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# Seroprevalence of avian influenza virus and its relationship with increased mortality and decreased egg production

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**To evaluate the occurrence of avian influenza viruses (AIV) infections in southern Pakistan, an enzyme-linked immunosorbent assay was initially used to screen sera from broiler, layer and broiler breeder flocks for the presence of antibodies to type A influenza viruses. Data from this survey showed high levels of AIV antibodies, indicating unrecognized AIV infection occurring in these flocks. Based on this information a second investigation was undertaken in selected broiler-breeder, broiler and layer flocks. In this investigation, nine H9N2 AIV isolates were recovered. Serological data from this investigation indicated that chickens in flocks with a previous history of respiratory tract infection and some without overt clinical respiratory signs had seroconverted to H9N2 AIV.**

## Introduction

In northern Pakistan the first detected outbreak of avian influenza (AI) occurred in 1994 to 1995. This was caused by an H7N3 type A influenza virus that lead to high mortality in the field and was shown to be highly pathogenic in the laboratory (Naeem & Hussain, 1995). The outbreak was subsequently controlled within 4 to 5 months by vaccination, along with de-population and adopting strict biosecurity measures. In 1998, a second AI outbreak occurred in northern Pakistan. This outbreak was caused by H9N2 type A influenza virus of low pathogenicity, and infections resulted in sudden drops in egg production, mortality of 2 to 3% per day and respiratory lesions (Naeem *et al*., 1999). Vaccination and biosecurity were used in this regard to control this outbreak. The absence of reported AI outbreaks and avian influenza virus (AIV) isolations from southern Pakistan raised the question whether AI infections were going unrecognized in that region of the country. This epidemiologic investigation was therefore conducted to determine whether undetected AIV infections were occurring in southern Pakistan.

## Materials and Methods

### *First serological survey*

Chicken sera from two breeder flocks, two broiler flocks and one layer flock in southern Pakistan (Figure 1) were tested for the presence of serum antibodies to specific antigen of type A influenza viruses. For this analysis, commercially available enzyme-linked immunosorbent assay (ELISA) kits (IDEXX, Portland, ME, USA) were used according to the protocol suggested by the manufacturer. Each serum sample was diluted 1:500 in the accompanied sample diluent and 100 μl diluted serum was used for testing. The results were read using an ELISA reader and the data were recorded accordingly. The data were subsequently analysed using FLOCKCHEK software provided by IDEXX. A sample containing ELISA antibody titres $\geq 500$ was considered positive.

The selected flocks had the following histories:

- 36-week-old broiler breeder (BB) flock, designated GP-1 with a history of a sudden decrease in egg production; 20 blood samples collected.

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**Figure 1.** *Map of Pakistan showing southern cities of Sukkur, Hyderabad and Karachi, from where the sampling was done.*

- Clinically normal 21-week-old BB flock, designated GP-2; eight blood samples collected.
- 25-day-old broiler flock with respiratory disease, designated GP-3; 15 blood samples collected.
- 17-day-old broiler flock with respiratory disease, designated GP-4; 15 blood samples collected.
- 35-week-old flock with no history of respiratory disease or drop in eggs production, designated GP-5; 42 blood samples collected.

There was no history of AIV vaccination in all these flocks from where the samples were collected.

*Second serological survey*

Once sera antibodies to the type-specific antigen of type A influenza virus were demonstrated in the first serological survey; a second serological survey was conducted to determine whether antibodies to haemagglutinin subtype H9 of influenza A viruses could be demonstrated in chickens raised in southern Pakistan. For this investigation, arrangements were made with the Pakistan Poultry Association to visit flocks in person and to collect flock histories and blood samples. Blood samples were collected from chickens in broiler flocks with histories of respiratory disease (six flocks), broiler flocks with no history of respiratory disease (seven flocks), BB flocks with a history of respiratory disease (seven flocks), BB flocks without a history of respiratory disease (five flocks) and layer flocks without any history of respiratory disease (eight flocks). None of these flocks was vaccinated against AIV.

Generally, eight to 12 blood samples were collected from each flock, except group B where 18 samples were collected from each flock. The haemagglutination inhibition test (HIT) was used to detect antibodies to the influenza A virus H9 subtype (Beard *et al*., 1989). Here, an HIT $\geq 5$ was considered positive. The reagents for HIT were obtained from Poultry Health Centre (Doorn, Netherlands).

*Virus surveillance and detection of type A influenza virus antigen in tissues*

Type A influenza and virus isolation attempts were made on tissues collected from chickens raised in southern Pakistan. Chickens included in this investigation had signs of respiratory disease and were submitted to the M/S Poultry Diagnostic Laboratory, Rawalpindi. Organs evaluated for the presence of virus included the lungs, trachea, kidneys and spleen. Virus isolation attempts were made on a total of 12 broiler breeder and eight layer flocks.

*Virus isolation*

The spleen, trachea, kidneys and lungs were collected from the 34 flocks (four BB flocks, 10 layer flocks, and 20 broiler flocks) showing respiratory tract infection. The organs were triturated together in Hank's balanced salt solution. The material was frozen and thawed thrice, and centrifuged at $1500 \times g$ for 15 min. A $10 \times$ stock solution of streptomycin (10 mg/ml), gentamycin (5 mg/ml) and nystatin (5 mg/ml) was added to the supernatant at a $1 \times$ final concentration, and the samples were incubated at room temperature for 1 h. These were centrifuged again at $1500 \times g$ for 5 min at 4°C. The supernatant was filtered through a 0.2 μm syringe filter and the filtrate was inoculated into the allantoic cavity of 9-day-old embryonated chicken eggs (0.1 ml/egg). The eggs were incubated at 37°C with 55% relative humidity for 5 days, with daily examination to identify the dead embryos. The embryos were chilled at 4°C and the allantoic fluid was collected after opening the eggs. It was further tested for haemagglutination (HA) activity, and in cases of being positive was treated with an equal quantity of antiserum against Newcastle disease virus and incubated on ice for 30 min. It was centrifuged at $1500 \times g$ for 10 min at 4°C. The supernatant was collected and HA was performed. The samples with positive HA activity were subsequently treated with H9 antiserum to confirm the presence of H9 following standard protocol (Beard *et al*., 1989). The

isolates were subsequently sent to CVL, Weybridge, UK, for neuraminidaze typing.

## Results

Table 1 presents the ELISA antibody titres against AIV in 36-week-old BB with a history of respiratory disorders. All the samples from these groups, except GP-5, were positive for AIV antibodies. Out of 20 samples from GP-1, most samples showed high AIV antibody titres ranging from 3551 to 9550. The geometric mean titre (GMT) was 6320, with a coefficient of variance (CV) of 24.2% and a standard deviation of 1596. Here the low CV means less variation in the titres, thereby indicating an old infection in the absence of AI vaccination. In flock GP-2, variable AIV antibody titres (1500 to 9000) were observed, with a GMT of 2963. The flock was 21 weeks old, without any clinical signs of respiratory tract infection. A high CV of 50.1% was recorded in this group, which indicates a recent field exposure to AIV.

In the case of 25-day-old and 17-day-old broilers (GP-3 and GP-4), the AIV antibody titres ranged between 2551 to 12 550 and 1001 to 13 000, respectively. A CV of 41.1 and 46.3%, respectively, among these flocks was also recorded (Table 1). A CV value above 40% is indicative of recent field exposure of AIV. On the other hand, in the case of a layer flock (GP-5) with no history of respiratory tract infection or dropped egg production, only five samples out of 42 showed ELISA antibody titres $\leq 500$. The high CV of above 52.9% is indicative of recent AIV field exposure.

The serum samples subsequently collected from the aforementioned region were specifically examined for the presence of antibodies against AIV H9 by HIT (Table 2). Here group A showed AIV H9 antibodies in broilers exhibiting respiratory disorders. From the serum samples collected for the HIT, 55 out of 72 samples were found suitable for carrying out the test. The results showed an antibody titre range of 20 to 80. In group B the titres ranged between 5 and 80. This group included broiler flocks without any sign of respiratory tract infection. In group C, a BB with a past history of respiratory tract infection showed an antibody titre range of 10 to 160, with a GMT of 49. The BB of group D, which did not have any history of respiratory tract infection, showed AIV antibody titres ranging between 10 and 160, with a GMT of 35. In the case of healthy layers in group E, the H9 antibody titre varied between 5 and 80, with a GMT of 6.

Out of 34 samples propagated for virus isolation, only nine were found positive for the presence of AIV. The isolates were typed to be H9 in this laboratory and were subsequently typed as N2 by Veterinary Laboratories Agency, Weybridge, UK. In most of the *in ovo* inoculations, the embryos were found alive even up to the fifth day of incubation. Out of nine AIV H9N2 isolates recovered from the field, three were from 14 broiler samples, four out of 12 BB samples and two out of eight layer samples.

## Discussion

Keeping in view the previous history of AIV in northern part of Pakistan over the past 6 years, it was necessary to investigate its prevalence in southern part of the country. The northern part has a mild temperature (up to 15°C) in summer, whereas in the southern region it ranges between 25 and 45°C in this season. Initially, a very small sample of population was subjected to ELISA-based serologic evaluation. All the flocks, except GP-5, showed 100% seroconversion against AIV. Here the data from the breeder flock (GP-1) indicated high levels of ELISA antibodies against AIV. As the flock had a history of decreased production, the high titre against AIV was indicative of recent flock exposure to AIV. On the other hand, data of the flock GP-2 also reflected exposure to AIV, although the flock did not have any history of respiratory tract infections. Similarly, the broiler and layer flocks also showed increased levels of ELISA antibody titres against AIV. As no vaccination against AIV was done in

**Table 1.** *Detection of antibodies to the type-specific antigen of influenza viruses in the sera of chickens in southern Pakistan using a commercial ELISA kit*

| Group | Flock identification | Range of AIV antibody titres[a] | Number of samples tested | Geometric mean titre | CV (%) | Standard deviation | % positive |
|---|---|---|---|---|---|---|---|
| GP-1 | BB, 36 weeks old, decreased egg production | 1500–10 000 | 20 | 6320 | 24.2 | 1596 | 100 |
| GP-2 | BB, 21weeks old, healthy flock | 1500–9000 | 8 | 2963 | 50.1 | 2441 | 100 |
| GP-3 | Broilers, 25 days old, suffering from respiratory tract infection | 2550–13 000 | 15 | 5702 | 41.1 | 2576 | 100 |
| GP-4 | Broilers, 17 days old, suffering from respiratory tract infection | 1000–13 000 | 15 | 5717 | 46.3 | 3046 | 100 |
| GP-5 | Commercial layers, 35 weeks old, healthy flock | 500–3000 | 42 | 1083 | 52.9 | 918 | 88 |

[a]ELISA antibody titres $\geq 500$ were considered positive.

**Table 2.** *Distribution of haemagglutination inhibition antibody titres against subtype of avian influenza virus in chickens in southern Pakistan*

| Group | Flock identification | Number of flocks examined | Number of samples tested | Number of HIT[a] positive samples | HI antibody titre range | Geometric mean titre | % positive |
|---|---|---|---|---|---|---|---|
| A | Broilers with a history of respiratory tract infection | 6 (30 000) | 55 | 30 | 20–80 | 43 | 54.54 |
| B | Broiler without a history of respiratory tract infection | 7 (20 000) | 144 | 101 | 5–80 | 7 | 70.14 |
| C | BB with a history of respiratory tract infection | 7 (40 000) | 84 | 70 | 10–160 | 49 | 83.33 |
| D | BB without a history of respiratory tract infection | 5 (40 000) | 51 | 42 | 10–160 | 35 | 82.35 |
| E | Layers without a history of respiratory tract infection | 8 (25 000) | 94 | 60 | 5–80 | 6 | 63.83 |

[a]Haemagglutination inhibition titres $\geq 5$ were considered positive.

the area of investigation, these results indicate widespread avian influenza infection in the flocks caused by AIV of low pathogenicity (Swayne *et al*., 1997).

The second investigation focused on detecting antibodies to H9 AIV and recovering AIV from clinically ill chickens. The HIT data detected a high prevalence of antibodies against AIV serotype H9N2 in chickens with and without any history of respiratory disease. Subsequently, H9N2 virus was isolated from a few clinical specimens collected from chickens suffering from respiratory tract infections.

The present study indicates that in southern Pakistan H9N2 AIV infection may be occurring in proportion in the flocks but has remained undiagnosed until now. Occasional reports of respiratory tract infections among the flocks in the area under investigation must now include AI in the differential diagnosis. Also, reports of the drastic decline in egg production in the area, with no record of any specific investigation, may have been caused by AIV infections. Now, with the high prevalence of antibodies against H9N2 AIV and the isolation of H9N2 AIV from clinical specimens in this study, it can be suggested that AIV H9N2 may be the major cause of earlier reported respiratory tract infections as well as a decline in egg production in this area.

This conclusion is further supported by a number of unpublished observations in this country and other countries in this region about the benefits of AIV killed vaccines. The AIV H9 vaccines have been employed during the first week of age in broilers and BB, followed by two more vaccinations at the eighth and 18th weeks in the breeder flocks, with good protection to the flocks against this virus. These vaccinations were, however, accompanied by strict biosecurity measures in the affected areas.

The role of H9N2 in the public health management is also of major concern these days. In 1999, H9N2 influenza viruses were isolated from two children in Hong Kong, and unconfirmed reports of an additional five such cases from southern China have also appeared (Guan *et al*., 1999). Analysis of these H9N2 AIV from children reveal that they are closely related to H9N2 influenza viruses isolated from poultry, which was circulating in the live bird markets in Hong Kong during 1997. Here the molecular analysis of H5N1, which caused human disease among 18 confirmed cases, and H9N2 viruses has also revealed that they share six internal gene segments (Guan *et al*., 1999). Another study carried out on the genetic analysis of H9N2 isolates from Pakistan has revealed that these isolates are closely related to all eight genes of H9N2 AIV that caused human deaths in Hong Kong (Cameron *et al*., 2000). Despite the aforementioned information, more evidence is required to identify the poultry-originated H9N2 AIV as a donor of virulence factor to human influenza viruses. On the other hand, owing to its involvement in a number of outbreaks in poultry in this region (Sluis, 2000), we need to continuously monitor the prevalence and pathogenic potential of AIV H9N2 in the poultry population in this country.

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## References

Beard, C.W., Purchase, H.G., Arp, L.H., Domermuth, C.H. & Pearson, J.E. (1989). Serologic procedures. In H.G. Purchase, L.H. Arp, C.H. Damermuth & J.E. Pearson (Eds.), *Laboratory Manual for the Isolation and Identification of Avian Pathogens* (3rd edn, pp. 192–200). Kennett Square, PA: American Association of Avian Pathologists.

Cameron, K.R., Gregory, V., Banks, J., Brown, I.H., Alexander, D.J., Hay, A.J. & Lin, Y.P. (2000). H9N2 subtype influenza A viruses in poultry in Pakistan are closely related to the H9N2 viruses

responsible for human infection in Hong Kong. *Virology*, *278*, 36–41.

Guan, Y., Shortridge, K.F., Krauss, S. & Webster, R.G. (1999). Molecular characterisation of influenza viruses H9N2: were they the donors of the internal genes of H5N1 viruses in Hong Kong? *Proceedings of National Academy of Science USA*, *96*, 9363–9367.

Naeem, K & Hussain, M. (1995). An outbreak of avian influenza in poultry in Pakistan. *Veterinary Record*, *137*, 439.

Naeem, K., Ullah, A., Manvell, R.J. & Alexander, D.J. (1999). Avian influenza A subtype H9N2 in poultry in Pakistan. *Veterinary Record*, *145*, 560.

Sluis, W.V. (2000). Avian influenza in South East Asia and Middle East, *World Poultry*, *November*, 25.

Swayne, D.E., Bech, J.R., Garcia, M., Perdue, M.L. & Brugh, M. (1997). Pathogenicity shifts in experimental avian influenza virus infections in chicken. In D.E. Swayne & R.D. Slemons (Eds.), *Proceedings of the 4th International Symposium on Avian Influenza* (pp. 171–181). University of Georgia, Athens, GA.

## RÉSUMÉ

**Séroprévalence de l'influenza aviaire et relation avec une augmentation de la mortalité et une chute de ponte**

Pour évaluer la prévalence des infections à virus influenza aviaires (AIV) au Sud du Pakistan, une immunoadsorption à enzyme conjuguée (ELISA) a été initialement utilisée pour tester les sérums des troupeaux de poulets de chair, de reproducteurs de type chair et de type ponte afin de détecter la présence d'anticorps anti virus influenza de type A. Les données de cette enquête ont montré des taux élevés d'anticorps AIV, indiquant une infection occulte d'AIV chez ces troupeaux. A partir de ces informations une deuxième investigation a été entreprise chez des troupeaux de reproducteurs de type chair, de pondeuses et de poulets de chair. A partir de cette étude, neuf isolats d'AIV H9N2 ont été isolés. Les données sérologiques de cette étude ont indiqué que les animaux des troupeaux qui ont présenté antérieurement des problèmes respiratoires et d'autres qui n'ont pas montré de symptômes respiratoires évidents, ont séroconverti vis à vis de l'AIV H9N2.

## ZUSAMMENFASSUNG

**Seroprävalenz von aviärem Influenzavirus im Zuhammenhang mit erhöhter Mortalität und Legeleistungsrückgang**

Zur Bestimmung des Vorkommens von aviären Infuenzavirus(AIV)-Infektionen in Südpakistan wurde anfänglich ein Enzymimmunotest (ELISA) benutzt, um Seren von Broiler-, Legehennen- und Broilerelterntierherden auf das Vorhandensein von Antikörpern gegen Typ A-Influenzaviren zu überprüfen. Die Untersuchung ließ hohe AIV-Antikörpertiter erkennen, was auf das Vorkommen unerkannter AIV-Infektionen in diesen Herden hinwies. Auf diesen Erkenntnissen basierend wurde eine zweite Untersuchung in ausgewählten Broilerelterntier-, Broiler- und Legehennenherden durchgeführt. In dieser Untersuchung wurden neun H9N2-AIV-Isolate entdeckt. Die serologischen Ergebnisse aus dieser Untersuchung wiesen darauf hin, dass Hühner in Herden mit der Vorgeschichte einer respiratorischen Infektion und einige auch ohne offensichtliche klinische respiratorische Symptome seropositiv gegen H9N2-AIV geworden waren.

## RESUMEN

**Seroprevalencia frente a virus de influenza aviar y su relación con un incremento de la mortalidad y una disminución en la producción de huevos**

Para evaluar la presencia de infecciones por virus de influenza aviar (AIV) en el sur del Pakistán, se utilizó inicialmente un técnica de ELISA para testar los sueros de lotes de pollos de engorde, ponedoras, reproductoras y detectar la presencia de anticuerpos frente a virus de influenza tipo A. Los datos de esta inspección demostraron niveles altos de anticuerpos frente a AIV, lo que indicaba infecciones no reconocidas de AIV en estos lotes. En base a esta información, se realizó una segunda investigación en lotes seleccionados de pollos de engorde, reproductoras y ponedoras. En este trabajo, se aislaron nueve AIV subtipo H9N2. Los datos serológicos de esta investigación indicaron que los pollos de lotes con historia previa de infección del tracto respiratorio y algunos sin claros síntomas clínicos respiratorios habían seroconvertido al AIV H9N2.

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