



# Molecular characterisation of an avian influenza (H5N8) outbreak in backyard flocks in Al Ahsa, Eastern Saudi Arabia, 2017–2018

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## ABSTRACT

**Background** Avian influenza viruses are still causing major concern not only to the poultry industry but also to human health across the globe. The live poultry markets and the small-scale local breeding of various species of birds in backyards are still playing important roles in the sustainability of most virulent influenza viruses, especially H5N8.

**Methods** The authors investigated an outbreak of highly pathogenic avian influenza H5N8 in backyard flocks in Al Ahsa, Eastern Saudi Arabia that occurred in 2017–2018.

**Results** A range of poultry including chickens, ostriches, ducks, pigeons and turkeys were clinically affected. Phylogenetic analysis suggested that this was a common source outbreak caused by a virus closely related to H5N8 viruses causing outbreaks elsewhere in Saudi Arabia in early 2018.

**Conclusions** Small backyard flocks are still contributing to the epidemiology and transmission of H5N8.

## INTRODUCTION

Avian influenza viruses (AIVs) belong to the family Orthomyxoviridae. Currently, 16 haemagglutinin (HA) and 9 neuraminidase subtypes of influenza viruses infect birds.<sup>1</sup> Highly pathogenic AIVs (HPAIV) are usually associated with H5 and H7 subtypes while the other subtypes are mainly low pathogenic avian influenza. HPAIV subtypes H5N1 and H5N8 were recently reported in poultry populations in many countries in the Middle East and Asia including Saudi Arabia, Iran, Lebanon, Iraq, Bangladesh and India.<sup>1–6</sup> HPAIV H5N8 was recently reported in different species of birds from 10 provinces in Saudi Arabia.<sup>1</sup> The main goals of the current study were (1) to isolate strains of the H5N8 from various species of birds (ostriches, pigeons, ducks, turkeys and chickens) in 10 foci of backyard poultry, (2) to document the clinical presentation in different species of birds, (3) to phylogenetically characterise the viruses causing this outbreak.

## MATERIALS AND METHODS

### Outbreak description

The outbreak was sporadic and occurred in small-scale backyard poultry flocks in Al Ahsa in the Eastern Province of Saudi Arabia between December 2017 and May 2018. A total of 43 cloacal, tracheal swabs and tissue homogenates were collected from 10 backyard flocks containing different species of poultry showing sudden death and high mortality ([table 1](#), [figure 1](#)). Oropharyngeal and cloacal swabs were collected in viral transport medium which comprised Dulbecco's minimum essential medium tissue culture, fetal bovine sera, and penicillin and streptomycin. The collected swabs were processed as previously described.<sup>7</sup> Briefly, sterile swabs were introduced into the oral cavity of the bird that was then allowed to absorb the fluid from the oropharyngeal passage. The collected swabs were transferred into viral transport media then stored at  $-80^{\circ}\text{C}$  for further processing. The collected swabs were vortexed and the fluid was collected and centrifuged at 6297 g for 5 min at  $4^{\circ}\text{C}$ . The supernatant was collected and stored at  $-80^{\circ}\text{C}$ .

Samples of organs (trachea, lung, stomach, spleen, liver and brain) were also collected from dead birds. Ten per cent tissue homogenates were prepared as previously described<sup>8</sup> and they were stored at  $-80^{\circ}\text{C}$  for further testing. Briefly, the authors collected 1 g per organ in a separate sterile tube then crushed them using sterile scissors in a sterile mortar. The authors added 9 mL of sterile water to each sample and minced them thoroughly. The tissue suspensions were centrifuged at 6297 g for 5 min at  $4^{\circ}\text{C}$  then the supernatant was collected and stored at  $-80^{\circ}\text{C}$  for further testing.

**Table 1** Summary of the collected specimens from birds in the eastern region of Saudi Arabia during the H5N8 outbreak in 2018

Map ref. no.*	Date of sampling	Type of birds in flock	Type of samples	Total number of birds	Number of sick birds	Number of dead birds	Mortality (%)	Severity/duration of disease
1	Mar-18	Ostrich	OPS	5	4	3	60	Acute
2	Feb-18	Chicken	TH	200	20	120	60	Acute
3	Feb-18	Pigeon	TH	200	15	90	45	Acute
4	Feb-18	Chicken	OPS	75	13	30	40	Acute
5	Jan-18	Chicken	TH	120	15	80	67	Acute
6	Jan-18	Chicken	OPS	80	9	53	66	Per acute
7	Dec-17	Chicken	–	300	60	125	42	Acute
		Duck	OPS					
		Pigeon	–					
		Turkey	–					
8	Apr-18	Duck	TH	50	11	23	46	Acute
9		Chicken	OPS	175	25	122	70	Acute
		Duck	–	25	–	19	76	
		Pigeon	–	15	–	9	60	
10	Jan-18	Chicken	–	80	–	80	100	Per acute
		Turkey	–	20	–	20	100	
		Duck	–	50	5	0	0	
		Pigeon	–	50	10	10	20	
		Totals		1445	187	784	54	

\*Figure 1 Locations of the backyard flocks.  
–, not tested; OPS, oropharyngeal swabs; TH, tissue homogenates.

### Isolation of H5N8

Samples in viral transport medium were inoculated into specific pathogen-free, 9–10-day-old, embryonated chicken eggs for virus isolation in a biosafety level 3 facility at the University of Hong Kong. After incubation at 37°C for 48 hours, allantoic fluid was harvested and tested by haemagglutination assay for the presence of influenza virus.

### multiple sequence alignment program - CBRC

#### Extraction of the viral RNA and NGS sequencing

Total viral RNA was extracted from the collected swabs and tissue homogenates using the Qiagen QIAamp viral RNA mini kits as per the manufacturer's instructions. To sequence the full genome of the positive culture isolates, viral RNA was amplified by multisegment RT-PCR as described.<sup>9</sup> The RT-PCR products obtained were sequenced using Illumina HiSeq platform (PE150) with the Nextera library preparation method.

### Phylogenetic analysis

Raw sequence reads were mapped to a reference genome or assembled de novo. Virus genome sequences were deduced from the consensus of the aligned raw reads with at least 100× sequencing raw read coverage. Virus gene segments were aligned by using MAFFT and phylogenetic analysis by PhyML.<sup>10</sup> The sequences were deposited on the GenBank under the accession numbers (MN687475 - MN687570).

## RESULTS

### RT-PCR detection of influenza A H5N8

A total of 1445 birds from 10 local backyard flocks in Al Ahsa were observed for signs of H5 infection during



**Figure 1** A map showing the 10 sampling sites in Al Ahsa involved in this study.

the AI outbreak in Saudi Arabia in 2017–2018 (figure 1 and table 1). All the flocks were located within a distance of 5–15 km from each other. The first location had ostriches, which showed mortality rate up to 60 per cent. Locations 2, 4, 5 and 6 had native breed local chickens with a high mortality rate reaching up to 67 per cent. Locations 7, 9 and 10 had mixed types of birds such as chickens, pigeons, turkeys and ducks with high mortality rates reaching up to 100 per cent.

### Clinical features and postmortem lesions of the H5N8 infection in different species of birds in eastern Saudi Arabia between 2017 and 2018

Clinical inspection of various species of birds in the affected flocks revealed typical AI clinical signs and postmortem lesions. In some cases, the per acute form of AI infection was reported, especially in chickens and turkeys (figure 2). In this form, sudden onset of high mortality and death of large number of birds up to 100 per cent with no obvious pathognomonic signs was reported. In other instances, less acute or milder forms of AI infection were observed. The affected birds showed decreased food and water consumption associated with respiratory and/or nervous manifestations. Some birds showed depression and oedema (figure 2A). Cyanosis of combs, wattles as well as the non-feathered parts of the skin were seen (figure 2B,C). Coughing, gasping and diarrhoea and ecchymosis of the shanks and feet were sometimes noted. Neurological signs such as paralysis of the wings and legs were also noticed in some populations (figure 2D). Postmortem inspection of some native breed chicken showed congestion and haemorrhage in the internal organs, particularly in the ovaries and oviducts (figure 2E). Petechial haemorrhage in the epicardium of the heart (figure 2F), congestion and ulceration in the caecal tonsils and intestinal mucosa (figure 2G) and congestion and enlargement of the spleen (figure 2H) in native breed chickens was reported. Depression and paralysis in the native ducks (figure 2I) was observed. Affected pigeons showed anorexia, depression, greenish diarrhoea as well as respiratory and neurological signs such as paresis and paralysis of wings, torticollis, opisthotonos and circling (figure 2J,K). Congestion and haemorrhage of the brain of pigeons was also reported (figure 2L). Infected waterfowls such as ducks and geese showed depression, anorexia, nasal discharges and diarrhoea. Some of these birds showed neurological manifestations such as paralysis, incoordination, head shaking and torticollis. Necrosis in the pancreas in the native breed ducks was also reported (figure 2M). This was in addition to the congestion and haemorrhage in the pancreas and duodenal blood vessels of some affected turkeys (figure 2N).

### Phylogenetic analysis

Samples with high viral load (CT values < 30) in influenza M gene specific RT-qPCR Ct values were inoculated into specific-pathogen-free embryonated eggs in a biological safety level 3 laboratory for virus culture. A total of 12 H5N8 influenza viruses from birds including ostriches,



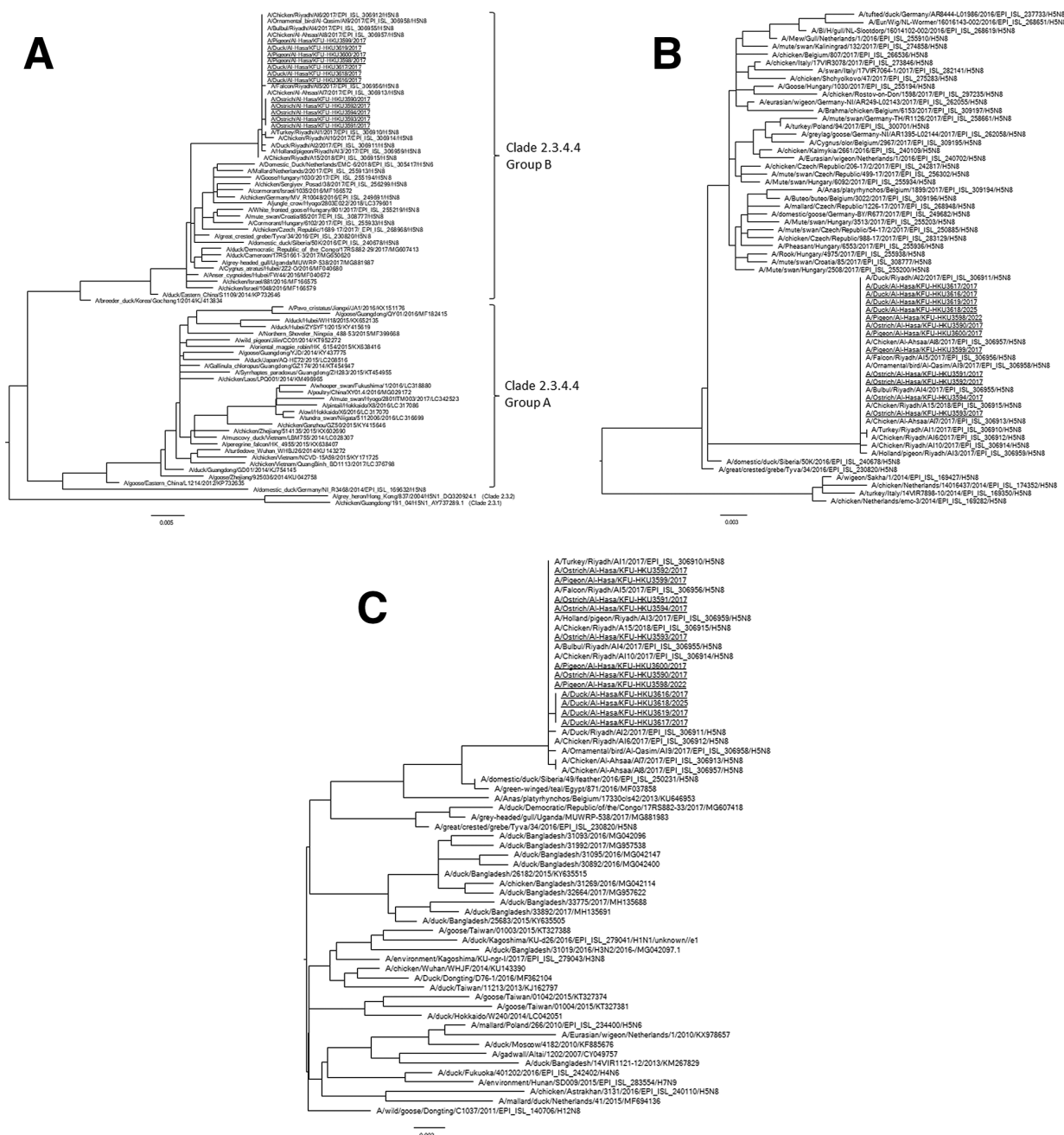
**Figure 2** Clinical signs and postmortem lesions of the H5N8-infected birds during the 2018 outbreak.

pigeons and ducks were isolated. Full genomes of these virus isolates were sequenced using Next Generation Sequencing (NGS) methods. Phylogenetic analysis was performed for all the eight gene segments of the isolated H5N8 viruses together with sequences downloaded from public influenza sequence database. The HA had the motif RKRRKR at the HA1-HA2 cleavage site typically associated with HPAIVs. The HA genes of viruses from this study form a single cluster in H5 clade 2.3.4.4 group B. All eight gene segments of viruses from this study were closely related to each other and also closely related to H5N8 viruses reported previously in Riyadh in year 2017. All the gene segments of viruses from Riyadh in 2017 formed a monophyletic group as shown in the trees constructed (figure 3 and online supplementary figure). The pairwise genetic similarities of virus genomes generated in this study were higher than 99.95 per cent, while these genomes compared with other previously reported H5N8 viruses in Riyadh in 2017 have nucleotide sequence similarities ranging from 99.87 per cent to 99.98 per cent. These findings indicate that the H5N8 viruses from backyard poultry flocks in Riyadh, in this and the previous report, were from the same source which has caused the outbreak in avian hosts in year 2017.

### DISCUSSION

The 12 influenza A H5N8 viruses isolated from this study were all genetically highly related and they formed a closely related monophyletic sublineage within H5 clade 2.3.4.4. Included in this sublineage were H5N8 viruses reported previously from avian species including ornamental birds, ducks, chicken, bulbul, falcon, Holland pigeon and turkey collected in Riyadh in 2017. The authors now also report the involvement of ostriches in this outbreak. Phylogenetic analysis revealed that all H5N8 viruses from this outbreak formed a monophyletic sublineage within this outbreak and were closely related to virus sequences from Riyadh. This





**Figure 3** Phylogenetic analyses of haemagglutinin (HA), neuraminidase (NA) and nucleoprotein (NP) genes of H5N8 influenza viruses detected in specimens from birds in eastern Saudi Arabia, 2017–2018. Viruses isolates sequenced in this study were underlined. Bootstrap values were showed at major nodes.

sublineage of viruses was a sister group to viruses from other countries in the Middle East and in Europe where outbreaks of H5N8 in avian hosts has been reported. These findings indicated that the H5N8 viruses in Riyadh and Al Ahsa was likely from a single source of virus introduction subsequently spreading within the country. These findings also highlight the important role of the backyard poultry system on the epidemiology of HPAIV in terms of risk of spread to other poultry production sectors as well as to human beings, hence integrating backyard poultry in any control programme.

**Contributors** MH, AAI, MP designed the experiments, conducted data analysis, AAB conducted field study, DC and SC conducted laboratory experiments.

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**Competing interests** None declared.

**Ethics approval** All animal experiments were conducted according to the Animal Ethics protocols of the National Committee of Bio-Ethics, King Abdul-Aziz City of Science and Technology, Royal Decree No. M/59.

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**Data availability statement** Data are available upon reasonable request. All data relevant to the study are included in the article or uploaded as supplementary information.

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