Sequence of the gene coding for the p26 protein from a Cuban strain of Equine Infectious Anemia Virus

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ABSTRACT

Equine Infectious Anemia Virus (EIAV) is the causal agent of equine infectious anemia. Plasma virus RNA levels and the fraction of infected cells are both very low during the chronic stage of EIAV infections, turning the amplification of viral genes into a technically demanding task. In this work, DNA was extracted from the spleen of a chronically infected animal previously immunosuppressed with prednisolone, amplifying by nested PCR and sequencing the segment of the gag gene that codes for the p26 protein. Phylogenetic analysis placed the Cuban strain closest to three Canadian strains. The translated protein sequence exhibits 7 differences with the most related Canadian strain (Can7) and 13 with the Wyoming strain. This is the first report of a partial nucleotide sequence from EIAV in Cuba and the Caribbean.

Keywords: Equine Infectious Anemia Virus, PCR, p26, sequence, spleen, Cuba, capsid

Introduction

Equine Infectious Anemia Virus (EIAV) is a member of the Lentivirus genus, in the Retroviridae family. Its genetic organization closely resembles that of type 1 Human Immunodeficiency Virus (HIV-1) [1, 2]. EIAV is the causal agent of equine infectious anemia (EIA), a disease restricted to members of the Equidae family that is distributed worldwide, causing important economic losses [2, 3]. The EIAV virion contains two glycoproteins in its envelope and four non-glycosylated internal proteins forming its capsid. Protein p26 is the most abundant capsid protein, exhibiting the highest sequence conservation not only among EIAV gene products, but among the capsid proteins of all retroviruses [4, 5].

Acute EIAV infections are characterized by frequent cycles of fever and intense viral replication, whose intensity and frequency gradually taper off until the host becomes an asymptomatic carrier for the remainder of its life. The absence of clinical symptoms, undetectable levels of viral RNA in plasma and low frequencies of infected cells are all signs of the strong control exerted at this stage on viral replication by the host immune system, which restricts the virus to organs of the lymphopoietic system [2, 6-9].

It has been shown that the administration of immuno-suppressive drugs to asymptomatic carriers leads to the appearance of clinical signs resembling an acute infection and to increased viral replication [9-11].

No prophylactic vaccines are available against this disease. Since control of EIA takes a heavy financial toll, consisting mainly in the detection and elimination of infected animals [12, 13], a number of countries prefer to isolate these horses in segregated facilities. Such a counter-epizootic measure requires, however, the availability of sensitive and reliable diagnostic means. Agar gel double radial immunodiffusion (AGID), also known as Coggins test, is the recommended test for this purpose by the International Office of Epizootics.

The application of molecular biology techniques such as reverse transcription followed by the polymerase chain reaction (RT-PCR), the latter often performed as a nested PCR, has allowed detecting and quantifying viral RNA in the plasma of recently or persistently infected animals. Nested PCR designs have also been employed to detect proviral DNA in monocytes/macrophages [14-18]. These techniques are also useful for genotyping wild strains and for monitoring the emergence and spread of pathogenic EIAV strains [14-18].

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lowing up experimental infections, and can replace se-
erological tests in the presence of interfering colostral
antibodies [5, 9, 17].

Little is known, however, about the molecular epi-
demiology of this retrovirus, as the low levels of viral
replication in chronically infected animals pose a for-
midable challenge to viral RNA detection in plasma
[19-20]. Sequence databases contain a measly total
of 17 sequences obtained from independent strains,
originating in just 6 countries.

In Cuba, this disease has been detected in the pro-
vinces of Pinar del Río, Sancti Spíritus, Ciego de Ávila,
Camagüey, Las Tunas, Holguín and Granma. Some of
them relocate positive horses to segregation farms as
an epizootic countermeasure, and the authorities in
fection-free provinces usually prefer to sacrifice
these horses they suspect might be infected. EIA is
traditionally diagnosed by AGID, and no sequence in-
formation is available on circulating strains.

The purpose of the present study was to begin the
genetic characterization of EIA strains circulating in
Cuba. Genomic DNA (gDNA) was isolated for this
purpose from peripheral blood mononuclear cells
(PBMC) and splenocytes of EIA-infected horses that
were previously subjected to an immunosuppressive
therapy. Then, nested PCR was used to isolate a frag-
ment of the gag gene coding for the p26 protein that
was then sequenced. This constitutes the first re-
port of the sequence of a circulating EIAV strain for
Cuba and the Caribbean basin.

Materials and methods

Horses and clinical parameters

Four horses naturally infected with EIAV were includ-
ed in this study. They were all asymptomatic carriers
from a segregation farm in the Pinar del Río province,
first diagnosed with equine infectious anemia (EIA)
by AGID, and declared negative for Leptospira, Bru-
cella and blood parasites. These horses were subjected
to a transitory immunosuppressive treatment intended
to activate viral replication.

Morning and afternoon rectal temperature readings
were taken one week before, and during the immuno-
suppressive treatment.

Immunosuppression

Two of the animals were administered daily intramus-
cular doses of 2 mg of prednisolone per kilogram of
bodyweight, during five days. The other two animals
received intravenous doses of 3.45 mg of prednisol-
one per kilogram of bodyweight, administered every
other day. Blood samples were drawn immediately af-
after the appearance of symptoms of the disease (fever,
lethargy, anorexia and others, but mainly increased
body temperature). The animal with the highest body
temperature was euthanized at the end of the study.

Sample extraction and processing

A total of 10 mL of venous peripheral blood were
drawn from the jugular vein, using the Vacutainer sys-
tem with EDTA as anticoagulant (Becton Dickinson,
USA). All horses had anti EIAV antibody titers above
1:16 by AGID.

Erythrocytes were lysed by osmotic shock with a
lysis solution composed of 0.03 M ammonium chlo-
ride, 0.002 M KHCO₃, 0.02 mM EDTA. The result-
ing peripheral blood mononuclear cells (PBMC) were
washed twice with PBS before proceeding to the ex-
traction of gDNA.

DNA purification

Genomic DNA (gDNA) was extracted from periphe-
ral blood mononuclear cells (PBMC) and splenocytes
from one chronically infected individual and another
undergoing immunosuppression, using Tri-Reagent
(Sigma, USA) and following instructions from the
manufacturer. One milliliter of Tri-Reagent was used
for each sample of five million PBMC. Spleen tissue
samples (50 mg) were processed in a Tissue Lyser II
(Qiagen, USA, manufactured by Retsch), also using
1 mL of Tri-Reagent per sample. Absorbance at 260 nm
and the 260/280 absorbance ratio were determined
with a NanoDrop 1000 spectrophotometer (Thermo
Scientific Nano Drop Technologies LLC, USA), and
the purified nucleic acids were visualized by electro-
phoresis in 1% agarose gels.

Polymerase Chain Reaction

Amplification by PCR of the pcna reference
gene

PCR amplification reactions were set up for the pcna
(proliferating cells nuclear antigen) housekeeping
gene in every analyzed sample, following the protocol
described by Schiller et al. and the oligonucleotide
primers described therein. These primers generate a
242 bp amplification product. The target gene is high-
conserved across all mammalian species [21].

Amplification by nested PCR of a fragment
of the gag gene

The first reaction employed the oligonucleotides de-
scribed by Langemeier et al. [17], which yield an
853 bp-long amplification product. Published ampli-
fication conditions were modified to implement a
touch-down PCR as follows: an initial denaturation
at 94 °C for 10 min, followed by ten cycles of de-
naturation at 94 °C (30 s), annealing at 60 to 51 °C,
decreasing temperature by 1 °C per cycle (30 s) and
extension at 72 °C (1 min), followed in turn by 25 cy-
dles of denaturation at 94 °C (30 s), annealing at 50 °C
(30 s) and extension at 72 °C (1 min). An extension
step of 72 °C for 7 min was added at the end of the
reaction.

The primers described by Rosatti et al. [22] were
modified for the second PCR reaction, which yields
a 705 bp-long amplification product. Their sequenc-
es were: sense primer- 5'-CCAATCATGATAGAT
GGGGCTGGAAACAG-3'; antisense primer: 5'-AAG
TCTTTTGGCAATAACATCATCTTTTG-3'.

Conditions for the second PCR included an initial
denaturation at 94 °C for 10 min, followed by 30 cy-
dles of denaturation at 95 °C (30 s), annealing at 54 °C
(30 s) and extension at 72 °C (30 s). An extension step
of 72 °C for 7 min was added at the end of the reaction.

All PCR reactions were performed in a total volume
of 25 µL, including 1 µL of template DNA containing
approximately 500 ng, 12.5 µL of PCR Master Mix
2X, (Promega, USA), and 25 pmol of each oligonu-
cleotide. Amplified products were visualized by elec-
trophoresis in 0.8% agarose gels at 120 V for 30 min.

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in the presence of ethidium bromide (0.5 μg/mL) and using Bromophenol Blue as running dye. Electrophoretic runs always included a DNA ladder (Promega, USA) as molecular weight marker, negative controls from the PCR, reagent controls and a positive control, prepared with leukocyte DNA from peripheral blood of an animal with an experimental infection of the Wyoming (Wy) reference strain.

**DNA sequencing**

The amplified product was diluted to a concentration of 50 ng/μL and sent to Macrogen (South Korea) for further purification and sequencing, using the same primers utilized for PCR, diluted at 5 pmol/μL.

**Phylogenetic analysis**

Nucleotide sequences were aligned using Mega 3.1 [23]. This software application is based on the Clustal X algorithm, which uses neighbor-joining [24] with Kimura’s two-parameter nucleotide substitution algorithm. The 705 nucleotide sequence of the amplified gag gene sequences was compared with existing EIA GenBank sequences corresponding to this part of the gene. Their accession numbers, strain code and origin are: EU240733.1 (Ita-1), EU375543.1 (Ita-2), EU375544.1 (Ita-3) and EU741609.1 (Ita-4), all from Italy; AF327878.1 (CHDLV) and AF327877.1 (CHN) from China; EF418584.1 (Can-7), EF418582.1 (Can-1), EF418583.1 (Can-3) and EF418585.1 (Can-10) from Canada; AB008196 (V70) and AB008197.1 (V26) from Japan; and AF170989 (EIAV-ID), AF170984 (EIAV-TX), AF033820.1 (Wy), AFO16316 (EIAVuk), M16575 (WE-EIA VOG), AF172139.1 (WSU-5) and AF247394.1 (Wy) from the United States.

**Results and discussion**

Although all four horses had clinical EIA symptoms (increased body temperature, anorexia and weakness), these were intense only in animal 5-04 (body temperature of 39.5 °C), indicating that higher doses of immunosuppressants or longer treatments must be employed in the future. Blood samples were drawn from all animals one day after concluding the treatment.

The samples were used to obtain PBMC, which were in turn used to extract genomic DNA. Template quality was assessed by PCR amplification of the pcna gene from gDNA. A product of the expected size for this species (242 bp) [21] was observed in all cases, thereby ruling out poor sample quality as a factor accounting for failed EIA amplifications.

These samples, however, failed to yield detectable amounts of the expected p26 amplicon upon EIAV-specific PCR, despite using the same conditions and oligonucleotides that successfully detect proviral copies in gDNA from PBMC obtained from horses experimentally infected with the Wyoming reference strain.

Other authors have had similar problems in the past [7, 19]. The amount of proviral DNA in PBMC from asymptomatic horses chronically infected with EIAV is known to be very low, bordering on the undetectable for all but the most sensitive of PCR setups [25-28]. We failed to obtain the p26 amplicon even in gDNA of PBMC from animals previously subjected to immunosuppressive treatments.

Harrold et al. [7] described differences in proviral DNA load for several organs (proviral DNA levels in spleen tissue, for instance, were 10- to 20-fold higher than anywhere else). Their results, in addition, confirmed that the levels of proviral DNA during chronic EIAV infections are extremely low, even in horses experimentally infected with the Wyoming strain that survive the acute stage of the disease.

It was decided, therefore, to try to amplify the proviral p26 sequence from gDNA extracted from the spleen of horse 5-04, which exhibited the most pronounced clinical symptoms. An amplicon with an electrophoretic mobility consistent with that of the pcna band was observed when this sample was amplified with the corresponding oligonucleotides (Figure 1). When subsequently amplified by means of a nested PCR with the same primer sets failing to produce a signal with PBMC gDNA, this sample yielded an amplicon whose electrophoretic mobility matched that of the expected 705 bp band (Figure 2).

The amplified fragment was sequenced with the same primers used for the second PCR amplification. The obtained nucleotide sequence was denominated CUBPR001, and can be retrieved from GenBank under access number HQ853234.

Worldwide availability of EIAV sequence data is restricted to a total of only 17 independent strains, and most EIAV GenBank sequences correspond to viruses derived from the Wyoming reference strain. The only countries that report sequence information from indigenous EIAV strains corresponding to the gag segment amplified in this work are USA, Canada, Argentina, Italy, China, Japan and Thailand. A phylogenetic tree obtained by comparing the obtained 705 bp nucleotide sequence with that from these strains shows the genetic relations between the Cuban isolate and independent EIAV isolates from around the world (Figure 3).

**Figure 1. Amplification by polymerase chain reaction (PCR) of the pcna housekeeping gene. Agarose gel electrophoresis (1.5%) of PCR amplification products: 1) Sample template gDNA; 2) Equine control gDNA; 3) no-template negative control; 4) Molecular weight markers (1- Hin III, Promega, USA).**
It should be borne in mind that accuracy in phylogenetic studies increases with the number of sequences included in the analysis [29]. Our results are, therefore, constrained by the scarcity of available sequence data from independent strains.

However, phylogenetic tree reconstruction and estimation of evolutive parameters can also be improved by using longer sequences. In this sense, the 705 bp-long sequence used in the alignments is appropriate for obtaining reliable results when estimating genetic relatedness with distance-based methods, such as neighbor joining [24]. The analysis showed that strain CUBPR001 clustered closest to three of the ten Canadian strains, although bootstrap values in this node were low. Bootstrapping, used to estimate the level of confidence for internal branches of phylogenetic trees, is considerably simplified by the speed of the chosen analysis method [30].

Aiming at studying how the detected genetic differences translate into amino acid variability, it was decided to also prepare sequence alignments of translated p26 segments from the Cuban strain, the Wyoming strain, and the most closely related Canadian strain (Figure 4). There were 13 amino acid changes between the Cuban and Wyoming strains, and 7 between the Cuban and Can1 strains; confirming that the obtained p26 fragment is most similar, at the amino acid level, to viruses circulating in Canada.

The nested PCR setup used in this study has allowed obtaining, for the first time, sequence information from the gag gene of an indigenous Cuban EIAV strain. Combining PCR with a previous immunosuppressive treatment and extracting gDNA from spleen tissue seems to be the most effective procedure for the molecular characterization of new field isolates from chronically infected animals. Immunosuppression must, however, be further optimized, as increasing viremia to the point that amplification of viral sequences from PBMC becomes feasible would make...
invasive procedures, such as spleen biopsies, totally unnecessary, and would altogether obviate the occasional need to sacrifice the animal.

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