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# Investigating the epidemiology of EI epidemic spread in the Province of Khyber Pakhtunkhwa, Pakistan in 2015–16

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ABSTRACT

EI in non-vaccinated population causes disruption and economic losses. To identify the risk factors associated with the EI epidemics in equids in Pakistan, a 1:1 matched case control study was conducted during 2015–2016. Including a total of 197 laboratory confirmed cases and negative controls, matched on the basis of geography, time of sampling, specie and age. A piloted questionnaire was used to collect data regarding risk factors associated with the occurrence of EI in face to face interviews. Conditional logistic regression was performed to analyze the data. A total of 16 out of 23 variables were found associated as risk factors in Univariable conditional logistic regression analysis. Multivariable conditional logistic-regression analysis was also performed. Monthly removal of manure doubles the risk of EI (EI) compared to its daily removal. Due to lack of vaccination; the spread of disease was favored by high equine density. Investigating the index-case it was recorded that infected cases were imported from Afghanistan. Most of these risk factors related to biosecurity and management were due to low awareness level regarding EI amongst the respondents. These findings are in line with the results of many other studies identifying similar risk factors for EI infection in various countries. Adopting protective practices, vaccination and controlling the risk factors identified in the present study could reduce the spread and future outbreaks of EI in Pakistan.

## 1. Introduction

EI (EI) virus is an influenza A virus from a family known as, the *Orthomyxoviridae*. These viruses are negative sense, having a single-stranded RNA genome, having eight gene segments (Rash et al., 2014). Originally, thought to have transmitted from birds, the subtype EI A (H3N8) virus was first isolated during an outbreak widespread in the United States in the year 1963 (Rash et al., 2014). Since then spread throughout the world causing several major epidemics in equine population. It could be transmitted through direct or indirect means. Equines infected for the first time can shed the virus 24 h after infection, in nasal secretions, and last shedding for up to 10 days (Murcia et al., 2011). Recent studies on animal influenza viruses have reported the risk factors for interspecies transmission (Harris et al., 2017). Another study from China also reported studied risk factors for influenza A viruses (Ai et al., 2013). Girmay and Mebrahtu (2017) reported several risk factors responsible for the occurrence of EI. Most of the cases in the Australian four-month EI epidemic during which around 70,000 horses got infected, were either linked to two events, participation in equestrian-or-due to the local spread over a distance of around 2–8 km from the regions infected in the first weeks of the outbreak (Davis et al., 2009).

Equine industry in Pakistan can be divided into two subtypes i.e. equines used for working and sports, including imported and indigenous breeds. Imported breeds consist of Arabian and thoroughbred as the leading breeds. While indigenous includes “Balochi”, “Hirzai”, “Morna”, “Siaen (Shiaen)”, “Anmol”, “Kajlan”, and “Topras” breeds (Pakistan Economic Survey, 2014). Identification of the locally important risk factors associated with infection spread is important in development of the surveillance and control strategies. The only study conducted in Pakistan reported an outbreak of EI infecting all the equine species, was based on a sero-prevalence study in 2012–2013

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(Sajid et al., 2013). Despite the significance of equestrian pursuits in Pakistan, the endemic infectious equine diseases (e.g., strangles and glanders) and the occurrence of EI as an emerging infectious threat, the research to date on the risk factors and outbreak investigation has been limited. The present study was designed to investigate the risk factors associated with the EI epidemic in equine population during 2015–1016 in Pakistan. Specifically on the border areas of Khyber Pakhtunkhwa Province with Afghanistan. The purpose of the current study, as the first ever epidemiological investigation study on EI in Pakistan, was intended to improve our understanding of the basic epidemiology of the current EI epidemic. The information gathered here will be helpful for disease control authorities in future in implementing the appropriate control strategies in events of the EI epidemics. These findings, may also provide insight into the mechanism of EI spread in Asia.

## 2. Materials and methods

### 2.1. Study design

We conducted a cross sectional survey based 1:1 matched case control study. Eligible cases were equines those met the screening criteria as per recommendation of OIE. A potential cases inclusion criteria was that, if detected positive either on hemagglutination inhibition (HI) assay against EI (H3N8) A virus or detected by RT-PCR. As all the sampled equine population was unvaccinated against EI; therefore all the samples were considered potential cases having titre $\geq 2$. In screening criteria matching of cases and controls were also performed. Matching was done based on age (young = 1–3 years, adult = > 3 years), specie (Mule for Mule, horse for horse), Neighborhood (within 2 kilometers) and time (sampling week). Samples were collected from four randomly selected districts (Fig. 1) equine population of Khyber Pakhtunkhwa province in Pakistan. This region is having border with Afghanistan, across this border equines are imported and exported all the year without any biosecurity control measures.

### 2.2. Sample size

A sample size of 139 cases and controls were calculated to provide 95% confidence of detecting an odds ratio of one with 80% statistical power, assuming a 1:1 ratio of case to control premises and a minimum of 10% of control premises exposed to the factor of interest (Thrusfield, 2007). The estimated sample size *n* for matched case-control study was calculated following (Kasiulevičius. et al., 2006).

### 2.3. Matching and laboratory confirmation

Initially 376 suspected cases and physically healthy controls were sampled; matched based on “age”, “specie”, “geography”, and “time of sampling”. At the time of interviews some of the cases and controls were lost or dropped from the study. At the end total of 197, laboratory confirmed matched cases and controls were included in the final analysis. The number of cases and controls lost during the surveillance are given in detail in Fig. 2. Total of 25 cases were detected positive on RT-PCR and rest of the cases were included based on serology. 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 Terrestrial Manual, 2016). Spot HA was performed after harvesting of embryonated eggs to check the agglutination activity of the sample being inoculated. For RNA extraction Mag MAX™-96 Viral RNA Isolation Kit was used. Complete procedure used for RNA extraction is given in detail (Table S1). PCR products for HA1 gene was obtained by adopting the method described previously by Hoffmann et al. (2002). In this purpose, the primer pair described by (Hoffmann et al., 2002) were used. PCR product from RT-PCR suitable for the sequence analysis were produced using the gene specific primers, tagged with the M13 sequence primers following the procedure (Table S3) as described previously (Rash et al., 2014). Amplification products for all the reactions were visualized on 1% agarose gels for all genes, using Gel-Red nucleic acid-stain (Biotium), then purified by using the QIA-quick PCR purification kit (Qiagen) 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).

### 2.4. Questionnaire and interviewing

Two interviewers (AK and SHF) collected data via structured face to face interviews, on a “predesigned questionnaire” that was piloted on 25 equine owners, farmers and veterinarians, and modified after their comments accordingly. The intended respondent (owner or farmer) was the person who was directly responsible for management of the equines at the time of outbreak. This questionnaire, which is available from the corresponding author on request, contained 23 closed ended questions aimed to capture data on:

- demography and locality case or control,
- Management,
- Biosecurity,
- Signs of EI in equines at time of sample collection,
- Other measures.

### 2.5. Data entry

Data from questionnaires was entered into a database set using a purpose-built sheet or form in Microsoft Access 2010 (Microsoft Corporation, WA, USA). Basic data manipulation and data cleaning was conducted in this database.

### 2.6. Statistical analysis

Statistical analyses were conducted using SPSS version 20.0. The outcome variable was of binary status as an interest variable, according to the presence or absence of EI in a matched form.

#### 2.6.1. Variables studied

A comprehensive list of 23 explanatory variables explored here is presented in Tables 1 and 2. Geographical variables (Spatial) were derived through connecting the database to the spatial layers in ArcMap 10.5 (ESRI, Redlands, CA, USA). The exact locations of respondents (cases and controls) were collected by using hand-held GPS devices throughout interviews for validation. Several variables were considered a *priori*, as probable to confound between the occurrence of EI and variables likely to either prevent or promote the spread of influenza virus. Some variables were not included in analyses-such as “vaccination status” because no vaccination against EI never done in Pakistan. A method known as “dummy variable adjustment” was adopted to manage the missing data on the predictor variables in the regression models (Chaudhry et al., 2015). For each predictor with missing data, a dummy/indicator variable was created to indicate whether or not data are missing on that predictor. All such dummy/indicator variables were included as predictor in regression (Allison, 2001). Those dummy/indicator variables were coded with a constant value for missing data. For the construction of dummy/indicator variable “Nested IF function in Excel” was used. It was coded as “1” if the value was missing and “2” if the answer was “No” and “3” if the answer was “Yes”.

#### 2.6.2. Univariable analysis

Conditional logistic regression analysis was conducted to analyze the data following (Breslow et al., 1978). The normality in data was determined using Shapiro-Wilk test (Thode, 2002). Linearity of