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Analysis of mixed foot-and-mouth disease virus infections in Saudi Arabia: prolonged circulation of an exotic serotype

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SUMMARY

Plaque purification of foot-and-mouth disease (FMD) type O viruses isolated from cattle in Saudi Arabia showed the presence of mixed serotype infections. Sixteen out of 31 samples collected between 1985 and 1991 also contained Asia 1 virus, a serotype which had previously only been isolated from a single outbreak in that country in 1980. Nucleotide sequences of the Asia 1 component of all these samples revealed little variation and showed that they were closely related to both a Russian lapinized vaccine virus strain (Asia 1/Tadzhikistan/64), and to a field isolate from Turkey (Asia 1/TUR/15/73). Although mixed FMD infections have been observed previously this is a first report of a serotype, considered to be exotic to a country, co-existing undetected for an extended period of time.

INTRODUCTION

Foot-and-mouth disease (FMD) is endemic in most Middle Eastern countries with serotype O being the most prevalent. Outbreaks due to serotypes A and Asia 1 occur sporadically whereas those due to serotypes C, SAT 1 and SAT 2 are rare. Some epizootics have swept through the Middle East to threaten parts of Europe, notably SAT 1 in 1962–5, A_{22} in 1964–5 [1, 2] and Asia 1 in 1973 [3] and 1983–4 [4]. Recent studies have shown that the Middle East strains of FMDV serotype O are serologically distinct from those in Europe and South America. During the period 1987–8 an antigenic shift appears to have occurred [5].

In Saudi Arabia outbreaks of FMD attributable to types O, A, C, Asia 1 and SAT 1 have occurred. Recent control campaigns have utilized vaccines incorporating serotypes O, A, C and Asia 1. Since 1971, when samples were first submitted to the OIE/FAO World Reference Laboratory (WRL) for FMD from Saudi Arabia, serotype O has been recorded in 1971–3, 1978, 1980–93; serotype A in

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1973, 1976, 1984, 1986–7 and 1991–3; serotype Asia 1 in 1980 and 1992; serotype C in 1984; and serotype SAT 1 in 1962 and 1970. During 1988 the number of reported outbreaks increased significantly particularly in the large dairy farms around Riyadh.

Antigenic analysis of serotype O viruses isolated from Saudi Arabia showed that while some isolates reacted strongly with a monoclonal antibody (MAb) to O_1/Lausanne/Switzerland/65 others exhibited little or no reactivity [6]. The reactivity of this MAb has been mapped to the G–H loop on $1D^{cap}$ (VP1) which lies between amino acid residues 129 and 165 [7]. It was considered that plaque purification and sequencing of these phenotypically different strains might help to identify the cause of this phenomenon. This paper reports results obtained during such a study.

MATERIALS AND METHODS

Cell cultures

Monolayers of primary bovine thyroid (BTy), secondary calf kidney (CK) or continuous pig kidney (IB-RS-2) cells were used in the original isolation procedures. Subsequently baby hamster kidney (BHK-21) cells were also used. All the cells were grown in Eagle's MEM containing 5% normal bovine serum and antibiotics. Serum-free medium was used during virus growth.

Viruses

The origin of the virus isolates used in this study are shown in Table 1. Stock viruses were passaged on 175 cm^2 flasks of IB-RS-2 monolayers. When CPE was complete the supernatant was clarified at 2000 g for 5 min, an equal volume of sterile glycerol added and the virus stored at −20 °C.

Plaque purification

Six-well tissue culture plates confluent with IB-RS-2 cells were washed twice with calcium and magnesium-free PBS. Virus dilutions were made in the range 10^{-1}–10^{-6} in Eagle's MEM. Aliquots of the virus dilution (200 μl) were added and left to adsorb for 30 min at 37 °C. The inoculum was removed and the cell sheet washed twice with PBS and overlayed with 3 ml of Eagle's MEM containing 1·6% agar and 1% normal bovine serum at 43 °C. When the agar had set the plates were covered in 'cling film', inverted and incubated for 18 h at 37 °C. Plaques were picked using a pasteur pipette and resuspended in 1 ml of Eagle's MEM. Aliquots (200 μl) were inoculated onto flasks containing confluent monolayers of IB-RS-2 cells. When CPE was complete the supernatant was treated as described above. These viruses were subjected to a further two plaque purification procedures. In some of the later procedures a 1/50 dilution of either anti-O_1/BFS 1860/UK/67 or anti-Asia 1/PAK/1/54 guinea-pig serum was added to the overlay medium to suppress the growth of the homologous serotype.

Monoclonal antibody analysis

A panel of MAbs raised against the reference strain O_1/Lausanne/Switzerland/65 [8, 9] and characterized by sequencing neutralization escape mutant viruses [7, 10], was used in a trapping (capture) ELISA [11] to obtain antigenic profiles.

Table 1. *Foot-and-mouth disease virus isolates analysed in this study*

WRL ref. no.	Geographical location	Date collected§	Animal	Serotypes isolated
SAU/2/83	Al Kharj, Saudi Arabia	00/00/83	Cattle	O
SAU/4/83	Riyadh, Saudi Arabia	00/00/83	Cattle	O
SAU/11/85	Al Kharj, Saudi Arabia	26/11/85	Cattle	O, Asia 1
SAU/17/86	Al Kharj, Saudi Arabia	07/09/86	Cattle	O, Asia 1
SAU/1/87	Al Jouf, Saudi Arabia	00/00/87	Sheep	O, Asia 1
SAU/34/87	Haradh, Saudi Arabia	00/00/87	Cattle	O
SAU/1/88	Al Quatif, Saudi Arabia	19/01/88	Cattle	O, Asia 1
SAU/8/88	Al Kharj, Saudi Arabia	28/09/88	Cattle	O, Asia 1
SAU/24/88	Al Kharj, Saudi Arabia	29/10/88	Cattle	O, Asia 1
SAU/28/88	Al Kharj, Saudi Arabia	01/01/88	Cattle	O, Asia 1
SAU/30/88†	Riyadh, Saudi Arabia	100/10/88	Cattle	O
SAU/33/88	Riyadh, Saudi Arabia	15/12/88	Cattle	O
SAU/34/89	Al Kharj, Saudi Arabia	00/00/89	Not known	O, Asia 1
SAU/35/89	Al Kharj, Saudi Arabia	00/00/89	Not known	O, Asia 1
SAU/36/89	Al Kharj, Saudi Arabia	00/00/89	Not known	O, Asia 1
SAU/40/89	Al Kharj, Saudi Arabia	00/00/89	Not known	O
SAU/56/89	Todhia, Saudi Arabia	25/08/89	Cattle	O, Asia 1
SAU/17/90	Al Jouf, Saudi Arabia	00/00/90	Sheep	O
SAU/18/90	Afif, Saudi Arabia	00/00/90	Sheep	O
SAU/19/90	Quatif, Saudi Arabia	00/00/90	Cattle	O
SAU/25/90	Thadiq, Saudi Arabia	29/07/90	Cattle	O
SAU/26/90	Al Hair, Saudi Arabia	26/11/90	Cattle	O
SAU/30/90‡	Al Kharj, Saudi Arabia	11/09/90	Cattle	O
SAU/35/90	Al-Majmaa, Saudi Arabia	05/12/90	Sheep	O
SAU/36/90	Riyadh, Saudi Arabia	10/12/90	Cattle	O, Asia 1
SAU/3/91	Al Medyan, Saudi Arabia	19/02/91	Cattle	O
SAU/7/91‡	Al Kharj, Saudi Arabia	05/01/90	Cattle	O, Asia 1
SAU/15/91	Al Kharj, Saudi Arabia	29/06/91	Cattle	O, Asia 1
SAU/16/91	Al Kharj, Saudi Arabia	30/06/91	Cattle	O, Asia 1
SAU/40/91	Quasim, Saudi Arabia	10/08/91	Cattle	O
SAU/42/91	Gizan, Saudi Arabia	27/08/91	Cattle	O, A, Asia 1
LIB/6/88	Bin Walid, Libya	23/08/88	Not known	O
TUR/8/88	Oguzeli, Gaziantep, Turkey	11/05/88	Cattle	O
EGY/2/89	Ismailia, Egypt	00/00/88	Buffalo	O
TUN/3/89	Tunisia	00/00/89	Cattle	O
ALG/3/90	Algeria	15/05/90	Cattle	O
MOR/1/91	Morocco	00/00/90	Sheep	O

* OIE/FAO World Reference Laboratory for Foot-and-Mouth Disease reference number.
† nomadic animals.
‡ carrier animals.
§ 00, date not known.

Briefly, viruses to be tested were reacted with a panel of MAbs and the reactivity compared to that of the homologous virus. Results were expressed as a percentage of the homologous reaction.

Enzyme-linked immunosorbent assay

Typing assays were performed using antisera prepared against all seven serotypes of FMDV as previously described [12].

The viruses were compared by one-way liquid phase blocking sandwich ELISA

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using sera from vaccinated cattle according to the method of Kitching and colleagues [13]. The assay measures the residual antigen remaining after overnight reaction between dilutions of a reference antiserum and pre-titrated antigen prepared from the field isolate or the homologous reference strain. The serum titre obtained at 50% of the maximum optical density is used to calculate the relationship (r) value and interpreted according to the criteria described by Samuel and colleagues [5].

Nucleotide sequence analysis

Two oligonucleotide primers designated pNK10 (5′-GAAGGGCCCAGGGT-TGGACTC) complementary to the last 15 nucleotides of the 2A gene and the first six nucleotides of the 2B gene and pCP14 (5′-CCTTCACAAATCTGTCC) complementary to nucleotides 108–124 in the $1D^{cap}$ gene were used.

The sequence of approximately 170 nucleotides at the 3′ end of the $1D^{cap}$ gene was determined for all the plaque isolates by direct RNA sequencing using the dideoxy chain-termination method [14–16]. Additionally the sequence of the region spanning the 1C/1D junction was determined for one of the isolates.

Sequences were analysed using computer programs written by one of the authors (NJK) and from the PHYLIP phylogeny package [17].

RESULTS

Plaque morphology

Virus isolated on BTy cells from the original epithelium of the sample designated O/SAU/8/88 was subjected to plaque purification and 10 plaques were picked. Five had a large plaque phenotype (4 mm diameter) and were designated P1–P5 and five had a small plaque phenotype (1 mm diameter) and designated P6–P10.

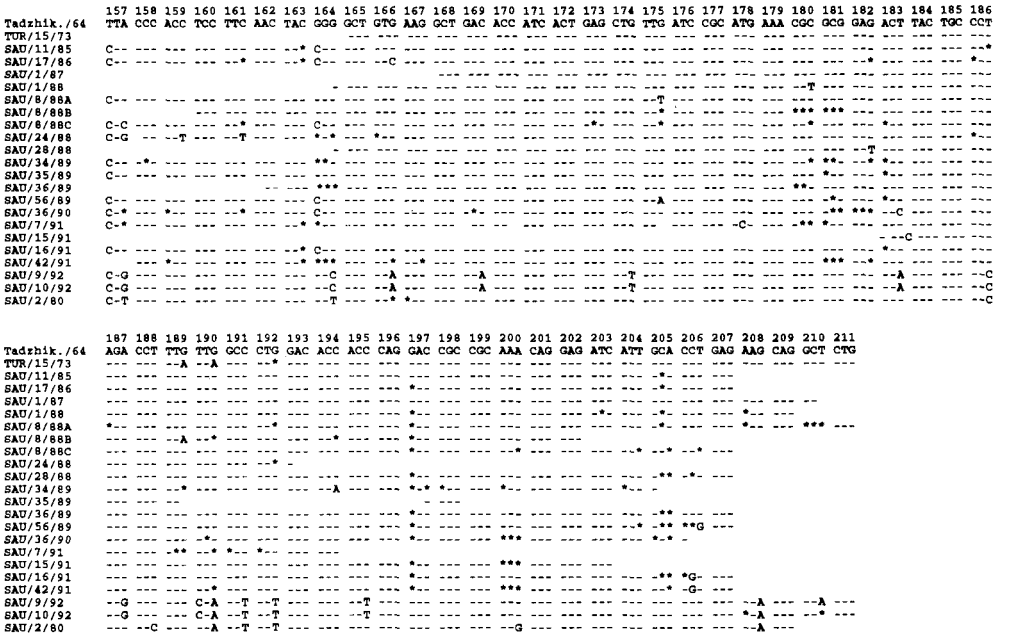
Monoclonal antibody analysis (antigenic profiling)

Plaque isolate SAU/8/88 (P1) exhibited reduced binding to all MAbs and approximately one third of the expected binding to the polyclonal positive control. Plaque isolate SAU/8/88 (P7) showed expected binding levels on the antigen controls (approx. 1·2 o.d.) and to the MAbs.

Enzyme-linked immunosorbent assay

SAU/8/88 (P1) and SAU/8/88 (P7) were chosen as representative plaque isolates for examination by ELISA. They were compared with the O_1/Manisa/Turkey/69 and O_1/BFS 1860/UK/67 reference strains. Sucrose density gradient purified material that had been quantified spectrophotometrically was used in the ELISA so that a known amount of each virus was reacted in the assay. Isolate P7 was found to be most closely related to the O_1/Manisa/Turkey/69 reference strain ($r > 1{\cdot}0$). The reactivity of SAU/8/88 (P1) in the assay was too low for useful comparisons to be made. It was concluded that this isolate was so different antigenically that the polyclonal trapping antibody failed to bind the virus at sufficient levels.

(*a*)



(*b*)

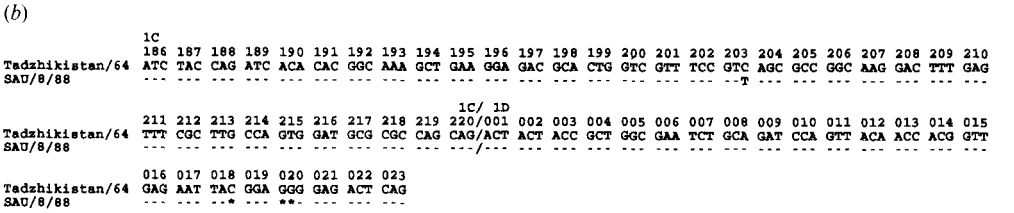


Fig. 1. (*a*) Nucleotide sequences of the 3′ end of the 1D gene of the FMDV Asia 1 isolates from Saudi Arabia. (*b*) Nucleotide sequences of the 3′ end of the 1C gene of the FMDV Asia 1 isolate SAU/8/88 compared to that of Asia 1/Tadzhikistan/64. *Sequence ambiguity (identity of nucleotide could not be determined).

Nucleotide sequence analysis

The nucleotide sequence of the 3′ end of the ID^{cap} coding region of the virion genome was determined and the sequences obtained for SAU/8/88 (P1) and SAU/8/88 (P7) were compared. The nucleotide sequence analysis revealed that whilst the SAU/8/88 (P7) sequence was the same as that previously determined for the original unplaqued O/SAU/8/88 isolate [6], the SAU/8/88 (P1) sequence showed that this isolate belonged to the Asia 1 serotype (Fig. 1).

The nucleotide sequence obtained for SAU/8/88 (P1) was compared to other available Asia 1 sequences and was shown to be most closely related to the sequence of a virus isolated from Tadzhikistan SSR in 1964 [18] and to TUR/15/73 [19]. These viruses were not closely related to either SAU/2/80, the first occurrence of Asia 1 in Saudi Arabia, or to SAU/9/92 and SAU/10/92 which were isolated from samples received during the progress of this investigation (Fig. 2).

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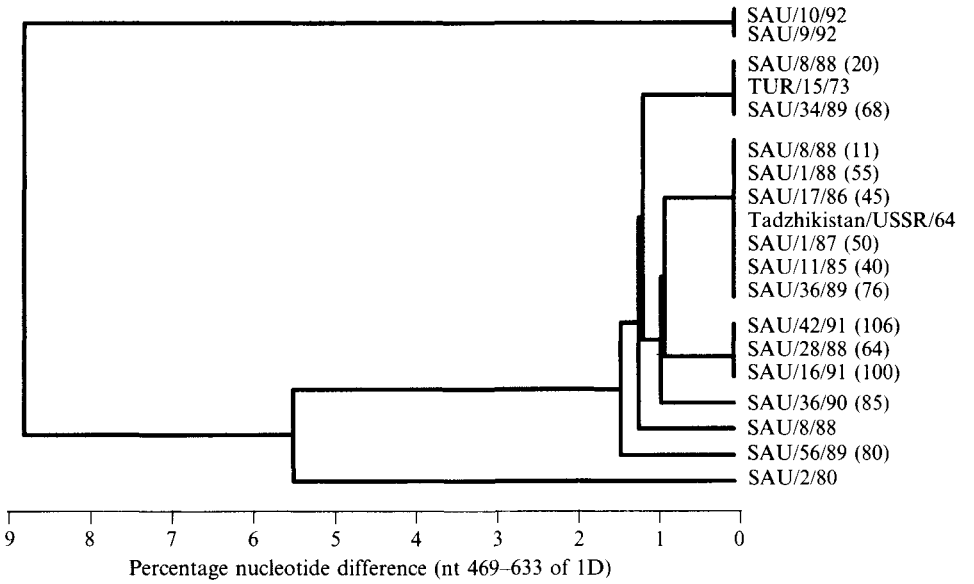

Fig. 2. Dendrogram showing the relationships between the Saudi Arabian FMD Asia 1 viruses, Asia 1/Tadzhikistan/64 and Asia 1/Turkey/15/73.

Serotyping by ELISA

Virus typing was performed on the 10 plaque isolates of SAU/8/88 (P1–P10). Results showed that plaques P1–P5 (the large plaque phenotype) were of serotype Asia 1 and plaques P6–P10 were serotype O.

Passage on different cell cultures

The isolate SAU/8/88 was serially passaged five times on either BTy, CK, IB-RS-2 or BHK-21 cells and the supernatant fluids were then serotyped by ELISA as described by Roeder and Le Blanc Smith [12]. All samples typed as serotype O. The Asia 1 component had not been amplified to an extent where it was detectable by ELISA.

Examination of additional FMDV type O isolates

The study was expanded to include virus isolates from different regions within Saudi Arabia and during 1983–91 (Table 1; Fig. 3). Because serotype O strains circulating in North Africa are related to the Saudi Arabian group of isolates (A. R. Samuel and N. J. Knowles, unpublished data), viruses from Algeria (O/ALG/3/90), Tunisia (O/TUN/3/89), Morocco (O/MOR/1/91), Libya (O/LIB/6/88), Turkey (O/TUR/8/88) and Egypt (O/EGY/2/89) were subjected to the plaque isolation procedure described using type O antiserum. In no case was Asia 1 virus isolated.

Many of the samples from Saudi Arabian farms were negative for dual infection (Table 1). However, one sample (SAU/42/91) from Gizan in the south-west of the country contained three different serotypes – O, A and Asia 1. FMDV type Asia 1 was isolated from samples that had originally been typed as O during successive

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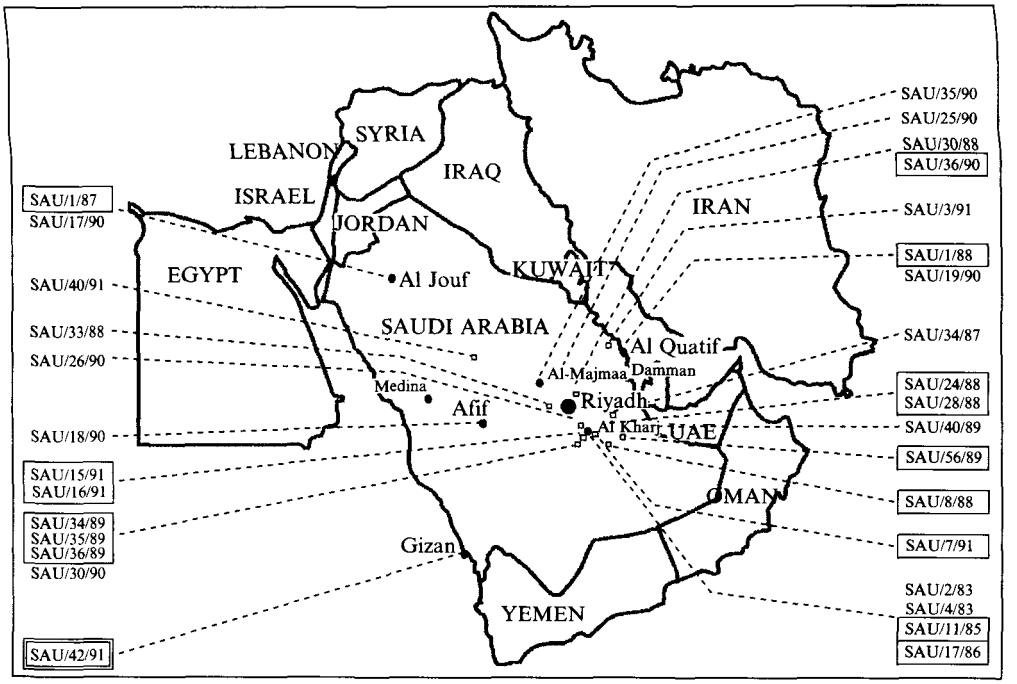

Fig. 3. Map showing the origin of the samples from Saudi Arabia which were tested for mixtures of FMDV serotypes. Type O viruses were isolated from all the samples, types O and Asia 1 from those contained in a single box and types O, A and Asia 1 from the isolate in a double box.

years from 1985 until 1991. Most of these samples were from farms around the Riyadh and Al Kharj areas, although SAU/1/87 was from Al Jouf in the north, SAU/1/88 from Quatif in the east and SAU/42/91 in the south west (Fig. 3).

DISCUSSION

The phenomenon of mixed serotype FMDV infections is not new. Hedger [20] showed that it was possible to isolate viruses of serotypes SAT 1, SAT 2 and SAT 3 from oesophageal/pharyngeal fluid collected from African buffalo (*Syncerus caffer*) in Botswana. Here, however, we show for the first time that a serotype thought not to be present in a region has persisted undetected in cattle alongside another endemic serotype. It has been reported that during the course of an epizootic of FMD the serotype of the virus changed. It had been postulated that high mutation rates could be responsible or that latent infections had been triggered when a secondary infection of FMDV had occurred [see refs in 21]. Also, serological tests which gave reactivities with two or more serotypes were sometimes explained as being due to non-specific cross-reactions. It has been usual to passage a virus isolate showing minor cross-reactions in serological assays (complement fixation or ELISA) until a clear typing was obtained.

The possibility of laboratory contamination was discounted by repeating the plaque isolation from the SAU/8/88 sample after re-isolation from the original epithelial material received by the WRL. Isolation and plaquing from the

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epithelial material was also carried out independently. In all cases, the viruses were initially identified as serotype O, yet Asia 1 virus was recovered from the samples.

All the plaque isolates examined were closely related to each other and to a rabbit attenuated Russian vaccine virus strain [18] which originated in Tadzhikistan in 1964 and to an isolate from an outbreak in Turkey in 1973 (TUR/15/73) [19] (Fig. 2). They were not closely related to isolates of Asia 1 received from FMD outbreaks in Saudi Arabia in 1992 (> 9% nucleotide difference). These 1992 isolates are most closely related to another vaccine strain Asia 1/IND/8/79 [19]. Interestingly, some of these samples were typed by ELISA as mixtures of type O and Asia 1. The plaque isolates were not related to the isolate Asia 1/SAU/2/80 which was isolated from the first outbreak of Asia 1 in Saudi Arabia in 1980 [22].

Finding type A in one of the isolates was less surprising since outbreaks due to this serotype have occurred in Saudi Arabia since 1991. Sequence analysis showed that the type A component of the SAU/42/91 isolate was most closely related to a group of viruses which caused outbreaks in Turkey during 1991–2 [23] and Saudi Arabia in 1991 (A. R. Samuel, unpublished data). The Saudi Arabian viruses were not closely related to the previous type A outbreak strains from 1986–7 [24] which were more closely related to viruses from the Indian sub-continent [25]. Recent samples received from Saudi Arabia in 1993 have been typed as mixtures of type O and A and similarly samples from two outbreaks in Turkey in 1991 have also been found to be mixtures of serotypes O and A. During the 1984 outbreak of FMD type C in Saudi Arabia, one isolate, SAU/1/84, was found to be a mixture of types C and O (WRL records). Table 2 lists samples from cattle which have been received by the WRL since 1967 and which were identified as mixtures of two or more serotypes; however, the possibility that some were laboratory contaminants cannot be discounted.

Preliminary animal experiments at this institute using SAU/8/88 have shown that both the O and Asia 1 components of the sample can transmit together (F. Davidson, J. Salt, unpublished data).

Possible origins of the Saudi Arabian Asia 1 virus include: (i) the virus has persisted in a sub-clinical form since its initial introduction into Saudi Arabia in 1980; (ii) the virus has been introduced on one or a number of occasions from an area which has been using the Asia 1/Tadzhikistan/64 strain (or a closely related virus), either as a live attenuated vaccine or as an improperly inactivated vaccine; (iii) the virus has been circulating as a sub-population of the type O virus for some years; (iv) parts or all of the capsid-coding region of an inactivated Asia 1 vaccine strain have been rescued by recombination with a type O field virus.

It is improbable that the Asia 1 virus has persisted in Saudi Arabia since 1980 because the genetic difference between the viruses isolated between 1985 and 1991 and the field virus from 1980 (SAU/2/80) is so large (*c.* 5·5% nucleotide difference) and because of the close relationship between the 1985–91 isolates and Tadzhikistan/64 (< 2% nucleotide difference). Asia 1 viruses similar to Tadzhikistan/64 have, so far, not been recovered from type O infections occurring in countries other than Saudi Arabia, even from very closely related type O viruses. Although evidence of experimental and natural heterotypic recombination has been documented [26, 27] and genome fragments may be recovered from

Table 2. *Previously identified mixed serotype infections of foot-and-mouth disease virus**

Year	Country	WRL ref. no.	Serotypes isolated
1971	India	IND/7/71	A+O
1972	Afghanistan	AFG/1/72	O+Asia 1
1972	Uganda	UGA/81/72	O+SAT 1
1973	Malawi	MAL/43/73	SAT 2+A
1984	Saudi Arabia	SAU/1/84	C+O
1990	Nepal	NEP/115/90	O+C
1991	Kenya	KEN/22/91	O+A+SAT 2
1991	Kenya	KEN/26/91	A+SAT 2
1991	Kenya	KEN/31/91	O+SAT 2
1991	Saudi Arabia	SAU/46/91	O+Asia 1
		SAU/50/91	
1991	Saudi Arabia	SAU/32/91	O+A
1991	Turkey	TUR/9/91	O+A
		TUR/12/91	
1992	Saudi Arabia	SAU/7/92	O+Asia 1
		SAU/8/92	
		SAU/11/92	
1992	Saudi Arabia	SAU/34/92	O+A
		SAU/35/92	
		SAU/36/92	
		SAU/42/92	
1993	Saudi Arabia	SAU/6/93	O+A
		SAU/7/93	
		SAU/9/93	

* Compiled from the records of the OIE/FAO World Reference Laboratory for Foot-and-Mouth Disease.

nactivated vaccines by polymerase chain reaction amplification [28] the viruses ecovered show no relationship to Asia 1 vaccine strains known to have been used n Saudi Arabia between 1985 and 1991. We therefore feel that the second ossibility is the most likely explanation.

Because the Asia 1 serotype is co-infecting and is able to survive within these opulations of animals in the farms around Riyadh, the potential exists for the sia 1 to cause disease when the opportunity presents itself. At the moment there s no evidence that any outbreaks of FMD have been caused by this virus even hough Asia 1 has been isolated from samples from as early as 1985. However, it ould be possible that the clinical signs of this particular strain are so mild that nfection occurs but remains undiagnosed or are not reported to the authorities. We plan to experimentally infect animals at Pirbright with one of the Asia 1 laque isolates to ascertain its pathogenicity.

Animals in Saudi Arabia are immunized with vaccines containing an Asia 1 omponent and this may have suppressed outbreaks of this serotype. However, he danger of outbreaks due to this Asia 1 strain could increase if it were to spread o areas where an Asia 1 strain is not included in the vaccine used to control lisease, as is the case in North Africa where outbreaks of type O have recently een extensive.

Demonstration of the phenomenon of dual infections in FMD samples from

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cattle in endemic areas shows the need for further virological investigation when diagnostic laboratories find cross-reactivity in serotyping tests such as the ELISA. This policy has now been adopted by the WRL.

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