

Molecular Evaluation of Equine Influenza Virus in Horse Farms at Saudi Arabia

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Abstract: Equine influenza imposed itself strongly and wounded a number of horses in the Kingdom of Saudi Arabia in 2009 since these animals are sensitive and easily showed weaken immunity when they are infected with the virus through inhalation. There are many procedures and precautions under way to tackle the disease before it turns into an epidemic between horses and could extend its risks for citizens in light of the ease of transmission to humans, especially that caused by the virus species which could infect human and here lies the danger. Equine influenza virus (EIV) surveillance is important in the management of this disease. This work aimed to provide data on the current status of equine influenza infection at KS using recent technique. A total number of 507 horse was examined and 100 animals showing influenza symptoms were selected for the current study. Samples of blood and nasal swab were collected from these animals. Reverse transcription-PCR (RT-PCR) was carried out for evaluation of nasal swab samples using the matrix primers. Results revealed that the nasal swabs from 100 horses samples suffering from viral respiratory disease were negative for equine influenza viruses. It was concluded that RT-PCR using the matrix primers is the most sensitive methods for the identification of virus

Key words: Equine Influenza • EIV • PCR • RT • PCR

INTRODUCTION

Influenza A virus infection of equines has been reported worldwide with the exception of a small number of island countries including New Zealand and Iceland. Equine influenza (EI) is endemic in Europe and America. Other parts of the world such as Japan, South Africa, India and Hong Kong suffer occasional incursions but the disease is not endemic. Typical outbreaks of (EI) are characterized by pyrexia, coughing and nasal discharge. Although the mortality rate associated with equine influenza virus (EIV) infection is very low it is considered the most important respiratory virus of horses. This is because it is highly contagious and has the potential to cause significant economic loss due to the disruption of major equestrian events. The equine population is highly mobile and horses travel long distances by road and air for competition and breeding purposes. When an

infected horse is introduced into a susceptible population virus spread can be explosive. The incubation period can be less than 24 hours in naïve horses and the continuous coughing which is a major feature of the disease, serves to release large quantities of virus into the environment. The virus is spread by the respiratory route, personnel, vehicles contaminated with virus and fomites. Large outbreaks are often associated with high density stabling and the congregation of horses at equestrian events and their dispersal over a wide geographic area after the event [1].

The clinical signs are less severe and disease spread is slower in partially immune populations. The majority of outbreaks in endemic populations are contained with limited spread between premises. The severity of the disease depends primarily on the immune status of the horses at the time of exposure, the environment and the stress created by continuing to work or train. (EI) can be

controlled by vaccination and sub unit vaccines. In endemic countries the economic losses due to (EI) can be minimized by vaccination of highly mobile horses and many racing authorities and equestrian bodies have mandatory vaccination policies that serve as an insurance for business continuity. In well vaccinated race horses or competition horses the predominant sign may be sub-optimal performance and many horses may be sub clinically infected. Many countries have experienced (EI) epizootics related to the importation of such horses [2, 3].

Two subtypes of (EI) have been reported in horses, H7N7 and H3N8 [4]. The first reported outbreak of equine respiratory disease to be confirmed as equine influenza occurred in 1956 in Eastern Europe. The virus isolated was characterized as H7N7. Subsequently H7N7 viruses were identified as the cause of outbreaks in Europe, Asia and the United States. Although H7N7 viruses co-circulated with H3N8 viruses in horses for many years, it is generally accepted that these viruses have not been active for a long period and may be extinct. Phylogenetic analysis of nucleoprotein genes suggest that the H3N8 equine 2 virus genome originated in the late 19th century but the first isolation of a virus of this subtype took place in Florida in 1963. Since then H3N8 influenza viruses have been responsible for epizootics in all continents. Antigenic drift occurs less frequently in equine influenza viruses than in human viruses but the H3N8 subtype has evolved into two distinct lineages designated the (EI) viruses have the potential to cross species barriers and have been associated with outbreaks of respiratory disease in dogs (primarily but not exclusively, greyhounds and dogs in shelters) in North America, quarry hounds in England and dogs on premises with horses affected by (EI) in Australia. Interspecies transmission of (EIV) to dogs maintained in the same stable as experimentally infected horses was demonstrated but there is to-date no evidence of (EI) transmission from dogs to horses. During 2004-2006 swine influenza surveillance in central China 2 equine H3N8 influenza viruses were isolated from pigs. Despite the successful experimental infection of human volunteers with (EIV) and the occasional identification of seropositive persons with occupational exposure there is currently little evidence of zoonotic infection of people with (EI) [1, 5].

The aims of this study is to reduce the economic impact of (EI) by maintaining the awareness of emergence

and international spread of antigenic variants by using the prevalence of Equine influenza virus in horse's samples taken the intake from horses farms in KSA.

MATERIALS AND METHODS

Samples Collections: A total number of 507 horses was examined at KSA during 2009 and 2010. Out of these animals 100 horse showed influenza symptoms and were selected for the current investigation. Blood samples and nasopharyngeal swabs were collected from these horses. Serum samples were separated and tested by hemagglutination inhibition test [6]. 97% of these animals were positive for influenza virus (data were not shown in this work). A part of samples were transported to molecular virology laboratory at King Abdul AL-Aziz University. Serum samples were kept at -20°C till uses. Nasopharyngeal swabs were immediately placed in vials containing 5 ml of phosphate-buffered saline (PBS) with 10% glycerol and antibiotics.

Molecular Testing: Reverse transcription-PCR (RT-PCR) assay was carried out for identification of the virus:

- RNA extraction reagents: Viral RNA extraction reagent kit was purchased from Qiagen (QIAamp; Qiagen, Hilden, Germany) Cat No.52904.
- Diethylpyrocarbonate (DEPC) was purchased from Sigma; Deisenhofen, Germany) Cat No. 1609-47-8.
- Reverse transcriptase (M-MLV) Cat No. M1701, *Taq* polymerase Cat No. M3001 and deoxynucleotide triphosphate Cat. No. U1330 were obtained from Promega (Madison, USA).

Oligonucleotide Primers: For H7, the following forward (F) and reverse (R) primers were designed based on previously published sequence, using the Lasergene sequence analysis software (DNASTAR Inc., Madison, USA) to amplify partial sequence of a 244 bp segment, PCR products of 552 bp (outer nucleoprotein primers), 241 bp (inner nucleoprotein primers) and 244 bp (matrix primers) were visualized on 1.2 or 1% agarose gels stained with ethidium bromide [6].

H7-F: 5'-ATGAGYCTTYTAACCGAG GTC GAAACG-3'
H7-R: 5'-TG GACAAAN CG TCTACGCTGCAG-3' [7].
N7-F: 5'-AGCAAAAGCAGGGTAGATAA-3'
N7-R: 5'-TCCTTGCATCAGAGAGCACA-3' [8].

Virus RNA Extraction from the Vaccine: Viral RNA extraction was accomplished using Viral RNA extraction reagent kit. Manufacturer's instructions were followed. About 140 µl of the virus sample was mixed with 560 µl AVL buffer containing carrier RNA in an RNase free tube and vortex for 15sec and then incubated at room temperature (RT) for 10 min. Mixture was then centrifuged briefly and the absolute ethanol (560 µl) was added and vortexed for 15 sec and then briefly centrifuged. A 630µl of the mixture was carefully applied to the spin column attached to 2 ml collection tube and centrifuged at 6000g (8000 rpm) for 1 min. Tube containing the filtrate was discarded and transferred the spin column into a fresh 2ml collection tube, closed the column to avoid cross contamination while centrifuging at 6000g (8000 rpm) for 1 min to get rid of the residual fluid. Repeated the above step till the whole sample mix pass through the filter. 500µl buffer AW1 was added to the spin column and closed the cap and centrifuged 6000g (8000 rpm) for 1 min and discarded the tube containing the filtrate. Then Placed the spin column into a fresh 2ml collection tube and added 500µl buffer AW2, closed the cap and centrifuged at maximum speed for 3 min and discarded. The column was Placed in a RNase free 1.5ml tube and 60 µl buffer AVE was added (RNA elution buffer; RT), after that incubated at RT for 1min then centrifuged 6000g (8000 rpm) for 1 min. The extracted RNA was divided into 10 µl aliquots and frozen at -80°C till used.

Reverse Transcription of Extracted RNA: Reverse transcription of extracted RNA was performed for synthesis of cDNA. Following the method of Squazza *et al.* [9] was used. According to mixture of 9 µl viral RNA, 1 µl Uni12 reverse primer (200 nMol/µl) and 5µl DEPC treated water was first heated at 70°C in the heating block of the DNA-thermal cycler for 5min. Such mix was chilled on ice followed by adding 1 µl of 10mM dNTPs, 1µl RNase inhibitor (40U), 5µl of 5X RT-buffer, 2µl DEPC-water and 1µl M-MLV-RT (20U). Mixture was returned to the heating block of the DNA-thermal cycler. The cDNA synthesis program included reverse transcription stage at 42°C for 90 min linked to RT-inactivation stage at 94°C for 10 min.

Polymerase Chain Reaction (PCR) of H7 and N7: According to the method of Martella *et al.* [10] with modification, the synthesized cDNA (5 µl) was mixed with 1 µl of 10 mM of dNTPs, 1 µl of each of the F and R primers (200 nMol/µl), 10 µl of 5 X Taq buffer, 2 µl of 25

mM MgCl₂, 1 µl (5U) Taq DNA polymerase and 29 µl DEPC-treated water giving a final reaction volume of 50 µl in 0.2 ml Eppendorf tube. The tube was placed in the DNA-thermal cycler and applied for amplification program included the following stages: initial denaturation at 94°C for 3 min linked to 40 cycles each consisted of 3 stages including denaturation at 94°C for 1min, annealing for 1min (at 58°C for H7 and at 55°C for N7) and extension at 72°C for 1min. The last cycle was linked to a final extension step at 72°C for 10 min.

Visualization of RT-PCR Product: A volume of 2 µl of 6X gel loading dye was mixed with (8 µl) of the PCR products. Mixture was loaded on to the 2% agarose gel. 5 µl of Diluted Marker was also loaded on the same gel. Agarose gel was placed in the electrophoresis apparatus and attached to the power supply set up as a voltage of 1-5 volt/cm and the electrophoresed for 30-45min. The resolved bands were visualized on the gel documentation system. The bands were analyzed in comparison to the 100bp DNA marker using the Lab image analyzer software version 2.7.0

RESULTS

According to the latest statistical recorded for the year 1433 H of the King Abdul-Aziz Center for Arabian Horses in Dirab (kaahcfao) the number of horses registered in the Kingdom of Saudi Arabia is 10628 horses ([www. Kaahcfao.com](http://www.Kaahcfao.com)). A total of 507 blood samples was tested for equine influenza viruses (EIV). Samples were collected from different regions of KSA (Eastern, Central, Western, Southern and Northern).

Out of 507 Samples, There Was 100 Nasopharyngeal Molecular Assay

Amplifying H7 and N7 Partial Sequences: Samples of equine influenza viruses available for PCR included fluids of extracts of nasal swabs from 100 horses. RNA was extracted, viral DNA was produced by reverse transcription and PCR was performed for 35 cycles. Inactivated whole influenza vaccine was used as a positive control and influenza B. Sichuan were used as a negative control. Amplification products were electrophoresed on 2% agarose, stained with ethidium bromide and visualized with UV light. Results in fig. 1 show that products of about 244 bp were obtained with influenza vaccine but not with all samples containing extracts from the horses.

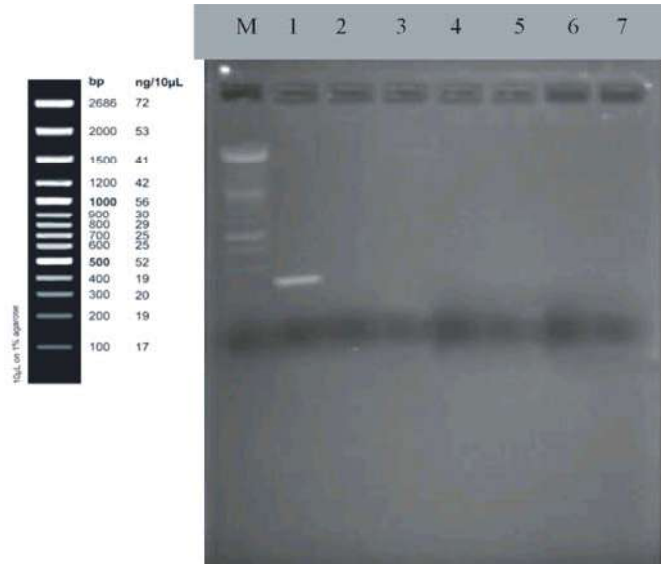


Fig. 1: RT-PCR Amplification products of equine influenza A on an agarose gel stained with ethidium bromide, Lane M: DNA Marker ladder 100 bp. lane1: the whole virus vaccine of H7N7, lane 2,3,4,5 and lane 6 the samples, lane 7 the control negative.

DISCUSSION

Equine influenza virus (EIV) is a leading cause of respiratory disease in horses. It induces a long-term immunity to re-infection. Recent strategies of vaccination aim to mimic this immunity by stimulating both antibody and cellular immune responses. Cell-mediated immunity (CMI) to influenza is well defined in man, but little has been done to characterize the responses in the horse. Additionally, the development of reliable assays for the measurement of equine CMI has lagged behind serological methods and vaccine development [11].

Equine Influenza is the most economically important and most frequently diagnosed respiratory disease affecting the horse [12]. Outbreaks of disease, considered to be influenza, have been recorded affecting horses since the 17th century. In 1872, a major epidemic of equine influenza occurred in North America. The Great Epizootic, as it was called, was first noticed near Toronto, Canada and in 90 days had spread across the continent. It spread down the Atlantic seaboard to Havana, Cuba, whereas another branch raced west to the Pacific. The overall mortality rate in horses was probably between 1 and 2%, although in some areas up to 10% of horses were said to have died from the disease. At that time, horses were vital to the economy of North America and the outbreak forced men to pull wagons by hand, while trains and ships full of

cargo sat unloaded, tram cars stood idle and deliveries of basic community essentials were no longer being made. While few countries are dependent on the horse for transportation today, the horse is part of the social fabric of most, if not all, nations. Horses travel internationally for competition, exhibition and breeding and while such travel is ostensibly regulated by health examinations and quarantines, these are often insufficient to prevent influenza from being introduced to the resident horses. Accordingly, new variants often spread quickly to other continents, most commonly with the movement of racehorses. Despite widespread use of vaccines, most countries consider equine influenza to be endemic in its equine population although the horse populations of New Zealand and Iceland have never been affected by influenza.

As mentioned previously, the first isolation of an equine influenza virus did not occur until 1956, when a subtype H7N7 virus was isolated from the affected horses in the Czech Republic (A/Equine/1/Prague/56) [13]. The H7N7 virus was subsequently identified as the cause of outbreaks of influenza in horses in many parts of the world. A second subtype of virus, A/Equine/2/Miami/63 H3N8 was isolated in 1963 from horses that had traveled from South America to Florida. For several years, both viruses caused equine influenza outbreaks worldwide: however, H7N7 virus has not been identified as a cause of clinical disease since the end of the 1970s. While there

were some serological data in the 1990s indicating that it was still circulating in horses in Central Asia and Eastern Europe, this virus is now considered to have gone extinct in the natural environment.

While, the H7N7 virus may have died out, horse populations throughout most of the world are still affected by variants of the H3N8 equine influenza virus first isolated in 1963. This virus has evolved considerably since its first isolation and at one point two distinct lineages, American and European, existed [14]. The American lineage has since split into South American, Kentucky and Florida lineages with the Florida lineage becoming the dominant global lineage in recent years [15]. To further complicate matters, the Florida and Kentucky sublineages are reportedly evolving in parallel in the United States, such that these viruses alternately circulate in the equine population [16].

Saudi Arabia in 2009 escalated complaints horse owners due to exposure of a number of horses for equine influenza subtype H7N7 and who moves quickly between horses and horse owners fear exposure to significant losses. Therefore it is necessary to hold immunizations for EIV prevention according to the International Organization for Animal Health OIE.

In 2002, a strain of H3N8 virus within the Florida lineage emerged in North America and quickly spread to other countries. Existing vaccines were found to be less effective against this variant [17]. In 2007, this virus spread widely in Asia and was ultimately introduced into Australia later that year by infected horses from Japan through ineffective quarantine of imported horses [18]. Sequence comparisons indicated that six of the eight viral gene segments were closely related to avian influenza viruses [4, 19]. Fortunately, the H3N8 Jilin virus did not appear to spread beyond China and seems to have gone extinct. It was noted at the time that the appearance of this new equine virus in China emphasized the potential for avian influenza viruses to successfully infect mammalian hosts and that this example of species jumping should serve as a warning for the appearance of new pandemic influenza viruses in humans [20]. This prescient warning was several years before the first case of H5N1 avian influenza in humans in Subbarao *et al.* [21].

In this study, the nasal swabs from 100 horses samples with suspected viral respiratory disease were negative for equine influenza viruses by RT-PCR. One hundred and seventy-one nasopharyngeal swabs submitted over a two year period from cases of suspected

viral respiratory disease were tested by Michelle *et al.* [22] for the presence of equine influenza virus, Virus was isolated from eight horses (4.7%) [23]. Isolated equine influenza virus from 117 out of 548 nasal samples of suspected viral respiratory disease (21.3%). Our negative results may be due to the small sample size.

It was concluded that RT-PCR using the matrix primers is the most sensitive methods for the identification of virus

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REFERENCES

1. Callinan, I., 2008. Equine Influenza: The August 2007 outbreak in Australia. Commonwealth of Australia (www.equineinfluenzaenquiry.gov.au). Centers for Epidemiology and Animal Health, USDA: APHIS: VS, attn. NAHMS, 2150 Centre Ave., Bldg. B, MS 2E7, Fort Collins, CO 80526-8117. 2001.
2. Daly, J., A. Daas and B./Mehr-Gross, 2007. Collaborative study for the establishment of a candidate equine influenza subtype 2 American-like strain A/EQ/South Africa/4/03-horse antiserum biological reference preparation. Pharmeuropa Bio., (1): 7-14.
3. United states department of agriculture, 2001. Animal and plant health inspection service. October 2001-1175.
4. Webster, R.G., W.J. Bean, O.T. Gorman, T.M. Chambers and Y. Kawaoka, 1992. Evolution and Ecology of Influenza A Viruses. Microbiol., 56(1): 152-179.
5. Lamb, R.A. and R.M. Krug, 2001. Orthomyxoviridae: the viruses and their replication. In Fields Virology (4th edn) (D.M. Knipe, *et al.*, eds), pp: 1487-1531, Lippincott-Raven.
6. (WHO) World Health Organization, 2002. manual on animal influenza diagnosis and surveillance.
7. (WHO) World Health Organization, 2009. information for laboratory diagnosis of pandemic (H1N1) 2009 virus in humans-revised.

8. Michelle, Q., C. Ann, N. Maura, M. Kees, H. van Jacco and A. Sean, 2004. Comparison of Sensitivities of Virus Isolation, Antigen Detection and Nucleic Acid Amplification for Detection of Equine Influenza Virus. Virology Unit, Irish Equine Centre, Johnstown, Naas, County Kildare, 1 and Department of Life Sciences, University of Limerick, Limerick, 4 Ireland and Animal Health Service, 7400 AA Deventer, 2 and Intervet International BV, Department for Virological Research and Development, 5830 AA Boxmeer.
9. Sguazza, G.H., N.A. Fuentealba, M.A. Tizzano, C.M. Galosi and M.R. Pecoraro, 2009. Complete genome amplification of equine influenza virus subtype 2. *Rev Argent Microbiol.*, 41(4): 207-11.
10. Martella, V., G. Elia, N. Decaro, L.D. Trani, E. Lorusso, M. Campolo, C. Desario, A. Parisi, N. Cavaliere and C. Buonavoglia, 2007. An outbreak of equine influenza virus in vaccinated horses in Italy is due to an H3N8 strain closely related to recent North American representatives of the Florida sub-lineage. *Vet. Microbiol.*, 121: 56-63.
11. Paillot, R., J.H. Kydd, S. MacRae, J.M. Minke, D. Hannant and J.M. Daly, 2007. New assays to measure equine influenza virus-specific Type 1 immunity in horses. *Vaccine.*, 25(42): 7385-98.
12. Landolt, G.A., H.G. Townsend and D.P. Lunn, 2006. Equine influenza infection. In: D.C. Sellon and M.T. Long (eds) *Equine Infectious Diseases*. Missouri: Saunders.
13. Paul, E., J. Gibbs and C. Tara, 2010. Equine and canine influenza: a review of current events. Anderson College of Veterinary Medicine, University of Florida, Gainesville, FL 32608, USA *Animal Health Research Reviews*, Page 1 of 9.
14. Daly, J.M., A.C. Lai, M.M. Binns, T.M. Chambers, M. Barrandeguy and J.A. Mumford, 1996. Antigenic and genetic evolution of equine H3N8 influenza A viruses. *J Gen Virol.*, 77(Pt 4): 661-671.
15. Lai, A.C., T.M. Chambers, R.E. Holland, P.S. Jr. Morley, D.M. Haines, H.G. Townsend and M. Barrandeguy, 2001. Diverged evolution of recent equine-2 influenza (H3N8) viruses in the Western Hemisphere. *Arch Virol.*, 146: 1063-1074.
16. Lai, A.C., K.M. Rogers, A. Glaser, L. Tudor and T. Chambers, 2004. Alternate circulation of recent equine-2 influenza viruses (H3N8) from two distinct lineages in the United States. *Virus Research*, 100: 159-164.
17. Toulemonde, E.C., J. Daly, T. Sindler, P.M. Guigal, J.C. Audonnet and J.M. Minke, 2005. Efficacy of a recombinant equine influenza vaccine against challenge with an American lineage H3N8 influenza virus responsible for the 2003 outbreak in the United Kingdom. *Veterinary Record*, 156: 367-371.
18. Jacob, C., 2008. Dealing with infectious disease: lessons of equine influenza in Australia. *Veterinary Record*, 165: 550.
19. Liu, S., J. Ji, Chen, D. Tai, W. Jiang, G. Hou, L. Ji and B. Huang, 2009. Panorama phylogenetic diversity and distribution of type A influenza virus. *PloS One*, 4: e5022.
20. Guo, Y., M. Wang, Y. Kawaoka, O. Gorman, T. Ito, T. Saito and R.G. Webster, 1992. Characterization of a new avian-like influenza A virus from horses in China. *Virology*, 188: 245-255.
21. Subbarao, K., A. Klimov, J. Katz, H. Regnery, W. Lim, H. Hall, M. Perdue, D. Swayne, C. Bender, J. Huang, M. Hemphill, T. Rowe, M. Shaw, X. Xu, K. Fukuda and N. Cox, 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science*, 279: 393-396.
22. Michelle, Q., D. Eugene, R. Fergus, A. Sean and C. Ann, 2005. Real-Time Reverse Transcription PCR for Detection and Quantitative Analysis of Equine Influenza Virus. Virology Unit, Irish Equine Centre, Johnstown, Naas, County Kildare, Ireland; Department of Life Sciences, University of Limerick, Limerick, Ireland; and Department of Biological Sciences, Dublin Institute of Technology, Dublin, Ireland. *Journal of Clinical Microbiology*, Oct. 2005, pp: 5055-5057, 43: 10.
23. Mika, I., N. Makoto, H. Yuji, K. Hirofumi, M. Naruto, Y. Syouchi, A. Shingo, S. Yoshihiro and K. Hiroshi, 2008. Genetic Analyses of an H3N8 Influenza Virus Isolate, Causative Strain of the Outbreak of Equine Influenza at the Kanazawa Racecourse in Japan in 2007. Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan 920-3105.