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RESEARCH ARTICLE

Molecular Epidemiology of the Two Internal Genes of Equine Influenza H3N8 Virus Isolated in Pakistan 2015-16

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ABSTRACT

An outbreak of equine influenza was detected in Khyber Pakhtunkhwa during the years 2015-16. The isolated viruses were typed for the first time in Pakistan as H3N8 and grouped with the Clade 1 viruses of Florida sub-lineages on the basis of Hemagglutinin (HA) and Neuraminidase (NA) gene sequence analysis. Here we described the genetic analysis of two internal genes (Nucleoprotein and Matrix) of the Pakistani isolates. The primer pairs were designed to amplify the NP and M internal genes of the 19 equine H3N8 influenza A viruses identified and isolated in this outbreak. Phylogenetic analysis of the derived sequences was performed and compared with the contemporary available sequences online. Comparison of the nucleotide sequences of the two internal genes of the Pakistani strains showed high similarity (99.7-100%) with the avian influenza A viruses (Avian/Pakistan/H7N3/ 2004) isolated in Pakistan. Two major substitutions i.e. F63L and K243R were recorded in Matrix gene of the isolates from the closely related Chicken/Pakistan/04 H7N3 virus. Notably NP gene did not acquire any amino acid substitution on comparison with Chicken/Pakistan/04. All isolates shared 99.5-99.9% homology with the reference sequences on alignment for M1 and NP amino acid sequences, respectively. Phylogenetic analysis revealed clustering of the Pakistani isolates with the avian viruses isolated in Pakistan in previous outbreaks in commercial and backyard poultry. Epidemiological investigation of the outbreak suggested that reassortment of equine H3N8 viruses with avian Influenza A H7N3 most probably, might be due to the mix farming system here. In conclusion, Phylogenetic analysis of the internal genes reported here will be helpful in early detection and control of EI.

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INTRODUCTION

Equine influenza (EI) is an important disease of equines, and it manifests as a rapidly transmitting infectious respiratory disease associated with lethargy, fever and coughing. Such a syndrome has been described in literature for centuries (Morens *et al.*, 2010). Equine influenza A virus (EIV) was isolated for the first time from equines during an epidemic in Czechoslovakia in 1956 (Rash *et al.*, 2014).

Briefly, the NP genes of EIV H3N8 were found phylogenetically being distinguished from those of the other animal viruses, although the PB2, NS, and M genes were closely related to the North American avian influenza viruses. Additionally, internal proteins of the eqPra56 (H7N7) were different from those of other animal influenza viruses (Lindstrom *et al.*, 1998). Influenza A viruses shows rapid evolution and complex molecular dynamics due to its diverse hosts, rapid replication and high mutation rates (Murcia *et al.*, 2011; Khan *et al.*, 2017). These viruses have also shown propensity to escape host immunity because of the continuous antigenic drift. Furthermore, influenza viruses can also cross species barriers due to genetic drift, genome re-assortment, rapid mutation, simply transmitted from one host to another (Holmes, 2010; Yondon *et al.*, 2013).

Each of the influenza A viral genes plays an important role in interactions with the hosts and subsequent infections; therefore, understanding the evolution of each viral gene can provide new insights into epidemiological dynamics of the influenza A viruses (Fourment *et al.,* 2010). Moreover, this will be equally useful in predicting periodicity of the future EI epidemics and Pandemics, and its genetic basis (Pybus and Rambaut, 2009). Internal genes i.e. nucleoprotein (NP) plays significant role in the host adaptation and virus replication, though molecular evolutionary dynamics of NP lineages are less well understood. NP gene encodes protein with almost 500 amino acids; playing a vital role in assembly and budding of the influenza A virus, also having a putative role in the host range (Ruigrok *et al.*, 2010; Alves *et al*., 2016).

Previously, in Pakistan Equine influenza epidemic was reported by Sajid *et al.* (2013) in the same province of Khyber Pakhtunkhwa reporting seroprevalence of 7-10 % in horses and donkeys. The aim of our study was to provide the complete profile of the protein variability of M and NP gene segments of the Pakistani H3N8 equine influenza viruses; isolated here in the outbreaks detected during 2015-16. In order to achieve our objectives, three primer pairs were designed and phylogenetic analysis was conducted for their proteins (M and NP).

MATERIALS AND METHODS

Study design and area: An active surveillance study was conducted in 2015 and 2016 for a period of one and a half year from Khyber Pakhtunkhwa (KP) province of Pakistan. KP is the Northern Province of Pakistan, having a large working equine population. KP is located at a latitude of 34°0' North and longitude of 71°35' East. The climate of KP varies enormously for an area of its size, having most of the climate forms found in Pakistan. Most of the equines brought to livestock markets (LMs) here in district Peshawar are imported from Afghanistan across the border.

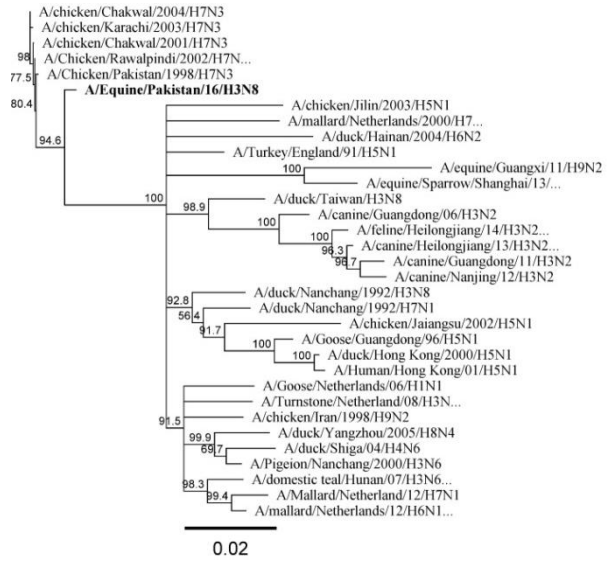

Fig. 1: Phylogenetic analysis of NP-gene nucleotide sequences of A/equine/Pakistan/2016 in bold. Maximum likelihood tree (1000 bootstrap) of the isolated virus with the contemporary sequences. Scale bar indicates the nucleotide substitution per site.

Sample source: Serum and nasopharyngeal swabs samples were collected from suspected equines in four randomly selected districts of Khyber Pakhtunkhwa, to isolate and characterize circulating equine influenza viruses during the outbreak. Vaccination against equine influenza is not practiced in Pakistan.

Diagnosis and virus isolation: Equine influenza viral (EIV) RNA was detected through qPCR and RT-PCR directly from 25 out of 376 nasopharyngeal swabs, collected during different EI epidemic waves in four districts of Khyber Pakhtunkhwa, Pakistan. Seven samples were positive from district Peshawar, three from Mardan, one from Charsadha, and 14 from district Swat. After three serial passages, the virus was effectively isolated from 19 samples in 9-11 days old embryonated eggs. These isolates were identified as a reassortant equine influenza virus originated from different species. Further molecular characterization of the virus was conducted.

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. Complete procedure used for RNA extraction following Rash *et al.* (2014). 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 Hoffman *et al*. (2001), on all the qPCR positive (25/376) samples to diagnose and identify the virus as equine influenza H3N8 virus. In this purpose, the following primer pair described by Hoffman *et al.* (2001) was used.

Bm-HA-1: (5′-3′) TATTCGTCTCAGGGAGCAAAAGC AGGGG

Bm-HA-890R: (5′-3′) ATATCGTCTCGTATTAGTAGA AACAAGGGTGTTTT

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.

Genome sequencing: PCR products from qPCR for M and NP gene suitable for sequence analysis were produced using the gene specific primers (Table 1), tagged with the M13 sequence primers following the procedure, as described previously (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 recommendations. Analysis of nucleotide sequences, nucleotide and amino acid alignments, and maximum likelihood phylogenetic trees construction was performed using an advance bioinformatic software "Geneious version R 10 (Kearse *et al*., 2012; Markowitz *et al.*, 2012).

RESULTS

In last decade, equine influenza (EI) epidemics were witnessed undetected and unreported throughout Pakistan in equine population almost every year especially in the Northern areas of Khyber Pakhtunkhwa province, including the tropical plane areas of District Peshawar (KP). KP is comprised of many of coldest regions in the country located

at the border with Afghanistan. EI for the first time was reported in 2013 in Pakistan (Sajid *et al*., 2013), detecting and reporting several seropositive cases for H3N8, H7N7 and H1N1 without a successful isolation of EI viruses. Here we isolated the virus for the first time from horses and equines during the current outbreak in the province of Khyber Pakhtunkwa, in the extreme north of Pakistan.

Outbreak description: Clinical cases of EI were first reported in December 2015 in district Peshawar. During collection of epidemiological data from equine owners and managers, we also came through some respondents reporting typical clinical signs for equine influenza in equine population of Afghanistan at the same time. Shortly after this wave of cases finished, further cases of EI were reported in district Mardan and Charsadha in the draught horses and donkeys with poor health status. On 11th January 2016, two horses from the same premises (within 50 meters) were reported showing typical influenza like clinical signs. Samples collected from these horses were positive by RT-PCR for EIV. On 22nd January, severe spread of EI was reported in district Swat in equine population from four different union councils. Analysis of epidemiological data collected during sampling showed that these equines were used to mix with other equines during work in the rivers and ponds from different regions. High rate of morbidity was recorded during this course of epidemic. Almost 327 equines were suspected for EI during this wave of epidemic. Equine farmers and owner's managemental practices supported the continuous spread of EI in the area. Descriptive analysis of data showed that 47% (177/376) equine farmers were keeping mixed equine species in the same indoor stables with 8-10 square feet area for each animal. 32% (120/376) respondents were also keeping dogs in the same stable with their equines, while 11% (41/376) respondents were having domesticated water fowls and backyard poultry in the same premises with their equines.

Comparison of M and NP gene: For the purpose of molecular characterization and determining the probable origin of the isolates, the phylogenetic analysis (Fig. 1) of M and NP amino acid sequence of equine/Pakistan/16 was performed. The derived sequences from the two segments encoding M1 and NP of equine/Pakistan/16 were aligned with corresponding most relevant amino acid sequences from different species based on blasting against each specie separately, to check the most probable possibility of interspecies transmission and reassortment. The observed substitutions in the amino acid alignments are concluded in Table 2. Comparison of these sequences indicated that equine/Pakistan/16 had acquired the nonsynonymous mutations in M1 and NP segments compared to various influenza A strains from different species including equine, canine, avian and humans. Four consistent amino-acid substitutions were observed (N86S, K102R, R112G and Q209R) in the M1 gene on comparison with multiple species. The additional substitutions were found in chicken/Pakistan/1/04 (F63L and K243R), and equine/Katra-Jammu/7/08 (V16I, D31N R96K and Q209K). NP gene of equine/Pakistan/16 showed 99.7% highest degree of similarity with chicken/Pakistan/1/04 (H3N7). These results revealed that A/equine/Pakistan/16 virus was a reassortant with the M1 and NP gene significantly correlated to the corresponding genes from Chicken/Pakistan/1/04 (H7N3) (degree of similarity 99.6 to 100%), canine/Beijing/10 (H3N2) (similarity 97.4%), canine/Liaoning/10 (H3N2) (similarity 97.0%), muskrat/Russia/63/14 (H2N2) (similarity 96.8%), Italy/3/13 (Human, H7N7) (similarity 94.3%).

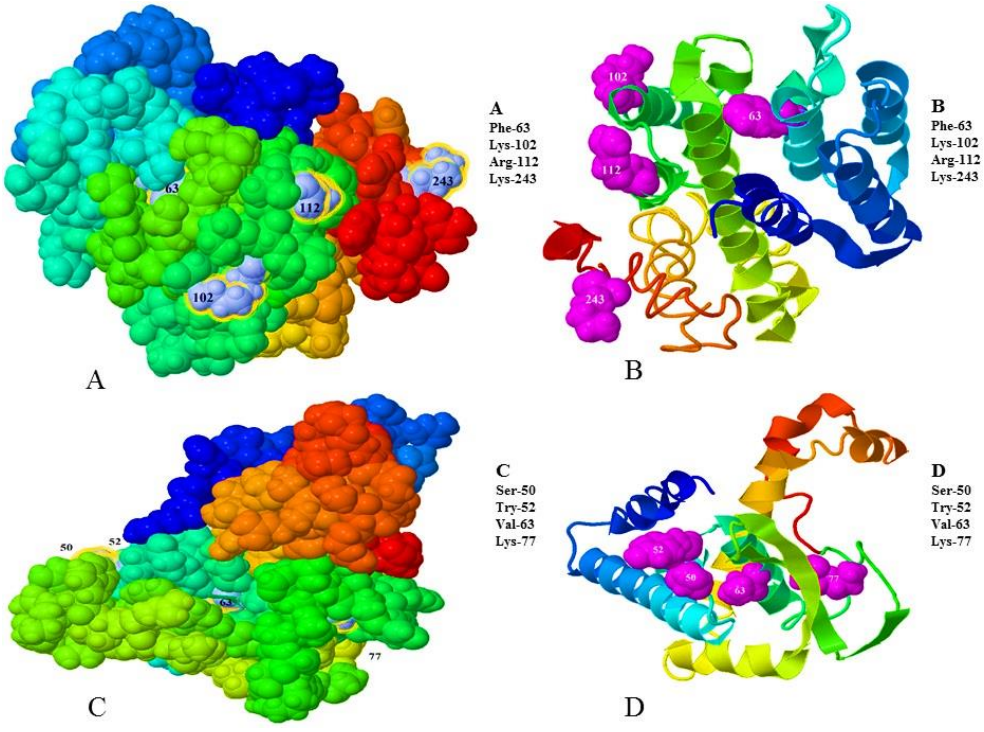

Fig. 2: Two views of three-dimensional space fill and cartoon model's structures for M1 and NP genes of the equine/Pakistan/2016 isolates. (A) Space fill structure for M1 gene representing amino acid (aa) substitutions. (B) Cartoon model structure for M1 gene. (C) NP gene space fill structure with marked aa substitutions. (D) Cartoon structure for NP gene representing aa substitutions comparing with Chicken/Pakistan/04 and FC-1 strains.

Table 1: Primer sequences with their annealing temperatures used for the genome sequencing of equine/Pakistan/16 isolated in 2015-16 equine influenza outbreaks in Pakistan

Primer Name	Primer sequence (5′-3′)	Approximate nucleotide coverage (5′-3′)	Annealing temperature used (^{0}C)
NP/AF	GC GTAAAACGACGGCCAGT	1-570	50
NP/AR	AGCGAAAGCAGGGTAGATAATC		
	GC AACAGCTATGACCATG CCGTGGGAGGGTTGAGCC		
M/AF	GC GTAAAACGACGGCCAGT	1-654	50
M/AR	AGCGAAAGCAGGTAGATATTTAAAG		
	GC AACAGCTATGACCATG CTAGCCTTACTAGCAAC		
M/BF	GC GTAAAACGACGGCCAGT CAGTACCACGGCTAAAG	571-1027	50
M/BR	GC AACAGCTATGACCATG		
	AGTAGAAACAAGGTAGTTTTTTAC		

Table 2: The difference between internal genes amino acids sequences of the reasortant A/equine/Pakistan/16 and the most relevant recently isolates from different species, including humans, canine, avian recently and equine in the neighborhood countries of Pakistan

Gene	M						Gene	NP								
Amino acid portion	63	86	102	112	209	243	Amino acid position	9	34	41	50	52	63	77	125	159
A/Equine/Pakistan/16 (H3N8)	F	N	K	R	Q	K	equine/Pakistan/16 (H3N8)	S	G	I	S	Y	V	K	N	M
Chicken/Pakistan/04 (H7N3)	L	.	.	.	.	R	Ch./Pakistan/04 (H7N3)	.	.	.	.	.	.	.	.	.
Muskrat/Russia/14 (H2N2)	.	.	R	G	.	.	Canine/Nanjing/12 (H3N2)	.	.	.	N	H	I	.	G	L
Canine/Beijing/10 (H3N2)	.	.	R	G	.	.	Canine/Guangdong/06 (H3N2)	.	.	.	.	H	I	.	.	.
Human/Italy/13 (H7N7)	.	.	R	G	.	.	Human/Hong Kong/01 (H5N1)	P	.	.	.	.	I	.	.	.
Human/England/96 (H7N7)	.	.	R	G	.	.	Human/Italy/13 (H7N7)	.	.	.	.	.	I	R	.	.
Equine/New York/07 (H3N8)	.	S	R	G	R	.	equine/Guangxi/11 (H9N2)	.	S	.	N	H	I	R	.	.

Phylogenetic analysis of NP gene (Fig. 1) of equine/ Pakistan/16 revealed that it was most likely originated from the homologous genes of chicken/Pakistan/1/04 (H7N3) and was also found closely related to canine and human influenza A virus (canine/Beijing/10 (H3N2) and Italy/3/13 (H7N7)). While the two surface glycoproteins HA and NA of equine/Pakistan/16 viruses were found similar to the classical H3N8 viruses of that FC-1 sub-lineage, closely related to Tennessee/14 or Malaysia/15 (based on the percent pairing similarity). These findings suggested, that the equine/Pakistan/16 virus was most probably originated from the re-assortments of H3N8 and avian origin H7N3. The current isolate was found genetically distinct from the other H3N8 EI viruses, due to the existence of NP and M genes different from the basic H3N8 genes. The predicting 3D models of the NP and M genes have given in Fig. 2 in details. Phylo-genetic tree of the M gene was similar with that of NP tree.

DISCUSSION

Molecular characterization of the H3N8 EIV isolated for the first time from equines (9 horses and 14 donkeys), revealed that these equine/Pakistan/16 viruses were circulating in Khyber Pakhtunkhwa province of Pakistan in 2015-16. The isolated strain represented genotypically a novel reassortants, containing Tennessee/14 like, Malaysia/15 like, chicken/Pakistan/04 (H7N3) like canine/Beijing/10 (H3N2) like, and H2N2 Muskrat/ Russia/14-like gene segments. Phylogenetic analysis suggested that the equine/Pakistan/16 might be initially produced in the backyard poultry or domesticated ducks, prior to being directly transmitted to equines. As previously reported H9N2 outbreaks in donkeys and horses in China (He, 2012), H5N1 in donkeys in china, and in other countries has revealed that avian influenza viruses could establish themselves in equine species. The mix farming system in Pakistan, keeping backyard poultry, canine, domesticated ducks, and equine species under one roof in indoor stables adds in the occurrence of interspecies transmission. Ultimately, genetic and antigenic re-assortments chance increases, as also reported previously (Guan *et al*., 1999; Perglione *et al.,* 2016). Notably, antibodies against viruses from poultry origin (H1N1) in equines have also been reported in the same study region in past (Sajid *et al*., 2013). Consequently, it can be seen that the novel reassortant equine/Pakistan/16 viruses were circulating in different equine species in KP province of Pakistan during 2015-2016 giving rise to a countless uncertainty for the present ecosystem of EIV and other influenza A virus in Northern regions of Pakistan.

Here we described the genetic analysis, including the determination of two internal genes sequences, for the representative isolates of the EIV epidemics in Pakistan in 2015-16. Results of the phylogenetic analysis suggested that the internal genes, M1 and NP of segments A and B, were descended from chicken/Pakistan/04 (H7N3) like viruses, which might have been circulated at the same time, supported by the previous reports for H5N1 (Xu *et al*., 2007) and NP gene was also found closely related to Hong Kong/01 human influenza A isolate. Consequently, these equine/Pakistan/16 viruses were most likely the result of multiple-re-assortments between possibly co-circulating H7N3 like (chicken/Pakistan/04) and H3N2-like (canine/Beijing/10) viruses. It had a close evolutionary association to the H5N1-like influenza A-viruses in Avian in NP complex genes. Similar kind of evolutionary behavior had been reported in H9N2 isolates, which contained some of its gene fragments being derived from varying influenza A-viruses from distinct lineages (Dong *et al*., 2011). Such evolutionary behaviors were also reported by Li *et al.* (2004). If these novel geno-typical equine/Pakistan/16 (H3N8) reassortant continues to evolve in the diverse hosts, it highlights the risk of EIVs to establish themselves in poultry and might also cause infection in human population in future. Due to the lack of phylogenetic data regarding these viruses and lack of interest in the continuous surveillance of influenza A-viruses in equine and avian species in Pakistan, it is difficult to draw a clear image of the current reassortant viruses.

Molecular characterization of internal genes demonstrated that all the equine/Pakistan/16 viruses maintained the same M and NP connecting peptide motifs with the terrestrial backyard poultry viruses (mostly H7N3), of low pathogenicity in the chickens. It suggests that internal genes of these equine influenza H3N8 viruses

might have been transmitted to the equines population from backyard poultry, also supported by the previous findings of Tai *et al*. (2016). They reported H9N2 outbreaks in equines and canines in China in 2012 consecutively circulating for two years. It is important to explore the influenza viruses through continuous surveillance in different avian species and equines because farming system and managemental practices in these regions supports re-assortment. All these species might have a considerable role in re-assortments and to define the evolutionary pathways in the local territory influenza A virus's ecosystem. The most suspected evolutionary pathway source of virus for the current reassortants could also be the migratory birds including ducks mostly, been regarded as natural reservoirs for the influenza A virus, with reported to date sixteen-HA and nine-NA subtypes (Rash *et al*., 2014). However, it is essential to note that current analyses cannot eliminate that a single co infected mammal or avian could have introduce more than one clades of influenza A virus into Pakistan, as the incidence of co infection among equines in our study is unknown, and represents a fundamental area for future research. Additional sequencing of equine influenza viruses in other regions of Pakistan and most importantly Afghanistan is important. Because, the imported horses from Afghanistan were supposed to be the key source of current EI epidemics in Pakistan. Taken together, these results showed that NP and M gene amino acid sequence of equine/Pakistan/16 responsible for the current equine influenza epidemics in Pakistan is mostly related to chicken/Pakistan/ 04 H7N3 viruses.

The role and possibilities of mix infections in the current re-assortment: In the context of epidemiological and geographical data obtained during the surveillance investigating the current outbreak, one of the most striking possibilities might be the incidence and range of the mixed-infections. The mixed infections of influenza A-viruses from distinct clades, as previously reported (Halbherr *et al*., 2015), probably could be a consequence of long distance equine transportation across the borders, especially without any border disease control strategies. Apart from this, the free movement of imported horses throughout Pakistan mixing earlier at livestock markets. Mixed-infections could result from co-infections (i.e., from multiple independent transmission equestrian events. A simple 'SEIR' model suggests that level of re-infection is a highly dependent procedure on latency and the infectious periods. If the latency and infectious periods stays long (Collins *et al*., 2014), like in the case of non-vaccinated equine population in Pakistan, all the infections will involve the mixed-infections rapidly (Cowled *et al*., 2009).

Conclusions: In conclusion, we characterized the two internal genes the isolated equine influenza viruses which caused respiratory disease in equine population in Pakistan. Moreover, phylogenetic analysis of the internal genes represented that the isolated equine influenza viruses here are re-assorted with avian influenza viruses. These results represent a potential threat of future outbreaks in equine species in Pakistan.

Authors contribution: AK conducted the whole research; MHM supervised the research and helped at each step of the research; MUA helped in the data analysis and methodology designing; JN contributed in the field work and data analysis; ZF contributed in the data collection and field work; AK contributed in the data collection; SHF contributed in the data entry and write up.

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