JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1995, p. 440–444 Vol. 33, No. 2
0095-1137/95/$04.00+0

Rapid Detection and Characterization of Foot-and-Mouth Disease Virus by Restriction Enzyme and Nucleotide Sequence Analysis of PCR Products

F. LOCHER,† V. V. S. SURYANARAYANA,‡ AND J.-D. TRATSCHIN*

Institute of Virology and Immunoprophylaxis, CH-3147 Mittelhäusern, Switzerland

Received 7 April 1994/Returned for modification 15 August 1994/Accepted 19 October 1994

Reverse transcription coupled with PCR was used for the detection of foot-and-mouth disease virus serotypes A, C, and O in organ extracts from experimentally infected cattle. Primers were selected from conserved sequences flanking the genome region coding for the major antigenic site of the capsid located in the C-terminal part of viral protein 1 (VP1). Because this region of the capsid is highly variable its coding sequence is considered to be the most appropriate for the characterization of virus isolates and, therefore, for the determination of the epidemiological relationships between viruses of the same serotype. For differentiation between serotypes and for detailed characterization of individual virus isolates restriction enzyme cleavage and nucleotide sequence analysis of the respective PCR products were carried out. In order to minimize the time required for sample preparation from clinical material, viral RNA was released from particles by heating the sample for 5 min at 90°C. Finally, an air thermocycler was used, which allows performance of a PCR of 30 cycles in approximately 20 min. The results show that reverse transcription PCR followed by restriction enzyme analysis and/or nucleotide sequence analysis of the PCR products is useful for the rapid detection and differentiation of foot-and-mouth disease virus.

Foot-and-mouth disease viruses (FMDVs) or aphthoviruses are picornaviruses which cause a highly contagious and economically important disease of cloven-hoofed animals. There are seven serotypes of the virus, known as serotypes A, O, C, SAT1, SAT2, SAT3, and Asia1. Type A, C, and O viruses are also referred to as the European serotypes. In Europe foot-and-mouth disease has been successfully controlled for several decades by extensive vaccination of the cattle population, but in 1991 the European Community as well as other European countries including Switzerland switched to a policy of nonvaccination. Consequently, in the case of future outbreaks, infected as well as contact animals will be slaughtered in order to eradicate the disease. Recently, this approach has been applied in Italy, when an epizootic comprising a total of 54 outbreaks occurred between February and April 1993 (1, 9a). In order to confine the spread of foot-and-mouth disease in any outbreak a reliable and rapid laboratory diagnosis of the disease is very important. Also, the immediate determination of the specific serotype of field viruses is required in order to administer emergency vaccination with the appropriate antigen. Finally, the characterization of virus isolates at the nucleotide level and comparison with the nucleotide sequences of strains collected in previous outbreaks are useful in order to track the origin of a disease outbreak.

Several reports on the rapid detection and differentiation of FMDV isolates by reverse transcription PCR (RT-PCR) with primers which allow amplification of sequences within the gene coding for the viral RNA polymerase gene have been published recently (8, 11, 16). Differentiation was carried out in a separate PCR with serotype-specific primers derived from sequences coding for the structural protein VP1 (8, 16).

Here we report on amplification by PCR of the highly variable sequences coding for the C-terminal part of capsid protein VP1 by using primers which recognize FMDV serotypes A, C, and O. This approach requires only one reaction for the detection of FMDV and then differentiation and characterization of virus isolates by restriction enzyme analysis and direct sequencing of the PCR-generated DNA. The RT-PCR procedure that we have applied is rapid and simple because samples prepared from clinical material can be used directly without prior RNA extraction (21).

MATERIALS AND METHODS

Virus samples. FMDV reference virus strains A5 Allier, C1 Vosges, and O1 Lausanne were grown in BHK-21 cells infected at a multiplicity of infection of 0.1 50% tissue culture infective dose per cell. Swine vesicular disease virus was replicated in PK15 cells. Virus was extracted by repeated freezing and thawing of infected cells and subsequent removal of the cell debris by centrifugation. The organ material used in the study consisted of frozen tongue epithelium derived from cattle experimentally infected with various FMDV isolates which have been collected at the Institute of Virology and Immunoprophylaxis since 1950. Virus was extracted by homogenizing 1 g of frozen material in 10 ml of phosphate-buffered saline (pH 7.5). Cell debris was removed by low-speed centrifugation, and the supernatant was collected for further analysis.

Primers. Three oligonucleotide primers, SV1, A2, and P1, were used. The sense primer SV1 (5′-GCGCCACACCGCGTGTTGG-3′; nucleotides 3337 to 3356) and the antisense primer A2 (5′-GCTTTGATTGCACCATAGTT-3′; nucleotides 3485 to 3466) are derived from conserved regions of the FMDV genome located within the 1A gene, which codes for the major structural protein VP1. The numbering used throughout this report refers to the sequence of strain O1K reported by Kurz et al. (7). Both primers were designed by comparison of the following published nucleotide sequences from serotypes A, C, and O: A5, Beck et al. (2); A10, Thomas et al. (19); A12, Robertson et al. (14); A22, Onishchenko et al. (12); C1, Villanueva et al. (20); C3, Gebauer et al. (3) and Piccone et al. (13); O1, Kurz et al. (7) and Makoff et al. (9); and O2, Krebs et al. (6). Because the primers were selected from genomic sequences which were highly conserved between the viruses compared, they were expected to detect most if not all FMDVs of serotypes A, C, and O. The second antisense primer, P1 (5′-GAAGGGCCCAGGGTTGGACTC-3′), has been described by Höfner et al. (5). It is derived from the highly conserved 2A region and corresponds to nucleotides 3669 to 3649 of the FMDV genome. With the primer pair SV1-A2

* Corresponding author. Phone: (41) 31 848 93 48. Fax: (41) 31 848 92 22. Electronic mail address: jdt@ivi.ch.

† Present address: Drug Safety Assessment, Toxicology, Sandoz Pharma Ltd., CH-4002 Basel, Switzerland.

‡ Present address: Indian Veterinary Research Institute, Bangalore 560024, India.

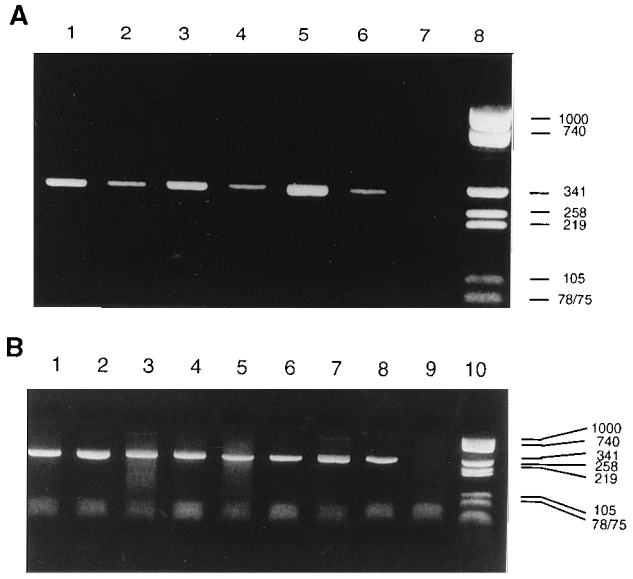

FIG. 1. RT-PCR with primer pair SV1-P1 performed in the water bath thermocycler. Fragment sizes (in base pairs) are indicated on the right. Lanes 7 (A) and 9 (B), supernatant of PK15 cells infected with swine vesicular disease virus as a negative control; lanes 8 (A) and 10 (B), *Sau*3AI-cleaved pBluescript II SK$^+$ as a size marker. (A) Supernatants from BHK-21 cells infected with FMDV serotypes A (lanes 1 and 2), O (lanes 3 and 4), and C (lanes 5 and 6) were diluted either 1:8 (lanes 1, 3, and 5) or 1:40 (lanes 2, 4, and 6). (B) Organ extracts derived from animals infected with FMDV isolates serotype C1 Tölz (lanes 1 and 2), C1 Sevelen (lanes 3 and 4), C1 Noville (lanes 5 and 6), and C1 Vosges (lanes 7 and 8) were diluted either 1:8 (lanes 1, 3, 5, and 7) or 1:40 (lanes 2, 4, 6, and 8).

PCR products of between 149 (O1) and 137 (C1) bp were expected, whereas the size of the fragment expected for the primer pair SV1-P1 was between 333 (O1) and 321 (C1) bp, depending on the virus serotype.

RT-PCR. To release the viral RNA, 5 μl of virus sample and 50 pmol of the reverse primer (A2 or P1) in a total volume of 40 μl of RT-PCR buffer (25 mM Tris-HCl [pH 8.4], 75 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM [each] deoxynucleoside triphosphate) were heated at 90°C for 5 min. cDNA synthesis was performed for 30 min at 37°C by the addition of 10 U of RNase inhibitor (Boehringer Mannheim, Indianapolis, Ind.) and 50 U of Moloney murine leukemia virus reverse transcriptase (BRL Life Technologies, Bethesda, Md.) in 5 μl of RT-PCR buffer. For the PCR 50 pmol of the reverse primer SV1 and 1.25 U of *Thermus aquaticus* DNA polymerase (Super Taq; Stehelin, Basel, Switzerland) in 5 μl of RT-PCR buffer were added and the samples were incubated for 30 cycles of amplification in a thermocycler. PCR products were analyzed by electrophoresis on 3% agarose gels (NuSieve 3:1; FMC BioProducts, Rockland, Maine) run in TBE buffer (100 mM Tris base, 50 mM boric acid, 2 mM EDTA). Gels contained ethidium bromide for visualization under UV light.

Thermocyclers. Two different types of programmable thermocyclers were used. In the water bath thermocycler (bio-med Thermocycler 60; Gesellschaft für Biotechnologie und Medizintechnik, Theres, Germany) the PCR conditions were the following: denaturation for 15 s at 95°C, annealing for 15 s at either 55 or 60°C, and elongation for 45 s at 72°C, with an increment of 1 s after each cycle. In the air thermocycler (Air Thermo-cycler 1605; Idaho Technology, Idaho Falls, Idaho) the samples (50 μl) were contained in glass capillaries and the reaction temperature was controlled with hot air (22). The same temperatures but shorter incubation times when compared with those used in the water bath thermocycler were used: 5 s each for denaturation and annealing and 20 s for elongation. In both thermocyclers PCR was carried out for 30 cycles.

Restriction enzyme analysis. The following restriction enzymes were used to analyze the DNA amplified by PCR: *Alu*I, *Apa*II, *Ban*I, *Bgl*I, *Bst*EII, *Dde*I, *Hph*I, *Nru*I, *Sac*I, and *Sca*I (New England Biolabs, Beverly, Mass.). Briefly, 5 μl of the PCR sample was digested for 2 h with 8 to 20 U of restriction enzyme in a total volume of 20 μl by using the appropriate restriction enzyme buffer and incubation temperature. Parallel reactions to which 1 μg of plasmid pBluescript II SK$^+$ DNA (Stratagene, La Jolla, Calif.) was added served as a control for proper cleavage by the restriction enzyme. Aliquots of 10 μl of each reaction mixture were separated on a 4% agarose gel as described above for PCR products, and the restriction patterns were visualized under UV light.

Nucleotide sequence analysis. To determine the nucleotide sequence, single-stranded DNA was synthesized from the primary PCR product in an asymmetric PCR and was subsequently sequenced (4) by the dideoxy termination method of

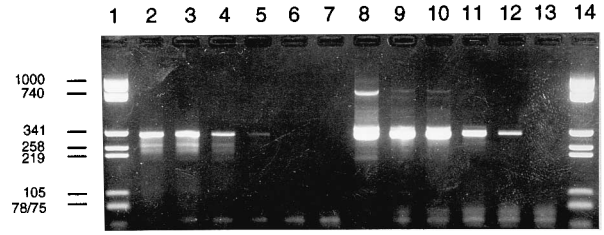

FIG. 2. Comparison of sensitivity and specificity of the PCR performed in the air thermocycler (lanes 2 to 7) or the water bath thermocycler (lanes 8 to 13). Serial dilutions of virus suspensions obtained by extraction of cell cultures infected with FMDV type C1 Vosges were used. PCR with the primer pair SV1-P1 was run for 30 cycles, and the samples were analyzed on a 3% agarose gel: lanes 2 and 8, 1:5; lanes 3 and 9, 1:40; lanes 4 and 10, 1:320; lanes 5 and 11, 1:2,560; lanes 6 and 12, 1:20,480; lanes 7 and 13, 1:163,840; lanes 1 and 14, *Sau*3AI-cleaved pBluescript II SK$^+$ as a size marker.

Sanger et al. (17). Briefly, the PCR product to be amplified asymmetrically was obtained by piercing the ethidium bromide-stained band in the agarose gel with a pipette tip and transferring the DNA molecules adhering to the tip into the reaction tube. PCR was carried out in the water bath thermocycler under the same conditions described above except that the respective primers were used at a ratio of 100:1 (200 and 2 nM). The number of cycles was 40, and the respective times and temperatures were 30 s at 95°C, 60 s at 50°C, and 90 s at 72°C. For sequencing a 20-μl sample of the asymmetric PCR was mixed with 1.2 pmol of ^{32}P-end-labelled primer (SV1, A2, or P1) and 1 U of *Taq* polymerase in a total volume of 30 μl of RT-PCR buffer. Aliquots of 7 μl were transferred into tubes containing 2.5 μl of each of the four termination mixes (A, G, C, T). The reaction mixtures were incubated for 5 min at 72°C, and the reactions were then stopped with 4 μl of stop solution containing formamide. Sequencing reactions were analyzed on 8% sequencing gels and were visualized by exposure of the wet gels to X-ray film.

RESULTS

Reverse transcription and amplification of FMDV RNA. To evaluate protocols for the extraction of viral RNA as well as for the PCR, FMDV obtained from BHK-21 cells infected with the respective reference viruses of serotypes A5, C1, and O1 were used. Swine vesicular disease virus grown in PK15 cells served as a negative control. RNA was not extracted separately from the virions. Instead, the release of viral RNA by heating, cDNA synthesis with reverse transcriptase, and PCR were carried out consecutively in a single tube. The PCR was performed in the water bath thermocycler. As shown in Fig. 1A (lanes 1 to 6), all three serotypes were detected with primer pair SV1-P1 when supernatants of cell extracts were used at dilutions of 1:8 or 1:40, respectively. Similar results were obtained when the primer pair SV1-A2 was used (data not shown). The same protocol was successfully applied for the detection of FMDV of all three serotypes in organ extracts (shown for viruses of serotype C in Fig. 1B, lanes 1 to 8).

Sensitivity and specificity of the RT-PCR. The sensitivity of the RT-PCR was analyzed by using primer pair SV1-P1 and serial eightfold dilutions of an extract with a titer of 10^6 50% tissue culture infective doses per ml which was prepared from BHK-21 cells infected with FMDV serotype C1 Vosges. For the PCR either the water bath or the air thermocycler was used. Because of the rapid heat transfer to and from the samples the latter type of PCR apparatus allows a significant reduction in the incubation time at each of the three temperatures. Also, because of the short transition times between individual temperatures a reduction in the amount of nonspecific products in the PCR was expected. The results of this experiment are shown in Fig. 2. The highest dilution giving a positive PCR result was 1:20,480 for the water bath thermocycler (lane 12). A band of comparable strength was obtained at a dilution of 1:320 for the air thermocycler (lane 4), corre-

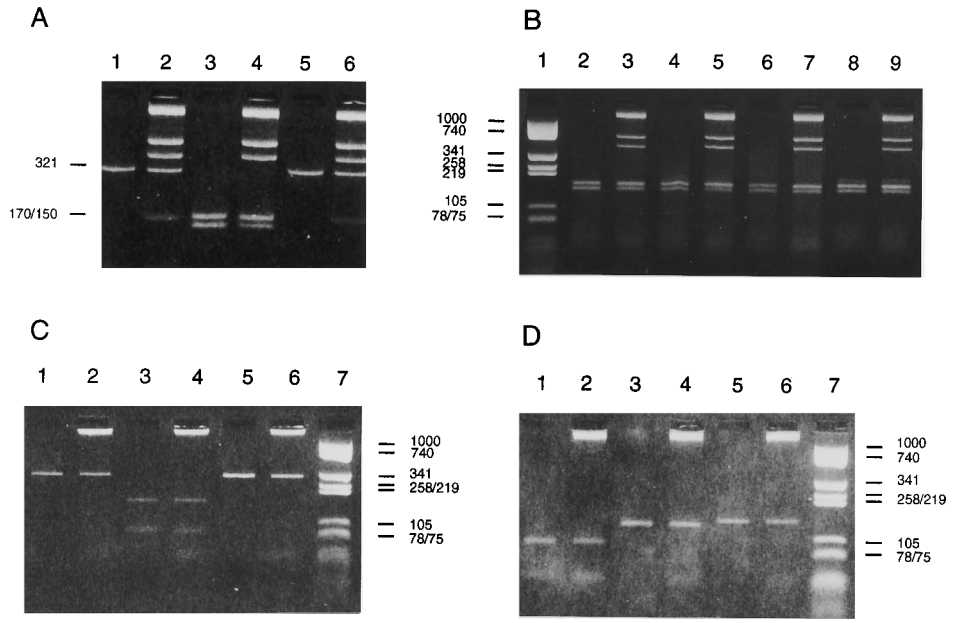

FIG. 3. Characterization of FMDV serotypes A, C, and O by restriction enzyme analysis of PCR products. Molecular size markers (B to D) or the approximate sizes of the PCR fragment and restriction products (A) are indicated. (A) PCR products obtained by amplification of RNA from reference viruses A5, C1, and O1 with primer pair SV1-P1 and cleaved with *Dde*I. Lanes 1 and 2, A5; lanes 3 and 4, C1; lanes 5 and 6, O1; lanes 2, 4, and 6, pBluescript II SK$^+$ DNA was added to the cleavage reaction. (B) PCR products obtained by amplification of RNA from four FMDV isolates belonging to serotype C1 and cleaved with *Dde*I. Lanes 2 and 3, strain Tölz; lanes 4 and 5, strain Sevelen; lanes 6 and 7, strain Noville; lanes 8 and 9, strain Vosges; lane 1, *Sau*3AI-cleaved pBluescript II SK$^+$ as a size marker; lanes 3, 5, 7, and 9, pBluescript II SK$^+$ DNA was added to the cleavage reaction. (C) PCR products obtained by amplification of RNA from reference viruses A5, C1, and O1 with primer pair SV1-P1 and cleaved with *Bst*EII. Lanes 1 and 2, A5; lanes 3 and 4, O1; lanes 5 and 6, C1; lane 7, *Sau*3AI-cleaved pBluescript II SK$^+$ as a size marker; lanes 2, 4, and 6, pBluescript II SK$^+$ DNA was added to the cleavage reaction. (D) PCR products obtained by amplification of RNA from reference viruses A5, C1, and O1 with primer pair P1-A2 were cleaved with *Sca*I. Lanes 1 and 2, A5; lanes 3 and 4, C1; lanes 5 and 6, O1; lane 7, *Sau*3AI-cleaved pBluescript II SK$^+$ as a size marker; lanes 2, 4, and 6, pBluescript II SK$^+$ DNA was added to the cleavage reaction.

sponding to a virus concentration of 0.25 and 16 50% tissue culture infective doses per reaction, respectively. Therefore, the sensitivity of the PCR performed in the air thermocycler was approximately 64-fold lower. In contrast, the specificity of the reaction performed in the air thermocycler was increased. This is indicated by the absence of DNA fragments longer than the expected 321 bp at virus dilutions of 1:5 to 1:320 when the PCR was done in the air thermocycler (lanes 2 to 4). PCR of similar virus dilutions in the water bath thermocycler resulted in the production of nonspecific high-molecular-weight DNA fragments (lanes 8 to 10).

Differentiation of serotypes A, C, and O by restriction enzyme analysis. The possibility of differentiating FMDV of serotypes A, C, and O by restriction enzyme analysis of the respective DNA fragments obtained by RT-PCR was evaluated. The published sequences listed in Materials and Methods were used to search for restriction enzymes which cleave between one and three times in the respective PCR products SV1-P1 or SV1-A2. After testing a total of 10 restriction enzymes, 3 of them (*Dde*I, *Bst*EII, *Sca*I) were selected. They proved to be suitable for the differentiation of the three serotypes (Fig. 3). Thus, the enzyme *Dde*I was found to cleave the serotype C-specific SV1-P1 PCR product into two fragments of approximately 150 and 170 bp (Fig. 3A, lanes 3 and 4). In contrast, the corresponding PCR products obtained from the A and O viruses were not cleaved by *Dde*I (Fig. 3A, lanes 1 and 2 and lanes 5 and 6, respectively). Figure 3B shows the same type of restriction analyses with *Dde*I with the SV1-P1 PCR products of four different C1 isolates, which all resulted in the same pattern characteristic for type C virus. Similarly, *Bst*EII exclusively cleaved the PCR fragment SV1-P1 generated from serotype O virus (Fig. 3C, lanes 3 and 4), and *Sca*I cleaved only the PCR fragment P1-A2 obtained from serotype A virus (Fig. 3D, lanes 1 and 2). In order to confirm proper cleavage by the restriction enzymes, we performed parallel reactions in which 1 μg of pBluescript DNA was added (lanes 2, 4, and 6 in Fig. 3A, C, and D and lanes 3, 5, 7, and 9 in Fig. 3B). *Dde*I is expected to cleave pBluescript DNA into four fragments of 1,846, 540, 409, and 166 bp, respectively. Restriction patterns corresponding to fragments of this size are shown in lanes 2, 4, and 6 of Fig. 3A and lanes 3, 5, 7, and 9 of Fig. 3B, indicating that complete cleavage has occurred. Cleavage of pBluescript DNA with *Sca*I generated a linear molecule of 2,961 bp which was too large to be resolved on a 4% agarose gel, as shown in Fig. 3D, lanes 2, 4, and 6. However, cleavage was confirmed in a separate analysis of these restrictions with a 1% agarose gel (data not shown). Finally, pBluescript DNA is not cleaved by *Bst*EII (Fig. 3C, lanes 2, 4, and 6) and therefore is not useful for testing for proper cleavage by this particular restriction enzyme. However, we have repeatedly shown that the SV1-P1 fragment obtained from type A and type C virus is not digested by *Bst*EII.

Characterization of FMDV isolates by nucleotide sequence analysis of PCR products. Determination of the nucleotide sequence of the PCR product is the most specific approach to the characterization of an individual virus isolate. In order to have these data available within the shortest time possible direct sequencing of the PCR products was applied. Synthesis of single-stranded DNA in an asymmetric PCR and subsequent sequencing by the dideoxy chain termination method proved to be the most reliable and convenient method. By sequencing from both ends of the fragment by using SV1 and

```
        1
        AACGGT*AGT *CAGGTACAG CAGAAATGCT GTGCCCAA** TGAGAGGTGA
O1 L.   ------G--- G--------- --------------------CT ----------
O2 B.   ------C--- C--------- --------------------TG ----------
O2 N.   ------G--- C--------- --------------------TG ----------
O2 S.   ------G--- C--------- --------------------TG ----------
        51
        CCT*CAGG*G *TG***CAAA AGG*GGC**G *ACGCTGCC* ACCTC*TTCA
O1 L.   ---T----T- T--GCT---- ---T---AC- G--------T -----C----
O2 B.   ---C----C- C--GAC---- ---C---GA- A--------C -----T----
O2 N.   ---C----C- C--AAC---- ---C---GA- A--------C -----T----
O2 S.   ---C----C- C--GAC---- ---C---GA- A--------C -----T----
        101
        ACTACGGTGC CAT*A*AGC* AC*C*GGTCA C*GAG*TGCT *TACCGGATG
O1 L.   ---------- ---C-A---G --C-G----- -C---T---- T---------
O2 B.   ---------- ---T-G---A --T-A----- -T---C---- C---------
O2 N.   ---------- ---T-G---A --T-A----- -T---C---- C---------
O2 S.   ---------- ---T-G---A --T-A----- -T---C---- C---------
```

FIG. 4. Partial nucleotide sequence of the PCR fragment SV1-P1 for viruses of serotype O1 Lausanne (L.), O2 Brescia (B.), O2 Normandie (N.), and O2 Spain (S.). Only the sequences corresponding to nucleotides 3367 to 3516 of the FMDV genome are shown. The region coding for the major antigenic site and corresponding to amino acids 138 to 160 of VP1 is underlined. The consensus sequence is given on top, stars indicate positions which are not identical in all four sequences, and boldface letters indicate nucleotides not conserved in the O2 viruses compared.

P1 as sequencing primers the complete nucleotide sequence except approximately 25 nucleotides at each end of the PCR fragment generated with primer pair SV1-P1 could be determined. This genome region codes for the major antigenic site of VP1, which is considered the most variable part of the viral capsid. To test the diagnostic value of such an analysis, reference strain O1 Lausanne was compared with three isolates of the subtype O2 (O2 Brescia, O2 Normandie, O2 Spain) extracted from organ material. One hundred fifty nucleotides of the SV1-P1 fragment including the region coding for the major antigenic site were aligned. As shown in Fig. 4, the O1 sequence was found to differ from the O2 sequences at a total of 22 positions. Inspection of the O2 isolates alone revealed only two single positions where the sequences were not identical in all three viruses.

DISCUSSION

The application of RT-PCR for the rapid diagnosis of FMDV has been described before (8, 11, 16). However, those investigators used primers selected from the viral RNA polymerase gene, which represents a highly conserved sequence of the FMDV genome for initial virus detection. Meyer et al. (11) were able to amplify a 454-bp fragment from the respective genes of all seven FMDV serotypes. For the differentiation of FMDV isolates serotype-specific primers which were derived from sequences coding for the highly variable carboxy-terminal end of the structural protein VP1 were used. By these means Rodriguez et al. (16) could differentiate a total of 15 FMDVs of serotypes A, C, and O.

Our goal was to establish a method which would provide for the diagnosis, differentiation, and characterization of FMDV in the shortest possible time. This was achieved by using a single primer pair, either SV1-P1 or SV1-A2, for detection of the virus and subsequent analysis of the respective PCR product by restriction enzyme cleavage and nucleotide sequence determination.

A protocol permitting very rapid amplification of viral cDNA by RT-PCR directly from clinical material, which we have successfully applied before to detect classical swine fever virus in organ samples (21), was used. In this procedure, release of RNA from the virus is obtained by heat denaturation, and furthermore, cDNA synthesis and PCR are performed in the same buffer. In addition, when an air thermocycler was used PCR could easily be performed in 20 min, resulting in a considerable reduction in the time required for laboratory diagnosis when compared with the time required to perform the procedures available so far. The specificity of the PCR proved to be superior for the air thermocycler (Fig. 2), and although the sensitivity was considerably lower, we did not encounter any problems in detecting virus in the organs of experimentally infected animals. Hence, we assume that the sensitivity reached in the air thermocycler is adequate for the diagnosis of FMDV in clinical specimens from acutely infected animals and therefore can be used at least for the initial screening of the samples.

Using the sense primer SV1 together with either of the antisense primers P1 or A2, we were able to amplify the expected DNA fragments from our reference viruses of serotype A5, C1, and O1 as well as from a series of viruses isolated from frozen organ material which was available in the laboratory (serotypes A5, C1, O1, and O2). These findings together with the fact that the primers used represent regions of the FMDV genome which, according to our alignment of published sequences, are highly conserved indicate that these primers are suitable for the detection of viruses of serotypes A, C, and O. However, the possibility that a given primer pair misses a particular virus isolate can never be excluded because a single point mutation at a critical site can prevent binding of the primer to the viral nucleic acid. Negative samples should therefore always be tested in a second PCR with different primers or by an alternative diagnostic method.

The genome region that we chose for amplification contains the sequence coding for the major antigenic site of the virus capsid located between amino acids 138 and 160 of VP1 (18). This site, which is exposed at the surface of the virion, is responsible for the induction of serum-neutralizing antibodies and represents the most variable part of the capsid (15). Considerable antigenic heterogeneity (10) as well as sequence variation (2) have been reported even between virus strains belonging to the same serotype. Thus, analysis of this region of the genome was expected to be suitable for the differentiation of FMDV isolates.

Restriction enzyme digestion of the DNA generated by PCR with selected endonucleases which generate fragment patterns diagnostic for a particular serotype does provide valuable information within hours after the PCR product is available. However, this method presumably does not always permit unambiguous classification of virus isolates because a single point mutation within the amplified sequence can either abolish an existing restriction site or generate a new restriction site. In contrast, determination of the nucleotide sequence of the PCR fragment which contains the sequence encoding the major antigenic site of VP1 is expected to provide the information required to unequivocally determine the serotype of an FMDV isolate. This sequence is contained in both PCR fragments that we amplified, but because SV1-P1 is almost twice as long as SV1-A2 determination of the sequence of this fragment is more likely to allow the differentiation of viruses which are more closely related, i.e., viruses of the same type or subtype. The example shown in Fig. 4 for different viruses of serotype O indicates that subtypes such as O1 and O2 can easily be differentiated even by comparison of a short sequence within the

genome region coding for the major antigenic site. In contrast, the sequences of three isolates of the same subtype (O2 Brescia, O2 Normandie, and O2 Spain) proved to differ only at two nucleotide positions, even in this region which is known to be highly variable. Although we do not know the exact origin of the O2 isolates compared in the present study these findings suggest that virus isolates of the same subtype might indeed be differentiated only by detailed sequence analysis.

The protocol presented here for the detection, differentiation, and classification of FMDV isolates is expected to be a very powerful tool in the case of a sporadic disease outbreak in which a rapid diagnosis is required in so that the appropriate measures can be taken.

ACKNOWLEDGMENTS

F. Locher was supported by the Swiss Federal Veterinary Office.

We are grateful to M. A. Hofmann, D. B. Mitchell, and N. Ruggli for support in the laboratory. We thank K. McCollough, K. Stärk, and H. K. Müller for helpful discussions and C. Griot and N. Ruggli for critical reading of the manuscript.

REFERENCES

1. **Anonymous.** 1993. Bulletin of the Office International des Epizooties (OIE) **105:**163, 235, 431.
2. **Beck, E., G. Feil, and K. Strohmaier.** 1983. The molecular basis of the antigenic variation of foot-and-mouth disease virus. EMBO J. **2:**555–559.
3. **Gebauer, F., J. C. de la Torre, I. Gomes, M. G. Mateu, H. Barahona, B. Tiraboschi, I. Bergmann, P. Augé de Mello, and E. Domingo.** 1988. Rapid selection of genetic and antigenetic variants of foot-and-mouth disease virus during persistence in cattle. J. Virol. **62:**2041–2049.
4. **Hofmann, M. A., and D. A. Brian.** 1991. Sequencing PCR DNA amplified directly from a bacterial colony. BioTechniques **11:**30–31.
5. **Höfner, M., W. C. Carpenter, and A. I. Donaldson.** 1990. Detection and identification of foot-and-mouth disease virus in clinical samples using the polymerase chain reaction (PCR) method: a rapid molecular biology technique of exquisite sensitivity. *In* Report to the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease, Lindholm, Denmark.
6. **Krebs, O., H.-G. Berger, and O. Marquardt.** 1991. The capsid protein-encoding sequence of foot-and-mouth disease virus O2 Brescia. Arch. Virol. **120:**135–143.
7. **Kurz, C., S. Forss, H. Küpper, K. Strohmaier, and H. Schaller.** 1981. Nucleotide sequence and corresponding amino acid sequence of the gene for the major antigen of foot and mouth disease virus. Nucleic Acids Res. **9:**1919–1931.
8. **Laor, D., H. Torgerson, H. Yadin, and Y. Becker.** 1992. Detection of FMDV RNA amplified by the polymerase chain reaction (PCR). J. Virol. Methods **36:**197–208.
9. **Makoff, A. J., C. A. Paynter, D. J. Rowlands, and J. C. Boothroyd.** 1982. Comparison of the amino acid sequence of the major immunogen from three serotypes of foot and mouth disease virus. Nucleic Acids Res. **10:**8285–8295.

9a. **Maragon, S., E. Facchin, F. Moutou, I. Massirio, G. Vincenzi, and G. Davies.** 1994. The 1993 Italian foot-and-mouth disease epidemic: epidemiological features of the four outbreaks identified in Verona province (Veneto region). Vet. Rec. **135:**53–57.

10. **Mateu, M. G., J. L. daSilva, E. Rocha, D. L. deBrum, A. Alonso, L. Enjuanes, E. Domingo, and H. Barahona.** 1988. Extensive antigenic heterogeneity of foot-and-mouth disease virus of serotype C. Virology **167:**113–124.
11. **Meyer, R. F., C. C. Brown, C. House, J. A. House, and T. W. Molitor.** 1991. Rapid and sensitive detection of foot-and-mouth disease virus in tissues by enzymatic RNA amplification of the polymerase gene. J. Virol. Methods **34:**161–172.
12. **Onishchenko, A. M., N. A. Petrov, V. M. Blinov, S. K. Vassilenko, L. S. Sandakhchiev, A. N. Burdov, V. N. Ivanyushchenkov, and N. A. Perevozchikova.** 1986. The DNA-copy primary structure for the gene of the FMDV A-22 serotype VP1-protein. Bioorg. Khim. **12:**416–419.
13. **Piccone, M. E., G. Kaplan, L. Giavedoni, E. Domingo, and E. L. Palma.** 1988. VP1 of serotype C foot-and-mouth disease viruses: long-term conservation of sequences. J. Virol. **62:**1469–1473.
14. **Robertson, B. H., M. J. Grubman, G. N. Weddell, D. M. Moore, J. D. Welsh, T. Fischer, D. J. Dowbenko, D. G. Yansura, B. Small, and D. G. Kleid.** 1985. Nucleotide and amino acid sequence coding for polypeptides of foot-and-mouth disease virus type A12. J. Virol. **54:**651–660.
15. **Robertson, B. H., D. M. Moore, M. J. Grubman, and D. G. Kleid.** 1983. Identification of an exposed region of the immunogenic capsid polypeptide VP1 on foot-and-mouth disease virus. J. Virol. **46:**311–316.
16. **Rodriguez, A., E. Martinez-Salas, J. Dopazo, M. Davila, J. C. Saiz, and F. Sobrino.** 1992. Primer design for specific diagnosis by PCR of highly variable RNA viruses: typing of foot-and-mouth disease virus. Virology **189:**363–367.
17. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
18. **Strohmaier, K., R. Franze, and K.-H. Adam.** 1982. Location and characterization of the antigenic portion of the FMDV immunizing protein. J. Gen. Virol. **59:**295–306.
19. **Thomas, A. A. M., R. J. Woortmeijer, W. Puijk, and S. J. Barteling.** 1988. Antigenic sites on foot-and-mouth disease virus type A10. J. Virol. **62:**2782–2789.
20. **Villanueva, N., M. Davila, J. Ortin, and E. Domingo.** 1983. Molecular cloning of cDNA from foot-and-mouth disease virus C-1-Santa Pau (C28). Sequence of protein-VP1-coding segment. Gene **23:**185–194.
21. **Wirz, B., J.-D. Tratschin, H. K. Müller, and D. B. Mitchell.** 1993. Detection of hog cholera virus and differentiation from other pestiviruses by polymerase chain reaction. J. Clin. Microbiol. **31:**1148–1154.
22. **Wittwer, C. T., C. G. Fillmore, and D. J. Garling.** 1990. Minimizing the time required for DNA amplification by efficient heat transfer to small samples. Anal. Biochem. **186:**328–331.