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Application of nested-PCR for detection of foot-and-mouth disease viral sequences in tonsil of slaughtered cattle with clinically normal appearance in Iran

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BAHARI, A., T. TAGHIPOUR-BAZARGANI, O. MARQUARDT, S. A. GHORASHI, S. BOKAIE: Application of nested-PCR for detection of foot-and-mouth disease viral sequences in tonsil of slaughtered cattle with clinically normal appearance in Iran. Vet. arhiv 77, 299-306, 2007.

ABSTRACT

Persistent or inapparent infection, the so-called carrier state, is an important feature of foot-and-mouth disease (FMD) in ruminants. This may occur in non-vaccinated as well as in vaccinated ruminants following exposure to infectious FMD virus. Although the amount of infective virus that can be recovered from carriers is small, the virus can be present in some ruminants for months and in cattle for years. FMD is enzootic in Iran. The present study was carried out for determination of FMD viral genome in clinically normal cattle slaughtered at Zyaran Abattoir. A total of 133 tonsil tissue samples was collected and total RNA was extracted from each sample individually. RT-PCR and subsequently nested-PCR were carried out on each sample using FMDV specific primers from the 3D-3A region. A 222 bp DNA fragment was amplified from positive samples. Of the 133 tonsil tissue samples, 46 were found positive. Results indicated that the frequency of FMD carriers among tested animals is 34.59%. Statistical analysis did not show any significant differences ($P>0.05$) between positive samples in relation to sex, age and breed. The high frequency of carriers could be due to extensive FMDV circulation among susceptible animals. This finding suggests that, at least at present, full vaccination coverage is required for domestic ruminants in Iran to increase their resistance to field virus exposures.

Key words: foot-and-mouth disease (FMD) virus, tonsil, cattle, persistent infection, carrier state, nested-PCR

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Introduction

Foot-and-mouth disease virus (FMDV), a member of the family *Picornaviridae*, genus *Aphthovirus*, is the causative agent of the economically most important cloven-hoofed animal viral disease worldwide. Although mortality with FMD is usually low, the disease decreases livestock productivity, and affected countries cannot participate in the international trade of animals and animal products (DOMINGO et al., 2002). FMD is present in many regions of Africa, South of America and Asia (KNOWLES and SAMUEL, 2003), and is enzootic in Iran. Persistent or inapparent infection is a common sequel to clinical and sub-clinical infection of susceptible and vaccinated ruminants by foot-and-mouth disease virus, the so-called carrier state. A carrier of FMDV is defined as an animal from which virus can be recovered for more than 28 days post-infection (ALEXANDERSEN et al., 2002; KITCHING, 2002). Field evidence that implicated the role of carrier cattle in a number of outbreaks has been described (ALEXANDERSEN et al. 2002).

Currently, identification of FMD carrier ruminants depends on the isolation of virus from oesophageal-pharyngeal fluid (OPF) samples, collected with a 'probang'- a metal cup passed into the oesophagus (SUTMOLLER and GAGGERO, 1965). However, several investigators have reported the use of nucleic acid amplification methods for detecting viral RNA genome in samples collected during persistent FMDV infection. ROSSI et al. (1988) detected FMDV genomes by nucleic acid hybridization in OPF samples of carrier cattle taken at 180 and 560 days post-infection, while it was not possible to recover infectious virus by conventional methods. PRATO MURPHY et al. (1994) showed that the RT-PCR technique is more sensitive than standard virus isolation techniques and may be used for the rapid detection of FMDV in tissues from persistently infected cattle. BERGMANN et al. (1996) have shown presence of FMDV-specific genomic sequences from several tissues, such as tonsils of cattle using in situ hybridization.

The aim of this study was to detect FMDV-specific genomic sequences in clinically normal slaughtered cattle, as persistently infected animals, at Zyaran Abattoir in Qazvin Province, Iran.

Material and methods

Specimens. Tonsils were taken from two native-bred cattle (1. a variety of *Bos taurus* and 2. Sistani cattle as a variety of *Bos indicus*), as well as from Holstein-Friesians and their cross-breeds. The animals, of both sexes and of various ages, were clinically normal. Each sample was transferred into a tube containing PBS-glycerol (1:1) and kept in frozen until tested at the Federal Research Centre for Virus Diseases of Animals, Tübingen, Germany.

RNA isolation. A small piece (c. 30-50 mg) of every tonsil was minced and lysed in RLT buffer supplied with the RNA extraction kit RNeasy (QIAGEN, Germany). RNA was extracted from aliquots of 350 µl of tissue homogenate, as recommended, and re-suspended in a volume of 20 µl of DEPC-H2O. Five µl of extracted total RNA were used for RT-PCR reaction immediately. Distilled water was used as a template in negative controls.

Selection of oligonucleotides. The primer sequences were selected from the conserved genomic sequences of the viral RNA polymerase gene. MOSS and HASS (1999) described that the region (derived from the genome of strain O1 Kaufbeuren, C-terminus of NS protein 3A to N-terminus of polymerase 3D) exhibits little variation among FMDV serotypes and is ideal for amplification and detection of all seven serotypes. The name and sequences of primers were as below:

PCR (external) primers: 3C1 antisense 5'-CGC TCT TCC ACA TCT CTG GT-3' (nucleotide position O1 Kaufb.: 6329-6348) and 3A1 sense 5'-CCA CAA GCT GAA GGA CCC T-3' (nucleotide position O1 Kaufb.: 5450-5468).

Nested-PCR (internal) primers: 3C3 antisense 5'-GGC CTC ACC AGA GAA AAT CA-3' (nucleotide position O1 Kaufb.: 6054-6073) and 3C5 sense primer 5'- TAG AGC CAT GAC AGA CAG TG-3' (nucleotide position O1 Kaufb.: 5851-5871).

PCR. Samples were amplified in a one-step RT-PCR in a final volume of 25 µl containing 5 µl extracted RNA and 1 µl of each external primer using QIAGEN® OneStep RT-PCR kit, as recommended by manufacturer. Thermocycling was performed in an MJ PTC-100 apparatus with the following program: (1) 30 min. at 50 °C, (2) 15 min. at 95 °C, (3) 45 s. at 94 °C, (4) 45 s. at 55 °C, (5) 1 min. at 72 °C, (6) 10 min. at 72 °C; steps (3)-(5) were repeated for 40 cycles. One µl of the RT-PCR product was then amplified by nested-PCR. For the second amplification, 3C3 and 3C5 primers were used. The nested-PCR thermal cycling conditions were similar to those described above, except step 1 (reverse transcription). Each reaction was analysed by agarose gel electrophoresis and ethidium bromide staining (4µg/ml). The nested-PCR products were purified as recommended by FREIBERG et al. (1999) and subjected to fluorescent dye deoxy-terminator cycle sequencing (MARQUARDT and ADAM, 1990) for confirmation of specificity of test (data not shown).

Statistical analysis. Statistical data analysis was done based on Fischer's exact test using SPSS 11 software.

Results

Nested-PCR samples producing a band of the expected size (222 bp) were considered positive (Fig. 1). Viral RNA was detected in 46 of the 133 tonsil tissue samples. Sampled

animals were divided into 5 age groups based on dental formulation. Results are shown in Table 1 according to the breed and age for male- and female-tested animals.

Table1. The frequency of FMD viral genome detected in tonsil tissues of male and female cattle with respect to the number of samples tested according to the breed (1. Native; 2. Sistani; 3. Holstein-Freisians; 4. Cross bred of Holstein-Freisians×Native) and age groups based on dental formulation (a - Nil; b - 1 pair permanent; c - 2 pairs permanent; d - 3 pairs permanent and e - 4 pairs permanent and older)

Breed	Age group	Pos.	Neg.	Total
1	a	11	18	29
	b	3	6	9
	c	3	8	11
	d	1	3	4
	e	2	7	9
	total	20	42	62
2	a	1	2	3
	b	2	3	5
	c	2	2	4
	d	1	1	2
	e	2	3	5
	total	8	11	19
3	a	0	3	3
	b	1	6	7
		1*	0*	1*
	c	0	3	3
		1*	0*	1*
	d	0	1	1
	e	1	0	1
		5*	11*	16*
	total	9	24	33
4	a	4	4	8
	b	2	3	5
	c	2	1	3
	d	1	1	2
	e	0	1	1
	total	9	10	19
Total		46	87	133

* female results

Discussion

The detection of FMDV carrier ruminants can be a relatively frequent finding in an area where FMD is enzootic. However, little is known about the incidence of the carrier state under natural conditions. In this study, the total frequency of positive samples was found to be 34.59%, although statistical analysis found no significant relationship (P>0.05) between sex, age and breed of tested cattle. A few studies have been carried out

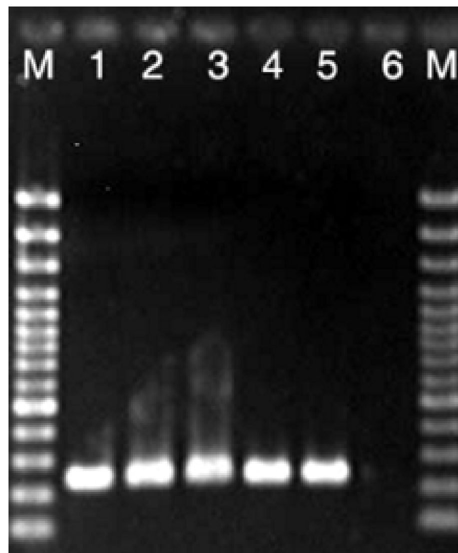

Fig. 1. Nested-PCR for detection of FMD viral genome in tonsil tissue samples. M: DNA molecular marker (Ladder 100), Lane 1-5: Amplification of a 222 bp DNA fragment in positive tonsil tissue samples, Lane 6: Negative control.

on OPF samples using virus isolation to determine the frequency of FMDV carriers in enzootic areas. A survey in Asiatic Turkey found 15-20% of cattle and sheep to be carriers (GURHAN et al., 1993). HEDGER (1968) reported a frequency of 20% of virus isolation from *Bos indicus* in Botswana.

The prevalence rate of carriers in a population depends on the species, the incidence of the disease (or infection) and the immune status of the population. Effective vaccination, even though it does not directly protect against the development of the carrier state in an exposed animal, has been suggested to reduce the prevalence of carrier animals by suppressing the amount of FMDV released or discharged in the environment by limiting the number of diseased animals in the population, and then, decreasing the overall challenge of the animals under field conditions (ALEXANDERSEN et al., 2002). SUTMOLLER and CASAS OLASCOAGA (2002) noted that after intensified vaccination in the 1980's, probang sampling of several hundred cattle in the endemic area of Brazil resulted in only a very small number of positive samples. The presence of FMD viral genome in our tested samples demonstrates the extensive exposure of the virus. In Iran, however, the control of FMD is effected by zoosanitary measures and vaccination.

At the present time, cattle are vaccinated regularly (at 4-monthly intervals) with an inactivated vaccine (Razi Institute, Karaj-Iran) containing three FMDV serotypes, O, A and Asia1, which are isolated from clinical specimens in the field. Pure-bred Holstein-Friesian cattle (approximately 20% of cattle population) are kept in industrialized and intensive dairy units which in the main are located around the large cities and are fully controlled and covered by this vaccination program. Other cross-breeds and indigenous cattle (approximately 80% of cattle population) which are located in villages, are under partial vaccination coverage because control systems for identification of individual animals may not be as effective as in intensive units (GARLAND, 1998). Moreover, the epizootiological role of sheep and goats cannot be excluded. Domesticated small ruminants are important in rural economies in Iran. Large numbers of indigenous breeds of sheep and goats are kept under nomadic and transhumant systems and are not usually vaccinated against FMDV. Thus, they are considered as one of the factors of the multiplication and spread of FMDV in the environment.

In conclusion, the authors suggest that full vaccination coverage of ruminant populations will be necessary in order to decrease carriers, which are the reflection of the FMDV situation and virus circulation in fields. The strategy should be to maintain high vaccination coverage, especially in borderlines for providing buffer zones, for a sufficient length of time in order to reduce the incidence of FMD and hence the carrier state of cattle.

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BAHARI, A., T. TAGHIPOUR-BAZARGANI, O. MARQUARDT, S. A. GHORASHI, S. BOKAIE: Primjena ugniježđene lančane reakcije polimerazom za dokaz specifičnog slijeda genoma virusa slinavke i šapa u tonzilama klinički zdravih zaklanih goveda u Iranu. Vet. arhiv 77, 299-306, 2007.

SAŽETAK

Perzistentna ili inaparentna infekcija, odnosno kliconoštvo, važna je značajka slinavke i šapa (SiŠ-a) u preživača. Ona se može javiti u necijepljenih i cijepljenih životinja nakon izlaganja infekciji virusom SiŠ-a. Iako je količina infektivnoga virusa koji se izlučuje iz životinje kliconoše mala, virus može biti prisutan u nekih preživača mjesecima, a u goveda godinama. SiŠ se u Iranu javlja enzootski. Istraživanje je provedeno da bi se dokazao genom virusa SiŠ-a u klinički zdravih goveda zaklanih na klaonici Zyaran. Pretražena su 133 uzorka tkiva tonzila. Svaki uzorak bio je pretražen najprije lančanom reakcijom polimerazom uz prethodnu reverznu transkripciju, a potom ugniježđenom lančanom reakcijom polimerazom upotrebom specifičnih molekula

početnica od 3D do 3A područja. Odsječak DNA od 222 bazna para bio je umnožen iz pozitivnih uzoraka. Od 133 pretražena uzorka, 46 je bilo pozitivnih. Kliconoštvo je bilo ustanovljeno u 34,59% pretraženih životinja. Nije ustanovljena statistički značajna razlika (P>0,05) u pozitivnih životinja s obzirom na spol, dob i pasminu. Velika učestalost kliconoštva može se pripisati znatnom kruženju virusa među prijemljivim životinjama. Rezultati upućuju na zaključak da je zasada potrebno cijepiti sve domaće preživače u Iranu protiv SiŠ-a radi povećanja njihove otpornosti prema terenskom virusu.

Ključne riječi: slinavka i šap, virus, tonzile, govedo, perzistentna infekcija, kliconoša, ugniježđena lančana reakcija polimerazom

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