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BRIEF REPORT

# Characterization of isolates of equine infectious anemia virus in Brazil

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**Abstract** Equine infectious anemia is an important infectious disease that affects equidsworldwide. Control of the disease is currently based on detection of anti-p26 EIAV byAgar Gel Immunodiffusion (AGID). In this work, 62 animals were examined by AGIDand nested-PCR using primers for the gag gene. Fifty-three samples (85.5%) werepositive by nested-PCR, whereas only 33 samples (53%) were positive for AGID.Fifteen amplicons obtained by nested-PCR were sequenced and the aligned resultssubjected to phylogenetic analysis. The analysis suggests that the Brazilian EIAV forma cluster with WSU5, EIAVUK and Wyoming strains from United States.

Equine infectious anemia (EIA) is an infectious and persistent but noncontagious disease restricted to members of the family *Equidae* (horses, mules and donkeys) found almost worldwide. It is caused by equine infectious anemia virus (EIAV), which belongs to the subfamily *Orthoretrovirinae* and genus *Lentivirus* in the family *Retroviridae* [9, 11]. In Brazil, EIA was diagnosed for the first time in 1968, in the states of Rio Grande do Sul and Rio de Janeiro [12]. Since 1981, Ministério de Agricultura Pecuária e Abastecimento (MAPA) has included the EIA in the list of diseases subjected to control measures, such as euthanasia of animals diagnosed positive by AGID, to limit the spread of the virus [3]. EIA has a direct economic impact due to the loss of livestock when diseased animals are euthanized, and also an indirect effect, since MAPA imposes embargoes for transit of horses, with barriers being imposed for markets of live animals and animal products, consequently also affecting expositions and equestrian sports. Altogether, the economic effects have considerable importance [16, 29]. The clinical signs of the disease are highly variable. It varies from an acute febrile episode to a chronic or long-term unapparent course. The chronic form of EIA is characterized by recurring cycles of viremia with fever, pronounced thrombocytopenia, severe anemia, weight loss, jaundice, tachypnea and ventral edema [2, 9]. During febrile episodes, the virus replicates actively on macrophages present in spleen, liver, lymph nodes, lungs and kidneys [31], although research has shown that other cells, such as endothelial cells, may also be susceptible to the virus [23]. Animals that survive the cyclic episodes of anemia and fever become asymptomatic virus carriers [11]. The virus has tropism for monocytes, which results initially in a non-productive infection. The differentiation of monocytes into macrophages activates viral replication, an event called "replication restriction" [6, 21]. The blood is therefore an important source of transmission between infected and non-infected horses [23]. The virus can be transmitted mechanically, through the bite of bloodsucking insects, such as horseflies (*Tabanus* spp; *Hybomitra* spp) and stable flies (*Stomoxys* spp), which are mechanical vectors of the virus [10, 14, 35], by iatrogenic means through harnesses, spurs and needles or surgical instruments contaminated with blood, and also

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through nursing [33]. There is no treatment or effective vaccine for EIA, and the diagnostic is serological. The most widely used test for EIAV is an agar gel immunodiffusion (AGID) assay commonly referred to as the Coggins test [7]. The clinical diagnosis is difficult due to the absence of specific signs of the disease and because many animals become asymptomatic carriers between 12 and 24 months after infection [17]. Virological diagnosis is complicated, since virus isolation in cell culture depends on the viral load of the infected animal [15, 19]. The two single-stranded positive-sense RNAs of the EIAV genome are approximately 8.2 kb in length and contain three major coding regions: *gag*, encoding the viral capsid proteins; *pol*, encoding the enzymes protease, reverse transcriptase (RT), and integrase (IN); and *env*, encoding the two envelope glycoproteins: the surface glycoprotein (gp90) and the transmembrane glycoprotein (gp45) [18, 20]. The proviral genome also contains three additional ORFs, encoding *tat* and *rev*, which are present in all lentiviruses, and S2 [19]. The *gag* gene encodes a polyprotein that is cleaved by a virus-encoded protease to generate three major products: matrix (p15), capsid (p26) and nucleocapsid (p11), plus a low-molecular-weight protein (p9) that is responsible for recruiting components of the cellular endocytosis machinery for viral budding [5, 28]. The prevalence of EIA varies in different states of Brazil. Melo et al. [24] reported the prevalence in Pantanal to be 36%; in Minas Gerais it varied from 0.07% to 5.1% [1]; in Bahia, was reported to be 5.9% [13]; and in Mato Grosso, it was 5% [26]. The prevalence of EIAV and the genetic profile of the virus circulating in Bahia-Brazil needs to be further studied. The aim of this study was to detect EIAV by nested PCR and subsequently perform DNA sequencing of the proviral genome to characterize the Brazilian EIAV strain.

A total of 62 blood samples (57 from horses and 5 from mules) were subjected to peripheral blood monocyte cell (PBMC) isolation by Ficoll gradient (Ficoll Paque Plus™, GE Healthcare) according to the manufacturer's instructions. All animals were tested for EIAV-specific antibodies using AGID (Biovetech, Brazil). The PBMCs were resuspended in Dulbeco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Gibco-BRL) and antibiotics (100 UI penicillin G sodium and 100 mg of streptomycin sulfate per ml). The resuspended PBMCs (at least 600,000 cells/well) were placed in 24-well microplates and incubated for 24 h at 37 °C in a 5% $CO_2$ atmosphere. After this time, the plates were washed so that only monocytes would remain due to adherence, and DMEM supplemented with 10% FBS and antibiotics was added. The PBMC cultures were incubated at 37 °C in an atmosphere of 5% $CO_2$ for 8 days to obtain monocyte-derived macrophages (MDMs). After this period, cultures were stored at −70 °C until nested PCR was performed. Proviral DNA (n = 62) was extracted from MDMs using a QIAamp Kit (QIAGEN, Germany) according to the manufacturer's instructions. A partial sequence of the *gag* gene was amplified following the protocol described by Oaks *et al.* [27]. After amplification, PCR products (427 bp) were separated by agar gel electrophoresis, stained with ethidium bromide, and detected using a UV transilluminator. Nested PCR products were purified using QIAquick Gel Extraction Kit (QIAGEN), according to the manufacturer's instructions and then subjected to direct sequencing using a Big Dye Terminator® v 1.1 Cycle Sequencing Kit (Applied Biosystems, EUA). Sequencing was performed by ACTgene, Biotech Center of the Universidade Federal do Rio Grande do Sul (UFRGS). Nucleotide sequences were aligned and edited with Clustal W software available in the Bio-Edit program (http://mbio.ncsu.edu/bioedit.html) and then compared with the EIAV sequences published in GenBank, using the Basic Local Alignment Search Tool (BLAST) application. Phylogenetic analysis was performed using MEGA version 6 software [32], employing the neighbor-joining method [34]. Phylogenetic trees were constructed by the maximum-likelihood method with the Hasegawa–Kishino–Yano model (HKY + G + I) nucleotide substitution algorithm. Statistical support for the trees was evaluated by bootstrapping based on 1000 repetitions. Including gaps and excluding the primer regions, a 393-nucleotide-long sequence of amplified partial *gag* fragments were compared with existing EIAV GenBank sequences. The accession numbers, strain codes and origins are as follows: AF033820.1 (Wyoming), AF016316.1 ($EIAV_{UK}$), L06609.1 (WSU5), AB008196.1 (V70), AB008197.1 (V26), AF170894.1 (EIAV/TEXAS) and AF172098.1 (EIAVID) from the United States; JX003263.1 (Myiazaki2011-A) from Japan; AF327877.1 ($EIAV_{Liaoning}$), GU385365.1 (LN5) and GU385361.1 (FDDV3-8TM) from China; AB693824.1 (German) from Germany, HQ888862.1 (Ita-90) from Italy; JX193070.1 (Assebroek) and JX19307 2.1 (Warsage) from Belgium; EF418585.1 (Can-10) from Canada and JX480631.1 ($EIAV_{IRE}$) from Ireland. The sequences reported in this paper were deposited under GenBank accession numbers KC213776-KC213790 and are the first sequences of the *gag* gene isolated from Brazil.

Of the 62 samples tested by nested PCR, proviral DNA was detected in 53 (85.5%). Of those, 20 samples were negative by serologic testing (Table 1). Only two samples that were positive by AGID were not detected by nested PCR (Table 2). Statistical analysis showed that the nested PCR and AGID tests had reasonable agreement ($\kappa = 0.21$). PCR-generated amplicons from 15 nested PCR-positive MDM samples were subjected to sequencing. Using the BLAST program, it was confirmed that all resultant

**Table 1** Comparison of agar gel immunodiffusion (AGID) and nested PCR test results for the detection of EIAV-infected equids

| Nested PCR | AGID | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Positive | 33 | 20 | 53 |
| Negative | 02 | 07 | 09 |
| Total | 35 | 27 | 62 |

nucleotide sequences possessed significant identity to those previously published for EIAV. The amplified 393-nt region (nucleotides 945-1337) of the viral genome corresponds to the amino-terminal region of the p26 protein (residue 141-271). Taking into consideration the studied sequence, the phylogenetic tree topology shows that the strains from Bahia form a cluster with the strains WSU5, EIAVUK and Wyoming (USA) (bootstrap value D 95). This is also confirmed by the genetic distances between sequences from this study and the WSU5 strain (0.003–0.005). The largest distance (0.209) was observed with the strain F2 (Ireland) (Fig. 1). As can be seen in the phylogenetic tree (Fig. 1), the KC213778.1 sequence was genetically more distant from the others determined in this work. This sequence was obtained from a foal (negative by AGID) born to a positive female (KC213790.1). Although the sequence from KC213778.1 contained a 43-nucleotide substitution, it still maintained enough similarity for it to group in the same cluster with other Brazilian strains.

The nested PCR methods showed reasonable concordance ($\kappa = 0.21$) with the AGID test for identifying EIAV-infected animals. However, despite its excellent specificity, the serological test has a number of deficiencies, including a lack of sensitivity and the fact that interpretation of results can be highly subjective [8, 30]. The sensitivity of nested PCR for detecting proviral DNA in blood cells allowed detection of infection in healthy equines, in which the viral loads are generally very low [27]. Similar results were obtained that showed a moderate agreement ($\kappa = 0.441$) between the techniques of nested PCR and AGID [30]. Two samples, however, failed to yield detectable amounts of the expected amplicon upon EIAV-specific PCR. Other authors have had similar problems [8, 27]. The amount of proviral DNA in PBMCs is known to be very low, bordering on the undetectable for all but the most sensitive of PCR setups, which may be explained by 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 [18, 22]. In addition, the primers used may not be able to amplify EIAV strains with mismatches within the primer-binding sites. One or two mismatches near the 3′ end of the primer do not fully inhibit primer binding, whereas three mismatches at the 3′ end and more than two mismatches in both the reverse and forward primers hamper primer binding, preventing amplification [28]. The absence of contaminants in the non-template controls and the results of sequencing confirmed the specificity of the amplified products. According to Capomaccio *et al*. [4], who analyzed the phylogeny of EIAV isolates, the virus circulating in North and South America was introduced by European settlers, which may explain the homology of the isolated sequences in Brazil with USA strains. Because healthy animals carrying the virus may have inconspicuous or absent clinical signs, the

**Table 2** Results of nested PCR performed on MDM and AGID from 15 animals infected with EIAV

| Animal number | Farm number | GenBank acession number | Results of nested PCR | AGID |
|---|---|---|---|---|
| LABVIRO/UFBA/02 | 1 | KC213776.1 | + | + |
| LABVIRO/UFBA/03 | 2 | KC213789.1 | + | + |
| LABVIRO/UFBA/04 | 2 | KC213788.1 | + | − |
| LABVIRO/UFBA/05 | 3 | KC213787.1 | + | + |
| LABVIRO/UFBA/06 | 4 | KC213786.1 | + | + |
| LABVIRO/UFBA/08 | 5 | KC213785.1 | + | + |
| LABVIRO/UFBA/15 | 5 | KC213784.1 | + | + |
| LABVIRO/UFBA/16 | 5 | KC213783.1 | + | + |
| LABVIRO/UFBA/21 | 5 | KC213777.1 | + | + |
| LABVIRO/UFBA/23 | 5 | KC213782.1 | + | − |
| LABVIRO/UFBA/31 | 5 | KC213781.1 | + | − |
| LABVIRO/UFBA/12 | 6 | KC213790.1 | + | + |
| LABVIRO/UFBA/44 | 6 | KC213778.1 | + | − |
| LABVIRO/UFBA/39 | 7 | KC213780.1 | + | − |
| LABVIRO/UFBA/52 | 8 | KC213779.1 | + | + |

Sequence data for EIAV strains are available in the GenBank database

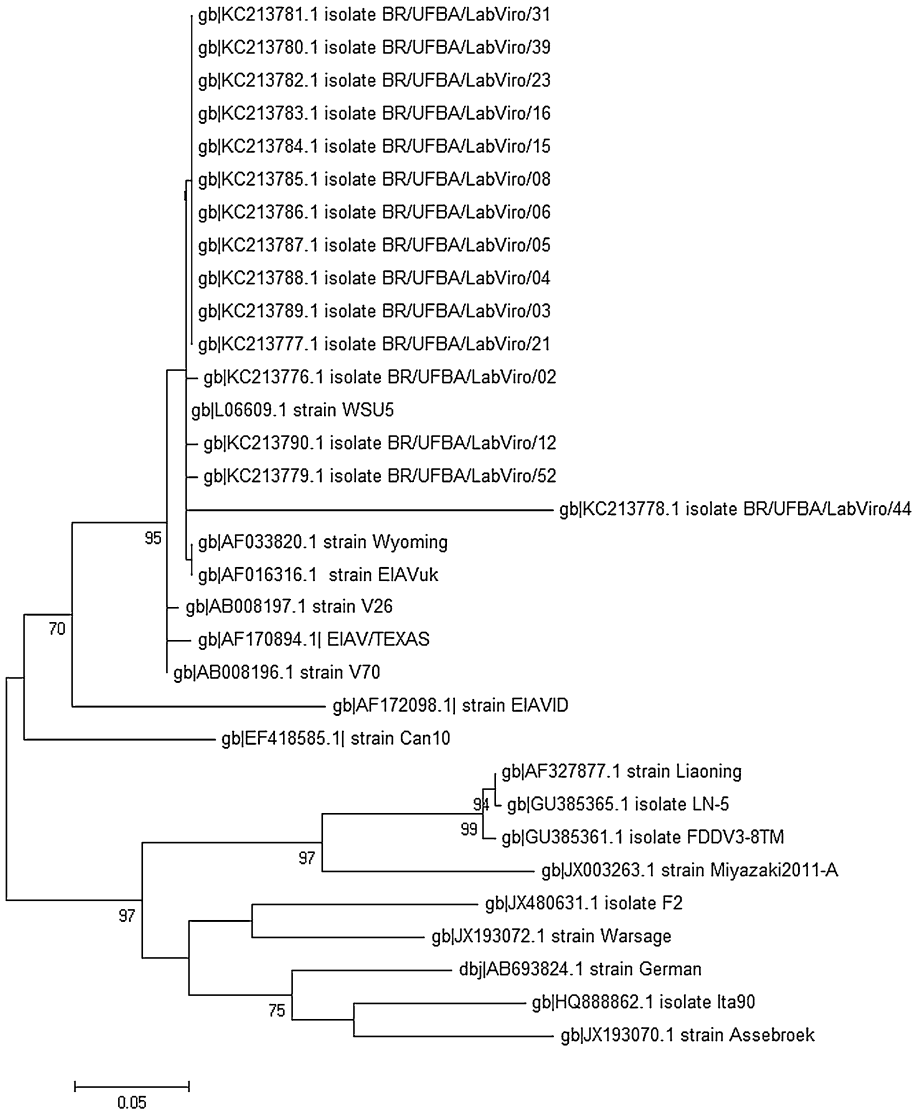


**Fig. 1** Phylogenetic tree based on a partial fragment of the *gag* gene. GenBank accession numbers and strain designations are shown

disease would have gone unnoticed by the European settlers. The variability in the sequence of the *gag* gene analyzed, although unusual, may represent polymorphisms, such as SNPs, deletions and insertions, that contribute to longer survival by evading immune surveillance and altered disease progression [25, 28]. It is characteristic of the lentiviruses to have high rates of small mutations, consequently giving rise to a population of different virus particles that share genetic, morphological and pathological features, commonly referred to as viral quasispecies [20]. Genetic variations lead to a viral persistence framework that allows the virus to escape the host immune response, leading to periods of recrudescence of disease [17, 20].

We studied the prevalence of EIAV infection and performed a phylogenetic analysis of the virus isolated in Bahia. Further studies are necessary in order to determine the prevalence of EIAV in other parts of the country and to investigate the genetic variability of the circulating virus. The study of genetic diversity of EIAV in Brazil allows molecular diagnostic methods to be evaluated and primers to be chosen to detect different field isolates.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** Not required by this journal: study based on samples collected from equids subjected to euthanasia due to their disease status.

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