparent freedom from disease. After 1977, FMD only recurred once during the 1980s, in 1983, when there was a limited epidemic of subtype A₅ which was linked to concurrent outbreaks in Portugal and Spain [2].

The epidemic of 1989-92

The major features of the 1989–92 epidemic are shown in Figure 1 which depicts the number of declared outbreaks in Tunisia, Algeria and Morocco over this period, the data from Egypt and Libya being insufficiently detailed for inclusion. There was a clear westward progression of the epidemic from Tunisia in the east to Morocco in the west (Fig. 2). The number of outbreaks declared each month showed an apparent seasonal variation. Except at the start of the epidemic, which in Tunisia occurred during the winter of 1989, the number of outbreaks tended to peak during early summer when animal movements were more frequent due to religious festivals. The peak monthly incidence decreased as the epidemic spread westwards, from a

maximum in December 1989 of 2201 declared outbreaks in Tunisia to a maximum in June 1992 of 36 outbreaks in Morocco, Tunisia and Algeria combined.

Egypt

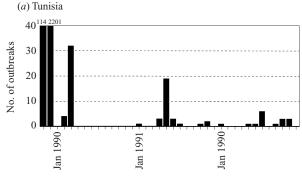
Reported outbreaks of FMD type O occurred in Egypt in 1987, 1989 and during December 1990 and the first quarter of 1991. The latter outbreak occurred in El-Sharkia province and affected only buffalo. Samples from these outbreaks were not submitted to the OIE/FAO World Reference Laboratory for FMD (WRL-FMD) at the Institute for Animal Health (IAH), Pirbright and so comparisons with earlier isolates were not possible. Foot-and-mouth disease was again reported in buffalo and cattle in 1993.

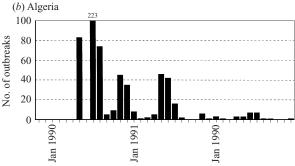
Libya

An outbreak of FMD due to serotype O occurred in Libya during August 1988 in an area 150 km from Tripoli. Further outbreaks occurred in 1989 and by mid-January 1990 a total of 34 outbreaks had been reported [34]. It is likely, however, that there was considerable under-reporting of disease during this period.

Tunisia

In November 1989, shortly after the occurrence of FMD type O in Libya, outbreaks of FMD occurred in Tunisia due to the same serotype. The first outbreaks occurred in the northwest of the country (Le Kef), rapidly followed by spread of the disease to provinces in the centre and west (Sidi Bouzid, Gafsa, Kasserine and Siliana). By January 1990, outbreaks had been declared in 22 out of 23 of the governorates comprising the Republic of Tunisia and in December alone over 2000 new outbreaks were declared [35] (Fig. 1). The epidemic affected sheep predominantly and, as the disease appeared during the lambing season, mortality amongst lambs was high, reaching 100% in some flocks. Although cattle were affected, morbidity rates were low and there was almost no mortality in this species. The epidemic was brought under control by a rapid and comprehensive vaccination campaign which had reached over 80% of the nation's susceptible stock by January 1990. Vaccination has continued since that time, albeit less intensively. The disease continued to be reported at a low level between 1990 and November 1992. There were no reports of outbreaks from November 1992





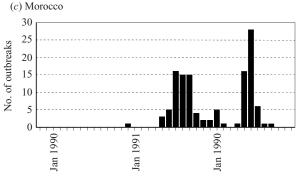


Fig. 1. Incidence of foot-and-mouth disease during the 1989–92 epidemic (a) Tunisia, (b) Algeria and (c) Morocco.

until May 1994, at which time there appeared to be a new introduction of FMDV type O. Two isolated outbreaks were reported involving cattle and sheep in the north of the country during May and sheep and goats in the south of the country during June. Whether the lack of declared outbreaks in the intervening period resulted from true freedom from FMDV or whether the virus continued to circulate at a low level in a subclinical form in a largely vaccinated population is uncertain.

Algeria

The appearance of FMD type O in Algeria was confirmed by the WRL-FMD in May 1990. Outbreaks were initially confined to provinces bordering Tunisia but the epidemic rapidly spread westwards. Transmission of the disease was accelerated due to the

extensive movement of animals which traditionally occurs at that time of the year in association with the religious festival of 'L'Aid el Kebir'. By June, 306 outbreaks had been declared and the disease had spread west beyond Algiers. After this time there was a dramatic fall in the numbers of declared outbreaks but the westward progression continued and the disease had reached the provinces bordering Morocco by November 1990. Vaccination and movement controls were the major measures used to arrest the progress of the epidemic. By August 1990 some four million susceptible stock had been vaccinated either in a buffer zone along the border with Tunisia or during ring vaccination around declared outbreaks. Figures for the species of animals affected during the early phase of the epidemic (up to August 1990) clearly demonstrated that sheep were the main species affected (total numbers affected were sheep 6293 (95%), goats 212 (3%) and cattle 130 (2%) (Diréction des Services Vétérinaires et Phytosanitaires, Algeria). Further outbreaks were declared during 1991 and 1992 and a similar seasonal incidence with a peak around May-July was observed. No outbreaks were declared during 1993 or 1994.

Morocco

During the course of 1990, faced with the spread of FMD type O from the east, the Moroccan authorities vaccinated susceptible livestock in the provinces bordering Algeria to create a 'buffer zone'. Despite this precaution, clinical disease consistent with FMD appeared in the eastern province of Oujda in December 1990 and the causative agent was confirmed as FMDV type O by the WRL-FMD early in January 1991. There followed a lull in the apparent progress of the disease until May 1991 when, again during the period of the festival of 'L'Aid el Kebir', there was a rapid progression westwards of the disease along the traditional trade route from Oujda through Taza, Fes, Meknes, and Kenitra towards Rabat. As observed in the other countries of the Mahgreb, sheep were the major species affected (records from the Diréction de l'Élevage of animals affected up to August 1991 showed: sheep 92.7%, goats 7% and cattle 0.3%). By September 1991 the disease had reached as far west as Ben Slimane to the south of Rabat. Disease continued to be reported at a low level until April 1992 when once again there was a rapid expansion of the disease. In view of the deteriorating situation, the decision was taken in August 1992 to vaccinate the entire national

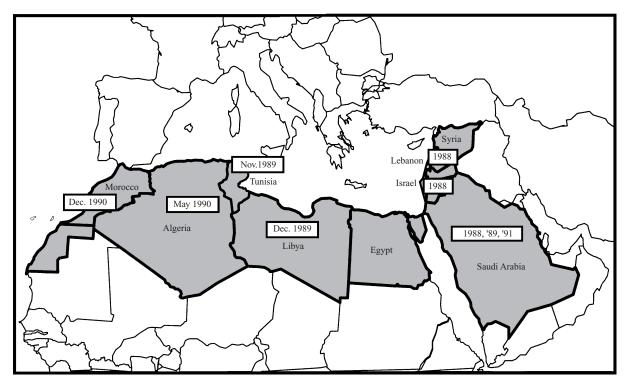


Fig. 2. Map showing the westward spread of FMDV type O in North Africa between 1989 and 1991. Dates represent the first isolation of the epidemic strain in each country.

sheep flock of some 16 million animals. Since that time no further outbreaks have been reported. Serological surveys conducted since 1993 have shown a progressive decrease in the number of animals seropositive for antibody to FMDV.

Nucleotide sequence analysis

The phylogenetic relationships between selected isolates of FMDV type O from the Middle East and North Africa, received by the WRL-FMD between 1987 and 1994, are shown in Figure 3. Strains which are less than 15% different over the region of genome sequenced are considered to belong to the same genotype and strains which differ by less than 5% are considered to be closely related [15, 36]. Bootstrap resampling (1000 replicates) was performed and a neighbor-joining tree constructed (data not shown). This analysis showed that the branch point separating the two groups of Middle East/North African viruses occurred at a frequency of 97.8%.

Although only relatively minor antigenic variation exists between strains from the Middle East [37], the genetic relationships between strains circulating and co-circulating in the region are complex. Some groups of virus strains appear quite widespread whereas others are confined to more discrete geographical

regions (A. R. Samuel, N. J. Knowles, unpublished observations). The complexity of the situation is reflected in the appearance of isolates from the same country (e.g. Israel) in numerous genetic groups (Fig. 3).

In the period up to 1988, FMD type O isolates from throughout the Middle East (O/ISR/2/85 and O/SYR/1/87) and from North Africa (O/LIB/6/88 and O/EGY/4/87) belonged to a number of different genetic groups (Fig. 3) which were only distantly related to the group which caused the epidemic of 1989–92. The North African epidemic of 1989–92 was caused by a distinct genetic lineage whose origin can be traced back to the Middle East. Chronologically, the genotype was first identified in strains received from Israel (O/ISR/1/88) and the Lebanon (O/LEB/A/88) in 1988. The first isolate of this type from North Africa was from Libya in 1989 (O/LIB/10/89). Thereafter, all the isolates received from Tunisia, Algeria and Morocco over the period 1989–92 belonged to this genotype. Whether the strain spread directly from Libya to Tunisia (or vice versa) or whether there was a second introduction of the Middle East strain into Tunisia is unclear, as some animals in the country were discovered to be originally of Turkish or Syrian origin (WRL-FMD records). The apparent spread of the virus strain from east to

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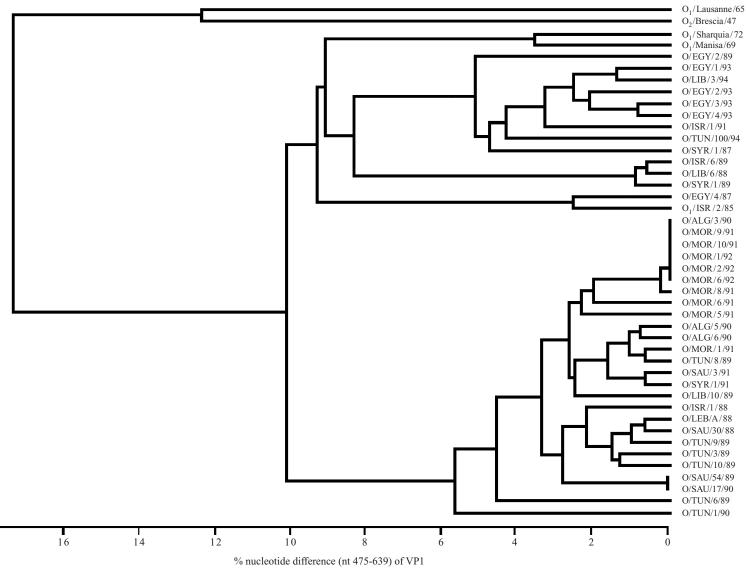


Fig. 3. UPGMA dendrogram showing the genetic relationships of foot-and-mouth disease type O viruses isolated in North Africa with those from the Middle East and with selected reference virus strains.

west can be seen by the close genetic relationships between the virus isolates, the chronological interval between their isolation and the epidemiological information which has been supplied from the field. At the same time this genetic lineage appears to have become widespread throughout the Middle East, being isolated from, amongst other countries, Syria (O/SYR/1/91) and Saudi Arabia (e.g. SAU/30/88, SAU/54/89). Isolates of FMDV type O collected from the region since 1993 (e.g. O/EGY/1/93, O/LIB/3/94, O/TUN/100/94) are more closely related to older strains from the Middle East and North Africa, isolated in the late 1980's, than to the strain responsible for the 1989-92 epidemic. The North African isolates were not genetically closely related to any of the vaccine strains with which they were compared $(O_1/Sharquia, O_1/Manisa, and O_1/$ Lausanne).

We have previously shown that many genetic lineages of FMDV type O may circulate within a country or geographic region [15]. In this study we have observed a single virus lineage spread from an endemic region (the Middle East) to cause an epidemic in an FMD-free region (western North Africa). This also suggests that the epidemic occurred as the result of a single introduction of the virus.

Foot-and-mouth disease type O viruses isolated from infected animals in the smaller 1994 epidemic in Libya and Tunisia were genetically closely related to viruses present in Egyptian cattle and buffalo between 1989 and 1993, but were distinct from viruses involved in the 1989–92 epidemic. The viruses studied from the 1994 epidemic (O/LIB/3/94 and O/TUN/100/94) were isolated from small ruminants, however, cattle were also affected. It is likely that only localized outbreaks were reported as the majority of cattle and sheep in Tunisia were receiving their annual vaccinations at this time. Studies conducted on oesophageal/pharyngeal samples collected from cattle in the outbreak which occurred in May showed that virus could be isolated from carrier animals as late as September 1994 (S. Hammami, D. K. J. Mackay, unpublished observations).

Epidemiological aspects

The nature of the relationships between strains of FMDV type O in the Middle East has previously been examined using antigenic techniques [37]. The application of molecular epidemiology, particularly in the context of the recent North African epidemic,

confirms the complexity of the situation. Viruses in the Middle East obviously pose a threat to Europe due to the close geographical proximity, trade links, indistinct national borders and the fact that FMD can be considered endemic in most of this region. Viruses from Asia have made incursions into Europe in the recent past: type O in Bulgaria (1991 and 1993), Italy (1993) and Greece (1994 and 1996) and type A in Albania (1996), Macedonia (1996) and the Federal Republic of Yugoslavia (1996), and there is no reason to think that further incursions will not occur in the future.

The North African epidemic of 1989-92 has a number of interesting epidemiological features, principal amongst which was the predilection of the virus for sheep. The epidemic caused massive losses of young lambs due to myocarditis, particularly in Tunisia, and in all the countries involved sheep were the major species affected. In the early stages of the epidemic (December 1989) disease was introduced around the main lambing season and so it was not surprising that considerable mortality occurred. However, later in the epidemic there was opportunity for the virus to affect cattle which were often reared in close proximity to the sheep. Although a small number of cattle demonstrated classical lesions, bovines remained largely unaffected. This can be attributed at least in part to the fact that cattle in Morocco were vaccinated annually against FMD using bivalent (O and A) or trivalent (O, A and C) vaccines. However, annual vaccination does not explain why cattle were so little affected in some of the other countries, e.g. in Tunisia where routine vaccination of cattle or sheep was not practised prior to 1989. Additionally, in previous epidemics, such as the 1983 type A outbreaks in Morocco, disease spread from affected sheep to cattle, despite annual vaccination of cattle against type A since 1976.

It therefore seems likely that this strain of virus has a particular predilection for sheep. This has been borne out in experimental infections at the IAH, Pirbright. In a pilot experiment cattle and sheep were directly inoculated with the strain O/MOR/9/92. The lesions produced in cattle were extremely variable, ranging from subclinical infection to the appearance of classical vesicles on the tongue and feet. In contrast, 4 out of a group of 5 sheep showed severe tongue and foot lesions and the virus was re-isolated from all 5 animals. Similar results have been obtained in sheep at the Community Coordinating Institute for FMD Vaccines, Lelystad, the Netherlands (Dr S. Barteling,

personal communication, 1993). A similar species tropism for type O FMD viruses in pigs in the Far East has been suspected for many years (N. J. Knowles, unpublished observations) and recently experimental infection of pigs and cattle has confirmed a marked predilection of one of these viruses for pigs [38].

Foot-and-mouth disease was eradicated from Europe by mass, annual vaccination of cattle as it was considered that the disease in sheep was self-limiting. The situation in North Africa appears to be different, as the disease was able to sustain itself for 4 years almost exclusively in sheep and to cause periodic epidemics within the countries affected over that time. In both Tunisia and Morocco it was not until mass vaccination of sheep was introduced that the disease was brought under control.

Control of FMD in the Mahgreb (Libya, Tunisia, Algeria, Morocco and Mauritania) is a regional and not a national problem. The borders between the various countries are administrative and not geographical. There is, therefore, massive and virtually uncontrolled movement of animals, particularly sheep, across the region. The traditions of the area are for free and frequent trade in animals, irrespective of national boundaries. Animals are frequently sold repeatedly in a short space of time and may be transported by lorry for long distances, frequently stopping to load or unload stock along the way. Trade and movement of animals can be increased due to the demands posed by certain religious festivals, such as that of 'L'Aid el Kebir' which coincided with a dramatic increase in the spread of FMD in Morocco in May 1991 and 1992 (Fig. 3). Superimposed on the movement of animals for trade is the migration of stock-holders and their animals from south to north in summer and vice versa in winter. In addition, there are the movements of true nomads and their animals throughout the region. Thus even if one country manages to control the disease through vaccination, re-entry of infection is almost unavoidable due to the illegal movements of animals from neighbouring, and possibly still infected, countries. The development of a regional programme for FMD surveillance and control is essential if the disease is to be eventually eradicated from the Mahgreb.

ACKNOWLEDGEMENT

This work was funded in part by the Ministry of Agriculture, Fisheries and Food, UK and by the Commission of the European Communities. We are grateful to the Diréction de l'Élevage, in Morocco and Algeria for information provided and to Dr S. Hammami of the Institut de la Recherche Vétérinaire de Tunisie for additional information and review of the manuscript. We would also like to thank David Ansell and Keith Marchant for valuable technical assistance.

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