Veterinary Microbiology 143 (2010) 224–237

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Equine influenza outbreak in India (2008–09): Virus isolation, sero-epidemiology and phylogenetic analysis of HA gene

Nitin Virmani *, B.C. Bera, B.K. Singh, K. Shanmugasundaram, B.R. Gulati, Sanjay Barua, R.K. Vaid, A.K. Gupta, R.K. Singh

National Research Centre on Equines, Sirsa Road, Hisar 125 001, Haryana, India

ARTICLE INFO

Article history:
Received 20 July 2009
Received in revised form 26 November 2009
Accepted 3 December 2009

Keywords:
Equine influenza
H3N8 subtype
Haemagglutinin (HA)
Sero-epidemiology
Phylogeny

ABSTRACT

An outbreak of equine influenza (EI) was reported in India in June, 2008 after a gap of two decades. The outbreak started from Jammu and Kashmir (Katra), northern state of India and spread to the other parts of the country affecting equines in 11 states. The virus (H3N8) was isolated from nasal swabs obtained from clinical cases in various locations in the country including Katra (Jammu and Kashmir), Mysore (Karnataka) and Ahmedabad (Gujarat) using embryonated chicken eggs. The virus isolates were identified as H3N8 by haemagglutination inhibition (HI) test titration with standard serum and by sequencing of full-length haemagglutinin (HA) gene and partial sequence of neuraminidase (NA) gene. Paired serum samples ($n = 271$) showing more than fourfold rise in antibody titres tested from 11 states confirmed equine influenza. Serum samples ($n = 2517$) of equines from 13 states of the country screened by HI test revealed 687 (26.85%) samples positive for antibodies to EI (H3N8). Phylogenetic analysis of the haemagglutinin (HA) gene confirmed the virus to be closely related to Clade 2 of the Florida sublineage in American lineage. Comparison of deduced amino acid sequence of HA gene with EIV isolates from various lineages showed substitutions in the antigenic regions C and D. HA1 gene sequence had highest amino acid identity to A/eq/Gansu/7/08 and A/eq/Hubei/6/08 isolates from China and Inner-Mongolia isolate, while the complete HA gene sequence was closest to A/eq/A/eq/Newmarket/5/03, A/eq/Bari/05 and A/eq/Kentucky/05/02 isolates. Recent outbreaks of Mongolia, China and India by clade 2 EI viruses imply their predominance in Asia in addition to Europe.

1. Introduction

Equine influenza (EI) is a highly contagious respiratory viral disease of equines caused by RNA virus – Influenza virus A – belonging to family *Orthomyxoviridae*. The EI disease is caused by two subtypes *viz.* H7N7 and H3N8. The outbreaks due to H7N7 are limited as the isolation of the virus has not been reported for more than two decades (Webster, 1993) and only serological evidence has been found (Madic et al., 1996). The virus of subtype H3N8 spreads very rapidly in the susceptible population (Bryant et al., 2009) and is known to cause severe clinical disease in equines, characterized by pyrexia, dyspnoea, dry hacking cough and serous nasal discharge that becomes muco-purulent due to secondary bacterial infections (Mumford, 1990; Daly et al., 2006). H3N8 subtype has diverged into two lineages *viz.* American and Eurasian (Daly et al., 1996). Both the lineages have been found to be circulating together without any propensity for the geographical locations. The American lineage has been further classified into Florida, Kentucky and South American sublineages (Lai et al., 2001). Analysis of the isolates of the H3N8 EI

* Corresponding author at: National Research Centre on Equines, Equine Health Unit, Sirsa Road, Hisar 125 001, Haryana, India. Tel.: +91 9416236367; fax: +91 1662276217.
E-mail address: nvirmani@gmail.com (N. Virmani).

0378-1135/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetmic.2009.12.007

viruses (EIV) isolated from United States since 1996, indicates that two distinct lineages of the virus i.e., Kentucky and Florida are circulating alternately in the population and are causing the outbreaks in the heavily vaccinated animals, despite of very little amino acid changes in the antigenic regions of these viruses (Lai et al., 2004). In 2007–08 outbreaks due to H3N8 were reported from Japan, Australia, Mongolia and China (Yamanaka et al., 2008; Callinan, 2008; OIE, 2008a,b). While EI infections are endemic in Europe and America, the disease re-emerged in India in last week of June, 2008 in the northern state, Jammu and Kashmir, 20 years after the first outbreak in 1987 (Virmani et al., 2008) and spread to various parts of the country. In 1987, India had experienced the epidemic of EI, when approximately 83,000 equine from the northern states of the country were affected by the disease (Uppal and Yadav, 1987a,b; Uppal et al., 1989). Two virus isolates (H3N8 subtype) *viz.* Bhiwani isolate and Ludhina/87 were confirmed from the outbreak in 1987. Since then, India has not experienced any outbreak of EI and continuous surveillance has negated seropositive reactors after 1998. The vaccination against EI is not mandatory in India. This report presents the detailed epidemiological picture of current EI outbreak across the country along with the phylogenetic analysis of the HA gene of the EI virus isolated from the affected animals during the outbreak of Jammu and Kashmir.

2. Materials and methods

2.1. Collection of clinical material

The information about the first outbreak of equine influenza was received from Katra (Jammu) in last week of June, 2008. The nasal swabs (28) in transport medium and serum samples (118) from animals exhibiting clinical signs of equine influenza and in-contact animals were collected and transported to the laboratory at 4 °C. Later from July, 2008 to May, 2009 the serum samples from the animals in acute stage of disease to convalescence (paired serum samples where ever possible) were collected along with nasal swabs in the transport medium from various states of India which included Himachal Pradesh, Delhi, Uttar Pradesh, Haryana, Rajasthan, Gujarat, Maharashtra, Jharkhand, Andhra Pradesh, Karnataka, West Bengal and Uttarakhand.

2.2. Virus isolation

Nasal swabs collected from the animals exhibiting respiratory signs were brought to the laboratory and processed for virus isolation in embryonated chicken eggs of 9–11 days of incubation. Briefly, the content from the nasal swab was squeezed in a 10 ml syringe and the fluid extracted into a sterile vial. The antibiotics (penicillin 50 IU/ml and streptomycin 5 μg/ml) were added to the aspirate followed by incubation at 4 °C for 30 min. The aspirate was cleared of the debris by spinning at 5000 rpm for 15 min and the supernatant was collected. An aliquot of 0.1 ml of this supernatant was inoculated into embryonated eggs undiluted as well as at 1:10 dilution in three eggs for each sample. The eggs were incubated at 35 °C for 72 h after which they were chilled overnight at 4 °C. The allantoic fluid was harvested from the eggs and subjected to haemagglutination (HA) test with chicken erythrocytes (0.5% in PBS) in microtitre plates. Each sample was subjected to five blind passages before declaring it negative for EI virus.

2.3. Haemagglutination inhibition (HI) test

Haemagglutination inhibition (HI) test was performed on the serum samples employing the OIE protocol (OIE, 2008c). The representative samples were counter-tested by using standard antigen for EIV H3N8 (A/eq/Miami/63, A/eq/Kentucky/1/81, A/eq/Newmarket/1/93, A/eq/Newmarket/2/93) and EIV H7N7 (A/eq/Prague/56), obtained from National Institute for Biological Standards and Control (NIBSC), UK.

2.4. Identification of the virus and antigenic characterization

The samples showing agglutination of chicken erythrocytes were identified and characterized using HI test employing standard antiserum against various strains of EIV H3N8 (A/eq/Newmarket/1/93, A/eq/Newmarket/2/93, A/eq/Kentucky/1/81, A/eq/Miami/63) and EIV H7N7 (A/eq/Prague/56), obtained from NIBSC, UK.

2.5. Genetic characterization of the virus

Viral RNA was extracted from the allantoic fluid (250 μl) employing AuPrep™ Viral RNA extraction kit (M/s Life Technologies (India) Pvt. Ltd., New Delhi, India) as per manufacturer's protocol. The synthesis of cDNA was carried out using random hexamer primer and AMV-reverse transcriptase in a 20 μl reaction volume. For the amplification of full-length HA gene, cDNA was subjected to PCR using the primer set {forward: 5′-CTGTCAATCATGAAGACAACC-3′ and reverse: 5′-TCAAATGCAAATGTTGC-3′}, designed on the basis of published sequence (X68437) and synthesized commercially from M/s Sigma–Aldrich Chemicals Pvt. Ltd., Bangalore, India. The reaction mix contained 5 μl cDNA as template, 20 pmole of each primer, 200 μM dNTPs and 0.5 μl (3 unit) Maxima Hot Start Taq DNA polymerase (Fermentas Inc., MD, USA) in a 50 μl reaction volume. The thermal cycling programme included initial denaturation at 95 °C for 4 min followed by 34 cycles of 95 °C for 1 min, 52 °C for 1 min, 72 °C for 2.30 min with final extension at 72 °C for 10 min. An aliquot of 5 μl of PCR amplified product was analyzed in a 1% (w/v) agarose gel to visualize the amplicon, after staining with ethidium bromide (0.5 μg/ml). The amplified product was sequenced by dideoxy method of sequencing by primer walking.

Neuraminidase (NA) gene was PCR amplified with the primers, forward: 5′-ATGAATC CAAATCAAAAGATA-3′ and reverse: 5′-CGTAAATTACATCTTATCGAT-3′ (Müller et al., 2005) using cDNA obtained as above as template. Thermal cycling condition was initial denaturation at 95 °C for 5 min followed by 34 cycles of 95 °C for 1 min, 50 °C for