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Subtyping of European Foot-and-Mouth Disease Virus Strains by Nucleotide Sequence Determination

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The VP1-coding regions of foot-and-mouth disease virus strains from 18 recent European outbreaks and of 9 strains isolated more than 20 years ago and used in part as vaccines were determined by direct cDNA sequencing. Comparison of the sequences revealed that most of the isolated outbreak viruses are closely related to the vaccine strains used. Isolates from the Italian epizootic of 1984 to 1985 correspond, for example, to the vaccine strain A5 Parma 62; the outbreak in 1984 in Bernbeuren, Federal Republic of Germany, was induced by A5 Allier 60; outbreaks in 1982 in Funen, Denmark, and in Murchin, German Democratic Republic, were caused by O1 Lausanne 65. Viruses isolated during the 1983 Iberian epizootic show a close relationship to the vaccine strain A5 Allier 60 but were probably derived from another not yet identified vaccine strain from Spain. Only two minor outbreaks in the Federal Republic of Germany, A Aachen in 1976 and O Wuppertal in 1982, did not correspond to the classical European strains but were obviously introduced from outside. We suggest that nucleotide sequence analysis should be used as a standard method of diagnosis, because when compared with other techniques it more clearly reveals the origin and course of epizootics and offers the possibility of preventing further outbreaks.

Foot-and-mouth disease virus (FMDV) exists in seven immunologically different types, each of which can be subdivided into a number of subtypes. The typing of FMDV strains, which is a prerequisite for vaccination programs, is usually carried out by immunological and serological methods. Since the introduction of the complement fixation test (26) and the virus neutralization assay in baby mice or tissue culture (28), new subtypes have continued to be detected.

As outbreaks in Europe are now markedly reduced compared with the situation aproximately 20 years ago, strategies for the control of the disease should not only be concerned with vaccination programs and controlling outbreaks, but should also attempt to trace the possible origins of the infectious viruses in order to eradicate putative sources of the disease.

Defined antisera are necessary to differentiate among types and subtypes, and only variations in the coat proteins which lead to alterations in the antigenic determinants can be recognized. Diagnosis by monoclonal antibodies, which have been obtained in several laboratories in recent years, may be helpful in identifying known strains but is probably not applicable in the characterization of new outbreak strains. Even in combination with other diagnostic techniques, such as oligonucleotide fingerprinting (5) and isoelectric focussing of the viral proteins (10), it may, in many cases, not be possible to establish an exact subtype in a reasonable time.

To obtain definitive data on the relationships among individual outbreak strains, we analyzed the nucleotide sequence encoding the capsid protein VP1, which is mainly responsible for the serospecificity of the virus (18, 24). As shown by the results, it was not only possible to detect unexpected similarities among certain strains, but it was also possible to detect subtle differences among other strains that could not be detected serologically since they exist at the nucleotide level only and have no effect on the amino acid sequence. The result of this study is most intriguing; it turns out that almost all outbreaks in middle Europe in recent years derive from a few basic types closely related to strains which were isolated more than 20 years ago and have since been used for the production of vaccines.

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MATERIALS AND METHODS

Purification of FMDV strains and RNA extraction. The origins of the individual viral strains are given in Table 1. The viruses were grown on BHK cells and purified as previously described (24,25). The virus solution was extracted twice with neutralized 80% (wt/wt) phenol containing 0.12% (wt/wt) hydroxychinoline and once with 3 volumes of chloroform–4% (vol/vol) isoamyl alcohol. The RNA was precipitated by the addition of 2 volumes of ethanol and stored in this form in several aliquots at −30°C.

Sequence analysis of the VP1 region. Three oligonucleotides were synthesized by the phosphoamidite method (6) with an automated oligonucleotide synthesizer (380A9; Applied Biosystems) by using the chemicals and procedures recommended by the supplier. The oligonucleotides were selected complementary to sequences most conserved among different serotypes at distances of 200 to 300 nucleotides on the viral genome (Fig. 1B, underlined). Although these oligonucleotides are complementary to the O1K sequence and do not fit in all positions to the corresponding sequence in the A strains, cDNA synthesis worked equally well with both serotypes.

For cDNA synthesis, the oligonucleotides were end-labeled with polynucleotide kinase (Boehringer Mannheim Biochemicals) and [γ-^{32}P]ATP (3,000 mCi/μmol; Amersham Corp.) as described previously (15). Samples (3 pmol) of labeled oligonucleotide were incubated with approximately 10 μg of viral RNA in a volume containing 30 μl of 50 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 6 mM $MgCl_2$, 0.5 mM each of the four deoxynucleotide triphosphates, 10 mM dithiothreitol, and 10 U of avian myoblastosis virus reverse transcriptase (Life Sciences, Inc.) for 10 min at 20°C and

TABLE 1. Sources of the FMDV strains analyzed

Short name[a]	Isolation location (country code)[b]	Date (mo/yr)	Animal	Origin of virus sample (collection designation)	Outbreak	Vaccine strains	Reference
O strains							
O1 Kb	Kaufbeuren (D)	1/1966	Cattle	BFAV[c]	Epizootic 1965/1966	X	4
O1 Ls	Lausanne (CH)	1965		Strain collection, BFAV	Epizootic 1965/1966	X	16
O1 Aul	Aulendorf (D)			Strain collection, BFAV		X	
O1 BFS	British field strain 67 (GB)			Strain collection, BFAV	Epizootic 1967	X	18a
O1 A Vac	Austrian vaccine strain (A)			BVH[d]		X	
O Zh	Zusmarshausen (D)	10/1984	Pig	BFAV	Isolated outbreak		[e]
O Mu	Murchin (DDR)	3/1982		FLI Riems[f]	Epizootic 1982		3
O Fu I	Funen (DK)	3/1982		SVIVR Lindholm[g]	Epizootic 1982		[h]
O Fu II	Funen (DK)	1/1983		SVIVR Lindholm	Isolated outbreak		[i]
O Wupp	Wuppertal (D)	5/1982	Pig	BFAV	Isolated outbreak		23
O Th	Thalheim (A)	3/1981	Pig	BVH	Isolated outbreak		11
O Isr	Golan (IL)	4/1981	Sheep	IFFA[j] (O Israel 81)			[k]
O Wien	Vienna (A)	3/1975	Cattle	BVH	Isolated outbreak		[l]
O2 Norm	Normandie (F)	1949		Strain collection BFAV			9a
A strains							
A5 Ww	Westerwald (D)	1951		Behring Werke	Epizootic 1951/1952	X	
A5 Fr	France			WRL[m] (A5 France 1/68)		X	21
A5 Al	Allier (F)	1960		strain collection BFAV	Epizootic 1960	X	
A5 Pa	Parma (I)	1962		IZSLE[n]	Epizootic 1962	X	
A Mod	Modena (I)	11/1984		IZSLE	Epizootic 1984/1985		
A Sal	Salerno (I)	1/1985		IZSLE	Epizootic 1984/1985		
A Bb	Bernbeuren (D)	6/1984	Cattle	BFAV	Isolated outbreak		23
A Sp	Madrid (E)	3/1983		IFFA A 88	Epizootic 1983		
A Mur	Murcia (E)	3/1983	Cattle	Lab. Sobrino[o] (P 13/83)	Epizootic 1983		
A Val	Valladolid (E)	3/1983	Cattle	Lab. Sobrino (P 14/83)	Epizootic 1983		
A Ler	Lerida (E)	3/1983	Pig	Lab. Sobrino (P 17/83)	Epizootic 1983		
A Port	Portugal (P)	1983		WRL (por 1/83)	Epizootic 1983		
A Mor	Morocco (MA)	1983		WRL (mor 1/83)	Epizootic 1983		
A Aach	Aachen (D)	12/1976	Pig	BFAV	Isolated outbreak		2
A OstD	Ostdeutschland (DDR)	1947		Strain collection BFAV			13

[a] For abbreviations not defined in the text, see legends to Fig. 1 and 2.
[b] International index mark.
[c] BFAV, Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen, Federal Republic of Germany.
[d] BVH, Bundesanstalt für Virusseuchenbekämpfung bei Haustieren, Vienna, Austria.
[e] Office International des Epizootiques (O.I.E.) Information Epizootiques D 84/44/233.
[f] FLI Riems, Friedrich Loeffler Institut, Insel Riems, German Democratic Republic.
[g] SVIVR Lindholm, State Veterinary Institute for Virus Research, Lindholm, Denmark.
[h] Danish Veterinary Service, Copenhagen, Denmark.
[i] Danish Veterinary Service and State Veterinary Institute for Virus Research, March 1983.
[j] IFFA, Institut Francais de la Fièvre Aphteuse, Mérieux, Lyon, France.
[k] O.I.E., Monthly epizootic circular no. 413, May 1981.
[l] O.I.E., note d'information no. 338(u), 1975.
[m] WRL, World Reference Laboratory, Pirbright, United Kingdom.
[n] IZSLE, Instituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia, Brescia, Italy.
[o] Lab. Sobrino, Laboratorios Sobrino S.A., Olot, Spain.

then for 30 min at 42°C. The reaction was stopped by the addition of 2 μl of RNase (2 mg/ml, DNase free; Boehringer) and incubated for another 15 min at 42°C. After a short electrophoresis on 1% agarose, cDNA longer than 800 base pairs was eluted from the gel. This sizing was essential for clear results in the subsequent sequence analysis, especially if the RNA preparation contained larger amounts of degraded material. In some cases, only 5 to 10% of the total cDNA was used.

The nucleotide sequences were determined by the method of Maxam and Gilbert (15). For several viral strains we used a solid support for the cDNA during the chemical modification (19). This latter method is less time-consuming and resulted, in our hands, in a higher resolution of the sequencing runs. cDNA sequences primed with the same oligonucleotide were run in parallel on the gels for easier detection of single-base exchanges between individual strains. The dideoxy sequencing method (22) led to unsatisfactory results, presumably owing to RNase contamination and the high degree of secondary structure of the viral RNA.

Nucleotide sequences were processed by using the computer programs of Osterburg et al. (17).

RESULTS

Source of FMDV strains analyzed. The viral strains analyzed in this study (Table 1) are a representative spectrum of the outbreaks in the Federal Republic of Germany during recent years. Only outbreaks of serotypes A and O were observed, since Europe was free of type C for 5 years until the recent outbreak in Italy (spring 1986). Included in this study were strains from some recent outbreaks in other European countries. In addition, we analyzed several classical type A and O vaccine strains to determine the immunogenic status of the vaccines used. The results are given in Fig. 1 and 2 for the O and A strains, respectively. In the O

types, long stretches of the sequence are invariable among all viruses analyzed. Therefore, only codons are shown which carry an exchange when compared with O1 Kaufbeuren (O1 Kb). For easier comparison, the complete VP1-coding sequence of this strain (12) is given in Fig. 1B.

The virus strains were usually amplified by passaging in BHK cells. Owing to the rapid genetic drift of FMDV, this may lead to a heterogeneity in the virus population, as demonstrated, for example, in cloned cDNA of type O1 Kb (9, 12), type A12 (20), type A5 (27), and type C1 (F. Sobrino, E. L. Palma, E. Beck, M. Davilo, J. C. de la Torre, P. Negro, N. Villanueva, J. Ortin, and E. Domingo, Gene, in press). Since we used viral RNA for the sequence analysis and not cloned cDNA, heterogeneous mixtures of viral mutants could have caused ambiguous results in certain positions of the nucleotide sequences. Such positions are marked by dashes in Fig. 1 and 2. This does not, however, interfere with the overall conclusions concerning the relationships among viral strains which were revealed by the nucleotide sequences.

Vaccine strains. The types O1 Laussane 65 (O1 Ls), O1 Kb, O1 British field strain 67 (O1 BFS), and until 1984, O1 Aulendorf (O1 Aul) were used as the O component for FMDV vaccines in the Federal Republic of Germany. We compared the nucleotide sequence of strain O1 Kb which was derived from highly passaged virus (64 tissue culture passages) with the primer-extended cDNA sequence from RNA isolated after passage 7 and found no nucleotide differences in the VP1-coding region, although the pathogenicity of the highly passaged virus for suckling mice was drastically reduced (unpublished results). This is also true for codon 138, where a heterogeneity was found in cloned cDNA (12).

The type O vaccine strains O1 Kb, O1 Ls, and probably also O1 Aul were derived from the same epizootic, which spread in western Europe from south to north in 1965 and 1966. The strain O1 BFS, isolated in 1967 in the United Kingdom, differs in several codons from these three strains but shows close relationship with the South American virus O1 Campos (7; see, for example, codons 41 and 94 in Fig. 1). In the VP1-coding sequence, the four vaccine strains differ overall by 10 single-base exchanges. Most of these exchanges are present in only one of these strains and do not appear in the other strains. Characteristic reference points are codons 64 and 137 for O1 Kb; codon 120 for O1 Ls; and codons 41, 94, and 149 for O1 BFS. O1 Aul is very similar to O1 Ls, differing only in codon 177. None of these nucleotide exchanges altered the amino acid sequence of VP1, except for a variation in codon 137 in O1 Kb.

In addition to these strain from the Federal Republic of Germany, we analyzed the O vaccine strain used in Austria. This strain has exactly the same sequence as O1 BFS, as determined by Makoff et al. (14), and does not contain the nucleotide exchange in codon 133 that is present in the O1 BFS sample stored in the FMDV strain collection in Tübingen, Federal Republic of Germany.

The subtype O2 was a component of the Federal Republic of Germany vaccines until 1966 and is still used in eastern Europe and in the German Democratic Republic (3). We analyzed the sequence of strain O2 Normandie (O2 Norm) as an example of this subtype. The nucleotide sequence of this virus differs markedly from the O1 types, leading to several amino acid exchanges in VP1, which explains the different immunological features of the two strains.

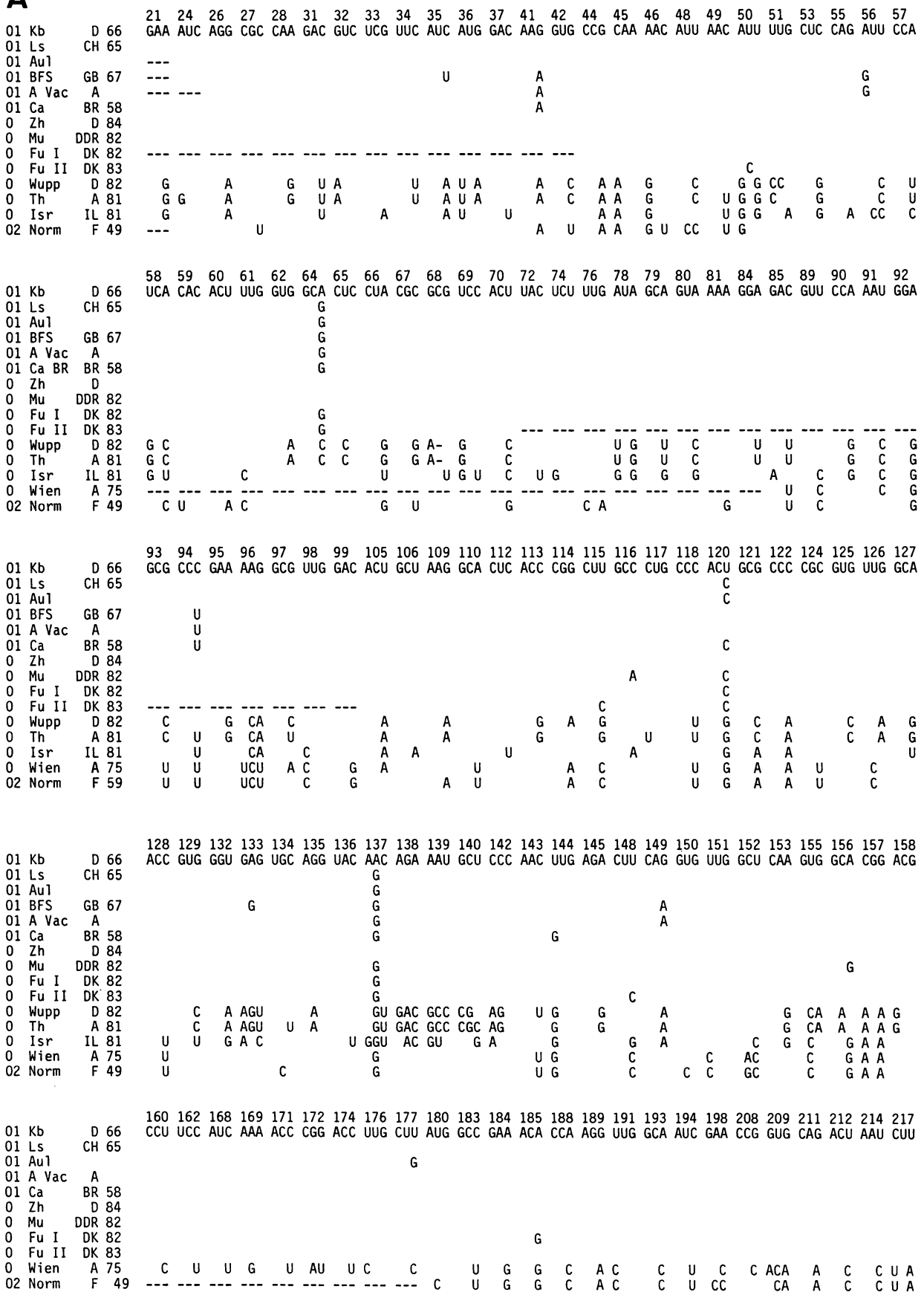
Most of the FMDV strains used as the A component in European vaccines belong to the subtype A5. The vaccine strain A5 Westerwald was isolated in 1951 during a large epizootic in the Federal Republic of Germany. This strain is clearly different from A5 Allier (A5 Al) that appeared in 1960 in France. A derivative of this strain, designated A5 France (A5 Fr), has been independently passaged since 1968 in different laboratories. The greatest deviation from the other type A5 vaccine strains is shown by A5 Parma (A5 Pa), which was isolated in 1962 in Italy. In the nucleotide sequence analyzed, the four strains differ from each other by 13 to 22 nucleotides each, except for A5 Fr and A5 Al which differ by only two nucleotides (Fig. 2, codons 145 and 203). The characteristic positions differing between these most common vaccine types are codons (underlined codons correspond to amino acid exchanges in VP1) 21, 62, 139, 149, 150, 170, and 210 for A5 Westerwald; codons 21, 35, 47, 88, 91, 139, 208, and 210 for A5 Fr and A5 Fl; and codons 21, 45, 50, 139, 140, 141, 147, 150, 178, 184, 210, and 212 for A5 Pa.

Type O outbreaks. The last reported outbreak of an FMDV O strain in the Federal Republic of Germany was in 1984 (O Zusmarshausen). According to our sequence analysis, the isolated virus is identical with the vaccine strain O1 Kb, except for one nucleotide exchange in codon 221 (GCA, eight codons downstream from the C terminus of VP1 [not shown in Fig. 1A]).

In the spring of 1982 in the German Democratic Republic and in Denmark, two serious outbreaks of the disease occurred simultaneously. The serological classification of subtype O1 (Danish Veterinary Service, Copenhagen, Denmark, 1982) was confirmed in both cases by the nucleotide sequence of three representative isolates, O Murchin (German Democratic Republic) and O Funen I (Denmark) and, from a later outbreak in January 1983, O Funen II. Apart from a few random exchanges, the three isolates are identical to the vaccine strain O1 Ls. Compared with this strain, O1 Murchin has exchanges in codons 64, 116, and 156; O Funen I has only one exchange (codon 185); and O Funen II has exchanges in codons 50, 115, and 148. Only one exchange in O Funen II (codon 50) results in an altered amino acid residue. In the three positions 64, 120, and 137 which are typically different between vaccine strains O1 Ls and O1 Kb, the sequences of the outbreak strains correspond to O1 Ls, except for codon 64 of O Murchin.

In 1982 another minor type O outbreak occurred in a pig breeding farm in Wuppertal, Federal Republic of Germany (O Wuppertal). According to the analysis of the World Reference Laboratory, Pirbright, United Kingdom, (information sheet 34, 1982), this strain is not subtype O1 but is closely related to a strain of an outbreak in Austria in March 1981 (O Thalheim). A serological relationship was also detected with a virus isolated in April 1981 in Israel (O Israel; M. Lombard, Institut Francais de la Fièvre Aphteuse, Merieux, communication 81/07/550, 1981). Analysis of the nucleotide sequences revealed that these three strains are indeed closely related and different from subtype O1. They show, however, a number of nucleotide exchanges with each other, leading to several amino acid alterations in the analyzed region between codons 20 and 150. These strains, therefore, do not descend directly from one another, and a connection between the outbreaks seems to be unlikely. The strains may, however, have a common origin.

We also analyzed the sequence of the last type O outbreak before 1981 in Austria (O Wien, 1975) to see whether this strain could be correlated with O Thalheim. This is clearly not the case, as indicated by numerous base exchanges. This virus obviously belongs to the subtype O2, as can be seen from the comparison with O2 Norm.


A

B

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1   [ACC ACU UCU GCG GGC GAG UCA GCG GAU CCU GUC ACC ACC ACC GUU GAA AAC UAC GGU GGC GAA ACA CAG AUC CAG    25
26   AGG CGC CAA CAC ACG GAC GUC UCG UUC AUC AUG GAC AGA UUU GUG AAG GUG ACA CCG CAA AAC CAA AUU AAC AUU    50
51   UUG GAC CUC AUG CAG AUU CCA UCA CAC ACU UUG GUG GGA GCA CUC CUA CGC GCG UCC ACU UAC UAC UUC UCU GAC    75
76   UUG GAG AUA GCA GUA AAA CAC GAG GGA GAC CUC ACC UGG GUU CCA AAU GGA GCG CCC GAA AAG GCG UUG GAC AAC   100
101  ACC ACC AAC CCA ACU GCU UAC CAC AAG GCA CCA CUC ACC CGG CUU GCC CUG CCC UAC ACU GCG CCC CAC CGC GUG   125
126  UUG GCA ACC GUG UAC AAC GGU GAG UGC AGG UAC AAC AGA AAU GCU GUG CCC AAC UUG AGA GGU GAC CUU CAG GUG   150
151  UUG GCU CAA AAG GUG GCA CGG ACG CUG CCU ACC UCC UUC AAC UAC GGU GCC AUC AAA GCG AGC CGG GUC ACC GAG   175
176  UUG CUU UAC CGG AUG AAG AGG GCC GAA ACA UAC UGU CCA AGG CCC UUG CUG GCA AUC CAC CCA ACU GAA GCC AGA   200
201  CAC AAA CAG AAA AUU GUG GCA CCG GUG AAA CAG ACU UUG]AAU UUU GAC CUU CUC AAG UUG GCG GGA GAC GUC GAG   225
226  UCC AAC CCU GGG CCC UUC UUU UUC UCC GAC GUU AGG UCG AAC UUC UCC AAA CUG GUG GAA ACC AUC AAC CAG AUG   250
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FIG. 1. (A) Comparison of the VP1-coding sequences of the FMDV O strains. The upper sequence corresponds to the nucleotide sequence of strain O1 Kb (12). The codons are numbered according to their positions in the VP1 gene. For the other strains, only nucleotides different from this sequence are shown. As long stretches of the sequence are invariable for all viruses analyzed, only the codons which carry an exchange in comparison with O1 Kb are shown. Regions not analyzed and uncertain positions are indicated by dashes. Abbreviations not defined in the text: O1 A Vac, O1 Austrian vaccine; O1 Ca, O1 Campos (7); O Zh, O Zusmarshausen; O Mu, O Murchin; O Fu, O Funen; O Wupp, O Wuppertal; O Th, O Thalheim; O Isr, O Israel. For an easier comparison, the complete VP1-coding sequence is shown in panel B. The locations of VP1 and of the primer oligonucleotides used for sequence analysis are indicated by brackets and lines, respectively.

Type A outbreaks. Two isolates from the most recent European type A outbreaks in Italy from 1984 to 1985 were analyzed, one isolate from the beginning of the epizootic in the north (A Modena, November 1984) and the other isolate from after the disease had spread to the south (A Salerno, January 1985). The two sequences differ from each other by five nucleotides. In each of these five positions, one of the two strains is identical with the vaccine type A5 Pa, except for codon 31 in which both outbreak strains differ from the vaccine strain. On the other hand, the three Italian strains all differ by 12 codons from the other European type A strains (codon 21, 45, 50, 139, 140, 141, 147, 150, 178, 184, 210, and 212).

Another outbreak strain was isolated some months earlier in 1984 in Bavaria, Federal Republic of Germany (A Bernbeuren). It does not show similarities to the Italian strains but is remarkably related to the vaccine strain A5 Al, differing in only three positions (codons 100, 144, and 210). In codons 145 and 204, which are characteristic for the difference between A5 Fr and A5 Al, A Bernbeuren is identical with A5 Al. A Bernbeuren differs much more from the other two A5 vaccine strains.

Several outbreaks in Spain, Portugal, and Morocco in 1983 represent another A5 complex which is clearly different from the strains isolated in Italy and the Federal Republic of Germany. We analyzed strains A Murcia, A Valladolid, and A Lerida, which were isolated within a period of 13 days in widely separated locations in Spain; A Spain, which originates from the Madrid region; and strains A Portugal and A Morocco, which were also isolated in the spring of 1983. The dates and places of the isolation of the latter two viruses are not exactly known. This group shows homologies to the vaccine strains A5 Fr and A5 Al in several characteristic positions (e.g., codons 47, 91, 139, 204, 208, and 210). There are, however, some common specific differences (positions 35, 76, 88, and 151), which speak against a direct connection between the outbreaks and these vaccine strains. The Iberian strains also differ clearly from the 1984 outbreak in Bernbeuren, Federal Republic of Germany, by several codons (78, 88, 100, 144, 204, and 210), which speaks against the suspected introduction of the disease from Spain to Bavaria.

The only outbreak of an A strain in the Federal Republic of Germany in the past 10 years which cannot be attributed to subtype A5 was in 1976 in Aachen. It is clear that the virus is not related to the European outbreak strains but rather to the extra-European A types whose VP1-coding sequence, as far as is known, is included in Fig. 2 downstream from codon 125 (see, for example, codons 157, 162, 168, 182, 183, 190, 202, 203, and 213). The best homology was found with A22 and A Venceslau. There are, however, many nucleotide differences, so that a direct lineage has to be excluded.

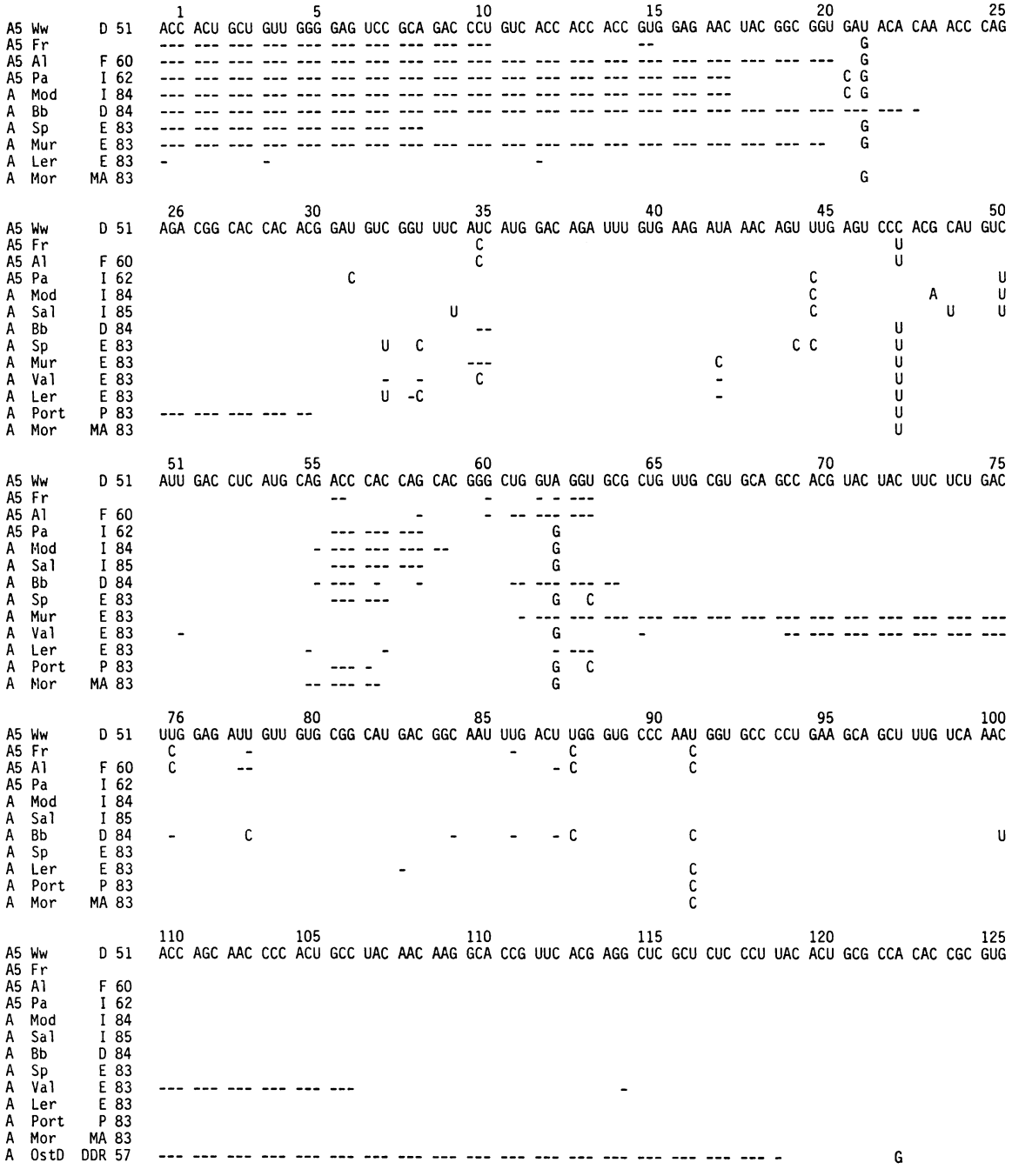
Finally, we analyzed a very old isolate contained in the strain collection in Tübingen, Federal Republic of Germany, which was derived from an outbreak in 1947 in the German Democratic Republic (A Ostdeutschland), since it was classified as subtype A5 (13). The sequence indicated a much closer relationship to the other analyzed A types than to A5, which makes the former classification highly questionable.

DISCUSSION

Successful control of FMD in Europe and other parts of the world requires that the sources from which the infection is spreading can be traced. This means that the diagnosis has not only to discriminate among serotypes and subtypes but also among different variants of a given subtype. The new diagnostic technique described here, i.e., the comparison of the primary genetic structures, allowed a much more precise evaluation of the degree of relationship among viral strains than do the serological and physicochemical methods used up to now. As shown by the results, it was possible to differentiate among vaccine strains derived from the same outbreak and containing none or only very few amino acid exchanges in the genomic region analyzed.

By using synthetic primer oligonucleotides, the results could be obtained within 1 week with RNA isolated from approximately 1 mg of purified virus. Previous knowledge of the specific serotype is helpful, although not absolutely necessary, for the selection of the correct primer oligonucleotides.

For mixed virus populations, as must be expected from field isolates, only the predominant type can be identified if no virus plaque purification has been performed. However, as the predominant component is expected to determine the immunogenic effect, knowledge of its sequence is more essential than knowledge of the sequence of an accidentally isolated clone.



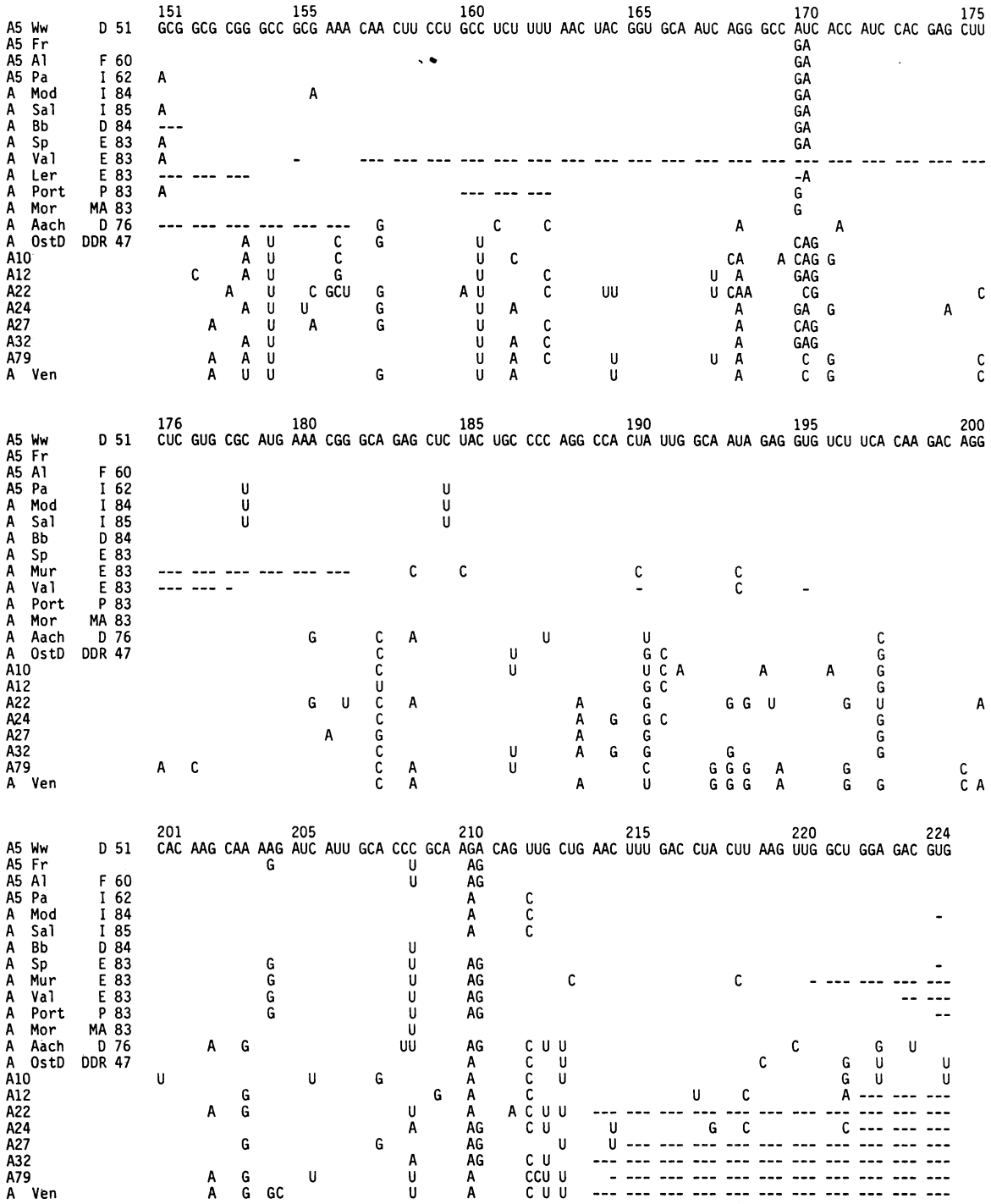


FIG. 2. Comparison of the VP1-coding sequences of the FMDV A strains. The upper sequence corresponds to A5 Westerwald. The previously reported sequence of this strain (1) has been corrected in codons 28, 29, 30. Base exchanges in the sequences of the analyzed strains compared with this sequence are indicated. Regions not analyzed and uncertain positions are indicated by dashes. Abbreviations not defined in the text: A5 Ww, A5 Westerwald; A Mod, A Modena; A Sal, A Salerno; A Bb, A Bernbeuren; A Sp, A Spain (IFFA A 88); A Mur, A Murcia; A Val, A Valladolid; A Ler, A Lerida; A Port, A Portugal; A Mor, A Morocco; A Aach, A Aachen; A OstD, A Ostdeutschland; A Ven, A Venceslau. The sequences of A10, A12, A22, A24, A27, A32, A79, and A Ven downstream from codon 126 are from reference 27.

The practicability of sequence analysis to characterize viral strains is already evident in the comparison of the vaccine strains. The fact that A5 Fr originated from A5 Al or that the Austrian vaccine strain originated from O1 BFS can clearly be seen, although the strains have been cultivated in different laboratories for many years. The detected relationship of O1 BFS with O1 Campos confirms earlier British suspicions that the infection was being introduced from overseas, possibly by frozen lamb imports from Argentina (18a). The relationship between O1 BFS and O1 Campos is

obvious, although there are nucleotide differences in a few positions. Owing to the rapid genetic drift of FMDV, alterations in the nucleotide sequence may occur continuously and differences among strains are therefore to be expected, although these strains derive from the same origin but have been passaged under different cultivation conditions.

It was thus all the more surprising that the virus of the last recorded occurrence (1984) of FMD in the Federal Republic of Germany (O Zusmarshausen) displayed exactly the same sequence as strain O1 Kb except for one nucleotide. The circumstances of this outbreak and its diagnosis speak for an infection by a laboratory strain adapted to replication in tissue culture rather than by a virulent field strain. A possible origin of this infection could not be detected. The O component of the vaccine used in this region in spring 1984 was strain O1 Aul, which can clearly be distinguished from O1 Kb.

Two preceding outbreaks of subtype O1 in Europe occurred two years before in Murchin in the north of the German Democratic Republic and simultaneously on the island of Funen in Demark. The comparison of the VP1 sequences of strains O Murchin and O Funen I and II shows that these two outbreaks were not independent of one another, and it is evident that these strains are very similar to the vaccine strain O1 Ls. It cannot be concluded from the sequence data whether the disease spread from one country to the other or whether both outbreaks derived from a common origin. In the German Democratic Republic, the last vaccination had been applied 6 months before with subtype O2, but this vaccine could obviously not prevent the outbreak. In Denmark, vaccination against FMDV is prohibited, and the country had been free of the disease for 12 years. The last European outbreak of the detected subtype occurred in 1981 in northern France, spreading later to the British Channel Islands (10). It is worth noting that the two outbreaks occurred while vaccination was being carried out in several European countries. Animals in the province of the Federal Republic of Germany neighboring Denmark were also vaccinated at that time, with vaccines including O1 Ls.

Our results clearly show that the European type A outbreaks in Italy from 1984 to 1985, in the Federal Republic of Germany in 1984, and on the Iberian Peninsula in 1983 (together with an outbreak in Morocco in 1983) were independent of each other and stemmed from separate sources. The two Italian outbreak strains are de facto identical to the vaccine type used (A5 Pa). From its antigenic structure, the vaccine strain should be highly protective against the virus causing the epizootic. This close relationship, on the other hand, raises the question as to whether the vaccine or the production plant is causally involved in the outbreak. Since vaccination in Italy is carried out from the autumn until the end of the year, a temporal coincidence cannot be disregarded.

The sequence of the virus from the outbreak in the Federal Republic of Germany 6 months before (A Bernbeuren) shows a remarkable similarity to that of the vaccine strain A5 Al, including the codons 145 and 204, in which this strain differs from A5 Fr. In the area of the outbreak, a Formalin-inactivated Frenkel vaccine was used a few months before which contained A5 Al as the A component. The trace of infections could be reconstructed up to the time of vaccination (23), so that the vaccine itself as the origin of infection is highly probable.

The sequences of the Spanish and Moroccan outbreak strains are in some characteristic positions identical with A5 Al and A5 Fr, but in another set of codons, the sequences are clearly different from these two and from the other analyzed vaccine strains. Several vaccine production plants provide the Iberian Peninsula with FMDV vaccines. Unfortunately, we could not obtain any of the corresponding vaccine strains for sequence analysis.

The only two outbreaks in the Federal Republic of Germany in recent years which are newly acquired strains are A Aachen and O Wuppertal. In both cases, the viruses were detected in pigs fed with leftovers from restaurants, which corresponds to a route of infection which would be expected for an imported disease. A Aachen is an independent strain with a VP1-coding sequence not previously observed. It shows similarities with strains from the Middle East (A 22) and South America (A Venceslau) (8) but has no identity with any known sequences.

The VP1 sequence of O Wuppertal differs in many positions from the O1 subtype. Interestingly, there was an outbreak, under similar circumstances, of an almost identical strain in Austria 1 year before (O Thalheim). The viruses revealed a very low pathogenicity, as most of the animals from both farms did not show clinical symptoms (11). As there are no indications of a direct connection between these two outbreaks, it must be assumed that the viruses were probably introduced independently from the same outside origin. A suspected descent from O Israel can be excluded, although a certain similarity exists at the nucleotide level. There is more nucleotide sequence homology to the O2 subtype than to O1. The virus from the previous type O outbreak in Austria, O Wien 1 exhibits a striking similarity to the strain O2 Norm. The low virulence of O Thalheim as well as the sequence homology between O Wien and O2 Norm suggests in both cases a descent from type O2 vaccine strains, which according to our information, are used in eastern European countries.

In summary, we found that of 18 isolates from recent FMD outbreaks, 14 were induced by a few types which have existed endemically in Europe for more than 20 years and are used for the production of vaccines. In general, close relationships among the outbreak strains and the vaccines used in the corresponding regions are obvious. A direct connection between a vaccine and an outbreak could be demonstrated when, in addition to the outbreak strain, the corresponding vaccine virus component used in the area was analyzed, as, for example, in the case of A Bernbeuren.

This finding confirms the results of an epizootiological investigation of Strohmaier and Böhm (23) on the origins of FMD outbreaks in the Federal Republic of Germany. In this study, 16 of 27 cases of primary outbreaks between 1970 and 1983 are classified as being derived from improperly inactivated vaccine charges and three further outbreaks, for which the source of infection was not evident, occurred during the vaccination period in the area. Four outbreaks occurred in the vicinity of commercial vaccine plants. All vaccines causing FMD outbreaks were Formalin-inactivated, except for one. This holds also for the outbreak in Bernbeuren. In the meantime, such vaccines are no longer licensed in the Federal Republic of Germany.

From the data presented, it is clear that most of the European outbreaks are "homemade" and not introduced from outside. Many of them are probably induced by vaccines, especially if Formalin-inactivated charges are used. To eradicate and not just control FMD in Europe, the first objective must be to prevent the use of Formalin-inactivated vaccines. It would, however, be shortsighted to change only the protocols of vaccine production. In many cases, the

infectious particles could have directly escaped from the vaccine production plants. Therefore, it is important to impose stricter precautions in handling the virus in vaccine production and in laboratories working with viable viruses.

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