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The Equine Influenza Outbreak in Pakistan 2016: Seroprevalence and Geo-Temporal Epidemiology of a Large Propagating Outbreak

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Authors' Contribution
MHM designed and supervised the study. Amjad K, Asghar K and SHF collected the data. Amjad K and JN analyzed the data. Amjad K wrote the manuscript while MHM, MUA and JN helped him.

Key words
Equine influenza, Seroprevalence, Epidemiology.

ABSTRACT

Khyber Pakhtunkhwa (KPK) province of Pakistan experienced an extensive epidemic of equine influenza (EI) in October 2015 in equine population. EI is among the OIE or WHO notifiable, contagious equine respiratory diseases. Due to lack of awareness and interest of all the equine industry stakeholders no vaccination against EI is performed, also the information regarding the epidemiology and occurrence of EI is lacking in Pakistan. We hereby attempted to determine the seroprevalence, investigating the EI outbreak 2015-16, and describing the demographic and management risk factors associated with seroprevalence of EIV. Using ELISA kit and HI as serological methods for diagnosis, we found a 24.13% (168/696) overall seroprevalence, where the seroprevalence for H3N8 was 14.51% (101/696), for H7N7 6.03% (42/696), and 3.59% (25/696) as mixed infections, suggesting EIV current and active circulation in equine population of Pakistan. Statistical analysis suggested predicting variables including "local equine density per 2 KM", "equines workings in ponds and rivers", and "geography of the equine" were significantly ($P<0.05$) associated with seroprevalence of EIV. Our findings of the EIV occurrence in equine population in Pakistan, suggests an under-diagnosis of this virus in Pakistan and warrant additional investigation and continuous surveillance at the molecular level to identify circulating strains for control and prevention of future outbreaks.

INTRODUCTION

Equine influenza (EI) is an important infectious respiratory disease in equids worldwide. The causative agent for which is an equine influenza virus (EIV), a highly contagious pathogen. Like other influenza viruses, the EIV has two-surface proteins, hemagglutinin (HA) and neuraminidase (NA). HA plays an important role in the virus entry to the host cell by attaching to sialic acid receptors on the cell surface and encouraging membrane fusion (Halbherr *et al*., 2015); it is the main target for neutralizing antibodies and consequently an essential component of the commercial vaccines. Having the sialidase activity, NA is also thought to play an important role in the entry as well as the exit of the virus. Antibodies produced against influenza NA glycoprotein are also identified to contribute in protection and have been recently shown to block sialidase activity as well as virus adsorption (Beuttemmüller *et al*., 2016), though their significance for immunity against the EIV remains unclear.

Two subtypes of influenza A virus are identified to infect equines, H7N7 and H3N8. The H7N7 EI was isolated for the first time in 1956 in Europe, followed by the H3N8 in 1963 (Sovinova *et al*., 1958; Waddel *et al*., 1963). These two subtypes co circulated, with reassortment, until H7N7 last time isolation in 1970 (Ito *et al*., 1999). Succeeding its emergence in the South America, most probably from the avian source EIV (H3N8) phylogenetically diverged into American and the Eurasian lineages (Woorbey *et al*., 2014), with adequate antigenic changes both have warranted their inclusion in vaccines. Then three sub lineages named Florida, Kentucky and South American emerged from the American lineage (Lai *et al*., 2001), and the Florida sub lineage further diverged into the two distinct clades 1 and 2, respectively (Bryant *et al*., 2009; Murcia *et al*., 2011). Florida clade-1 (FC-1) viruses are endemic in the North

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American region but have also caused major outbreaks in Australia, South Africa and Japan (Guthrie, 2006; Yamanaka *et al*., 2008; Cowled *et al*., 2009). In Asian and European countries viruses from Florida clade-2 (FC-2) predominate, causing major outbreaks in China, Mongolia and India recently and smaller scale outbreaks in European countries (Qi *et al*., 2010; Yondon *et al*., 2013; Virmani *et al*., 2010).

Antibodies against EIV (H3N8), H7N7 and H1N1 have already reported in Pakistan in the same study area in 2013 (Sajid *et al*., 2013). During 2015 and 2016, an extensive EI outbreak was detected in Pakistan, first identified in November 2015 in district Peshawar. The outbreak spread throughout the country rapidly in the non-vaccinated equine population. Here we describe the 2015-16 Pakistan EI outbreak seroprevalence, geo-temporal epidemiology, and phylogeny of the HA1 gene.

MATERIALS AND METHODS

Study design and area

An active surveillance was conducted for a period of one year from November 2015 to December 2016 in Khyber Pakhtunkhwa (KPK) province of Pakistan. KP is the northern province of Pakistan, having a large working equine population. KPK is located at an altitude of 34°0' North and longitude of 71°35' East (Fig. 1). The climate of KPK varies enormously for an area of its size, having most of the climate forms found in Pakistan. Most of the equines brought to live animal markets (LAMs) here in district Peshawar are imported from Afghanistan across the border.

Sampling procedure

Blood samples and nasopharyngeal swabs were collected from 696 healthy and suspected equines in four randomly selected districts of Khyber Pakhtunkhwa (Fig. 1), to determine the prevalence of EIV and molecular characterization of isolated viruses circulating during the outbreak. The sample size calculated here was 139, using the formula:

$$n = \log \alpha / \log (1-p)$$

Where, n stands for sample size, α = 1-confidence level, and p is minimum incidence worth detecting following Fosgate (2009). Paired sera samples were not collected because these were not important here as no vaccination is done against EIV in Pakistan.

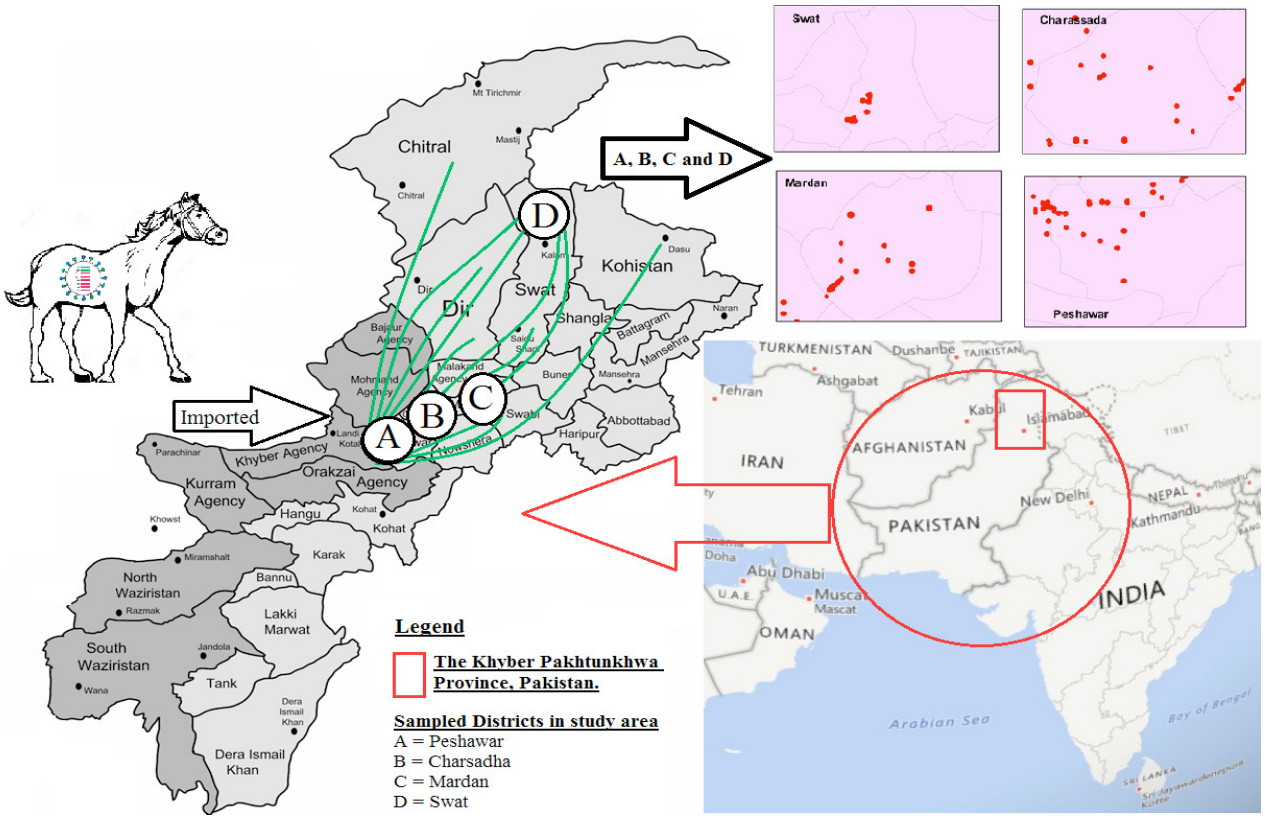

Fig. 1. Geographical distribution of seroprevalence and spread of EIV outbreak 2015-16 in Khyber Pakhtunkhwa, Pakistan.

Data collection

A predesigned piloted questionnaire was used to collect the basic epidemiological data on demography, geography, management type, clinical signs, and equines activities. The questionnaire contained a total of 37 closed questions. Upon evaluation during piloting the questionnaire by 15 professional field workers and 25 equine owners and farmers, some of the variables were dropped accordingly (discussed further in result section).

Diagnostic serology assay

All the serum samples (696) were tested using influenza-A antibody kit for detecting the antibodies, the ID screen influenza-A competition multi species, IDvet innovative diagnostics (Grabels, France), designed to detect the antibodies directed against internal nucleocapsid of the influenza-A virus, but it is not specific for subtyping. For subtyping of these antibodies, to evaluate the influenza virus against which they were raised (the virus causing the outbreak), Hemagglutination inhibition (HI-subtyping) assay was conducted following the standard procedures (Bryant *et al*., 2009). All serum samples were first treated potassium periodate (Beardmore *et al*., 1968), to get rid of all the non-specific inhibitors of the influenza A virus hemagglutination and to elevate the test specificity, in accordance with the OIE procedure. Equine antisera were obtained from Animal Health Trust Laboratory (AHT), and Gluck Equine Research Center. The protocols for HI used by Woodward *et al*. (2014) were followed. Dilutions equal or greater than 1:2 were considered positive.

Virus isolation and RNA extraction

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). For RNA extraction Mag MAX™-96 Viral RNA Isolation Kit was used. HA1 gene was amplified by adopting the method described previously by Hoffmann *et al*. (2001).

Genome sequencing

PCR products for HA1 gene from RT-PCR suitable for the sequence analysis were produced using the gene specific primers, tagged with the M13 sequence primers following the procedure. 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 Bio system) using the Big-Dye Terminator V3.1 (Applied Bio system). Edition of nucleotide sequences, nucleotide, and amino acid alignments, and maximum likelihood phylogenetic trees construction was performed using latest bioinformatics software "Geneious version R 10 (Kearse *et al*., 2012).

Statistical analyses

All the statistical analyses of collected data were performed using the statistical software (SPSS version 20.00). Seroprevalence was calculated with a statistical significance of 95% confidence interval (C.I) for each category included in each variable. Statistical significance of variation in the prevalence between these different groups was evaluated by the two-sided chi-square test. A regression model was also constructed with significant variables included in this model performed by the stepwise likelihood-ratio method. Hosmer and Lemeshow test was used to test the goodness of fit for the used regression model, which proved a good fit to the data as p=0.821 (>0.05) (Hosmer and Lemeshow, 2002).

Table I.- Seroprevalence for equine influenza (EI) virus H3N8, H7N7 and mixed infections during the 2015-16 EI outbreak in Khyber Pakhtunkhwa, Pakistan.

Prevalence	EIV H3N8	H7N7	Mix infection
Prevalence (Positive/total)	101/696	42/696	25/696
Prevalence %	14.51%	6.03%	3.59%

RESULTS

A total of 696 serum samples collected, including 258 (37.06%) donkeys, 342 (49.13%) and 96 (13.8%) horses, were tested for the presence of antibodies against different influenza A viral strains and subtypes. Considering the equine population characteristics, almost 99% of the sampled equines were kept for the working purpose consisting of Donkeys, Horses, and Mules. Geographically, 220 (31.60%) serum samples were collected from Peshawar, 145 (20.83%) from Mardan, 67 (9.62%) from Charsadha, and 264 (37.93%) from district Swat (Fig. 1). Of the 696 non-vaccinated equines, 101 (14.51%) were seropositive for H3N8, 42 (6.03%) were seropositive for H7N7 and 25 (3.59%) equines were positive as mix infections (Table I). The overall seroprevalence of equine influenza (EI) using commercially available Influenza-A enzyme linked immunosorbent-assay (ELISA) kit was 24.13% in the sampled equine species after the outbreak of 2015-16 in KPK. The kappa calculated between the

ELISA and HI results was 0.84 (P<0.05). The highest prevalence was observed in mules (29.16%) followed by donkeys (24.41%), and horses (22.51%). Geographically, the highest EI seroprevalence was recorded in district Swat (30.68%) followed by Mardan, Peshawar and Charsadha district as tabulated in Table II. Similarly, among the sampling sites, seropositive samples were mostly from the samples collected from the live animal markets as compared to those collected at civil veterinary hospitals (CVHs) or in the field while the equines were working.

Table II.- The seroprevalence and its association with various categories using a univariable statistical analysis, for 696 sampled equines from Khyber Pakhtunkhwa, Pakistan in 205-16.

Variables	Categories	Positive/Total (%)	Odd ratio (95% CI)	P-value
Equine species	Donkey	63/258 (24.41)	REF	
	Horse	77/342 (22.51)	1.14 (0.67-1.94)	0.62
	Mule	28/96 (29.16)	1.26 (0.75-2.12)	0.37
Geography	Peshawar	46/220 (20.90)	REF	
	Mardan	33/145 (22.75)	1.65 (1.08-2.50)	.01
	Charsadha	08/67 (11.94)	1.45 (0.90-2.33)	.12
	Swat	81/264 (30.68)	3.18 (1.45-7.00)	.004
Sampling site	LAMs	45/169 (26.62)	REF	
	CVH/field	123/527 (23.33)	1.19 (0.80-1.77)	0.38
Gender	Male	127/491 (25.26)	REF	
	Female	41/205 (20.0)	1.39 (0.93-2.07)	0.09
Keeping purpose	Working	166/684 (24.26)	REF	
	Sports	2/12 (16.66)	1.60 (0.34-7.38)	0.54
Origin of equine	Imported	33/155 (21.29)	REF	
	Country bred	135/541 (24.95)	1.22 (0.79-1.89)	0.34
Movement in Stable	Restricted	81/376 (21.54)	REF	
	Free	87/320 (27.18)	0.73 (0.51-1.04)	0.08
Canine kept with equines	Yes	58/218 (26.60)	REF	
	No	110/478 (23.01)	1.21 (0.83-1.75)	0.30
Domestic ducks kept with equines	Yes	26/110 (23.63)	REF	
	No	142/586 (24.23)	0.96 (0.60-1.56)	0.89
Backyard Poultry kept with equines	Yes	110/452 (24.33)	REF	
	No	58/244 (23.77)	1.03 (0.71-1.48)	0.86
Sheep or Goat sharing premises	Yes	44/154 (28.57)	REF	
	No	124/542 (22.87)	1.34 (0.90-2.01)	0.14
No of Equine Species kept together	single specie	114/430 (26.51)	REF	
	Two or more	54/266 (20.30)	1.41 (0.98-2.04)	0.06
Local equine density (total equines /2KM)	1-10/2KM	40/343 (11.66)	REF	
	>10/2KM	128/353 (36.26)	0.23 (0.15-0.34)	<0.000
Equines working in ponds having migratory birds setting in it	Yes	63/207 (30.43)	REF	
	No	105/489 (21.47)	1.60 (1.10-2.30)	0.01
Equestrian events	Yes	119/489 (24.33)	REF	
	No	49/207 (23.67)	1.03 (0.70-1.51)	0.85
Quarantine of infected equines	Yes	25/94 (26.59)	REF	
	No	143/602 (23.75)	1.16 (0.70-1.9)	0.54
Quarantine of newly arrived equines	Yes	58/201 (28.85)	REF	
	No	110/495 (22.22)	1.42 (0.97-2.05)	0.06

Variables dropped, vaccination status, body conditioning score, number of people attending equines, the source of the animal. These variables were dropped because a cell was having 0 number of equines. Age of animal was dropped because of having recall bias due to imported equines, respondents did not exactly know the age of the animal.

Univariate analysis found numerous factors to be statistically associated with the seroprevalence for EI (Table II). These factors included "Geography", "local equine density per 2 KM", and "equines working status in the ponds where migratory birds used to sit and stay". As to "geography" equines that reside in the district, Swat region was associated with the significant (P<0.05) higher seroprevalence for the EI occurrence that those kept in district Mardan, Peshawar and Charsadha district, respectively. In addition, equines from the densely-populated regions, having more equines per two kilometers had significantly higher seroprevalence as compared with those held in low populated regions (Table II). The most interesting one in the findings of the present study was the predicting variable of "Equines working status in ponds having migratory birds setting in it", showing a significant (P<0.05) association as a potential risk factor (Prevalence odds = 1.60, C.I = 1.10-2.30) occurrence of EI or in other words the prevalence of EI in the study area. Several predicting variables *i.e.* "Equine species", "Sampling site", "Gender", "Backyard poultry kept with equines", "Sheep and goat sharing premises with equines", "Number of equine species kept together", "Equestrian events", "Quarantine of infected equines", and "Quarantine of newly arrived equines" were found as potential risk factors (Prevalence Odds= >1) but did not establish a significant (P>0.05) association with the seroprevalence or occurrence of EI (Table II).

In the final logistic regression model, "Local equine density" and "Geography of equines" were recorded to be significant risk factors related to the seroprevalence or occurrence for EI (Table III). The increase in the population of equines per two Kilometer distance was found responsible for the increase in the seroprevalence of EI in that specific region. Here, the "Equine working status in ponds having migratory birds sitting on it" was found to be a potential risk factor (Prevalence odds = 1.41; 95% C.I = 0.72-2.78), but did not proved to be significantly (P >0.05) associated with the prevalence of EI (Table III).

Temporal epidemiology and spread of the EI outbreak

The outbreak started in October 2015 based on the clinical signs shown by the equine population in district Peshawar, of KPK. Sampling was started in the 3rd week of October 2015 (Fig. 2) from equine located in live animal market imported from Afghanistan and brought from other parts of the country. The outbreak continued to spread from to other parts of the province. Analysis of data showed that the outbreak was originated from horses imported from Afghanistan across the border and spread throughout the province (Fig. 1). The epidemic curve shows the severity of the outbreak elevating from 3rd week of November 2015 and reached to peak in the 2nd and 3rd week of Feb 2016 (Fig. 2).

Table III.- The statistical relation between equine influenza seroprevalence and variable categories (local horse density, equines working in ponds having migratory birds, and locality) determined through multiple variable analysis.

Variables	B	Standard error	Exp (B)	95% CI	*Sig
Local equine density**	1.48	0.20	4.430	2.96-6.62	<0.001
Equines***	0.349	0.34	1.41	0.72-2.78	0.310
Locality (districts)	1.53	0.49	4.61	1.75-12.12	0.002

*P-value; **Local equine density (total equines/2 KM); **Equines working in ponds having migratory birds setting in it.

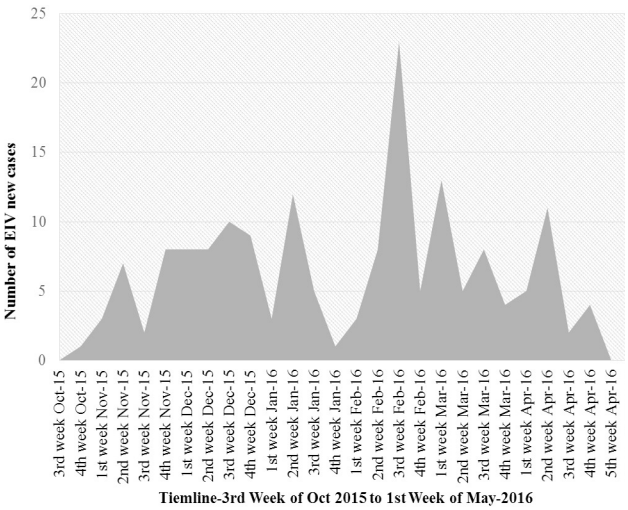

Fig. 2. Epidemic curve for the equine influenza outbreak in Khyber Pakhtunkhwa. The vertical columns show the new infections by a week of the outbreak 2015-16.

Genetic characterization and phylogeny

The viruses were isolated and sequenced. The H3N8 virus was successfully isolated but we did not succeed to isolate any H7N7 virus successfully. The OIE recommended EIV reference strain South Africa/4/03 for vaccine and the contemporary isolates from Florida clade-1 (FC-1) and clade-2 sequences were compared with the derived HA1 amino acid representative sequence for equine/Pakistan/16 (Fig. 3). The sequences from equine/Pakistan/16 were mostly identical to the FC-1 strains recently isolated, including Tennessee/14 and Malaysia/15. The phylogenetic tree concluded that the isolated strain was identical to the Florida clade-1 recent isolates.

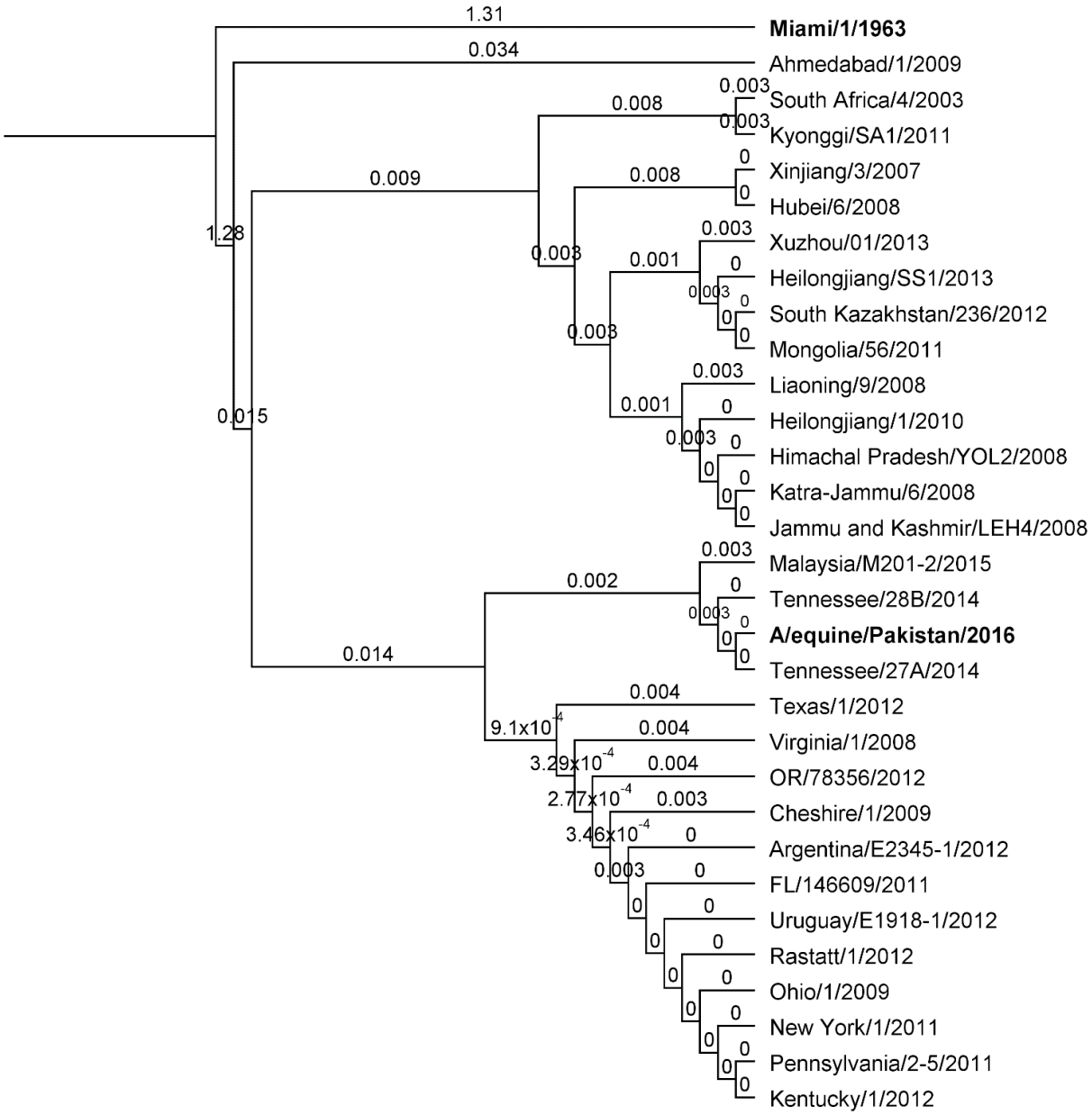

Fig. 3. Phylogenetic analysis of HA1 gene segment-amino acid sequence from Pakistan, 2016. Maximum likelihood tree was constructed. The ancestor root on top and the current isolate are in bold.

DISCUSSION

Using the ELISA kit and HI as a serological detection method, we demonstrated the active presence and circulation of the EIV in equine population in Pakistan with 24.13 % seroprevalence to H3N8 and H7N7, during 2015-16 outbreak. Analysis of data suggested that the outbreak lasts for 4-5 months in the winter season. H3N8 was isolated for the first time in Pakistan, the current outbreak investigated and reported here is the first of its nature.

Since late 1970, no H7N7 influenza A viruses have been detected in equine population (Cowled *et al*., 2009). However, the 6.03% H7N7 seroprevalence detected here in this study, supports the previous serologic surveillance studies indicating that these viruses still might be circulating at low prevalence in the equine population of Central Asia and some Eastern European states (OIE,

2014; Guthrie, 2006; Cowled *et al*., 2009). Nevertheless, instead of higher H3N8 relevance risk factor analysis conducted here on serological data was carried out for both the H3N8 and H7N7. In addition, as characteristic for the endemic infections, in which no greater scale outbreaks occur, an accumulation of probability is there to encounter these viruses with time, and consequently, the seroprevalence was revealed to positively relate with an increase in the age. As predicted for such an extremely contagious infection, of all the management, environmental and demographic risk factors investigated in the current study, the strongest statistical association was originated between the seroprevalence and local equine density. Higher seroprevalence was detected in equines residing in areas that enable meeting and exposure to other working equines or new individuals. Also, the variable "equine working in ponds having migratory birds setting in it" was recorded as a potential risk factor (Prevalence odds = 1.60; CI = 1.10-2.30), significantly associated ($P<0.05$) with seroprevalence of EI. These results are in accordance with the findings reported by Raz *et al*. (2014). The epidemiological information collected here shows that in these ponds a large number of other equines from different areas visited for work, and hence maximizing the exposure rate to the infected equines. This finding is well established that large epidemics of EI are usually related with the congregation of equines at different equestrian events, that finally leads to spatial widespread dissemination of the virus (Waddell *et al*., 1963), as previously occurred in United Kingdom in 1979 (Bryant *et al*., 2011), in 1989 in Ireland (Gildea *et al*., 2012), and in 2007 in Australia (Cowled *et al*., 2009).

Geographically the seroprevalence varied significantly ($P<0.05$), the higher seroprevalence was recorded in district Swat, followed by Mardan, Peshawar and Charsadha, respectively. The higher prevalence in district Swat may be due to the difference in the management, and its climate from the other districts. The equine kept in Swat region were mostly working equines used to work in ponds where migratory birds visited all over the year and also the climate of Swat is snowy and severely cold being a hilly and forested area, as compared to Mardan, Peshawar, and Charsadha, which is a plane and hot temperate. These findings are supported by the reported findings of Towers *et al*. (2013). However, considering geographical distribution of equines sampled in this study meticulously represents the calculated distribution of equines in Pakistan.

The other predictable variables studied here, regarding demography, management, biosecurity practices, equestrian events, and different other animal and bird species sharing premises with equines were found as risk factors but they were not found as significantly ($P>0.05$) associated with the seroprevalence of EI. It may be due to the free movement of healthy as well as infected equine without any restrictions. Keeping other species *i.e.* sheep, goat, domesticated ducks, backyard poultry and canines with equines under the same shed may play a major role in the reassortment of new influenza viruses. Such mix species keeping practice may also enhance the transmission and local spread of the virus among the susceptible population, as has been discussed in previous studies (Akhter *et al*., 2017).

CONCLUSION

Here we can conclude, based on the evidence of epidemiological data collected, that EIV equine influenza outbreak 2015-16 in equine population in Pakistan was originated in Peshawar in horses imported from Afghanistan. Several factors contributed as potential risk factors in the transmission and spread of EIV epidemic throughout the province. Concluding these factors free horse movement in the form of transportation and the equestrian events supported the spread of the outbreak and increase seroprevalence of EIV.

Statement of conflict of interest

Authors have declared no conflict of interest.

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