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Equine herpesvirus 1: characterisation of the first strain isolated in Colombia

A. Cano [1], C.M. Galosi [2, 3]*, G.P. Martin Ocampos [2, 4], G.C. Ramirez [1], V.J. Vera [1], L.C. Villamil [5] & J.G. Chaparro [1]

(1) Department of Animal Health, National University of Colombia, Av. Carrera Nº 45, Bogotá, Colombia
(2) Department of Virology, Faculty of Veterinary Sciences, National University of La Plata, 60 and 118, CC296, 1900 La Plata, Buenos Aires, Argentina
(3) Scientific Research Commission (CIC), 10 and 526, 1900 La Plata, Buenos Aires, Argentina
(4) National Research Council (CONICET), Av. Rivadavia 1917, C1033 AAJ, Buenos Aires, Argentina
(5) Post-graduate Department, Faculty of Veterinary Medicine, La Salle University, Carrera 9 A N° 128-40 Apto 1008, Bogotá, Colombia
* Corresponding author: E-mail: cmgalosi@fcv.unlp.edu.ar; cmgalosi@yahoo.com

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Summary
This paper describes the isolation and characterisation of equine herpesvirus 1 (EHV-1) in Colombia. The virus was isolated from a nasal swab and an aborted foetus of a pregnant mare imported from Argentina, with clinical signs of rhinopneumonitis. The new strain was characterised through culture and morphological, serological and immunocytochemical studies. Polymerase chain reaction and DNA restriction maps revealed an EHV-1 1P genome. This is the first report on the isolation and characterisation of EHV-1 in Colombia.

Keywords
Aborted foetus – Colombia – Equine herpesvirus 1 – First characterisation – Nasal swabs.

Introduction

Equine herpesviruses 1 and 4 (EHV-1 and EHV-4) are alphaherpesviruses that cause respiratory infections in horses. EHV-1 and EHV-4 are two genetically and antigenically distinct herpesviruses, with different patterns of epizootiology, pathogenesis and clinical disease (1, 3, 10, 12). The most severe problem following EHV-1 infection is late abortion in mares, which may result in an abortion storm. Abortion usually occurs without warning. The virus is transmitted as a respiratory infection and has become endemic throughout the world. In addition, EHV-1 causes paresis or paralysis. This neurological syndrome is associated with respiratory disease and abortion and can affect animals of either sex at any age, leading to lack of coordination, weakness, and posterior paralysis. Abortion caused by EHV-4 is considered to be rare because the EHV-4 infection does not cause viraemia (1, 3).

Equine herpesvirus 4 is genetically stable, whereas two types (1P and 1B) have been identified for EHV-1 (1). Allen *et al.* reported a sudden increase of the 1B type in the early 1980s in Kentucky (2). Recent surveys in Europe have

shown that the majority of EHV-1 isolates belong to the 1P type (9). Similar studies were reported in Australasia and the results reveal that 1P type was the most common cause of abortion (11). In Argentina, the first isolate of EHV-1 was reported in 1979 (4) and since then, numerous viral isolates have been obtained from aborted foetuses and neonatal disease (5, 7). The first studies demonstrated genetic homogeneity among the Argentine EHV-1 strains (6). The most recent results, obtained with 69 Argentine strains analysed by restriction endonuclease analysis, demonstrated that genome 1B has been present in Argentina since 1996 (8).

This study describes the characterisation of the first EHV-1 strain isolated in Colombia (in 2002) from a pregnant mare introduced from Argentina showing clinical signs of rhinopneumonitis.

Materials and methods

Virus strains

A nasal swab from the mare and samples of the lung from the aborted foetus were transported in Minimum Essential Medium (MEM) without foetal calf serum (FCS) and processed at the virology laboratory by standard methods for viral isolation in MDBK (Madin-Darby bovine kidney) cells. Supernatants of infected cell cultures were kept at –146°C and then lyophilised. The Argentine (1/01) strain and the HH1 Japanese strain provided by Dr T. Mikami (Tokyo University, Japan) were used as controls.

Cell cultures and virus isolation

Confluent monolayer cultures of rabbit (RK-13) cells grown in six-well plates were inoculated with the lyophilised samples reconstituted in distilled water. Plates were incubated for 60 min at 37°C in an atmosphere of 5% CO_2. After removing the inoculums, the cells were overlaid with maintenance medium (MEM 2% FCS). The cell cultures were reincubated at 37°C and examined daily for the appearance of viral cytopathic effects (CPE). When CPE was extensive the supernatant was separated by low speed centrifugation (3,000 *g* for 15 min at 4°C) and titrated in RK-13 cells using the Reed and Muench method. The titre was expressed at 96 h post infection as $CCID_{50}$ (cell culture infectious dose 50%) / 50 µl. Infected and non-infected RK-13 cells grown on coverslips were fixed for 30 min in cold acetone for subsequent routine immunocytochemical analysis.

Virus neutralisation test

To confirm the isolates as EHV-1, a micro-neutralisation test was performed. The reference sera used were EHV-1 antiserum (T. Kumanomido, Japan Racing Association, Tochigi, Japan 1990) and equine arteritis virus (EAV) Bucyrus strain antiserum (Fukunaga, Japan Racing Association, Tochigi, Japan 1990). Serial two-fold dilutions were then prepared in serum-free medium starting at a 1:2 serum dilution. These dilutions were then mixed with an equal volume of 100× $CCID_{50}$ of the viruses isolated from both samples. After 60 min of incubation in a 5% CO_2 atmosphere, 100 µl of RK-13 cells (3×10^5 cells/ml) were added.

Immunocytochemical analysis

Equine herpesvirus 1 and EAV monoclonal antibodies diluted 1:100 in phosphate-buffered saline (PBS) were used as primary antibodies at 37°C for 45 min. The avidin-biotin-complex (Vector Lab., Burlingame, CA, United States of America) was employed as the detection system.

Electron microscopy

Cell cultures were examined 24 h after viral inoculation and when a CPE was slightly evident, the infected cells were scraped, fixed in buffered glutaraldehyde and then treated with osmium tetraoxide. A Jeol, JEM 1200EX II (Japan) electronic microscope was used for the visualisation.

DNA extraction and restriction endonuclease analysis

For viral DNA extraction, confluent monolayers of RK-13 cells were grown and infected with the new isolates. When the CPE was extensive, the cells were scraped, washed with PBS and digested at 50°C for 4 h with Proteinase K at a final concentration of 0.2 mg/ml in buffer consisting of 100 mM Tris-HCl pH 7.5, 12.5 mM EDTA (ethylene diamine tetracetic acid) pH 8.0, 150 mM NaCl and 1% SDS. The lysate was extracted once with TE- (10 mM Tris-HCl pH 8.0 and 1mM EDTA pH 8.0) saturated phenol and once with a mixture of phenol, chloroform and isoamyl alcohol (25:24:1). Finally, the total DNA were precipitated with two volumes of 99% cold ethanol, rinsed twice with 70% cold ethanol, dried and dissolved in 40 µl of sterilised distilled water. The DNA solution was digested overnight with *Bam*HI at 37°C and then subjected to electrophoresis at 16V for 16 h on 0.5% agarose gel in TAE buffer (40 mM Tris-acetate pH 7.8, 5 mM sodium acetate, and 1 mM EDTA). Bacteriophage lambda DNA cleaved with *Hind*III was used as a size marker. After electrophoresis, the gel was stained with ethidium bromide and photographed under shortwave ultraviolet light.

Polymerase chain reaction

Polymerase chain reaction (PCR) was conducted on DNA extracted using a previously described method (6). It was performed using a pair of specific oligonucleotide primers derived from glycoprotein C (gC) of EHV-1:

– P1 5´-ACACCAACTCACACAACTCCGAATC-3´ and

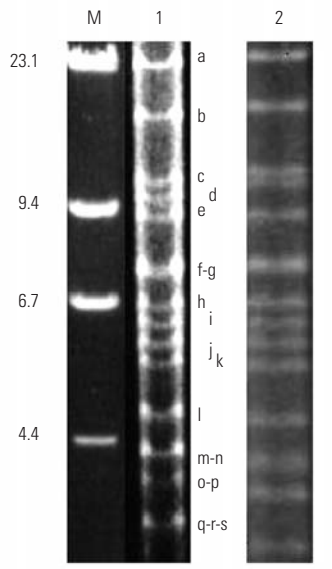
– P2 5´-GCATACAAGGACCACACGTAAATG-3´)

The amplification was carried out in a final volume of 50 µl containing 5 µl of DNA, 3 µl of $MgCl_2$ (25 mM), 5 µl of 10× concentrated PCR buffer, 1.25 U of *Taq* DNA polymerase, 1 µl of deoxynucleotide mix (0.2 mM each of deoxyadenosine 5´-triphosphate, deoxyguanosine 5´-triphosphate, deoxycytidine 5´-triphosphate, and deoxythymydine 5´-triphosphate) and 2 µl of each primer (20 pM each). The conditions for PCR amplification were: a) initial heating to 94°C for 5 min; b) 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 90 s; c) final extension at 72°C for 5 min. The PCR products (10 µl sample) were examined on 2% agarose gel in TBE buffer (50 mM Tris pH 8.0, 50 mM boric acid and 1 mM EDTA). The gels were examined under UV light following ethidium bromide staining. The molecular sizes of fragments were compared with those of a 100 bp (base pairs) ladder (Promega Lab, Madison, United States of America). A visible band of weight equal to 489 bp was considered a positive result. Complementary DNA (cDNA) of EAV was used as control.

Results and conclusions

Cytopathic effects consisting of foci of spherical cells and lysis were detected about five days after first inoculation with both samples on RK-13 cells. This time frame may have been the consequence of the low titres of virus in the lyophilised samples and of the adaptation of the virus to the new cells. The CPE increased after successive passages. Immunocytochemical analysis was positive for EHV-1. Electron microscopy revealed viral particles in the nucleus and in the cytoplasm and their ultrastructure was consistent with descriptions for EHV (7). The isolated viruses (designated B-C/02) were confirmed as EHV-1 by virus neutralisation test. The viruses were not neutralised by EAV antiserum. DNA extracted from cell cultures infected with two isolated viruses was specifically amplified by PCR and generated a product yielding a sharp visible band of 489 bp on an ethidium bromide gel. Identical bands were obtained from both positive controls. The negative control consisting of EAV cDNA produced no amplification band with the EHV-1 specific primer pair used in this study. Restriction endonuclease DNA fingerprinting with *Bam*HI confirmed that the virus recovered from the nasal swab and aborted foetus was EHV-1 1P (Fig.1).

This is the first report of the isolation and characterisation of an EHV-1 strain in Colombia. However, the infected horse was imported from Argentina where EHV-1 is endemic (4, 6, 7). Surveys to establish the serological evidence have not been carried out and no new strains have been isolated. This information and additional studies will contribute to the knowledge of epidemiology of EHV-1 infection in Colombia.



1: B-C/02 strain
2: HH1 Japanese reference strain
M: molecular weight marker fragments (λ DNA HindIII digested)

Fig. 1
Restriction fragment obtained with *Bam*HI of DNA obtained from RK-13 infected cells

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Herpèsvirus équin 1 : caractérisation de la première souche isolée en Colombie

A. Cano, C.M. Galosi, G.P. Martin Ocampos, G.C. Ramirez, V.J. Vera, L.C. Villamil & J.G. Chaparro

Résumé
Les auteurs décrivent l'isolement et la caractérisation d'une souche d'herpèsvirus équin de type 1 (EHV1) en Colombie. Le virus a été isolé à partir d'un écouvillon nasal et d'un fœtus avorté provenant d'une jument importée d'Argentine, qui présentait des signes cliniques évocateurs de rhinopneumonie. L'identification de la nouvelle souche a été obtenue par culture et par des méthodes de caractérisation morphologique, sérologique et immunocytochimique. L'amplification en chaîne par la polymérase et les cartes de restriction ont permis de mettre en évidence le génome de l'EHV-1 1P. Il s'agit du premier rapport décrivant l'isolement et la caractérisation de l'EHV-1 en Colombie.

Mots-clés
Colombie – Écouvillon nasal – Fœtus avorté – Herpèsvirus équin de type 1 – Première caractérisation.

Herpesvirus equino 1: caracterización de la primera cepa aislada en Colombia

A. Cano, C.M. Galosi, G.P. Martin Ocampos, G.C. Ramirez, V.J. Vera, L.C. Villamil & J.G. Chaparro

Resumen
Los autores describen el aislamiento y la caracterización de la primera cepa de herpesvirus equino 1 (EHV-1) aislada en Colombia. El virus fue aislado a partir de un hisopado nasal y de un feto abortado de una hembra preñada, importada de Argentina, con signos clínicos de rinoneumonitis. La cepa aislada fue caracterizada por sus propiedades culturales, morfológicas, serológicas y por estudios inmunocitoquímicos. La técnica de reacción en cadena de la polimerasa y los patrones de restricción de ADN confirmaron que pertenecía a EHV-1 1P. Esta es la primera descripción del aislamiento y la caracterización de EHV-1 en Colombia.

Palabras clave
Colombia – Feto abortado – Herpesvirus equino 1 – Hisopado nasal – Primera caracterización.

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