Virus Research 240 (2017) 56–63

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Molecular Epidemiology of a novel re-assorted epidemic strain of equine influenza virus in Pakistan in 2015–16

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ARTICLE INFO

Keywords:
Equine influenza
Epidemic
Re-assortment
Phylogenetic
Equine
Pakistan

ABSTRACT

Background: A widespread epidemic of equine influenza (EI) occurred in nonvaccinated equine population across multiple districts in Khyber Pakhtunkhwa Province of Pakistan during 2015–2016.
Objectives and methods: An epidemiological surveillance study was conducted from Oct 2015 to April 2016 to investigate the outbreak. EI virus strains were isolated in embryonated eggs from suspected equines swab samples and were subjected to genome sequencing using M13 tagged segment specific primers. Phylogenetic analyses of the nucleotide sequences were concluded using Geneious. Haemagglutinin (HA), Neuraminidase (NA), Matrix (M) and nucleoprotein (NP) genes nucleotide and amino acid sequences of the isolated viruses were aligned with those of OIE recommended, FC-1, FC-2, and contemporary isolates of influenza A viruses from other species.
Results: HA and NA genes amino acid sequences were very similar to Tennessee/14 and Malaysia/15 of FC-1 and clustered with the contemporary isolates recently reported in the USA. Phylogenetic analysis showed that these viruses were mostly identical (with 99.6% and 97.4% nucleotide homology) to, and were reassortants containing chicken/Pakistan/14 (H7N3) and Canine/Beijing/10 (H3N2) like M and NP genes. Genetic analysis indicated that A/equine/Pakistan/16 viruses were most probably the result of several re-assortments between the co-circulating avian and equine viruses, and were genetically unlike the other equine viruses due to the presence of H7N3 or H3N2 like M and NP genes.
Conclusion: Epidemiological data analysis indicated the potential chance of mixed, and management such as mixed farming system by keeping equine, canine and backyard poultry together in confined premises as the greater risk factors responsible for the re-assortments. Other factors might have contributed to the spread of the epidemic, including low awareness level, poor control of equine movements, and absence of border control disease strategies.

1. Introduction

Equine influenza (EI) is one of the main respiratory infectious diseases of equines and economically considered severely destructive. This is owing to the highly transmissible nature of the equine influenza virus (EIV), which facilitates its rapid spread among the susceptible equine population, with the consequent break of equestrian events (Rash et al., 2014; Halbherr et al., 2015). EIV is an Influenza A virus belonging to the family of *Orthomyxoviridae*, having a negative sense, single-stranded RNA viral genome, consisting of eight gene segments (Rash et al., 2014). EIV is endemic in most countries of the world. Like other flu viruses, EIVs has two surface glycol-proteins, the haemagglutinin (HA) and neuraminidase (NA). HA plays an important role in the virus entry into the host cell by attaching to the sialic acid receptors on it and

Abbreviations: EIV, equine influenza virus; FC-1, Florida clade 1; FC-2, Florida clade 2; HA, hemagglutinin; NA, neuraminidase; OIE, Office International des Epizooties (World Organization for Animal Health); M, Matrix gene; NP, Nucleoprotein; A/equine/Pakistan/16, A/equine/Pakistan/2016 and SEIR Susceptible, Exposed individuals in latent period, Infective, Recovered with immunity

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http://dx.doi.org/10.1016/j.virusres.2017.07.022
Received 26 March 2017; Received in revised form 15 June 2017; Accepted 26 July 2017
Available online 27 July 2017
0168-1702/

promoting the membrane fusion (Woodward et al., 2014); it is the main target of neutralizing antibodies.

Two influenzas A subtypes have recognized to infect equines, H7N7 and H3N8. The subtype H7N7 EIV for the first time was isolated in 1956 in Europe and H3N8 in 1963 (Woodward et al., 2014; Yondon et al., 2013). This two virus subtypes co-circulated, with re-assortment, until the H7N7 isolation for the last time in the late 1970s (Gildea et al., 2013; Paillot, 2014). In South America following its emergence, most probably from avian, the EIV (H3N8) diverged phylogenetically into two distinct lineages the American and Eurasian lineage (Murcia et al., 2011; Perglione et al., 2016), with enough antigenic variations to warrant the inclusion of both of them in vaccines. Then three sub-lineages named Florida, Kentucky and South American in Argentina emerged within American lineage (Lai et al., 2001), while Florida sublineage further diverged into two distinct clades, clade 1 and 2 (Bryant et al., 2009; Collins et al., 2014). Currently, strains from the Florida sublineage are prevalent throughout the world and inclusion of clade 1 and 2 as representatives are recommended for equine influenza vaccines worldwide (Markowitz et al., 2012). Florida clade-1 (FC-1) viruses are considered as endemic in the North America, but it has also caused major epidemics in Australia, South Africa, and Japan. In Asia and Europe Florida clade-2 viruses are predominant, causing severe epidemics in China (2010), India (2008-09), and Mongolia (2011) recently, and several smaller scale epidemics in numerous countries of Europe (Woodward et al., 2014; Yondon et al., 2013; Qi et al., 2010; Virmani et al., 2010). A well-established phenomenon exists amongst the mammalian Influenza viruses, that cumulative mutation and re-assortment results in the amino acid exchange in HA, which eventually allows escape of the emerging strains from humoral protection of hosts, and or acquired due to exposure previously to earlier strains (Tewawong et al., 2015; Xu et al., 2007). Additionally, re-assortment among the gene segments during the mixed influenza virus infections can contribute to new strains appearance, a process identified to have happened amongst recent strains (EIV) from distinct sublineages (Collins et al., 2014).

In late 2012, the only study conducted in Pakistan on equine influenza was, reporting seroprevalence of H3N8, H7N7, and H1N1 in horses in the Northern parts of the country (Sajid et al., 2013). In spite of a large population of equines and being the sole source of income for poor class human population especially females in the remote areas of the country, it has remained the most neglected part of research in this region. In 2015-16 a severe epidemic was identified in Khyber Pakhtunkhwa (Northern Province of Pakistan) equine population with severe morbidity and low mortality rate in young equines. Therefore, the aim of our study was to investigate the molecular epidemiology of the EI virus causing the outbreak.

2. Material and methods

2.1. Study design and geography

An equine influenza surveillance program for 2015 and 2016 for Khyber Pakhtunkhwa was designed by the authors in collaboration with Office of International Epizootics (OIE) Animal Health Trust Laboratory, a reference lab for equine influenza viruses. A questionnaire was designed having questions on the demography of animal, managemental practices, and biosecurity practices compliance. Khyber Pakhtunkhwa (KPK) is the northern province of Pakistan (Fig. 1), located at a latitude of 34°0′ North and longitude of 71°35′ East. The climate of KPK varies enormously for an area of its size, having most of the climate forms found in Pakistan. KPK stretching southwards from the Hindu Kush covering nearly six degrees of latitude, it is mostly a mountainous region. Its northern zone is very cold and snowy in winter with plenty of rainfall annually.

2.2. Samples collection

Nasopharyngeal swabs and paired sera samples were collected from 376 equines suspected for equine influenza during the outbreak, showing equine influenza cardinal clinical signs, including cough, nasal discharge, and pyrexia, as likely after the onset of clinical signs, most preferably within 3-5 days. All sampled equines were largely aged below 5 years irrespective of species and breed. Samples were stored in the field conditions according to the protocols recommended by OIE (2014), and transported for long term storage (at −70 °C or below) to the main diagnostic laboratory in University of Veterinary and Animal Sciences, Lahore, Pakistan.

2.3. Virus isolation

RT-PCR positive samples were used for virus isolation, simultaneously inoculated into 9–11 days old embryonated chicken eggs. For isolation, 0.1 ml of swab sample was inoculated into the allantoic fluid of 9-11-days old specific pathogen free (SPF) embryonated eggs, as previously described (OIE, 2014).

2.4. RNA extraction and qPCR

For RNA extraction Mag MAX™-96 Viral RNA Isolation Kit was used. A high quality and purity RNA was recovered from the samples that were further used for RT-PCR. For getting PCR products for M and NP genes qPCR was performed for all the samples. HA1 gene was amplified by adopting the method described previously by Hoffmann et al. (2001), on all the qPCR positive (25/376) samples for M and NP genes. In this purpose, the following primer pair described by (Hoffmann et al., 2001) were used.

Bm-HA-1: (5′–3′) TATTCGTCTCAGGGAGCAAAAGCAGGGG

Bm-HA-890R: (5′–3′) ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT

The advantage of using this ‘universal’ HA primer pair is that HA genes were amplified without the subtyping of the virus before the RT-PCR.

2.5. Genome sequencing and analysis

PCR products from qPCR for M and NP gene, and HA1 gene from RT-PCR suitable for the sequence analysis were produced using the gene specific primers (Table 1), tagged with the M13 sequence primers following the procedure; as described previously by (Rash et al., 2014). Amplification products for all the reactions were visualized on 1% agarose gel, using Gel-Red nucleic acid-stain (Biotium), then purified by using the QIA-quick PCR purification kit (Qiagen) as per manufacturer’s directions. Sequencing was accomplished on an ABI-PRISM® 3100 Genetic-Analyzer (Applied Biosystem) using the Big-Dye Terminator V3.1 (Applied Biosystem). Edition of nucleotide sequences, nucleotide, and amino acid alignments, and maximum likelihood phylogenetic trees construction was performed using an advanced bioinformatic software “Geneious version R 10” (Kearse et al., 2012). Maximum likelihood phylogenetic trees based on 1000 bootstrap and blasting were constructed showing the distance and bar scales.

2.6. Ethical approval

The current research work was approved by the Advance Studies and Research Board (ASRB) committee of the University of Veterinary and Animal Sciences Lahore, Pakistan. After approval samples were collected by signing a consent form by owners and managers of infected equines, allowing their animals for collection of samples.