

# Identification of Foot-and-Mouth Disease Virus Serotype O Strains Using Lineage-Specific Reverse Transcription-Polymerase Chain Reaction

## Amplification Assays

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### ABSTRACT

Foot-and-mouth disease type O is the serotype most frequently isolated at the World Reference Laboratory for FMD (located at the IAH, Pirbright), accounting for approximately 60% of the FMD-positive samples. We routinely select virus isolates for sequencing studies to monitor the occurrence and movement of different strains. This enables us to predict potential threats to various parts of the world and to discover the origins of epidemics. However, the number of virus isolates we select is limited and when large numbers (>10) are received from any one country only a few can be studied. Therefore we have developed lineage-specific RT-PCR assays for FMD type O viruses to enable many virus isolates to be screened and any unusual ones identified for further sequencing studies. A highly variable part of VP1 (nt 395 to 422) was chosen as a basis for designing the upstream lineage-specific primers which were combined with a universal FMDV primer located in 2B (NK61). Three specific primers were initially designed and named Hk94, Cam94 and PanAsia. Twenty two FMD type O viruses isolated from cattle, pigs and water buffalo in Vietnam in 1999 were chosen for study. Viruses from this country were selected because i) at least two different viruses were known to present in that country from previous studies in 1997 (N.J. Knowles and A.R. Samuel, unpublished data); and ii) a large number of samples had been submitted. The results of the screening assays were as follows: two of the isolates (one from a cow and one from a pig) were amplified by the Cam94/NK61 primer set indicating that they belonged to a group of viruses endemic in south-east Asia since at least 1994; nine (all from pigs) were amplified by the Hk94/NK61 primer set showing that they belonged to a lineage present in Hong Kong since 1985 (and which had spread to the Philippines in 1994 and Taiwan in 1997), and nine (seven from cattle, one from a buffalo and one from a pig) were amplified by the PanAsia/NK61 primer set indicating that they belonged to a recently recognised pandemic strain which has spread throughout southern Asia since 1990. Two samples appeared to contain mixtures of viruses; one from a pig was amplified by both the Hk94/NK61 and Cam94/NK61 primer sets and the other from a cow was amplified by the PanAsia/NK61 and Cam94/NK61 primer sets. Sequencing of these products using an internal primer (NK72) confirmed the specificity of the PCRs. These primer sets are currently being evaluated with a wider range of FMD viruses. Additionally specific primers have been designed to amplify two other FMDV type O lineages currently circulating, one from Myanmar and one from West Africa, however, PCRs using these primer sets await validation.

### INTRODUCTION

Foot-and-mouth disease (FMD) is an economically important disease of cloven-hoofed animals, principally affecting cattle, water buffalo, pigs, sheep and goats. It is caused by a small RNA-containing virus belonging to the family *Picornaviridae*, genus *Aphthovirus*. The species *Foot-and-mouth disease virus* (FMDV) is divided into seven immunologically distinct serotypes, O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1. These serotypes are not uniformly distributed geographically and occur with differing frequency. Serotypes O and A occur in much of South America, East and West Africa and southern Asia; type C occurs much more infrequently and usually only in South America and the Indian subcontinent; Asia 1 is endemic in the Indian subcontinent and south east Asia, but epidemics have occurred as far west as Greece and as far east as Hong Kong. The Southern African Territories (SAT) serotypes are normally restricted to sub-Saharan Africa, but SAT 1 has, on a number of occasions, caused epidemics in the Middle East. Serotype O is the most widespread and commonly occurring FMDV and accounts for approximately 60% of the viruses isolated each year by the World Reference Laboratory for FMD (WRLFMD). Since 1987, the WRLFMD has used nucleotide sequence analysis and phylogenetic reconstruction to study the epidemiology of FMDV. The recent pan-Asian spread of a type O FMD virus (see poster by N.J. Knowles and A.R. Samuel at this meeting) has led to the apparent disappearance of other type O strains, particularly in the Middle East, as judged by the sample of virus isolates examined by RT-PCR and nucleotide sequencing. However, as not all virus isolates are tested other strains may be missed. Another type O strain, which has been shown to be attenuated for cattle (Dunn and Donaldson, 1997) and was previously confined to China and Hong Kong, has in recent years spread to the Philippines, Taiwan and Vietnam (N.J. Knowles and A.R. Samuel, unpublished data). We have therefore developed strain-specific RT-PCRs to screen greater numbers of virus isolates and thereby target variants not detected by these primers for further study.

Total RNA was extracted either directly from epithelial suspensions or from cell culture grown virus using a commercial kit (RNeasy, Qiagen) and an RT-PCR was performed as described by Knowles and Samuel (1994) using primers ARS4 and NK61. The PCR amplicons were partially sequenced using a commercial kit (fmol, Promega) and an automated sequencer (ALFExpress II, Pharmacia) with Cy5-labelled primers NK72, 1D296F, 1C564F and ARS4. UPGMA trees were constructed using the PHYLIP package (Felsenstein, 1993). All these techniques are fully described in a laboratory manual (Knowles and Samuel, 1998) which is downloadable from our website:

<http://www.iah.bbsrc.ac.uk/virus/Picornaviridae/Aphthovirus/fmdv.htm>

Additional strain-specific primers, designed using alignments of sequences shown in Fig. 1, are shown in Table 1.

Table 1. Oligonucleotide primers used in this study.

Name	Primer sequence (5' to 3')	Length	Location		Specificity
			Gene	Nt. Position†	
NK72	GAAGGGCCAGGGTGGACTC	21	2AB	34-48/1-6	FMDV universal
NK61	GACATGTCTCTGCATCTG	20	2B	58-77	FMDV universal
ARS4	ACCAACCTCTTGTATGGCT	21	VP3	124-144	FMDV-O
O-1D395F	GGAAGTCCAAGTACGCTCAAG	21	VP1	395-416	Cam94
O-1D397F	AACCTGCAAGTAYGGCGAGARC	21	VP1	397-418	PanAsia
O-1D400F	AGYAAGTACGGYGRACCAGC	21	VP1	400-421	Hk94
O-1D400F2	TGTAGGTACAGTGGAGCTGTG	21	VP1	400-421	WestAfr99
O-1D401F	GCAAATACGCCGAGGGCYCAC	21	VP1	401-422	Mya98

\* Y = C and T, R = A and G  
† nucleotide position within the gene

Table 2. Results of ELISA, virus isolation and lineage-specific RT-PCR amplification assays.

WRLFMD Ref. No.	Species	Virus isolation		Main used for RT-PCR	RT-PCR / sequencing					Result	
		ELISA typing on ES	RTS		VP1	Seq	Hk94	PanAsia	Seq		Cam94
OVT/1/99	bovine	+	+	BTy1	++	+	-	-	+	+	Cam94
OVT/2/99	bovine	+	+	RS2	+	+	+	+	-	-	Hk94
OVT/3/99	bovine	+	+	RS2	+	+	+	+	-	-	Hk94
OVT/4/99	bovine	+	+	ES	-	+	-	-	-	-	Hk94
OVT/5/99	bovine	+	+	RS1	+	+	+	+	-	-	Hk94
OVT/6/99	bovine	+	+	RS1	++	+	+	+	+	+	Hk94/Cam94
OVT/7/99	bovine	+	+	ES	-	+	-	-	-	-	Hk94
OVT/8/99	bovine	+	+	ES	-	+	+	+	-	-	Hk94
OVT/9/99	bovine	+	+	ES	+	+	+	+	-	-	Hk94
OVT/10/99	bovine	+	+	ES	-	-	-	-	nd	nd	PanAsia
OVT/11/99	bovine	+	+	BTy1	-	-	-	-	-	-	PanAsia
OVT/12/99	bovine	+	+	ES	+	+	+	+	+	+	PanAsia
OVT/13/99	bovine	+	+	ES	+	+	+	+	+	+	PanAsia
OVT/14/99	bovine	+	+	ES	+	+	+	+	+	+	seq
OVT/15/99	bovine	+	+	ES	+	+	+	+	+	+	PanAsia
OVT/16/99	bovine	+	+	ES	+	+	+	+	+	+	PanAsia
OVT/17/99	bovine	+	+	ES	+	+	+	+	+	+	PanAsia
OVT/18/99	bovine	+	+	ES	-	-	-	-	-	-	Cam94
OVT/19/99	bovine	+	+	ES	-	+	+	+	+	+	Hk94
OVT/20/99	bovine	+	+	BTy1	++	+	+	+	+	+	PanAsia/Cam94
OVT/21/99	bovine	+	+	ES	+	+	+	+	+	+	PanAsia
OVT/22/99	bovine	+	+	BTy1	++	+	+	+	+	+	PanAsia
OVT/23/99	buffalo	+	+	ES	+	+	+	+	+	+	PanAsia
OVT/24/99	bovine	+	+	ES	++	+	+	+	+	+	Hk94

VP1 primer set ARS4/NK61  
Cam94, Vietnam 1997 bovine strain (primer set: 1D395F/NK61)  
PanAsia, virus pandemic strain (India-90) (primer set: 1D397F/NK61)  
Hk94, Hong Kong/China/Taiwan/Philippines 'pig-adapted' strain (primer set: 1D400F/NK61)  
ES: 1/10 epithelial suspension  
BTy: primary bovine thyroid cells  
RS: BRS-2, mouse kidney cell line  
\* non-specific band at 1.1 to 1.2 kb

### REFERENCES

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Knowles, N.J. and Samuel, A.R. (1998). 'RT-PCR and Sequencing Protocols for the Molecular Epidemiology of Exotic Virus Diseases of Animals'. Institute for Animal Health, Pirbright. <http://www.iah.bbsrc.ac.uk/virus/Picornaviridae/Aphthovirus/Protocol.pdf>

Fig. 1. Alignment of VP1 codons 132-150 of some recent FMD type O viruses. Strain-specific oligonucleotide primers designed from the variable region are shown in blue.

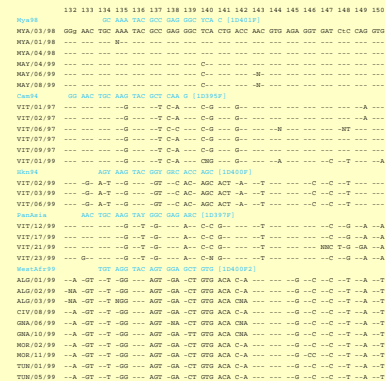
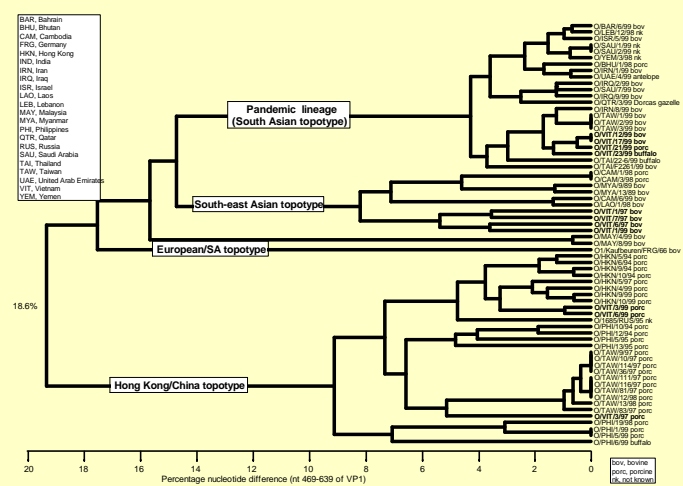


Fig. 2. Recent FMD type O viruses from northern Vietnam fall into three distinct topotypes



### RESULTS

Only three of the primer sets that we have designed have been evaluated so far, viz. PanAsia, Cam94 and Hk94. These lineage-specific RT-PCRs generally resulted in amplicons of a single size (data not shown). However, occasionally mis-priming resulted in bands larger or smaller than the correct product. Attempts at sequencing these bands, using an internal primer (NK72), were negative. Where isolates were positive with two of the specific primer sets, sequence analysis revealed the presence of two sequences. However, we cannot preclude the accidental contamination of samples prior to the RNA extractions, especially since samples may be batch-processed for serotyping and virus isolation when received together.

### CONCLUSIONS

Lineage-specific RT-PCR assays are useful for screening large numbers of FMDV isolates in order to confirm the presence of a single genetic lineage or to distinguish two or more variants active in a single geographical location. The primers used in these assays will have to change as the viruses evolve and will therefore have a finite life. Sometimes non-specific amplification may occur and the test should not be used without confirmation (at least on some isolates in a batch) by nucleotide sequence analysis. Similarly, where mixtures of viruses are indicated this should be confirmed by plaque cloning of each virus. Preliminary analysis of our FMDV sequence database suggests that type O virus may be classified by the sequence motifs in the region of the genome which we have used to design the specific primers. This is being investigated further.

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