CHRONOLOGICAL CORRELATION OF TRACHEAL METAPNEUMOVIRUS ANTIGENIC DISTRIBUTION TO LEVELS OF SPECIFIC HUMORAL ANTIBODIES IN BROILER BREEDERS AND THEIR OFFSPRING

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ABSTRACT

This study correlates chronologically the detection of antigenic distribution of Metapneumovirus in ciliated brush border of tracheal surface epithelial cells by direct immunofluorescence (IF) to detection of systemic humoral Metapneumovirus-specific antibodies by indirect ELISA in four broiler breeder flocks and their respective four broiler offspring flocks. The chronological ratios of percentage (%) of positiveness (+ve) for Metapneumovirus infection (% +ve by IF / % +ve by ELISA, P value for significance of correlation) in breeders were: 3 wks before swollen head syndrome (SHS) outbreak (0.0/0.0, $P < 0.05$), 2 wks before SHS (28.6 / 0.0, $P > 0.05$), 1 wk before SHS (71.4/0.0, $P > 0.05$), at initiation of SHS (67.9/0.0, $P > 0.05$), 1 wk after initiation of SHS (100/0.0, $P > 0.05$), 2 wks after initiation of SHS (100/17.9, $P > 0.05$), 3 wks after initiation of SHS (100/67.9, $P > 0.05$), and 4 wks after initiation of SHS (100/100, $P < 0.05$). However, the chronological ratios in broiler offspring flocks were: 4 wks before SHS outbreak (1 day of age) (0.0/100, $P > 0.05$), 3 wks before SHS (0.0/67.9, $P > 0.05$), 2 wks before SHS (17.9/0.0, $P > 0.05$), 1 wk before SHS (71.4/0.0, $P > 0.05$), at initiation of SHS outbreak (100/0.0, $P > 0.05$), 1 wk after initiation of SHS (100/0.0, $P > 0.05$), and 2 wks after initiation of SHS (100/25, $P > 0.05$). These findings are indicative of the higher sensitivity of direct IF over indirect ELISA in earlier diagnosis of Metapneumovirus infection in meat breeders and their offsprings.

Keywords: broiler breeders, offspring, swollen head syndrome, Metapneumovirus, immuno-fluorescence, Enzyme-linked immunosorbent assay

INTRODUCTION

A condition in broilers and broiler breeders known as swollen head syndrome (SHS) has been associated with turkey rhinotracheitis virus, which is now identified as Metapneumovirus (Cavanagh and Barret, 1988; Cook *et al.*, 1988; Cook and Cavanagh, 2002). Swollen head syndrome is reported in many parts of the world (Lister and Alexander, 1986; Lwamba *et al.*, 2002), including its recent appearance in Lebanon (Barbour *et al.*, 1998).

Evaluation of different technologies for detection of Metapneumovirus-specific antibodies (indirect) or its antigens (direct) is under continuous investigation (Cook *et al.*, 1988; Pattison *et al.*, 1989; Eterradossi *et al.*, 1995; Majó *et al.*, 1995; Mekkes and de Wit, 1988; Shin *et al.*, 2000; Cook and Cavanagh, 2002).

To our knowledge, this is the first attempt in literature to correlate the detection of antigenic distribution of Metapneumovirus in ciliated brush border of tracheal surface epithelial cells by direct immunofluorescence (IF) with detection of humoral pneumovirus-specific antibodies by indirect ELISA in four broiler breeder flocks and their respective four broiler offspring flocks. The correlations were chronologically established by sampling at different time intervals namely, before, at, and after the initiation of SHS signs.

MATERIALS AND METHODS

Birds and sampling

This study included four meat chicken breeder flocks (30,000 birds/flock), and their respective four offspring broiler flocks (5000 birds/flock).

Seven morbid birds were sampled weekly from each flock for cryostat tracheal sections and for brachial vein-blood collection. The breeder flocks were sampled between 25 and 40 weeks of age while their respective broiler offspring flocks were sampled between 1 and 42 days of age. The percent positive birds by immunofluorescence or ELISA technique is based on a total of 28 birds collected from 4 flocks per time.

Clinical signs of SHS

Record was taken of each flock during the period of SHS outbreaks. This included the percent morbidity and mortality. A record was also taken of the age of the flock at the initiation of the SHS, duration of the episode, and percent drop in egg production of the breeders. Clinical signs of the disease were recorded by examining 200 randomly chosen birds from each flock.

Indirect ELISA

A commercial indirect ELISA (Svanovir® Avian Metapneumovirus EIA, Svanova Biotech, Upssala, Sweden) was used for analysis of individual serum samples for detection of

Metapneumovirus infected birds. Samples with a percent inhibition greater than 40% were considered positive for antibodies to avian Metapneumovirus. More specifically, the wells in the microtiter plates are already coated with Metapneumoviurs. The more antibody in the serum specific to Metapneumoviurs, the more the coated antigen will be bound to antibodies in the serum, and the more will be the inhibition of the secondary antibody binding to the coated-antigen. Since the conjugate is labeled with an enzyme, then the less the presence of the enzyme in the well, and the less the development of the color in the well. An inhibition of 40% of the color development was determined by the Svanovir® company to be a reflection of a bird that is infected with Metapneumoviurs with presence of significant antibodies in it to inhibit the binding of the conjugate or the secondary antibody. The percent inhibition by a serum sample is calculated from the following formulae:

$$\text{Percent inhibition} = \frac{(\text{Negative control optical density} - \text{sample optical density})}{\text{Negative control optical density}} \times 100$$

The positive and negative control sera are provided by the commercial kit.

Direct immunofluorescence

A direct immunofluorescence procedure was applied to detect Metapneumovirus antigen(s) in the ciliated brush border of tracheal surface epithelial cells using previously described method (McNulty and Allan, 1984; Majó *et al.*, 1995). Briefly, three cryostat tracheal cuts, each of 3 µm thickness, were collected from the anterior, middle, and posterior portions of each individual trachea. The 3 cuts from each bird were fixed on one microscope slide by cold acetone. A Metapneumovirus specific fluorescein isothiocyanate-labelled antibody (Doorne Laboratories, Doorne, Holland), diluted 1/50 in phosphate-buffered saline (PBS) (pH 7.2), was added on the acetone-fixed preparations. The reaction took place at 37°C for 1 hour. The cryostat sections were then washed in PBS, mounted in glycerol saline (90% glycerol in 10% PBS) and examined under incident ultraviolet illumination of a Leica DMLS microscope (Wetzlar, Germany).

Statistics

The multiple regression was used to detect the significance of correlation between direct IF and indirect ELISA in detection of avian Metapneumovirus infection in examined birds. Data were analyzed by M-Stat computing statistical program (Michigan State University, Michigan, USA). The sample size of a total of 28 birds collected from 4 flocks per time is statistically adequate to study the correlation between results of IF and ELISA per collection time.

RESULTS

Clinical signs

The clinical sings of SHS appearing in the meat chicken breeders and their respective broiler offspring flocks are presented in Table 1. The average morbidity and mortality in the four broiler offspring flocks was higher than that of their parent flocks. The signs differed in the breeders from those manifested in their broiler offsprings; more specifically, the cerebral disorientation was present in breeders but not in their offsprings;

however, the respiratory signs of coughing, sneezing, and tracheitis were present in the broiler offsprings but not in their respective parent flocks. The sign common in breeders and their offspring was the presence of periorbital oedema. It is worth mentioning that the average egg drop in breeders during the episode was 5.8%, an economically significant loss.

TABLE 1
Clinical Signs of SHS Outbreaks in Meat Chicken Breeders and their Broiler Offspring

Chicken type	Signs
Breeders	Average morbidity of 4%. Morbid birds show cerebral disorientation, constant and repetitive head movements, arched necks with heads resting on their backs, torticollosis or opisthotonus. Episode starts at an average age of 34 wks and lasts for an average of 18 days, with an average egg drop of 5.8% and mortality of 3.5%. Heads are swollen as a result of periorbital oedema, often associated with cyanosis and oedema of the wattles.
Broilers	Average morbidity of 7% and mortality of 4.8%. Head swelling is more severe than in breeder flocks, caused by subcutaneous periorbital oedema around the eyes, extending over the head. The SHS was associated with respiratory signs including coughing, sneezing, and high rate of tracheitis. Episode usually starts at around 4 weeks of age and progresses until marketing.

IF and ELISA in breeders and broilers

The chronological correlation of direct IF and indirect ELISA for detection of Metapneumovirus infection in meat breeders and their broiler offspring is shown in Table II. No variation was noticed among the 3 tissue cuts from each bird regarding the presence of Metapneumoviurs. The direct IF technique was higher in sensitivity than the indirect ELISA in detecting Metapneumovirus infection in the breeder flocks at 2 weeks before the appearance of the SHS signs; however, ELISA detected infection at 2 weeks post the initiation of SHS outbreaks in the breeder flocks. The two methods had a significant positive correlation in detecting Metapneumovirus infection in breeders, only at four weeks after initiation of SHS outbreaks ($P<0.05$).

The chronological correlation of direct IF and indirect ELISA for detection of Metapneumovirus infection in broiler offspring flocks is shown in Table 2. The direct IF was higher in sensitivity than the indirect ELISA; the IF method detected Metapneumovirus infection at 2 weeks before the appearance of SHS signs in the broilers, while by indirect ELISA the infection was detected at 2 weeks after the initiation of SHS outbreaks. The two methods did not correlate significantly ($P>0.05$) in detecting Metapneumovirus infection in broiler at any of the studied chronological intervals. It is worth noting that the broilers at 1

and 2 weeks of age (3 and 4 weeks before the SHS outbreak) had maternal immunity, as detected by ELISA in 100.0 and 67.9% of the examined birds, respectively.

DISCUSSION

The clinical signs of SHS appearing in the meat chicken breeder flocks and their respective broiler offsprings (Table 1) were similar to those described in literature (Pattison *et al.*, 1989). Under field conditions, there is a possibility of exposure to pathogens other than the Metapneumovirus including paramyxoviruses, or thomyxoviruses, mycoplasmas, and other primary or secondary infections. The complexity of mixed infections under field situations could have created this spectrum of observed signs. Future investigations should include profiling for many other etiologic agents that could be involved in such complexity. However, the observed morbidity in breeders of 4% was higher by 3% than that reported previously (Pattison *et al.*, 1989). The SHS occurred in the breeders at around 34 weeks, 4 weeks later than those reported in literature (Pattison *et al.*, 1989); however, both studies reported the coincidence of SHS around the stress period of egg production peak. The percent drop in egg production was similar in both studies. The increased severity of head swelling in broilers compared to breeder chickens is in agreement with the previous observations (Pattison *et al.*, 1989).

The direct IF and indirect ELISA did not correlate in the early stage of Metapneumovirus infection, which is apparently due to the higher sensitivity of direct IF in detecting the Metapneumovirus antigen in the ciliated brush border of tracheal surface epithelial cells (Khehra and Jones, 1999) (Table 2). Both the direct IF and indirect ELISA detect infection by Metapneumovirus (specific) without having the ability to differentiate among subgroups of this virus namely, subgroups A, B, C, and D (Njenga *et al.*, 2003). The Metapneumovirus fluorescein isothiocyanate-labelled antibody used in direct IF is specific to common antigens present on all the 4 subgroups, thus confirming the presence of Metapneumovirus infection without differentiation of subgroups. Similarly, the indirect ELISA is coated with common antigens present in the four subgroups, thus having the ability to capture antibodies in the chicken sera with specificity to either one of the four subgroups of this virus (Cook and Cavanagh, 2002). Future investigations could target subgrouping of the etiologic agent of Metapneumovirus which is indispensable for development of control programs against such economic outbreaks. This subgrouping will depend on more sophisticated techniques to determine the shared percent nucleotide identify and percent predicted amino acid identity in Metapneumovirus strains (Shin *et al.*, 2002). Actually, Metapneumovirus antigen was also observed in previous studies in association with the cilia of epithelial cells of both the upper and lower respiratory system (Majó *et al.*, 1995; Catelli *et al.*, 1998). The localization of Metapneumovirus infection in the respiratory system and the pathogenesis of the virus could be responsible for the delay of the humoral immune system to respond to this type of infection, thus resulting in a direct IF procedure with higher sensitivity than indirect ELISA.

In contrast, both diagnostic methods started to insignificantly agree ($P>0.05$) at 2 weeks post initiation of SHS outbreaks in both the breeders and their broiler offsprings. However, it is worth noting that the agreement between the two methods in the breeders was getting higher in significance as the time period post initiation of SHS outbreaks increased

from 2 weeks (P>0.05) to 4 weeks (P<0.05). The short life span of broilers didn't allow for tracing the change in agreement between the two methods.

TABLE 2
Chronological Correlation of Direct IF[1] and ELISA[2] for Detection of Metapneumovirus Infection in Meat Breeders and their Broiler Offspring

Chicken[3]	Chronological sequence[4] (wks)	No. Positive[5] (IF/ELISA)	% Positive (IF/ELISA)	P Value[6]
Breeders	Before SHS outbreak			
	3 wks	0.0/0.0	0.0/0.0	P<0.05
	2 wks	8.0/0.0	28.6/0.0	P>0.05
	1 wk	20.0/0.0	71.4/0.0	P>0.05
	At initiation of SHS outbreak	19.0	67.9/0.0	P>0.05
	After initiation of SHS outbreak			
	1 wk	28.0/0.0	100.0/0.0	P>0.05
	2 wks	28.0/5.0	100.0/17.9	P>0.05
	3 wks	28.0/19.0	100.0/67.9	P>0.05
	4 wks	28.0/28.0	100.0/100.0	P<0.05
Broilers	Before SHS outbreak			
	4 wks	0.0/28.0	0.0/100.0	P>0.05
	3 wks	0.0/19.0	0.0/67.9	P>0.05
	2 wks	5.0/0.0	17.9/0.0	P>0.05
	1 wk	20.0/0.0	71.4/0.0	P>0.05
	At initiation of SHS outbreak	28.0/0.0	100.0/0.0	P>0.05
	After initiation of SHS outbreak			
	1 wk	28.0/0.0	100.0/0.0	P>0.05
	2 wks	28.0/7.0	100.0/25.0	P>0.05

[1]Direct immunofluorescence of Metapneumovirus antigen(s) in ciliated brush border of tracheal surface epithelial cells.

[2]Indirect enzyme-linked immunosorbent assay for detection of serum antibodies specific to Metapneumovirus antigen(s).

[3]Four breeder flocks each of 30,000 birds with an average age at SHS outbreak initiation of 28 wks; Four broiler offspring flocks, each of 5,000 birds with an average age at SHS outbreak initiation of 4 wks.

[4]Seven individual serum samples and seven tracheal organs were collected from 7 respective morbid birds of each flock at each time in the chronological sequence.

[5]No. positive out of 28 tested birds per time (4 flocks x 7 birds/flock = 28).

[6]P value indicate the significance level of correlation between IF and ELISA in detection of avian Metapneumovirus infection.

In conclusion, the SHS outbreaks coincided with early detection of Metapneumovirus antigens in the tracheal cilia by direct IF and late detection by indirect ELISA. The profiling by sampling of 7 birds per flock (trachea and blood) each week has helped in showing the advantage of IF over ELISA in early detection of the viral infection. This early detection by IF is by itself an observation that will help in quick biosecurity interception to prevent future spread of the SHS outbreaks in different countries. However, the indirect ELISA remains indispensable as a confirmatory diagnostic test (Wyeth *et al.*, 1987; Usami *et al.*, 1999) useful in monitoring chicken flocks within the framework of serological profiling programs of the modern poultry industry.

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