Veterinary Microbiology, 30 (1992) 13–20
Elsevier Science Publishers B.V., Amsterdam

Rapid identification and differentiation of the vaccine strain Rac H from EHV 1 field isolates using a non-radioactive DNA probe

H. Meyer[a], P. Hübert[b], C. Schwend[b] and W. Eichhorn[b]

[a]*Federal Armed Forces Medical Academy, Institute of Microbiology, Neuherbergstrasse 11, 8000 Munich 45, Germany*

[b]*Institute of Medical Microbiology, Infectious and Epidemic Diseases, Ludwig-Maximilians-University, Veterinärstrasse 13, 8000 Munich 22, Germany*

(Accepted 14 June 1991)

ABSTRACT

Meyer, H., Hübert, P., Schwend, C. and Eichhorn, W., 1992. Rapid identification and differentiation of the vaccine strain Rac H from EHV 1 field isolates using a non-radioactive DNA probe. *Vet. Microbiol.*, 30: 13–20.

A method for rapid differentiation between the EHV 1 live vaccine strain Rac H and field isolates is described. Total DNA was isolated from virus-infected small scale cell cultures. DNA fragments digested with restriction endonuclease BamHI were separated, transfered and immobilized on filter membranes. A Digoxigenin-labeled probe derived from EHV 1 was used for hybridization. This probe hybridized specifically to sequences of the inverted terminal repeat region which in case of Rac H include a deletion of 0.8 kb. By comparing the different migration patterns after blot hybridization it could be shown that in 65 isolates from cases of abortion the live vaccine strain Rac H was not involved.

INTRODUCTION

Equine herpesvirus 1 (EHV 1) is the major cause of equine abortion, which may occur as an epizootic, but occasionally it is associated with rhinopneumonitis. It is also recognized as a cause of perinatal foal mortality and encephalitis. EHV 4 is involved in acute upper respiratory disease, usually observed in foals in epizootic form. It has also been recovered from sporadic cases of abortion (for review see Allen and Bryans, 1986). All diseases mentioned cause severe economic losses in the horse-breeding industry. Although a wide range of vaccines has been developed, their efficacy is sometimes questioned. In Germany the high passage strain Rac H is widely used as an attenuated live vaccine (Mayr et al., 1968). In vitro markers for the differentiation of this vaccine strain from field isolates have been described. The ability of strain Rac H to produce plaques in a clone of mouse c-fibroblast-cells

(L-marker) has been evaluated by Borgen (1972). A different sensitivity to dithiothreitol treatment (DTT-marker) has been identified by Klingeborn and Dinter (1972). It has been shown that restriction endonuclease analysis of EHV DNA is an efficient tool for epidemiological studies (Allen et al., 1983; Studdert et al., 1981; Chowdhury et al., 1986b; 1988).

A fast method to detect EHV-specific restriction fragments in total DNA from infected cells has been described by Chowdhury et al. (1986a). After Southern blotting they used radioactively labeled EHV 1 DNA to detect restriction fragments. This allows typing of isolates. Meyer et al. (1987) demonstrated that the restriction endonuclease BamHI produced a unique DNA cleavage pattern of the vaccine strain Rac H different from the original wild type strain and abortigenic virus isolates. This pattern is characterized by i deletion of 0.8 kb at the left end of the genome, ii loss of a BamHI site without any detectable deletion and iii two deletions (0.8 kb each) in the inverted repeat (IR) region of the genome (Schwend, 1988).

In this paper we report a method which allows differentiation between the live vaccine strain Rac H and abortigenic field isolates.

MATERIALS AND METHODS

Viruses and cells

EHV 1 reference strain Kentucky D (Doll et al., 1954) was obtained from the American Type Culture Collection (ATCC). EHV 4 strain H45 (Shimizu et al., 1959) and EHV 3 isolate C175 (from a mare with signs of coital exanthema) were kindly supplied by K. Petzoldt (Hannover). The EHV 1 live vaccine strain Rac H originating from a fetal isolate had been attenuated by continuous propagation (256 passages) in porcine embryonic kidney cells (Mayr et al., 1968). Out of 30 cases of abortion which had been sent to our institute from Southern Germany during the last decade virus could be isolated on equine embryonic lung (EEL) or equine embryonic kidney (EEK) cells. From Northern Germany 35 isolates from aborted fetuses including one isolate from a calf suspected to be infected with EHV were supplied by Dr. Steinhagen (Neumünster). Cell cultures used were: EEL and EEK cells, and the equine dermal cell line L-337 (a gift of Dr. P. Thein, Wuppertal). The cells were grown as monolayers in Eagle's basal medium supplemented with 5% (L-337) or 15% (EEL and EEK) fetal bovine serum.

Isolation of DNA

Virus propagation and extraction of DNA from purified virions of EHV strains Kentucky D (EHV 1), H45 (EHV 4), C175 (EHV 3), and Rac H (vaccine strain) were carried out as described previously (Meyer et al., 1987). Propagation of EHV isolates from aborted fetuses and extraction of DNA was performed on the basis of small scale cell cultures (L-337) according to

Chowdhury et al. (1986a) with some modifications. Briefly, trypsinized L-337 cells were seeded in 24 well plates (Nunc) and incubated at 37°C and 5% CO_2 overnight. Then the medium was removed and the wells were infected with 0.4 ml virus suspension of the appropriate isolates. Incubation was continued until cytopathic effect had affected more than 80% of the cells (usually 24 hours). After freeze–thawing the content of each well including cellular debris was transfered into Eppendorf tubes. DNA was released with SDS–Proteinase K, followed by extraction with phenol and chloroform. Nucleic acids were resuspended in 1×BamHI reaction buffer (Boehringer, Mannheim).

Cloning of virus DNA

A 4.9 kb BamHI DNA-fragment of EHV 1 was prepared from an agarose gel and cloned into the plasmid vector pBR 322 (Maniatis et al., 1982). This plasmid, named pHSO2 was grown in *Escherichia coli* DH5 F′ (Pharmacia, Freiburg). According to the EHV 1 genomic map established for the restriction endonuclease Bam HI (Whalley et al., 1981) this fragment contains part of the IR-region and of the unique short region.

DNA hybridization

DNA from purified virions of EHV 1, 3, 4 and the vaccine strain as well as from small scale preparations of EHV isolates was digested with restriction endonuclease BamHI under conditions recommended by the supplier (Boehringer, Mannheim). Cleavage fragments were separated in 0.6% agarose gels, photographed and transfered to Hybond N^+ nylon membranes (Amersham) as described by Southern (1975). Plasmid pHSO2 was labeled by random primed incorporation of Digoxigenin-11-UTP (DNA labeling kit, non-radioactive; Boehringer, Mannheim).

Filters were prehybridized for 3 h at 40°C in a solution containing 50% (*v/v*) formamide; 5% blocking reagent; 5×SSC; 0.1% N-lauroylsarcosine; 0.02% SDS. Hybridization was carried out in prehybridization mixture containing Digoxigenin-labeled pHSO2 DNA for 14 h. After hybridization filters were washed 2×5 min at room temperature (2×SSC; 0.1% SDS) and 2×15 min at 65°C with 0.1×SSC; 0.1% SDS. Filter-bound labeled DNA was detected with an anti-digoxigenin–alkaline phosphatase conjugate. The location of the antibody–antigen conjugate was visualized by an enzyme-linked color reaction according to the manufacturer's protocol (DNA detection kit, non-radioactive; Boehringer, Mannheim).

RESULTS

Cleavage patterns of purified DNA of the EHV 1 strains Kentucky D and Rac H as well as EHV 3 and 4 after digestion with restriction endonuclease

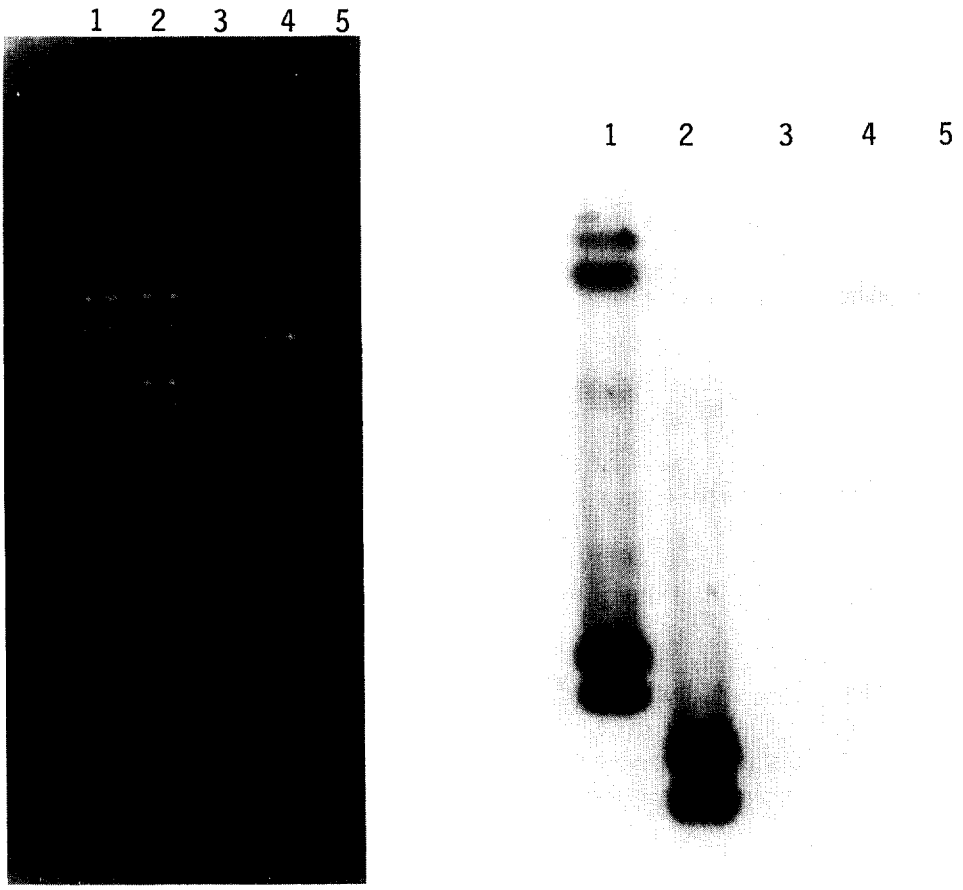
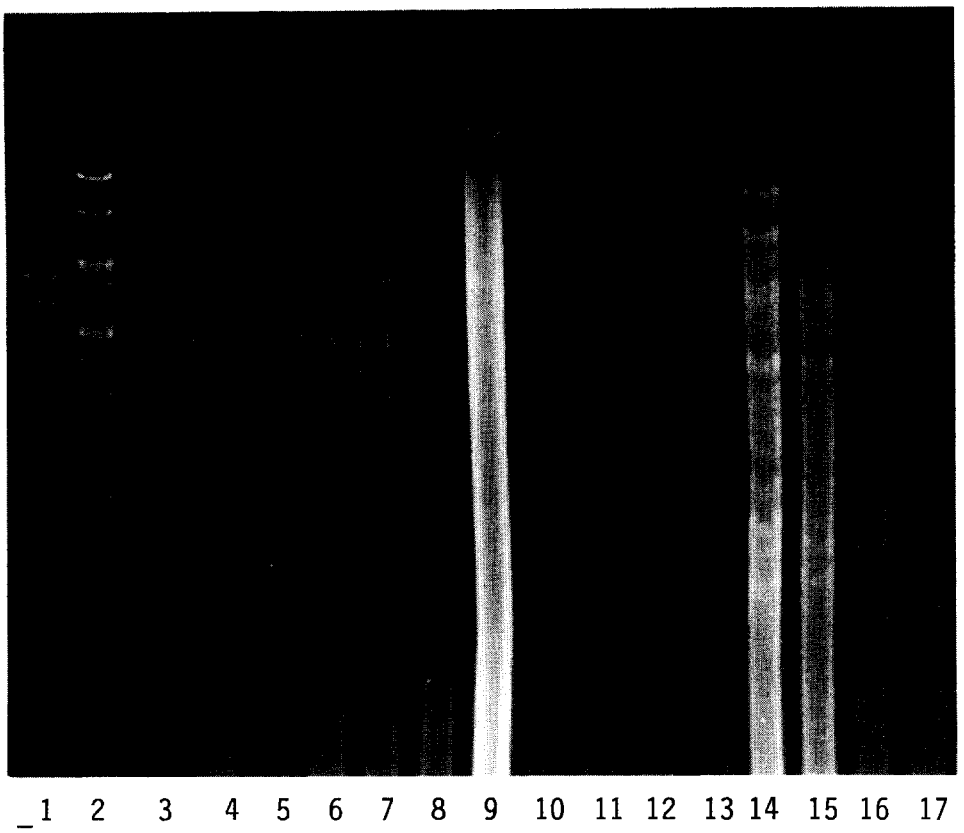
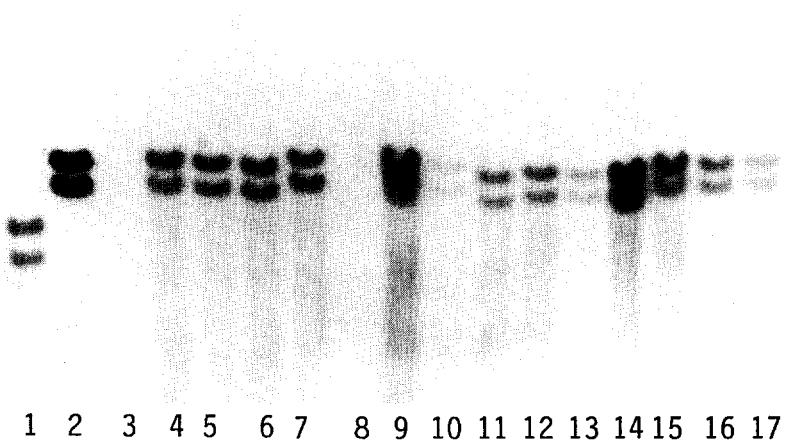


Fig. 1a. Electrophoresis of BamHI digests of DNA from purified virions of EHV strains: EHV 1 reference strain Kentucky D (lane 1); EHV 1 vaccine strain Rac H (2); EHV 3 strain C175 (3); EHV 4 strain H45 (4); lane 5 is a molecular weight marker (kb-ladder, BRL). Lane numbers are at the slot end of each gel.
Fig. 1b. This figure was prepared from the agarose gel of Fig. 1a after Southern-blot hybridization with the Digoxigenin-labeled probe pHSO2.

Fig. 2a. Electrophoresis of BamHI digests of EHV DNA: DNA of the EHV 1 vaccine strain Rac H and reference strain Kentucky D (lanes 1 and 2 respectively) was prepared from purified virions. DNA from isolates originating from cases of abortion during 1984–1989 (lanes 4–17) had been prepared from virus infected small cell cultures. DNA in lane 7 originates from a bovine fetal isolate. Slots are at the top of the figure.
Fig. 2b. This figure was prepared from the agarose gel of Fig. 2a after Southern-blot hybridization with the Digoxigenin-labeled probe pHSO2.


_1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

BamHI are shown in Fig. 1a. The strains display typical type-specific patterns which have been described for EHV 1 and 4 by Allen et al. (1983) and for EHV 3 by Atherton et al. (1982). The unique migration pattern of EHV 1 strain Rac H has been described by Schwend (1988).

After hybridization with the Digoxigenin-labeled probe pHSO2 a 4.9 kb and 4.5 kb band can be seen for the EHV 1 strain Kentucky D. For strain Rac H the homologous bands account for 4.0 kb and 3.6 kb (Fig. 1b, lanes 1 and 2, resp.). There was no reaction with EHV 3 and EHV 4 DNA. Additional bands of a higher molecular weight (lane 1) are caused by partially digested Kentucky D DNA.

Total DNA from cells infected with 14 different isolates from aborted fetuses has been digested with BamHI. After electrophoresis of such DNA smears of cellular DNA can be seen (Fig. 2a, lanes 4–17). In some lanes faint viral DNA fragments are visible.

After hybridization (Fig. 2b) two bands are detected for all isolates except isolate 5 (lane 8). All bands display only minor differences in size compared to the reference strain Kentucky D (lane 2), but are different from Rac H (lane 1). A similar migration pattern was observed for another 50 isolates from aborted fetuses (data not shown). There was no reaction with mock-infected cellular DNA or bovine herpesvirus type 1 DNA.

DISCUSSION

Our results indicate that in all cases of abortion investigated ($n=65$) the vaccine strain Rac H was not involved. The method described here uses a probe which developes signals to discrete viral DNA fragments allowing easy differentiation due to different migration distances of strain Rac H fragments. The method is suitable for analysis of large amounts of field isolates, because a single well of a 24 cluster plate yielded sufficient DNA for the investigation. After digestion with restriction endonuclease BamHI and gel electrophoresis, in most cases the EHV 1 specific DNA pattern was detectable, in some cases it was invisible due to a smear of cellular DNA. However, the rapidly-migrating fragments of interest could not be clearly evaluated for differentiation of the vaccine strain. Establishing a blot hybridization assay using a specific probe has overcome the disadvantage of further purification procedures. However, one isolate (Fig. 2b, lane 8) failed to react after blot hybridization even after repetition of the test. It also displays a cytopathic effect different from EHV 1 or EHV 4 isolates. The identification of this unknown agent is currently under study.

Of course, the method described can also be used for epidemiological studies and typing of isolates by utilizing a probe consisting of the entire EHV genome. We have applied this procedure for the bovine isolate (Fig. 2a and b, lane 7) and found a cleavage pattern typical of EHV 1 (data not shown).

Compared to a method developed by Chowdhury et al. (1986a) who used radioactively labeled DNA our method has several advantages. Probes can be produced in advance and hazards due to radioactive material are eliminated completely.

The probe we chose, detects both homologous IR-copies of EHV 1 (Fig. 1b). In the case of Rac H the corresponding fragments migrate simultaneously faster. This strongly indicates that 0.8 kb have been deleted from the IR-part, confirming the observed unique migration pattern of Rac H.

Recently polymerase chain reaction (PCR) has been applied to detect EHV 1 sequences in specimens from aborted fetuses (Ballagi-Pordany et al., 1990). EHV 4 strains could be detected as well, but by increasing the annealing temperature no band was further amplified in case of EHV 4. Based on our presented results we intend to establish a PCR using two sets of primers; one selected from a gene common to EHV 1 and 4 and the other located up- and down-stream of the 0.8 kb deletion. This would allow a differentiation of EHV 1, EHV 4, and the vaccine strain in one single assay.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the technical assistance of Doris Kronthaler and Gudrun Zöller. This work was in part supported by DFG grant Hu 404/1-1.

REFERENCES

Allen, G.P. and Byrans, J.T., 1986. Molecular epizootiology, pathogenesis and prophylaxis of equine herpesvirus-1 infections. Prog. Vet. Microbiol. Immun., 2: 78–144.

Allen, G.P. and Turtinen, L.W., 1982. Assessment of the base sequence homology between the two subtypes of equine herpesvirus 1. J. Virol., 44: 249–255.

ALlen, G.P., Yeargan, M.R., Turtinen, L.W., Bryans, J.T. and McCollum, W.H., 1983. Molecular epizootiologic studies of equine herpesvirus-1 infections by restriction endonuclease fingerprinting of viral DNA. Am. J. Vet. Res., 44: 263–271.

Atherton, S.S., Sullivan, D.C., Dauenhauer, S.A., Ruyechan, W.T. and O'Callaghan, D.J., 1982. Properties of the genome of equine herpesvirus type 3. Virology, 120: 18–32.

Ballagi-Pordany, A., Klingeborn, B., Flensburg, J. and Belak, S., 1990. Equine herpesvirus type 1: detection of viral DNA sequences in aborted fetuses with the polymerase chain reaction. Vet. Microbiol., 22: 373–381.

Borgen, H.C., 1972. Equine herpesvirus type 1; the 1-character in two of forty-three field isolates. Arch. Ges. Virusforschg., 36: 391–393.

Chowdhury, S.I., Hammerschmidt, W., Ludwig, H., Thein, P. and Buhk, H.J., 1986a. Rapid methods for the identification and screening of herpesviruses by DNA fingerprinting combined with blot hybridization. J. Virol. meth., 14: 285–291.

Chowdhury, S.I., Kubin, G. and Ludwig, H., 1986b. Equine herpesvirus type 1 (EHV-1) induced abortions and paralysis in a Lipizzaner stud: a contribution to the classification of equine herpesviruses. Arch. Virol., 90: 273–288.

Chowdhury, S.I., Ludwig, H. and Buhk, H.J., 1988. Molecular biological characterization of equine herpesvirus type 1 (EHV-1) isolates from ruminant hosts. Vet. Rec., 11: 127–139.

Doll, E.R., Richards, M.G. and Wallace, M.E., 1954. Cultivation of the equine influenza virus in suckling Syrian hamsters: Its similarity to the equine abortion virus. Cornell Vet., 44: 133–137.

Klingeborn, B. and Dinter, Z., 1972. Equine abortion (herpes) virus: strain differences in susceptibility to inactivation by dithiothreitol. Appl. Microbiol., 23: 1121–1124.

Maniatis, T., Fritsch, E.F. and Sambrook, J., 1982. Molecular cloning: a laboratory manual. New York, Cold Spring Harbor Laboratory.

Mayr, A., Pette, J., Petzoldt, K. and Wagener, K., 1968. Untersuchungen zur Entwicklung eines Lebendimpfstoffes gegen die Rhinopneumonitis (Stutenabort) der Pferde. J. Vet. Med., B, 15: 406–418.

Meyer, H., Hüber, P.H. and Eichhorn, W., 1987. Changes in restriction enzyme pattern of the equine Herpesvirus type 1 (EHV-1) strain Rac-H DNA during attenuation. J. Vet. Med. B, 34: 310–313.

Schwend, C., 1988. Das Genom des Equinen Herpesvirus Typ 1 (EHV 1) Stammes Rac H und seine Veränderung während der Attenuierung. Vet. Med. Diss, München.

Shimizu, T., Ishizaki, R., Kawakami, S., Kaji, R., Sugimura, K. and Matumoto, M., 1959. Isolation of equine abortion virus from natural cases of abortion in horse kidney cell culture. Jpn. J. Exp. Med., 29: 643–649.

Southern, E., 1975. Detection of specific sequences among dna fragments separated by gel electrophoresis. J. Molec. Biol., 988: 503–517.

Studdert, M.J., Simpson, T. and Roizman, B., 1981. Differentiation of respiratory and abortigenic isolates of equine herpesvirus 1 by restriction endonucleases. Science, 214: 562–564.

Whalley, J.M., Robertson, G.R. and Davison, A.J., 1981. Analysis of the genome of equine herpesvirus type 1: arrangement of cleavage sites for restriction endonucleases Eco RI, Bgl II and Bam HI. J. Gen. Virol., 57: 307–323.