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Enhanced laboratory diagnosis of foot and mouth disease by real-time polymerase chain reaction

A.E. Shaw, S.M. Reid, D.P. King, G.H. Hutchings & N.P. Ferris

Department of Exotic Disease Control, Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey GU24 0NF, United Kingdom

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Summary
The performance of an automated real-time reverse transcription polymerase chain reaction (RT-PCR) was compared to virus isolation (VI) in cell culture and antigen detection enzyme-linked immunosorbent assay (ELISA) for the laboratory diagnosis of foot and mouth disease (FMD). The World Reference Laboratory for FMD in Woking, the United Kingdom, examined a collection of 334 epithelia received from eighteen countries between August 2002 and January 2004. The results showed that all VI positive (n = 195) and VI and ELISA positive samples combined (n = 204) were also positive by RT-PCR. Depending on the cut-off used, FMD virus genome was detected in a minimum of an additional 60 samples (18% of all samples tested). Furthermore, the RT-PCR generated results in less than one day from test commencement in contrast to up to 4 days to define some positive and all negative samples by VI. The study demonstrates that real-time RT-PCR provides an extremely sensitive and rapid procedure for improved laboratory diagnosis of FMD.

Keywords
Diagnosis – Enzyme-linked immunosorbent assay – Foot and mouth disease virus – Rapidity – Real-time reverse transcription polymerase chain reaction – Sensitivity – Virus isolation.

Introduction

Foot and mouth disease (FMD) is caused by a virus of the *Aphthovirus* genus within the family *Picornaviridae* and is considered to be the most contagious virus of veterinary importance (2). There are seven FMD virus (FMDV) serotypes, namely O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1. Countries and zones within countries can be termed endemic, sporadic or free depending on the frequency of disease outbreaks. The disease causes serious economic losses due to reduction in productivity, mortality of young animals, the control measures required to curtail or eliminate the disease and the negative impact on exports of livestock and animal products.

Control of outbreaks is dependent upon a system of monitoring and early detection, which requires basic familiarity with clinical signs and the ability to characterise the strain of virus responsible by laboratory tests. The Institute for Animal Health, Pirbright Laboratory, hosts the Food and Agriculture Organization World Reference Laboratory (WRL) for FMD. The laboratory has the following remit:

– to investigate clinical specimens collected from outbreaks of vesicular disease to ascertain whether FMD or another vesicular virus is involved

– to characterise field virus isolates in order to select suitable vaccines for control

– to identify the possible origin of the disease, so that repetition of virus entry can be prevented

– to alert countries by providing information about the geographical location of outbreaks

– to assist other laboratories by supplying diagnostic reagents (4).

Definitive diagnosis of FMD requires the detection of virus, antigen or genome in clinical material. Ideally, the sample of choice should be vesicular epithelium from clinically affected animals since, during the acute stage of the disease, it is rich in virus. The WRL for FMD typically receives between 400 and 700 samples annually from overseas countries (Table I), including other sample types besides epithelia, e.g. epithelial suspensions, cell culture antigens, blood, throat swab (probang) and milk samples. For almost twenty years, the WRL for FMD has used an indirect, sandwich enzyme-linked immunosorbent assay (ELISA) (3, 7) to identify FMDV. However, the ELISA is not 100% sensitive. Consequently, suspensions of each specimen are also propagated in sensitive cell cultures (3) and the specificity of any isolated virus confirmed by the ELISA. Whilst such virus isolation (VI) methods are highly sensitive, they require four days before a negative result can be concluded (and reported as 'no virus detected' [NVD]). It is evident from Table I that FMDV antigen cannot be detected by ELISA and VI in approximately half the submitted samples. This has given cause for concern as to the efficiency of sample collection and dispatch and also with respect to the adequacy of the laboratory test procedures employed for their examination.

Recently, the development of a real-time reverse transcription polymerase chain reaction (RT-PCR) procedure has provided an additional tool which can be used for FMD diagnosis (9). Furthermore, this real-time RT-PCR method can be automated allowing increased throughput of samples with fewer user-dependent steps (10). The authors have compared the performance of a fully automated real-time RT-PCR (10) with VI and ELISA for the detection of FMDV on the majority of epithelium samples received at the WRL for FMD from overseas during a recent eighteen-month period (August 2002 until January 2004) and hereby report the findings.

Table I
Number of samples received by the World Reference Laboratory (WRL) for foot and mouth disease (FMD) from overseas between 1994 and 2003 and the numbers (and percentages) found to be positive or negative for FMD virus by passage in cell culture and antigen detection enzyme-linked immunosorbent assay

Year	Countries [a]	Number of samples		
		Total	Positive	Negative
1994	29	655	348 (53%)	307 (47%)
1995	29	697	421 (60%)	276 (40%)
1996	31	535	238 (44%)	297 (56%)
1997	29	526	334 (63%)	192 (37%)
1998	27	441	248 (56%)	193 (44%)
1999	43	595	357 (60%)	238 (40%)
2000	29	434	209 (48%)	225 (52%)
2001	32	619	197 (32%)	422 (68%)
2002	27	390	162 (42%)	228 (58%)
2003	19	475	262 (55%)	213 (45%)
Total		**5,367**	**2,776 (52%)**	**2,591 (48%)**

a) number of countries which submitted samples to the WRL for FMD

Materials and methods

Three hundred and thirty-four samples of vesicular epithelium were received from eighteen countries during the period of the study and were examined by ELISA, VI and RT-PCR (Table II).

Upon receipt, the pH of the transport buffer containing epithelial tissue was estimated using phenol red indicator. A suspension of the epithelium (ES) was made in 0.04 M phosphate buffer (ideally this should be done using a 10% concentration [w/v], but during this study a lower concentration was frequently used due to paucity of material). A 1.4 ml aliquot was taken for the antigen detection ELISA (3, 7) whilst 0.2 ml aliquots were used to inoculate cell cultures of primary bovine thyroid (11) and IB-RS-2 cells (1) (five tubes of each cell type per specimen). Additionally, 0.2 ml of ES were added to a 1 ml aliquot of Trizol solution and stored at temperatures of between –90°C and –50°C until assay. Real-time RT-PCR was usually performed on the ES within one to two days of preparation, with a diagnostic result typically being obtained within a single working day.

The real-time RT-PCR assay used in this study has been described elsewhere (10). Briefly, total nucleic acid was extracted from the solution of Trizol/ES using a fully automated robot system. This robot was then also used to pipette viral nucleic acid into a reverse transcription mix for reverse transcription and complementary deoxyribonucleic acid into PCR reaction mix (including an oligonucleotide primers/probe set targeting the internal ribosomal entry site of the FMDV genome). Thermal cycling and concurrent fluorescence detection were performed and a cycle detection threshold (i.e. the cycle at which a target sequence is detected [C_T]) was recorded for each test sample (8).

Results

The results achieved by ELISA, VI in cell culture and RT-PCR are summarised in Table II for comparison of the performance of the three assays.

The FMDV was detected in 195 samples by VI (58.4%), in 125 by antigen ELISA (37.4%) and in 204 samples by VI/ELISA combined (61.1%). These viruses represented

Table II
Detection of foot and mouth disease (FMD) virus in suspensions of submitted epithelia achieved by enzyme-linked immunosorbent assay (ELISA), passage in cell culture (VI), ELISA and VI combined and by reverse transcription polymerase chain reaction (RT-PCR) (August 2002-January 2004)

Country	No.	ELISA		VI[b]		ELISA/VI		RT-PCR for FMD virus (C_T[c] value)																	
		FMDV	NVD[a]	FMDV	NVD	FMDV	NVD	<35	<36	<37	<38	<39	<40	<41	<42	<43	<44	<45	<46	<47	<48	<49	<50	No.	C_T
Bhutan	58	2	56	17	41	17	41	36	1	–	–	3	2	2	–	2	–	–	–	–	–	–	–	12
Botswana	5	–	5	–	5	–	5	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	4
Burundi	7	4	3	5	2	5	2	1	1	1	1	1	–	–	–	–	–	–	–	–	1	–	–	1
Hong Kong	7	1	6	3	4	3	4	6	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Iran	43	17	26	31	12	31	12	32	–	–	1	–	1	–	–	–	–	1	–	–	–	–	–	8
Iraq	18	1	17	6	12	7	11	11	1	–	–	–	–	1	–	–	–	–	–	–	–	–	–	5
Laos	35	18	17	33	2	33	2	33	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Lebanon	5	3	2	3	2	5	–	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Libya	1	1	–	1	–	1	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Malaysia	12	8	4	12	–	12	–	12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Nepal	4	4	–	4	–	4	–	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pakistan	90	36	54	41	49	44	46	59	3	1	–	1	3	1	1	–	–	1	–	–	–	–	–	20
PAT	1	–	1	1	–	1	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Philippines	9	8	1	7	2	8	1	8	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Thailand	7	6	1	7	–	7	–	7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Turkey	24	10	14	17	7	18	6	21	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3
UAE	3	1	2	3	–	3	–	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Vietnam	5	5	–	4	1	5	–	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Total	**334**	**125**	**209**	**195**	**139**	**204**	**130**	**244**	**8**	**5**	**2**	**5**	**6**	**4**	**1**	**3**	**–**	**2**	**–**	**–**	**1**	**–**	**–**	**53**

a) NVD: no virus detected
b) VI: serotype of FMD virus isolated by passage (VI) in cell culture characterised by ELISA
c) C_T: threshold cycle value, showing additional samples scored positive as the number of cycles was increased
PAT: Palestinian Autonomous Territories
UAE: United Arab Emirates

four out of the seven FMDV serotypes (O, A, SAT 2 and Asia 1), although the majority (68.6%) were of serotype O. There was broad agreement between RT-PCR and VI for 265/334 (79.3%) and VI/ELISA combined for 274/334 (82%) of the epithelial samples tested when a cut-off C_T value of <39 was used to assign the samples as either FMDV positive or negative (results not shown). In general, VI positive samples corresponded to a real-time RT-PCR C_T value of <35, with only eight VI positive samples producing a C_T value in the range ≥35-<39. Furthermore, there were no false negative results obtained with RT-PCR since all samples assigned negative by RT-PCR were also negative by VI/ELISA. Of particular interest was the finding that RT-PCR was able to detect FMDV in an additional 60 (18%) of the samples tested. Fresh ribonucleic acid (RNA) was prepared from these epithelial suspensions and the RT-PCR was repeated with similar results confirming the presence of FMDV genome in these samples. In addition, there were 17 C_T values in the range ≥39 to <50. Repeat procedures were also performed for these samples with similar results, suggesting that FMDV was also present in these samples although the FMDV copy number was lower than the diagnostic threshold. The majority (96.9%) of VI positive samples were detected on the first passage in cell cultures (within two days). However, in order to ensure detection of infectious FMDV and to define NVD samples, a second cell culture passage was required (in total, up to four days); six samples required this second passage in cell culture before the presence of FMDV was demonstrated.

Calculations of the relative sensitivity and specificity of the RT-PCR at unit incremental increases in C_T value in comparison to either VI alone or to VI/ELISA combined are recorded in Table III. It shows that 100% relative sensitivity was achieved by the RT-PCR at a C_T value of <39, which justifies using this as the ideal diagnostic cut-off for the RT-PCR, since it is the lowest C_T value that achieves this.

Discussion

The ELISA and VI have been recommended laboratory procedures for FMD diagnosis for nearly twenty years based on their suitability to detect the presence of FMDV antigen in tissue samples. It is evident from the present study that these procedures are deficient for certain specimens. The results show that while all VI/ELISA

positive FMDV samples were also positive by real-time RT-PCR (100% sensitivity at a C_T cut-off value of <39) (Table III), FMD viral genome was detected in a significant proportion of the samples examined in which FMDV antigen was not.

The low relative specificity values (a range in value from 63.1% down to 38.1% depending upon cut-off) of the RT-PCR result from the doubtful premise that the combination of VI and ELISA is 100% efficient for detection of FMDV. The authors are confident that this is not the case, that the RT-PCR procedure has specific reaction for FMDV genome and that the low value is actually related both to the performance of the comparative assays and to the quality of the samples submitted.

If one considers what the VI and ELISA procedures actually measure then it is evident that their effectiveness for diagnostic use is inherently compromised. Virus isolation is dependent upon the presence of infectious virus in sample submissions and while the ELISA can detect both infectious and non-infectious FMD viral antigen, it is dependent upon the antigen being present in sufficient concentration (1 ng/ml to 2 ng/ml of antigen or 5 $\log_{10}$/ml to 6 $\log_{10}$/ml of live virus) to work (N.P. Ferris, unpublished results). If neither of these two conditions is met then FMDV will not be recognised.

Ideally, vesicular epithelium should be collected from an animal during the acute stage of FMD when the concentration of virus associated with the sample is high. Unfortunately, samples submitted to the WRL for FMD are very often collected late in the course of disease when the amount of virus may either be waning or indeed be absent after clearance. Delayed reporting of disease and late sample collection can arise for a variety of reasons, e.g. a lack of resources, communication difficulties in areas of rugged terrain or a low perceived importance of FMD. Delays can be worse depending on who has responsibility for sample collection; in some countries it is local staff and in others countries staff from a specialised laboratory (often many miles away) carry out the collection (5). Secondary bacterial infection is a common sequel to virus infection and can lead to a reduction in FMDV infectivity. Additionally, samples may be in transit for lengthy periods and subjected to physico-chemical stresses (e.g. elevated temperatures between collection and laboratory receipt) with the result that on arrival only small amounts of infectious virus, at best, may be present.

FMDV survival can be adversely affected by harsh environmental conditions, including excessive temperature, extremes of pH, disinfectants and desiccation. It is therefore advantageous to protect samples during the interval between collection and testing (especially for VI). Their dispatch to reference laboratories should follow specific guidelines to ensure their security (6). Small specimens are particularly vulnerable and should be placed in suitable transport medium, e.g. phosphate buffered glycerol (50%, v/v), pH 7.6. The FMDV survives best in a maintenance medium of between pH 6.8 and 7.8 and is readily disrupted outside this range, leading to loss of infectivity. Samples do not necessarily need to be transported cold (providing they arrive within one to two days of dispatch) but the presence of glycerol in the medium both aids antigen stability and reduces possible interference by inhibiting the growth of extraneous agents such as bacteria and fungi associated with the submission.

Table III
Relative sensitivity and specificity of the real-time reverse transcription polymerase chain reaction for foot and mouth disease virus at successive threshold cycle value cut-off points in comparison with virus isolation in cell culture (VI) or VI plus enzyme-linked immunosorbent assay combined (ELISA)

Threshold cycle value cut-off	Virus isolation [a]		VI/ELISA combined	
	Sensitivity	Specificity	Sensitivity	Specificity
<35	95.9	59.0	96.1	63.1
<36	97.9	56.1	98.0	60.0
<37	99.0	54.0	99.0	57.7
<38	99.5	51.1	99.5	54.6
<39	100.0	50.4	100.0	53.8
<40	100.0	46.0	100.0	49.2
<41	100.0	43.2	100.0	46.2
<42	100.0	42.4	100.0	45.4
<43	100.0	40.3	100.0	43.1
<44	100.0	40.3	100.0	43.1
<45	100.0	38.8	100.0	41.5
<46	100.0	38.8	100.0	41.5
<47	100.0	38.8	100.0	41.5
<48	100.0	38.1	100.0	40.8
<49	100.0	38.1	100.0	40.8
<50	100.0	38.1	100.0	40.8

a) virus isolation in cell culture with specificity of cytopathic effect confirmed by ELISA

Only two of the factors which can influence sample quality can be partly measured by the recipient laboratory, i.e. the pH of the transport buffer and (signs of) desiccation during transit. However, there did not appear to be a direct correlation between these measurements and the diagnostic results presently described. Out of 68 samples submitted in transport buffer of incorrect pH or else in a dry condition in bottles devoid of transport buffer, 19 were negative by both VI/ELISA and RT-PCR, 25 positive by both procedures and 24 VI/ELISA negative but RT-PCR positive. Clearly, some of the requirements for virus stability can be met by the sample itself, but it is interesting to note that of the nine samples which were positive for FMDV by ELISA (on epithelial suspension) but negative by VI, four were sent in transport buffer of incorrect pH and three were received dry. The measurements of pH give no

indication as to how the samples might have been stored before shipment to the WRL for FMD. Of the 60 samples which were VI/ELISA negative but RT-PCR positive, 36 were sent in transport buffer that was within the correct pH range and 24 were sent in transport buffer that was outside the correct range. In addition, it is not often clear from any accompanying submission documentation at what stage of the disease samples were collected. These features, which influence sample quality, are likely to be less important for the RT-PCR as it can detect a small fragment of FMDV genomic RNA, not just live virus.

The efficiency of the current RT-PCR may be compromised by the limited volume of sample which is analysed (in common with other PCR procedures). The spectrum of reaction against all likely FMD field virus strains is dependent upon the selection (and appropriateness) of the primer/probe sets employed and these may need to be refined with time with the likely emergence of new variants and in the light of increased sequence information. A further limitation is that the procedure does not identify the particular serotype of the causative FMDV. This is particularly limiting with samples that are RT-PCR positive but ELISA/VI negative. Serotyping such samples may possibly be accomplished by FMDV sequencing or by use of an RT-PCR which uses serotype-specific primer/probe sets.

Comparison between VI and the 'semi-quantitative' readout from the real-time RT-PCR allowed samples with a C_T value <39 to be classed as positive, whereas those with a C_T value ≥39 were classed as NVD. Based upon studies using RNA standards, a C_T value of 38 correlates to between 10 and 100 copies of the FMDV genome, therefore this is a conservative cut-off necessary to avoid false-positive results. Recent reports suggest that this positive/negative cut-off point may need to be re-considered for alternative sample types, such as oesophageal-pharyngeal (probang) scrapings (12). Preliminary results on a limited number of blood and milk samples received from overseas suggest similarly effective real-time RT-PCR assay performance and show that positive results can be achieved (data not shown). However, further validation is required and the results on other sample types (e.g. probang, blood and milk) are still under study.

In conclusion, the study demonstrates that real-time RT-PCR currently used at the WRL for FMD provides an extremely sensitive and rapid additional procedure for improved laboratory diagnosis of FMD. The RT-PCR generated results in less than one day from test commencement, in contrast to up to four days to define some positive and all negative samples by combined use of ELISA and VI. This is an important feature when definitive diagnostic results are required in a short timescale during emergencies.

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■

Amélioration du diagnostic biologique de la fièvre aphteuse par amplification en chaîne par polymérase en temps réel

A.E. Shaw, S.M. Reid, D.P. King, G.H. Hutchings & N.P. Ferris

Résumé

Les auteurs ont comparé une méthode automatisée d'amplification en chaîne par polymérase, couplée à une transcription inverse (RT-PCR) et effectuée en temps réel, à l'isolement viral sur culture tissulaire et au dosage immuno-enzymatique (ELISA) pour son efficacité dans le diagnostic biologique de la fièvre aphteuse. Le Laboratoire de référence mondial pour la fièvre aphteuse, établi à Woking (Royaume-Uni), a procédé à l'examen d'une collection de 334 prélèvements épithéliaux provenant de dix-huit pays entre août 2002 et janvier 2004. Tous les échantillons ayant donné des résultats positifs lors de l'isolement viral (n = 195) ou de l'isolement viral combiné à un dosage immuno-enzymatique

(n = 204) se sont également révélés positifs à l'épreuve RT-PCR. Selon la valeur limite utilisée dans l'étude, des gènes du virus de la fièvre aphteuse ont été dépistés dans au moins 60 autres prélèvements (18 % de l'ensemble des échantillons). En outre, alors que le test RT-PCR a fourni les résultats en moins de 24 heures, le dépistage par isolement viral des quelques échantillons positifs et de tous les échantillons négatifs a nécessité jusqu'à 4 jours. Cette étude démontre l'intérêt du test RT-PCR en tant que méthode très sensible et rapide permettant d'accroître l'efficacité du diagnostic de la fièvre aphteuse en laboratoire.

Mots-clés
Amplification en chaîne par polymérase et transcription inverse en temps réel – Diagnostic – Dosage immuno-enzymatique – Isolement viral – Rapidité – Sensibilité – Virus de la fièvre aphteuse.

Mejora del diagnóstico de laboratorio de la fiebre aftosa mediante la reacción en cadena de la polimerasa en tiempo real

A.E. Shaw, S.M. Reid, D.P. King, G.H. Hutchings y N.P. Ferris

Resumen
En este estudio se comparan los resultados obtenidos con la técnica de la reacción en cadena de la polimerasa con transcriptasa reversa (RT-PCR), automatizada y en tiempo real, con el aislamiento de virus (AV) en cultivos celulares y la detección de antígenos mediante la técnica inmunoenzimática (ELISA) para el diagnóstico de laboratorio de la fiebre aftosa. El Laboratorio Mundial de Referencia para esa enfermedad, situado en Woking, Reino Unido, examinó una colección de 334 epitelios enviados por 18 países entre agosto de 2002 y enero de 2004. Los resultados mostraron que todas las muestras examinadas mediante el AV que eran positivas (n = 195), así como las que resultaron positivas con el AV y el test ELISA simultáneamente (n = 204) también fueron positivas con la RT-PCR. En función de los límites utilizados, se detectó al genoma del virus de la fiebre aftosa en un mínimo de 60 muestras adicionales (18% de todas las muestras estudiadas). Además, con la RT-PCR los resultados se obtuvieron en menos de un día tras el inicio de la prueba, mientras que con el AV son precisos hasta cuatro días para determinar algunas muestras positivas y todas las negativas. El estudio demostró que la RT-PCR en tiempo real constituye un método sumamente sensible y rápido con el que se obtiene un mejor diagnóstico de laboratorio de la fiebre aftosa.

Palabras clave
Aislamiento de virus – Diagnóstico – Rapidez – Reacción en cadena de la polimerasa con transcriptasa reversa en tiempo real – Sensibilidad – Técnica inmunoenzimática – Virus de la fiebre aftosa.

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