The Journal of Basic & Applied Zoology (2013) **66**, 164–168

The Egyptian German Society for Zoology

The Journal of Basic & Applied Zoology

www.egsz.org
www.sciencedirect.com

Mutations and phylogenetic analysis of the equine influenza virus (H3N8) nucleoprotein isolated in Morocco

M. Boukharta [a], F. Zakham [a], N. Touil [b], M. Elharrak [c], M.M. Ennaji [a,*]

[a] *Laboratory of Virology, Microbiology and Quality/ETB, FSTM University Hassan II Mohammedia Casablanca, Morocco*
[b] *Département de Biologie, Hôpital Militaire d'Instruction Med V de Rabat, Université Mohammed V, Souissi, Rabat, Morocco*
[c] *Société de Produits biologiques et pharmaceutiques vétérinaires (Biopharma), Rabat, Morocco*

Received 25 May 2013; revised 19 June 2013; accepted 19 July 2013
Available online 17 September 2013

KEYWORDS

Equine influenza;
Nucleoprotein;
Equine leucocyte antigen

Abstract The equine influenza (EI) is a highly contagious respiratory viral disease of equines. The aim of the present study was to determine the amino acid mutation sequences of the partial nucleoprotein (NP), which includes four epitopes of the equine leucocyte antigen (ELA) of A/equine/Nador/1/1997 (H3N8). These epitopes are critical for their binding to major histocompatibility molecule complex (MHC) class I and recognition by specific Cytotoxic T Lymphocytes (CTLs). The isolate was subjected to RT-PCR amplifications followed by sequencing analysis. Phylogenetic analysis showed that Moroccan isolate belongs to equine host-specific lineage and more closely related to Italian strains A/equine/Rome/5/1991 and A/equine/Italy/1062/1991. Amino acid sequence comparison of the NP showed that the strain A/equine/Nador/1/1997 has twelve substitutions at the residues T/284/A, A/286/T, R/293/K, I/299/V, V/312/I, N/319/K, S/344/L, V/353/I, M/374/I, C/377/N, N/397/S and R/452/K. All substitutions concerned both the interaction domains NP–NP and NP–PB2. However, the mutation N319 K enhances the NP–PB2 interaction and polymerase activity in mammalian infected cells. S/344/L mutation was located on the FEDLRVSNFI epitope (aa 338–347), this substitution is likely to help the virus to overcome the barrier of cell-mediated immunity of the host. The identified mutations were grouped into two groups, one included residues that facilitate the adaptation and evolution of influenza viruses within the equine lineage such as A/286/T, R/293/K, S/344/L, V/353/I and R/452/K, while the second contained the substitutions which enhance the virulence as polymerase activity (N319 K) and muta-

* Corresponding author. Address: Laboratory of Virology, Microbiology and Quality/Eco-toxicology and Biodiversity, University of Hassan II, Mohammedia-Casablanca, Faculty of Science and Techniques, BP 146, Mohammedia 20650, Morocco. Tel.: +212 6 61 74 88 62/212 6 62 01 37 72; fax: +212 5 23 31 53 53.
E-mail address: m.ennaji@yahoo.fr (M.M. Ennaji).

Peer review under responsibility of The Egyptian German Society for Zoology.

ELSEVIER Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.jobaz.2013.07.007

tions that affect CTL epitopes, resulting in an escape from immune surveillance by specific CTLs (S/344/L).

Background

Equine influenza is an infectious and contagious disease of the upper respiratory tract of horses, donkeys and their cross products (Barquero et al., 2007; Gurkirpal, 1997). Two distinct subtypes of equine influenza viruses were identified: A/equine/Prague/1/56 prototypes (H7N7) and A/equine/Miami/1/63 (H3N8) (Sovinova et al., 1958; Waddell et al., 1963). These viruses are a member of the family Orthomyxovirus (Orthomyxoviridae), which consists of eight single-stranded viral RNA segments of negative polarity encoding a total of 11 proteins (Zhirnov et al., 2009). Among these genes, the NP gene (segment 5) plays a critical role in the species barrier and host adaptation of influenza A viruses (Bean, 1984; Gorman et al., 1990; Xu et al., 2011). These genomic RNAs are incorporated into virions as ribonucleoprotein (RNP) complexes, which consist of the viral RNA (vRNA) associated with three viral polymerase subunit proteins (PA, PB1, and PB2) and nucleoprotein (NP) (Li et al., 2009). The ribonucleoprotein complex (RNP) is essential for the transcription and replication of the genome in the cellular nucleus (Klumpp et al., 1997; Voeten et al., 2000).

Comma

Nucleoprotein, comprises a sequence of 498 amino acids, in which the oligomerization is mediated by the insertion of the structurally conserved tail loop (aa 402–428) of one NP molecule to a groove of another NP (Ng et al., 2012).

The NP sequence is highly conserved among influenza virus types and subtypes (Tarus et al., 2012). It contains a binding region of the viral RNA to its N-terminus (residues 1–181), two areas are responsible for auto-interaction NP–NP (residues 189–358 and 371–465) (Kobayashi et al., 1994; Albo et al., 1995; Elton et al., 1999) and three regions of NP (aa 1–161, aa 255–340 and aa 340–465) were found to interact independently with PB2 (Li et al., 2009; Ng et al., 2012).

Influenza viruses that cause severe infections are able to escape to neutralizing antibodies following the accumulation of mutations "antigenic drift" in their surface glycoproteins (HA) and also by the introduction of mutations in the conserved epitopes of viral proteins including NP and M1 (Epstein, 2003). The nucleoprotein includes the best preserved epitopes and appears to be the major target for the cytotoxic T lymphocytes response (CTL), which limits the replication of influenza virus and thus prevent the morbidity and mortality (Varich et al., 2009; Wahl et al., 2009). In humans, the antigenic peptides of viral nucleoprotein presented by infected cells are essential for binding to HLA (human leucocyte antigen) molecules class I of major histocompatibility complex (MHC) and specific CTL of the $CD8^+$ phenotype cross-reactive for all type influenza A viruses (Taylor and Askonas, 1986; Voeten et al., 2000).

Four NP epitopes of influenza A virus were analyzed and identified by several studies. They are in the form of short sequences recognized by cytotoxic T lymphocytes: aa 265–273, aa 338–347, aa 380–388 and aa 383–391 (Huet et al., 1990; DiBrino et al., 1993; Voeten et al., 2000)

Phylogenetic analysis of the NP gene sequences of influenza A virus indicates that the NP gene is highly conserved within the host-specific lineages (Thippamom et al., 2010) and has been shown to be a major determinant of host specificity (Shu et al., 1993). Eight distinct lineages were identified, including the human lineage, porcine, avian and equine (Xu et al., 2011).

In this paper, we studied the sequence of the nucleoprotein (NP) of the four previously described CTL epitopes (Equine Leucocyte Antigen (ELA)) of the equine influenza virus A/equine/Nador/1/1997, which was isolated on 31 December 1997 in a mule with severe respiratory signs (cough, nasal discharge, hyperthermia) in Nador in the north of Morocco.

Materials and methods

Viruses

A/equine/Nador/1/97 was isolated in Nador from a mule using 11-day-old specific-pathogen-free chicken eggs as described by Kissi (Kissi et al., 1998).

Viral RNA extraction and amplification

Viral RNA was directly extracted from isolates using a Purelink viral RNA/DNA-Minikit (Life Technologies) following the manufacturer's recommended protocol. cDNA was obtained by reverse transcription reactions by using the Superscript III Reverse transcriptase kit (Invitrogen, UK). PCR was performed by Platinum® PCR SuperMix High Fidelity kit (Invitrogen, UK) on obtained cDNA using primer specific NPF (ATGGCGTCTCAAGGCACCAAA) and NPR (TTAACTGTCAAACTCCTCAGC) at a final concentration of 0.5 μM for primers. Primer design is detailed by Muller et al., 2005. The thermal cycler was programmed as: incubation at 95 °C for 2 min, and then 35 cycles of denaturation at 95 °C for 30 s, 52 °C for 1 min for hybridization, 72 °C for 30 s.

Sequencing NP genes and phylogenetic analysis

The amplified PCR products were sequenced. Briefly, the PCR products were purified using EXOSAP-IT (USB) and bidirectionally sequenced by using ABI BigDye1 Terminator v3.1 (Applied Biosystems) on an ABI 3130xl sequencer (Applied Biosystems). Analysis of the produced electrophrogram was carried out with the sequencing Analysis Software version 5.3.1 (Applied Biosystems).

Phylogenetic analysis was performed on thirty virus influenza strains isolated from different animals (Human, Avian, equine, swine) (including Moroccan isolate) published in GenBank using the Neighbor Joining method (Saitou and Nei, 1987) in which the A/equine/Prague/1957 sequence was used