Descriptive epidemiology of equine influenza in India (2008-2009): temporal and spatial trends

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Summary

Equine influenza is a contagious viral disease that affects all members of the family *Equidae*, i.e. horses, donkeys and mules. The authors describe the pattern of equine influenza outbreaks in a number of states of India from July 2008 to June 2009. The disease was first reported in June 2008 in Katra (Jammu and Kashmir) and spread to ten other states within a year. All outbreaks of equine influenza in the various states were confirmed by laboratory investigations (virus isolation and/or serological confirmation based on haemagglutination inhibition [HI] assays of paired samples) before declaring them as equine influenza virus-affected state(s). The virus (H3N8) was reported from various locations in the country including Katra, Mysore (Karnataka), Ahmedabad (Gujarat), Gopeshwar and Uttarkashi (Uttarakhand) and was isolated in 9- to 11-day-old embryonated chicken eggs. The virus was confirmed as H3N8 by HI assays with standard serum and amplification of full-length haemagglutinin and neuraminidase genes by reverse transcriptase-polymerase chain reaction. Serum samples (n = 4 740) of equines from 13 states in India screened by HI revealed 1 074 (22.65%) samples as being positive for antibodies to equine influenza virus (H3N8).

Keywords

Donkey, Equine influenza, H3N8, Haemagglutination inhibition, Horse, India, Influenza, Mule, Outbreak, Virus.

Epidemiologia descrittiva dell'influenza equina in India (2008-2009)

Riassunto

L'influenza equina è una malattia infettiva virale che colpisce le specie della famiglia degli Equidi: cavalli, asini e muli. Gli autori descrivono la

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distribuzione dei focolai in alcuni stati dell'India, in un periodo compreso tra luglio 2008 e giugno 2009. La malattia è stata segnalata, la prima volta, nel giugno 2008 a Katra (Jammu e Kashmir), diffondendosi nei successivi 12 mesi in altri dieci stati. Prima di dichiarare i territori affetti dal virus dell'influenza equina, ogni singolo caso è stato confermato da indagini di laboratorio (isolamento del virus e/o conferma sierologica). Il virus (H3N8), isolato negli embrioni di pollo tra il nono e l'undicesimo giorno, è stato segnalato in diverse località del paese: Katra, Mysore (Karnataka), Ahmedabad (Gujarat), Gopeshwar e Uttarkashi (Uttarakhand). Il microrganismo è stato confermato per mezzo del test dell'inibizione dell'emoagglutinazione (HI) con prova sierologica standard e con i geni codificanti l'emoagglutinina e la neuraminidasi, utilizzando una catena di reazione mediante l'abbinamento dell'azione della trascrittasi inversa con l'amplificazione. I campioni di siero equino (4.740), provenienti da 13 stati dell'India, sono stati testati con la prova sierologia dell'inibizione dell'emoagglutinazione che ha evidenziato per 1.074 campioni (22,65%) la positività agli anticorpi del virus (H3N8).

Parole chiave

Asino, Cavallo, Focolaio, H3N8, India, Influenza, Influenza equina, Inibizione dell'emoagglutinazione, Mulo, Virus.

Introduction

Equine influenza, a viral disease of equidae caused by *Influenza A* virus, family *Orthomyxoviridae*, is caused by two subtypes H7N7 (equi-1) and H3N8 (equi-2). However, all the equine influenza outbreaks reported internationally during the last three decades have been due to H3N8. No outbreak associated with H7N7 has been reported (4, 13). Sporadic equine influenza outbreaks due to H3N8 continue to occur in Europe (1); the last major outbreak reported there was in 2003 (6). Between 2007 and 2008, outbreaks due to H3N8 were reported from Australia, China, Japan and Mongolia (2, 7, 15). New Zealand and Iceland are the only countries with significant horse populations which have never experienced an outbreak of equine influenza. The first recorded outbreak of the disease in India occurred between January and August 1987 and involved over 83 000 equines in north and central India (8, 9, 10). Subsequently, India remained free of equine influenza for about 20 years until it was detected again in June 2008 by the National Research Centre on Equines (NRCE) (national referral laboratory for equine diseases) (11, 12).

The equine influenza virus (EIV) is transmitted via the aerosol route, spreads very rapidly in the susceptible populations and has an incubation period of 1-3 days. EIV is known to cause severe clinical disease in equines, characterised by pyrexia, dyspnoea, a dry hacking cough and serous nasal discharge that becomes mucopurulent due to secondary bacterial infections (14). Laboratory confirmation of a suspected outbreak is required to identify the virus subtype and also for molecular characterisation by sequencing to assess the extent of genetic changes in relation to previous EIVs.

Outbreaks of equine influenza have had a significant economic impact in India as 1.58 million equines are the sole source of income of poor landless farmers and nomads who constantly move from one place to another in search of a livelihood. Although an inactivated vaccine incorporating the 1987 field isolate of EIV (A/equi/Ludhiana/87 [H3N8]) was developed after the outbreak of the disease in India in 1987, vaccination is not routinely practised. The aim of this paper is to provide information on the pattern of various outbreaks of equine influenza in India in 2008-2009 and trace their occurrence.

Materials and methods

Collection of clinical materials

Serum samples and nasopharyngeal swabs (16-18″ in length, made 'in-house' with cotton and gauze on flexible wire) were collected from animals that exhibited clinical signs of equine influenza and from animals that had been in contact with others involved in outbreaks in different states of India. These samples were placed in transport medium (40% glycerol in phosphate buffered saline,

pH 7.2) and transported to the laboratory at 4°C.

Virus isolation

Nasal swabs were processed for virus isolation in 9-11 day-old embryonated chicken eggs. Briefly, the content from the nasal swab was placed in a 10 ml syringe and the fluid extracted in a sterile vial. The antibiotic cocktail (penicillin 50 IU/ml and streptomycin 5 µg/ml) was added to the aspirate followed by incubation at 4°C for 30 min. The aspirate was cleared of debris by spinning at 5 000 g for 15 min and the supernatant collected. An aliquot of 0.1 ml of this supernatant was inoculated into embryonated eggs, both undiluted and at a dilution of 1:10 in three eggs for each sample. The eggs were incubated at 35°C for 72 h, after which they were chilled overnight at 4°C. The allantoic fluid was harvested from the eggs and subjected to the haemagglutination (HA) test with chicken erythrocytes (0.5% in phosphate buffered saline [PBS]) on microtitre plates. Each sample was subjected to five blind passages before declaring it negative for EIV.

Haemagglutination inhibition test

Haemagglutination inhibition (HI) test was performed on the serum samples using the protocol recommended by the World Organisation for Animal Health (*Office International des Épizooties*: OIE) (14). The representtative samples were counter-tested using standard antigen for EIV H3N8 (A/eq/Miami/63, A/eq/Kentucky/1/81, A/eq/Newmarket/1/93, A/eq/Newmarket/2/93) and EIV H7N7 (A/eq/Prague/56), obtained from the National Institute for Biological Standards and Control (NIBSC) in Potters Bar, Hertfordshire.

Virus identification and typing

The samples showing agglutination of chicken erythrocytes were identified and typed using the HI assay with standard antiserum against various strains of EIV H3N8 (A/eq/Newmarket/1/93, A/eq/Newmarket/2/93, A/eq/Kentucky/1/81, A/eq/Miami/63) and EIV H7N7 (A/eq/Prague/56), obtained from the NIBSC. Further, HA typing was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) using the methods described previously (12).

Neuraminidase (NA) typing was performed by RT-PCR of the NA gene. For this, viral RNA was extracted from the allantoic fluid (250 µl) using the AuPrep™ Viral RNA extraction kit (M/s Life Technologies [India] Pvt Ltd, New Delhi) in accordance with the protocol of the manufacturer. The synthesis of cDNA was performed using random hexamer primer and avian myeloblastosis virus (AMV)-RT in a 20 µl reaction volume. Using cDNA obtained as described above as the template, the NA gene was PCR amplified with the primers as follows:

- forward:
  5′-ATGAATCCAAATCAAAAGATA-3′
- reverse:
  5′-CGTAAATTACATCTTATCGAT-3′ (5).

Thermal cycling consisted of an initial denaturation at 95°C for 5 min followed by 34 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 2 min with a final extension at 72°C for 10 min. An aliquot of 5 µl of PCR amplified product was analysed in a 1% agarose gel to visualise the amplicon.

Results

Pattern of spread of equine influenza in various states of India

First equine influenza outbreak in Jammu and Kashmir

A few cases of respiratory illness in equines were reported by the Veterinary Hospital in Katra during the third week of June 2008. These animals had not responded to antibiotic treatment. Katra is a small hamlet in the northern most state of Jammu and Kashmir (bordering Pakistan, Afghanistan and China). In Katra, approximately 15 000 equines are kept in over-crowded conditions and are used to transport goods and carry pilgrims to a shrine, about 14 km from Katra. About 6.7 million devotees visit this shrine every year. The geographical coordinates of Katra are 32°58′48″N, 74°57′0″E, with an average altitude of 2 474 feet above sea level. Katra is situated on the foothills of the Trikuta

mountains of the Himalayan range. Nomads bring the equines, mostly mules and ponies, to this area from various parts of northern India during the peak pilgrimage season (April to August) to earn their livelihood.

Disease investigations conducted by a team from the NRCE revealed that initially a few animals showed clinical signs of high fever (39°C to 41°C), watery discharge from the nostrils (Fig. 1) and a deep dry hacking cough. Animals did not respond to antibiotics and, within 5-6 days, approximately 70% animals in that locality were showing the signs of watery to mucopurulent discharge, a dry hacking cough and high fever. Serum samples from 118 animals and nasal swabs from 29 animals were collected. EIV infection was confirmed on the basis of virus isolation from five nasal swabs and antibodies to EIV in the serum samples ($n = 80$) using the HI assay. The EIV isolates were typed as H3 subtype by HI assay using reference sera and amplification of the HA gene; the N8 subtype of the isolates was confirmed by RT-PCR.

Figure 1
Ponies showing watery to mucopurulent nasal discharge in an outbreak of equine influenza in Katra (Jammu and Kashmir)

The clinical form of equine influenza continued in Katra for about a month. Cases of disease were later reported in the adjoining areas of Jammu and Kashmir, such as Naoshera (27 June 2008), Gulmarg (9 July 2008), Srinagar (August 2008) and Zanskar valley in Ladakh (22 October 2008).

Outbreak in Rajasthan

Cases of equine influenza were reported from Jaipur in August 2008 where 73 horses gave positive results when tested for EIV antibodies. Subsequently, cases of equine influenza were reported from Ajmer and Jodhpur in December 2008, in Kota and Jhunjhunu district in January 2009 and in Hanumangarh in March 2009. These cases were confirmed by seroconversion for EIV antibodies in paired serum samples of representative animals. Some clinical cases were reported at an annual animal fair held at Tilwara (Barmer) during the third week of March 2009. From this location, animals moved to different districts, namely: Bikaner, Jodhpur, Pali and Jalor; clinical cases were reported during the first week of April 2009 and were confirmed by seroconversion (Fig. 2).

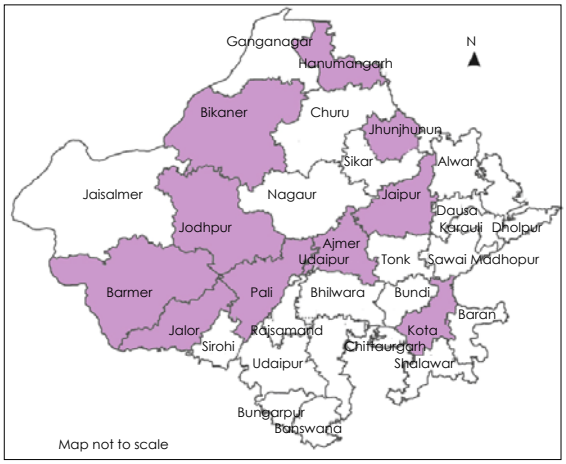

Figure 2
Rajasthan showing different districts affected by equine influenza, 29 June 29 June 2008

Rajasthan is a very large state and its equine population is spread across various districts and has no direct contact, but animals from different places come into contact with these animals during their participation in regular animal fairs held for animal trade purposes. The Mallinath fair at Tilwara (Rajasthan) is one of the largest animal fairs organised in Rajasthan.

Outbreaks in Delhi, Haryana and Himachal Pradesh

Between September and November 2008, cases of equine influenza were also reported in three states of Delhi, Haryana and Himachal Pradesh. In Delhi, the disease was reported during the last week of September in mules and ponies. Of 38 ponies tested, 15 were positive for EIV antibodies and paired serum samples from representative animals indicated seroconversion. Cases of disease were reported from horses on a farm in Hisar (Haryana) and subsequently in mules and ponies in Meham (Haryana). Similarly, cases in Himachal Pradesh were confirmed in December 2008 in Bilaspur, Hamirpur, Mandi and Palampur districts.

Outbreak in Maharashtra

The first outbreak of equine influenza in Maharashtra, the western state of India, was reported from Matheran in the Pune district during the second week of October 2008. Matheran is a small pedestrian hill station about 110 km from Mumbai and 118 km from Pune, where no vehicles are allowed for local transport and tourists can either walk or ride on ponies. There are about 400 ponies available at Matheran. These animals are generally procured from traders from northern India. Approximately 70% equines at Matheran exhibited typical signs of equine influenza, including high fever, a hacking cough and nasal discharge that subsided within one month. Although virus could not be isolated, EIV infection was confirmed as evident by fourfold or more rises in the EIV antibody titre in paired serum samples of equines tested by HI assay.

An outbreak of equine influenza was subsequently reported from adjoining areas in Pune district in November 2008. Pune is the hub of the organised racing industry of the country and thoroughbred horses are kept on different stud farms. Four of these stud farms reported clinical cases during the second week of November and 30 of 102 equines from these studs tested positive for EIV antibodies. No clinical cases were reported from other districts of Maharashtra. However, sero-surveillance conducted in the district of Nandurbar (Maharashtra) in December 2009, detected 58 out of 125 equines as being positive for EIV antibodies. No clinical cases were reported from Maharashtra after December 2008.

Outbreak in Karnataka

Clinical cases of equine influenza were reported from the Mysore and Bagalkot districts of the southern state of Karnataka during the third week of November 2008. EIV was isolated from nasal swabs of two horses from Mysore that was typed as H3N8 by HI assay and RT-PCR of the HA gene for haemaggutination, and amplification of NA gene for neuraminidase typing. Subsequently, EIV infection was confirmed in Mysore and Bangaluru during the second week of December 2008 by seroconversion in paired serum samples. No further cases of disease were reported from Karnataka.

Outbreak in Gujarat

The western state of Gujarat did not experience outbreaks in 2008, as indicated by serosurveillance of 570 equines for EIV antibodies using the HI assay. However, an animal purchased from Mallinath fair (Tilwara) in the adjoining state of Rajasthan in March 2009 and brought to the village of Kelyavasana (Ahmedabad district) developed clinical signs of equine influenza during the first week of April 2009 and spread infection to six in-contact animals which seroconverted. Within one week, the disease spread to other equine establishments in the Ahmedabad, Anand, Kheda, Kutch, Bhavnagar, Patan, Junagadh, Palanpur (Banaskantha), Navsari, Valsad, Amreli, Bharuch, Surat, Bhuj (Kachch) and Kalol (Gandhi Nagar) districts of northern Gujarat (Fig. 3). Of 1 670 equines tested, 167 were detected as being positive for EIV

antibodies. EIV was isolated from nasal swabs of an infected horse from Ahmedabad that was typed as H3N8 by HI assay and RT-PCR amplification of HA and NA genes.

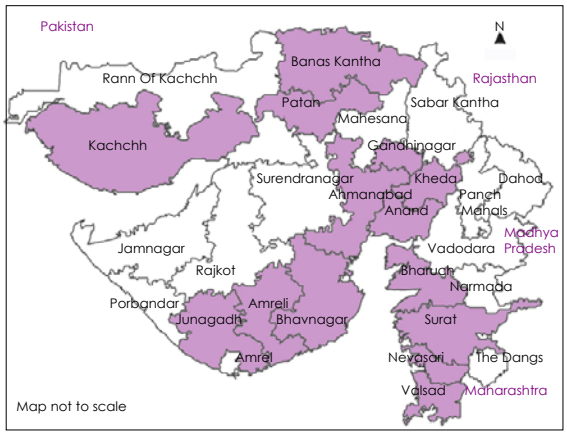

Figure 3
Gujarat showing different districts affected by equine influenza, April 2009

Outbreak in Uttarakhand

The first report of an outbreak of equine influenza from Uttarakhand was in May 2009 from Gaurikund (Rudrapryag district), an important Hindu pilgrimage site on the way to Kedarnath, 14 km uphill. About 6 000 mules and ponies congregate at Gaurikund during the last week of April from various places to transport of goods and to carry pilgrims from Gaurikund to the Kedarnath shrine, which is covered in snow during the winter and opens between May and October for pilgrims. During the first week of May 2009, between 60% and 70% of the animals at Gaurikund exhibited various signs of disease (dry hacking cough, high temperature and watery to mucopurulent nasal discharge). One important finding in this outbreak was that 50 animals died with complications of bronchopneumonia, owing to inclement weather conditions associated with low environmental temperature (4.5°C-10°C) and incessant rain. Post-mortem and bacteriological investigations performed on 4 mules revealed that death occurred due to secondary bacterial infections including *Streptococcus equi* (2), *Bordetella bronchiseptica* (1) and *Actinobacillus equuli* (1). Out of 133 equines from Gaurikund, 121 were detected as having EIV antibodies. EIV was isolated from the nasal swabs of two infected mules from Gopeshwar and typed as H3N8 by HI assay and amplification of HA and NA genes by RT-PCR.

Equine influenza spread during May and July 2009 to other districts of the state, namely: to Rudraprayag, Uttarkashi, Chamoli, Nainital, Udhamsinghnagar, Champawat, Haridwar and Dehradun. Out of 1 367 equines that were tested from Uttarakhand, 447 were detected positive for EIV antibodies (Fig. 4). EIV was also isolated from the nasal swab of one infected pony from Uttarkashi district and typed as H3N8 by HI assay and by RT-PCR of HA and NA genes.

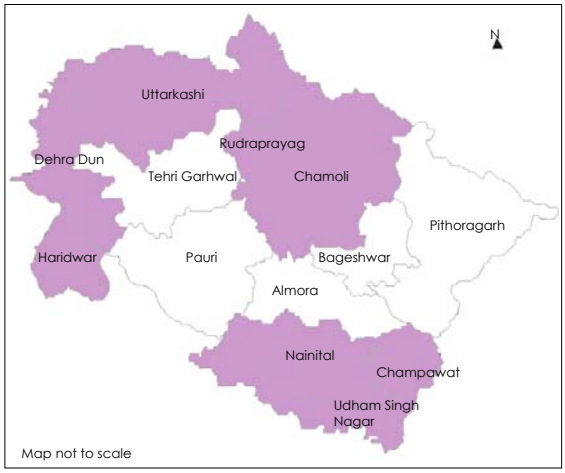

Figure 4
Uttarakhand showing different districts affected by equine influenza, May-July 2009

In 2008 and 2009, outbreaks of equine influenza were reported from 11 states in India (Fig. 5). The first outbreak was reported from Jammu and Kashmir in June 2008, followed by Rajasthan, Haryana, Delhi, Himachal Pradesh, Maharashtra, Karnataka, Gujarat, West Bengal Uttarpradesh and the last reported outbreak was from the state of Uttarakhand in July 2009. Overall, 4 761 equines from these states were tested for equine influenza infection during this period and 1 052 (22.19%) equines showed positive titres for EIV antibodies (Table I, Fig. 6). During this period, no EIV infection was reported in equines from other states in India.

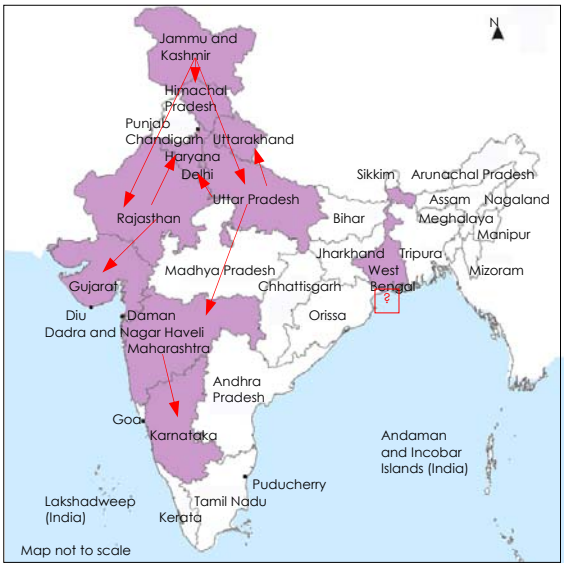

Figure 5
Different states of India that were affected by equine influenza and probable route of spread of infection (arrows)

Discussion

The previous outbreak of equine influenza reported in India in 1987 was due to EIV (A/equi/Ludhiana/87[H3N8]). The haemagglutinin 1 (HA1) gene sequence of Ludhiana/87 isolate closely resembled A/equi-2/Fontainebleau/79, A/equi-2/Miami/63 and the European (A/equi-2/Suffolk/89) isolate (3). India remained free of equine influenza for about 20 years although vaccination against the disease is not mandatory in India. From 1991 to 2007, routine serosurveillance of the Indian equine population by the NRCE revealed that India gave negative serological results for EIV antibodies (National Research Centre on Equines, unpublished data).

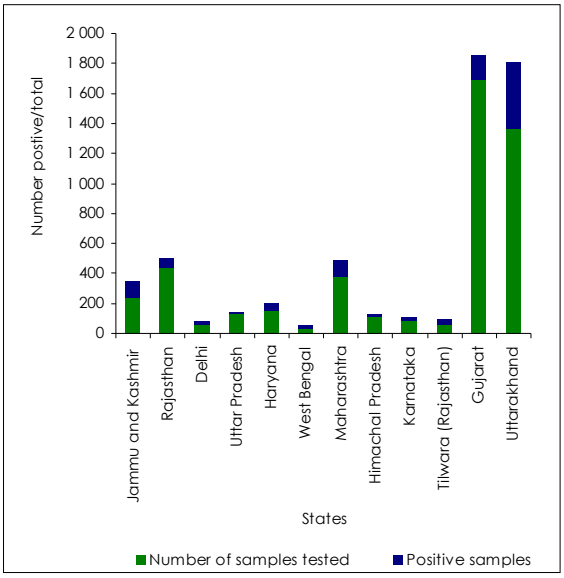

Figure 6
Percentage positivity of the serum samples for equine influenza in various states in India

Table I
Status of equine influenza in various states of India

Serial No.	States	Month	Number of samples tested	Positive samples	Positive samples in pair with more than four fold rise in titres
1	Jammu and Kashmir	Jun to Aug 2008	234	114 (48.7%)	98
2	Rajasthan	Aug 2008	438	62 (14.15%)	46
3	Delhi	Sep to Oct 2008	59	21 (35.6%)	3
4	Uttar Pradesh	Oct 2008	138	6 (4.3%)	4
5	Haryana	Oct to Nov 2008	150	51 (34%)	18
6	West Bengal	Nov 2008	30	22 (73.3 %)	19
7	Maharashtra	Nov to Dec 2008	380	108 (28.4%)	16
8	Himachal Pradesh	Dec 2008	109	18 (16.5%)	4
9	Karnataka	Dec 2008	89	19 (21.3%)	8
10	Tilwara (Rajasthan)	April 2009	56	39 (69.64%)	38
11	Gujarat	April 2009	1 690	167 (9.88%)	75
12	Uttarakhand	May 2009	1 367	447 (32.69%)	11
Total			4 740	1 074 (22.65%)	340

The current outbreak of equine influenza started in June 2008 and Katra (Jammu) was the focus of the outbreak which affected 70%-80% of the animals in the region. Equines from different places migrate here at this time and are used to transport equipment and to carry pilgrims to the shrine of Vaishno Devi. Outbreaks were subsequently confirmed in equines from Jammu, the Kashmir valley and Leh-Ladakh. Jammu and Kashmir is a land-locked hilly state with a porous border and unrestricted interstate and transborder animal movements for both trade and work. It is useful to mention that an outbreak of equine influenza occurred in China and Mongolia in 2007 (7). The HA gene sequence of EIVs isolated from various outbreaks in Katra (Jammu), Ahmedabad (Gujarat) and the Gopeshwar (Uttarkhand) outbreak revealed that the virus belonged to clade 2 of the Florida sublineage which itself belongs to the American lineage. Moreover, the sequences of these isolates most closely matched those from China and Mongolia (12), suggesting the possibility that the current infection was acquired from across the border.

Indigenous equines (ponies and mules) in India are primarily owned by nomads who migrate from one place to another across hilly terrain. Despite legislative measures to enforce movement restrictions, it is practically impossible to prevent nomadic interstate movement to places of animal trade. Although clinical cases of equine influenza subsided in Katra by August 2008, new outbreaks were reported from other states, such as Rajasthan (August 2008), Maharashtra (October 2008), Karnataka (November 2009), Gujarat (April 2009) and Uttarakhand (May 2009). Between these outbreaks sporadic cases were reported from Delhi, Haryana, Punjab, Himachal Pradesh and West Bengal (September-December 2008). In all, 11 states of India were affected by the equine influenza outbreak between 2008 and 2009.

An analysis of the 20082009 equine influenza epizootic in India requires knowledge of the demography of the Indian equine population and their uses from an Indian perspective. The states in India most heavily populated with equines are Rajasthan, Uttar Pradesh, Jammu and Kashmir, Uttarakhand, Himachal Pradesh, Maharashtra, Karnataka and Delhi where these animals are scattered across different regions. Maharashtra, Karnataka and Delhi have well established horse industries, primarily involving thoroughbreds. In other states, mules, ponies and donkeys are kept as for work purposes by the rural poor. In these states, equines constitute 98% of the total population and they are constantly on the move from one place to other with their owners in search of work. The movement of these animals does usually occur on the established roads; the animals are taken to various places along local trekking routes where movement controls are not possible. Animal owners normally procure their animals from traders in Uttar Pradesh and Rajasthan where animal fairs are organised year round. From these trading places, the disease spread to other parts of the country.

Equine influenza is an acute disease that is self-limiting. However, due to the highly contagious nature of EIV, coupled with unrestricted movements of equines, the virus spread to 11 states within 8 months of the first report of outbreak in Katra. In states such as Maharashtra, Delhi and Karnataka where equines are raised as part of an organised industry, the extent of disease spread was not high and it was controlled by a complete ban on the movement of horses. The northern state of Himachal Pradesh which is adjacent to Jammu and Kashmir, has equines in scattered locations and, although the outbreak occurred during the months of August and September, it only came to light in January 2009 as a result of retrospective studies on serum samples conducted in the region. The precise origin of the outbreak in West Bengal police horses could not be established as there had been no movement of horses. However, it seems logical to hypothesise that disease transmission could have been through people who had handled these horses. There was a brief lull from January 2009 until mid-March 2009 which could have been due to low environmental temperatures that led to the virus becoming dormant in northern India. During the second

week of March 2009, the state of Rajasthan organised a fair at Tilwara (south-west Rajasthan) where a few animals exhibited clinical signs that resembled equine influenza. These were later confirmed as such by laboratory diagnosis. The second surge of the virus in India was due to frantic selling and buying of equines at this fair, together with uncontrolled movements of equines suspected of being infected with the virus. Farmers who congregated at this fair panicked and took their animals hurriedly back to their native places, resulting in the outbreak in the states of Gujarat and Uttarakhand.

The 2008-2009 epizootic of equine influenza caught India unawares. However, the state governments, the government of India, the NRCE and the organised equine sector worked in tandem to contain the disease by implementing strict biosecurity measures and imposing bans on the movement of equines. Continuous surveillance and monitoring of the disease situation is being conducted by the NRCE. There have been no recent reports of clinical cases or diagnosis on the basis of serological (paired samples) testing or by active surveillance for the viruses. The data generated from the surveillance studies will be used for the process of de-notification of the disease from various states in the country and to obtain disease-free status from the World Organisation for Animal Health (*Office International des Épizooties*: OIE). In 1987, India was able to curtail infection by using strict biosecurity measures and by not adopting for mandatory vaccination. The same policy is still in place. Finally, research on upgrading inactivated equine influenza vaccine using latest EIV isolate from Katra is in progress at the NRCE for any future needs.

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