The document contains an introduction to the study of Equine Infectious Anemia Virus. It mentions that the virus is the cause of equine infectious anemia and describes the characteristics of the infection, including acute and chronic stages. The study highlights the importance of genetic analysis to understand the virus and its impact on the host. The text also discusses the potential use of reverse transcription followed by the polymerase chain reaction (RT-PCR) for detecting viral RNA. The study's methodology, including DNA extraction from the spleen of a chronic carrier, is described. The keyword list includes terms related to Equine Infectious Anemia Virus (EIAV), PCR, p26, sequence, spleen, Cuba, capsid, and the Caribbean.
lowing up experimental infections, and can replace serological tests in the presence of interfering colostral antibodies [5,9,17].

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

In Cuba, this disease has been detected in the provinces 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 infection-free provinces usually prefer to sacrifice those horses they suspect might be infected. EIA is traditionally diagnosed by AGID, and no sequence information is available on circulating strains.

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

Materials and methods

Horses and clinical parameters

Four horses naturally infected with EIAV were included 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, Brucella 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 immunosuppressive treatment.

Immunosuppression

Two of the animals were administered daily intramuscular 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 prednisolone per kilogram of bodyweight, administered every other day. Blood samples were drawn immediately 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 system 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 chloride, 0.002 M KHCO₃, 0.02 mM EDTA. The resulting peripheral blood mononuclear cells (PBMC) were washed twice with PBS before proceeding to the extraction of gDNA.

DNA purification

Genomic DNA (gDNA) was extracted from peripheral blood mononuclear cells (PBMC) and splenocytes of EIAV-infected horses that were previously subjected to an immunosuppressive treatment. Then, nested PCR was used to isolate a fragment of the gag gene coding for the p26 protein that was then sequenced. This constitutes the first report of a sequence of circulating EIAV strain for Cuba and the Caribbean basin.

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 highly conserved across all mammalian species [21].

Amplification by nested PCR of a fragment of the gag gene

The first reaction employed the oligonucleotides described by Langemeier et al. [17], which yield an 853 bp-long amplification product. Published amplification 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 denaturation 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 cycles 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-
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: EU240773.1 (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 AF172098 (EIAV-ID), AF170894 (EIAV-TX), AF033820.1 (WY), AF016316 (EIAVuk), M16575 (WY-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 (243 bp) [21] was observed in all cases, thereby ruling out poor sample quality as a factor accounting for failed EIAV 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 (λ-Hind III, Promega, USA).](image)
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

![Figure 2. Agarose gel electrophoresis (0.8%) of amplification products from the second reaction of the nested PCR procedure for p26. 1) DNA CUBPR001; 2) Positive control (gDNA from Wyoming strain); 3) No-template negative control for PCR 1; 4) No-template negative control for PCR 2; molecular weight marker (DNA ladder, Promega, USA).](image)

![Figure 3. Phylogenetic tree depicting relationships between existing EIAV strains, based on the amplified 705 bp nucleotide sequence. Gag sequences for the selected EIAV strains can be retrieved using the following access numbers: EU240733.1 (Ita-1), EU375543.1 (Ita-2), EU375544.1 (Ita-3), EU741609.1 (Ita+4), AF327878.1 (CHDLV), AF327877.1 (CHN), AF185841.1 (Can-7), AF185821.1 (Can-1), EIAV-TX-AF170894.1 (Can-10), AB081996 (V70), AB081971.1 (V26), AF172098.1 (EIAV-ID), AF172094.1 (EIAV-TX), AF033820.1 (WY), AF016316.1 (Wy-AF033820.1), M16575.1 (Wy-AF033820.1), AF172139.1 (WSU-5), AF247394.1 (WY).](image)

![Figure 4. Comparison of amino acid sequences for p26. The alignment includes the Cuban strain (CUBPR001), the Wyoming strain (WY) and the Canadian strain most closely related with the Cuban strain (Can-1).](image)
invasive procedures, such as spleen biopsies, totally unnecessary, and would altogether obviate the occasional need to sacrifice the animal.

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