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Short Communication

Case Report of Equine Influenza in Italy, in 2014

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ABSTRACT

Equine influenza (EI) is a highly contagious respiratory disease presently caused by subtype H3N8. As no active surveillance is ongoing in Italy, clinical observations of veterinary practitioners would indicate the infection as endemic. This study reports the isolation and genetic characterization of an EI virus (EIV) from a stable of Thoroughbred race horses. The episode was characterized by fever and dry cough and involved eleven, 2- to 3-year-old, flu vaccinated horses, four of which were found positive for EIV, by reverse-transcriptase real-time polymerase chain reaction (RRT-PCR) targeting the matrix gene. The EIV of two of the four RRT-PCR positive samples was successfully passaged in embryonated chicken eggs. Analysis of amino acid (AA) sequences of hemagglutination (HA) residues detected seven substitutions in the Italian isolate when compared with the prototype strain A/eq/Richmond/1/2007 (H3N8 American lineage, Florida clade 2). The constructed phylogenetic tree locates the present strain within the Florida clade 2, together with viruses isolated between 2011 and 2014 in Algeria, China, Eire, Germany, Japan, Mongolia, and the United Kingdom. Within this cluster, the Italian isolate presents 100% AA identity with A/eq/North Rhine Westphalia/1/2014, responsible of an outbreak in Germany around the same period. Even if vaccinated, only two of the 11 clinically involved animals, different from the EIV-positive animals, reacted positive in HA inhibition. Monitoring of the circulating strains of EIV is essential especially for verifying the appropriateness of the vaccine virus composition, as recommended by the OIE Panel of experts, on the basis of the genetic and antigenic characteristics of emerging strains.

1. Introduction

Equine influenza (EI) is a highly contagious respiratory disease caused by influenza virus (EIV), type A of the Orthomyxoviridae family. In susceptible animals, clinical signs include pyrexia, a harsh dry cough and a serous nasal discharge, often leading to secondary bacterial infections. Incompletely immune animals, condition that may be due to a previous infection with a different strain, an incomplete vaccination program, or vaccines containing strains different from those circulating, can contribute to the asymptomatic spread of EIV. Although rarely fatal, EI has a high impact on the equine racing industry, as a result of the long recovery, cost of therapy, and movement restrictions of sick and in contact horses [1].

In the past, two subtypes of EIV, H7N7 and H3N8, were associated with the disease in the horse. However, H7N7, first identified in 1956 in Eastern Europe, has not been isolated in the equine population since the late 70s [2]. On the contrary, H3N8, first documented in 1963, in Florida [3] is at present circulating throughout the world.

Starting from the early 90s, H3N8 diverged into two distinct evolutionary lineages, designated as European

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(A/eq/Suffolk/89-like) and American (A/eq/Newmarket/1/93-like), and the latter further evolving into the Kentucky, South American, and Florida sublineages, with the last containing clades 1 and 2 [4]. In the last decade, outbreaks of EI in the European horse population were chiefly due to the circulation of American lineage viruses and, more recently, clade 2 viruses of the sublineage Florida [5].

Although in Italy, there is no active surveillance for EIV, the infection is considered endemic, on the basis of clinical observations of field practitioners who, however, do not constantly forward diagnostic samples for the confirmation of the suspect. The latest three reported outbreaks of EI, in Italy, occurred between 2003 and 2005, and all isolated strains presented a high amino acid (AA) identity with the HA1 domain of the hemagglutinin (HA) gene of strain A/eq/SouthAfrica/4/2003, prototype member of the American lineage that was successively included in the sublineage Florida clade 1 [1,6].

As EIV, like other influenza viruses, undergoes antigenic shift or drift and therefore may evade antibody responses generated by divergent strains, continuous monitoring of circulating field strains is essential for the constant verification of the efficacy of the vaccines in use. This study reports the genetic characterization of an EIV, isolated in a training stable of Thoroughbred race horses, located within a track in Italy.

2. Materials and Methods

In January 2014, an investigation was conducted by the National Reference Center for equine diseases, after the onset of respiratory distress at a training stable. Field practitioners of stables nearby described a similar respiratory disease, but no biological samples were forwarded from these.

The animals of the affected stable principally presented with fever, respiratory distress, and a deep dry cough, involving 11 young horses of the 40 subjects present. The signs were confined in 2- to 3-year-old animals that had arrived at the stable at the end of 2013. These horses had been vaccinated for EI at the age prescribed by the immunization vaccine protocol receiving a ground immunization with two initial doses, followed by a booster, administered 5 to 6 months later. The last inoculation received by these animals was at around 6 months before the start of the episode.

During the clinical phase, nasal swabs and serum samples for each of the 11 symptomatic horses were submitted to our laboratory for preliminary tests. The presence of antibodies to EIV in serum samples was determined by hemagglutination inhibition (HI) assay carried out according to OIE standard procedure [7], using 1% chicken erythrocytes and the following prototype strain antigens: A/eq/Newmarket/1/93 (American lineage H3N8), A/eq/Newmarket/2/93 (European lineage H3N8), and A/eq/Praga/1/56 (H7N7).

On the basis of the clinical signs, screening tests were carried out on the nasal swabs using a panel of real-time (RT) PCRs for the detection of the principal equine respiratory viruses: EIV [8], equine arteritis virus [9], equine herpesvirus 1 and 4 (EHV 1 and 4) [10], equine rhinitis viruses A, equine rhinitis viruses B1, and B2 (ERAV, ERBV1, and ERBV2) [11].

Reverse-transcriptase real-time polymerase chain reaction positive samples for the matrix gene of EIV were subsequently inoculated into the allantoic cavity of 8-day-old embryonated chicken eggs (ECEs) and incubated at 35°C for 72 hours. The allantoic fluid was harvested from the eggs and examined by the HA test with chicken erythrocytes (0.5% in PBS) in microplates, using the standard method described by OIE [7]. The isolated virus was further characterized by sequence analysis of the HA1 domain of HA gene using amplification and sequencing primers described by Woodward et al [12]. The derived AA HA1 sequence was compared with those deposited in GeneBank using Blast, and a multiple alignment of similar HA1 sequences, isolated between 2011 and 2014, was performed with ClustalW software. Phylogenetic analysis was conducted using the program PHYML 3.1, and the tree was constructed using the maximum-likelihood method. The statistical robustness of the tree was assessed by bootstrap analysis with 100 replicates.

3. Results

The nasal swabs of four of the symptomatic horses were positive only for the screening EIV RRT-PCR, amplifying an area of the matrix gene, presenting cycle threshold values ranging from 29 to 36. The virus of two of the four EIV RRT-PCR positive samples was successfully replicated in ECEs. Summary of results of the diagnostic tests is given in Table 1 where the animals are numerically identified from 1 to 11.

Comparative AA sequence alignment with reference to clade 2, prototype strain A/eq/Richmond/1/2007, is illustrated in Fig. 1. The sequencing product consisting of 329 AAs is available in Genebank as A/eq/Rome/1/2014 with accession number KR534268. No nucleotide sequence differences were observed within the amplified region of the HA1 gene between the virus isolates after passaging and those present in the swabs, confirming that no significant selection had occurred within the sequenced portion.

Analysis of AA sequences of HA1 residues revealed seven substitutions (I46T, P103L, V112I, I179V, T192K, I282V, and E291D) in the strain responsible of the described outbreak when compared with clade 2, prototype strain A/eq/Richmond/1/2007. The only substitution mapped within an antigenic site corresponds to that located at position 192 (antigenic site B). On the other hand, although only one nucleotide substitution at 294 nt (C→T) was detected, a 100% AA identity was observed with the A/eq/North Rhine Westphalia/1/2014, strain isolated around the same period in Germany. Comparative AA sequences to strains isolated between 2011 and 2014 detected three to four, four to six, five and seven to eight AA changes for the respective years (Fig. 1). Within the constructed phylogenetic tree (Fig. 2), our isolates form a cluster with viruses isolated in Algeria, China, Eire, Germany, Japan, Mongolia, and the United Kingdom, between 2011 and 2014. The strains included in the phylogenetic tree were the first ones isolated relative to that year for each country.