Proceedings of the SIPA 43th Congress

Italian Society of Avian Diseases (SIPA), national branch of the World Veterinary Poultry Association (WVPA)

Forlì (Italy), October 7-8, 2004

Edited by

Rosanna Scipioni,
Editor-in-Chief

Guido Grilli, Antonio Lavazza
Guest Editors

Evaluation of an additive efficacy in broiler litter microbial level control in field: preliminary results

Giuseppe Tacconi, Patrizia Casagrande Proietti, Rossella Ventura, Rocco Arcaro, Matteo Pennacchi

Dipartimento di Scienze Biopatologiche Veterinarie. Università di Perugia, Italy

Corresponding author: Prof. Giuseppina Tacconi. Dipartimento di Scienze Biopatologiche Veterinarie. Facoltà di Medicina Veterinaria, Università di Perugia. Via S. Costanzo 4, 06126 Perugia, Italy – Tel. +39 075 5857736 – Fax: +39 075 5857738 – Email: gtacconi@unipg.it

ABSTRACT

The present study was conducted to evaluate in field the efficacy of an additive (SOP® C POULTRY), as an agent for the control of micro-organisms in broiler litter. The Total aerobic Microbial Count (TMC), *Staphylococcus* species (spp.), Coliforms, and *Salmonella* spp. in broiler litter samples of both the Houses, 2 (H2) and 3 (H3), were determined, and also at the end of each cycle the mortality rate was recorded. The results showed significant reduction of all the microbial counts: P= 0.0078 (CMT), 0,0021 (*Staphylococcus* spp.) and 0.0541 (Coliforms), and mortality (P= 0.00106) in treated litter samples H2 and the control H3.

Key Words: Litter additive, Environment, Broiler, Aerobic bacteria, Mortality.

RIASSUNTO

VALUTAZIONE IN CAMPO DELL'EFFICACIA DI UN ADDITIVO NEL CONTROLLARE IL LIVELLO MICROBICO DELLA LETTIERA: RISULTATI PRELIMINARI

Prove di campo sono state effettuate sulla lettiera di 2 capannoni (C2, trattato, e C3, controllo) di polli da carne per valutare l'efficacia di un prodotto igienizzante di nuova concezione (SOP® C POULTRY), in grado di controllare Carica Microbica Totale aerobia (CMT), Staphilococcus *spp.*, Salmonella *spp. e Coliformi; inoltre alla fine di ogni ciclo è stata valutata la percentuale di mortalità. I risultati hanno mostrato una significativa riduzione dei valori medi di tutti i parametri microbiologici valutati nei campioni di lettiera trattata rispetto al controllo (P = 0,0078 (CMT), 0,0021 (*Staphylococcus *spp.), 0,0541 (Coliformi) compresa la mortalità (P = 0,00106).*

Parole chiave: *Additivo per lettiere, Ambiente, Polli da carne, Batteri aerobi, Mortalità.*

Introduction

The environment in the poultry house is a combination of physical and biological factors which interact as a complex dynamic system of social interactions, husbandry system, light, temperature and the aerial environment (Sainsbury,1992; Kristensen and Wathes, 2000). The high stocking density in the modern poultry house may lead to reduced air quality with high concentrations of aerial pollutants (Curtis and Drummond, 1982; Maghirang *et al.*, 1991; Feddes and Licsko, 1993). Their concentrations in poultry houses approach, and sometimes exceed, recommended occupational

limits for humans (Kristensen and Wathes, 2000). Litter is considered one of the major sources of pollutants in poultry houses, then the need to ménage it using additives has been considered since the last past years (Ivanov, 2001) but has not yet been resolved conclusively. The present study investigated the use of an additive as an agent for the control of micro-organisms in broiler litter, and also the possible effect on the mortality.

Material and methods

Planning

This study was carried out from February 2002 to March 2004 in two large commercial broiler houses, H2 and H3 of one Umbrian Company farm, in which broilers were reared at the same conditions for feed (standard pellets broiler *ad libitum*), density (16 birds per m^2) to 7-8 weeks of age. The buildings were of conventional layout. The litter was cut wheat straw (about 5-10 centimetres thick). In H2 and H3 the ventilation system comprised 2 propeller fans of 40,000 m^3/hour and 26,000 m^3/hour capacity each, mounted on the windward side of the poultry house.

Treatment

Litter in H2 was treated, every 2 weeks, as follow (since the day before the arrival of the chicken): 2 grams (g.) of additive plus 25 g. of calcium carbonate per m^2 (1^{st} month), and after 1 g. per m^2 until the end of the cycle.

Additive

The field trials were performed with Calcium sulphate (gypsum) and essential oils (lemon grass and lavender) used as carriers. By the SIRIO OPERATING PROCESS® method such components are activated by an energetic modulation process and enriched with oxygen and specific information about litter components.

Samples

Litter was sampled one day during the first and the seventh week of each cycle. Composite samples of about 500 g. were obtained from ten different sites within each house and placed immediately into sterile plastic bags, sealed and refrigerated until microbiological evaluation was made.

Microbiological analysis

Twenty-five grams of each sample was transferred into a sterile plastic bag and 225 ml of sterile 1% buffered peptone water was added. After treatment with Stomacker Circulator 400 (PBI, Milan) the samples were allowed to sit for 30-45 min at room temperature with frequent shaking. One ml of this samples (1:10 dilution) was diluted serially via 10-fold dilutions (from 10^{-1} to 10^{-8}). Total aerobic bacteria, *Staphylococcus* spp. (*Staph.* spp.), *Salmonella* spp. and Coliforms in 1 g^{-1} of litter were determined by plating, in duplicated, 0.1-ml of appropriate dilution on SPGCA (Standard Plate Count Agar), BP (Baird Parker agar) and VRBA (Violet Red Bile Agar). The cultures were incubated at 37°C for 24-48 hr and the number of grown colonies was determined. The *Salmonella* spp. isolation procedure used in this study included Selenite-Cystine broth and Rappaport-Vassiliadis broth (Oxoid, Milan), as enrichment media and two plating media. The Selenite-Cystine Broth was incubated aerobically at 37°C for 24 hours and the Rappaport-Vassiliadis broth was incubated aerobically at 43°C for 24 hours. The composition of each selective medium is detailed in the Oxoid Manual. Mortality rate was recorded at the end of each eight cycles.

Statistical Analysis

The mean values of all parameters evaluated were compared by t-test.

Results and discussion

The results from eight cycles on treatment of litter are summarised in Table 1 and 2. Significant differences between experimental and control samples with regard both the microbial cell counts (Table 1), and the mortality (Table 2) were observed. Also the bacterial counts of the treated litter were reduced to about 70 % of the control values. Sex strains of *Escherichia coli (E. coli)* were isolated throughout the sampling period: 3 strains (1 from H2 and 2 from H3) during 2002-2003, and 3 strains (1 from H2 and 2 from H3) in 2004. Standard procedure were used to identify *E. coli* which do not differentiate between pathogenic and non pathogenic. The number of *E. coli* in the litter, like total aerobic bacteria, *Staphylococcus*

Table 1. Results of some microbial parameters in litter samples from H2 (treated with SOP® C POULTRY) and H3 (control) (mean values from eight cycles are expressed in CFU.g^{-1}).

Parameters	TMC	TMC	*Staph. spp.*	*Staph. spp.*	*Coliforms*	*Coliforms*
Dilution	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-6}	10^{-6}
Houses	treated	control	treated	control	treated	control
Mean	153.69	416.42	31.14	185.48	58.05	328.34
t test (P=)	0.0078		0.0021		0.0541	
%	-63.1		-83.21		-82.32	

% = reduced microbial concentration.

Table 2. Mortality rate (%) recorded at the end of eight cycles in H2 and H3 (treated with SOP® C POULTRY and control).

Cycles	1st	2nd	3rd	4th	5th	6th	7th	8th
Treated	5	4.3	3.1	8.4	3.4	3.1	3.3	3.9
Control	9	4.7	4.3	10.8	5.7	3.4	5.1	4.5

P= 0.00106

spp., and Coliforms resulted in treated litter lower than the control house litter.

The treatment of the litter proved to be effective in control of some microbial litter components. Of interest is the reduced mortality rate of broilers because in field conditions the health problems are known to be associated with litter.

Although all litter sampled was examined for *Salmonella* spp. none was found.

Several workers suggested that bird health is harmed by chronic exposure to modest burdens, especially in the presence of simultaneous challenge by respiratory pathogens (Oyetunde *et al.*, 1978; Carpenter *et al.*, 1986), but the concentration of most pollutants often rises in poultry houses as consequence of an increased generation rates from the major sources, that is the birds themselves and particularly the litter, which acts as a nutritious reservoir for micro-organisms (Conceição *et al.*, 1989).

Conclusions

The control of bacterial population in poultry houses is essential for better health and performance of birds.

The data of the present study seem to indicate a significant reduction of the bacteria evaluated in the treated litter. Based on the results of this field trials, it was concluded that the additive studied during this investigation has an inhibitory effect on the survivals of micro-organisms in broiler house litter.

The writers wish to express their gratitude to the SOP® S.r.l. for furnishing the additive used in this study and for providing financial support for the project.

REFERENCES

CARPENTER, G.A., SMITH, W.K., MACLAREN, A.P.C., SPACKMAN, D., 1986. Effects of internal air filtration on the performance of broilers and the aerial concentrations of dust and bacteria. Brit. Poultry Sci. 27:471-480.

CONCEIÇÃO, M.A.P., JOHNSON, H.E., WATHES, C.M., 1989. Air hygiene in a pullet house: Spatial homogeneity and aerial pollutants. Brit. Poultry Sci. 7:189-198.

CURTIS, S.E., DRUMMOND, J.G., 1982. Air environment and animal performance. In: M. Rechcigl Jr (ed.) Handbook of Agricultural Productivity. Vol. 2: Animal Productivity. CRC Press Inc., Boca Raton, FL, USA.

FEDDES, J.J.R., LICSKO, Z.J., 1993. Air quality in commercial turkey housing. Can. Agr. Eng. 35:147-150.

IVANOV, I.E., 2001. Treatment of boiler bitter with organic acids. Res. Vet. Sci. 70:169-173.

KRISTENSEN, H.H., WATHES, C.M., 2000. Ammonia and poultry welfare: a review. World Poultry Sci. J. 56:235-245.

MAGHIRANG, R.G., MANBECK, H.B., ROUSH, W.B., MUIR, F.V. 1991. Air contaminant distributions in a commercial laying house. T. Am. Soc. Agr. Eng. 34:2171-2180.

OYETUNDE, O.O.F., THOMSON, R.G., CARLSON, H.C., 1978. Aerosol exposure of ammonia, dust and Escherichia coli in broiler chickens. Can. Vet. J. 19:187-193.

SAINSBURY, D., 1992. Poultry Health and Management-Chickens, Turkeys, Ducks, Geese, Quail. 3rd ed. Blackwell scientific Ltd, Oxford, UK.

Efficacy in the field of two anticoccidial vaccines for broilers

Raffaella Ceruti[1], Viviana Ferrazzi[2], Luigi Gavazzi[1], Daniele Gallazzi[2], Guido Grilli[2]

[1] Gruppo Amadori. Cesena, Italy

[2] Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria. Università di Milano, Italy

Corresponding author: Dr. Guido Grilli. Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria. Università di Milano, Facoltà di Medicina Veterinaria. Via Celoria 10, 20133 Milano, Italy – Tel. +39 02 50318121 – Fax: +39 02 50318106 – Email: guido.grilli@unimi.it

ABSTRACT

We compared two attenuated anticoccidial vaccines, administered to broilers by spray into the incubator (88,000 males and 210,100 females). Vaccine A container five species of *Eimeria* and vaccine B three. Zootechnical performance was similar in the two groups, with mean lesion scores no higher than 1; vaccine A caused only duodenal lesions, while vaccine B also caused typhlitis. Maximum oocyst count was 23,000/g feces at age 28 days with vaccine A and 38,000 at 21 days with vaccine B. Broilers vaccinated with vaccine B had more frequent enteric symptoms, and *C. perfringens* isolation.

Key Words: Broilers, Coccidiosis, Vaccine, Field trial.

RIASSUNTO

EFFICACIA IN CAMPO DI DUE VACCINI ANTICOCCIDICI PER BROILERS

Sono stati confrontati due vaccini anticoccidici attenuati in broilers (88.000 maschi e 210.000 femmine) somministrati per spray in incubatoio. Il vaccino A conteneva 5 specie di Eimeria e il vaccino B 3 specie. Le performance zootecniche sono state simili nei due gruppi, lo score delle lesioni mediamente non superava il valore 1, anche se A dava lesioni solo duodenali, mentre con B era presente anche tiflite. L'emissione massima di oocisti era di 23.000 per grammo di feci (OPG) a 28 gg di vita in A e 38.000 OPG a 21 gg per B. Nei broilers vaccinati con B erano più frequenti sintomi enterici con isolamento di C. perfringens*, che ha richiesto terapia specifica per arginare la mortalità.*

Parole chiave: *Pollo da carne, Coccidiosi, Vaccinazione, Prova di campo.*

Introduction

Attenuated anticoccidial vaccines containing various species of *Eimeria* pathogenic for poultry are the only alternative to anticoccidial drugs (Chapman *et al.*, 2002). Live attenuated vaccines have been under investigation for several years now (Shirley, 1989). Most of these consist of a stabilized suspension of sporulated oocysts of *Eimeria* species from chickens, selected from precocious lines (shorter cycle, reduced reproductive potential, maintenance of the immunogenic capacity). Another method of attenuation is to produce lines of coccidia, especially *Eimeria tenella*, by passages in embryonated hen's eggs (Shirley and Bedrník, 1997). The *Eimeria* lines in commercial vaccines, even though attenuated, can cause some lesions to the intestinal mucosa (Williams and Andrews, 2001) and, in less than optimal breeding conditions, these can interact with other intestinal

pathogens, such as *Clostridium perfringens*, facilitating the onset of pathologies such as necrotic enteritis (NE) (Waldenstedt *et al.*, 1999).

This study compared the efficacy of two anticoccidial vaccines in eight commercial broiler farms.

Material and methods

Vaccines

We used two commercial vaccines, indicated here as A and B. Vaccine A contained five species of a precocious line of *Eimeria (E. acervulina, E. maxima* (two lines), *E. mitis and E. tenella)*. Vaccine B container two precocious lines, *E. acervulina* and *E. maxima*, and one egg-adapted line, *E. tenella*. Both were administered using a spray machine (Breuil model), following the manufacturer's instructions, directly into the incubator where all the chicks for controlled breeding were hatched.

Breeding establishments

Eight commercial chicken farms (numbered 1-8) were checked; four only bred males, and four only females, for a total of 298,100 broilers (hybrid ROSS 508). Vaccine A was given to the chicks in farms nos.1 (26,000 males), 2 (24,000 males), 3 (75,600 females), and 4 (46,000 females). Vaccine B was given to the chicks in farms nos. 5 (9000 males), 6 (29,000 males), 7 (37,500 females) and 8 (51,000 females). Chicks were housed between 31/05/04 and 10/06/04; the breeding density (male: 10; female: 16 chicks/sq.m) and feed were comparable in all eight farms.

Laboratory tests

From each shed we took 50 individual samples of feces, and counted the oocysts according to McMaster's method on days 7, 14, 21, 28 and 35 for males and females, and on days 42 and 49 as well for males. At the ages of 21, 28, 35 days for females and 21, 28, 35, 42, 49 days for males, we sampled ten chicks per farm to check for coccidial lesions, using the method of Johnson and Reid (1970). From six chicks for each age group we also removed samples of the intestine (duodenum, jejunum/ileus and cecum), which were fixed in 10% iso-osmotic formalin and examined histologically, according to routine methods. Semi-quantitative bacteriological counts were also done on the same animals, as recommended by Elanco Animal Health (Anonymous, 2002).

Results and discussion

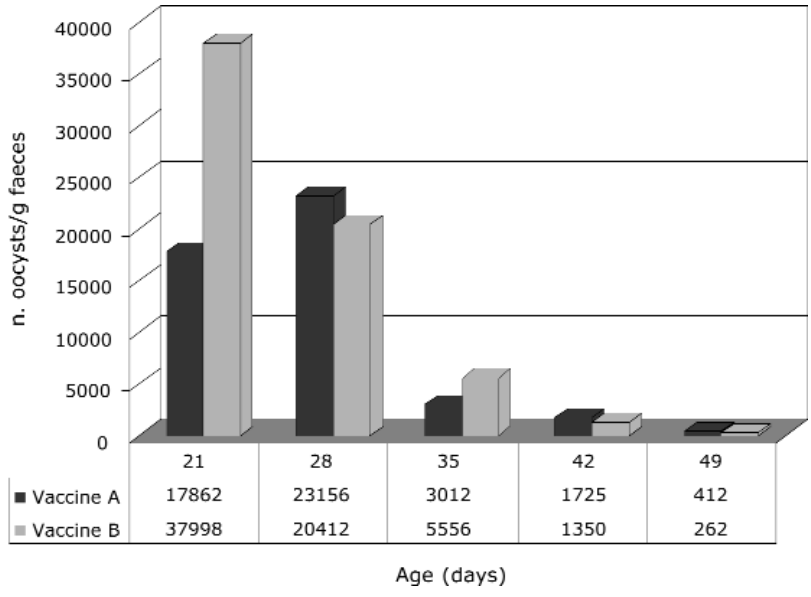
In the farms where chicks had been given vaccine A we found no clinical symptoms of intestinal pathology, and bedding remained in good condition. Table 1 shows the scores for lesions. Chicks vaccinated with A had few lesions due to *E. acervulina* and *E. maxima* and only very few to *E. tenella*, in accordance with the report by Williams and Andrews (2001). Semi-quantitative examination of the intestinal flora showed initial mild abnormality with a prevalence of Gram-negative microorganisms up to day 28; from then onwards until slaughter there was an increase in *C. perfringens*, but no gross or microscopic lesions due NE.

There are, however, reports of interactions between field coccidia or attenuated anticoccidial

Table 1. Mean lesion scores.

Age	*E. acervulina*		*E. maxima*		*E. tenella*	
days	Vacc. A	Vacc. B	Vacc. A	Vacc. B	Vacc. A	Vacc. B
21	0.725	0.65	0.35	0.15	0.1	0.125
28	0.75	0.25	0.55	0.45	0.1	0.575
35	0.15	0	0.45	0	0.2	0.05
42	0	0	0	0	0.02	0.05
49	0	0.15	0	0	0	0

Figure 1. Mean oocyst output in eight broiler breeding farms.



vaccines and NE (Williams *et al.*, 2003). Output of oocysts was near-nil in the first two weeks but peaked at 28 days, as already reported by Williams and Gobbi (2002), declining steeply thereafter (Figure 1).

In three of the breeding farms (nos. 6, 7 and 8) the broilers vaccinated with B had intestinal symptoms (catarrhal enteritis), with a rise in mortality, between the third and fifth weeks. The symptoms stopped only after repeated antibiotic treatments. Bacteriological tests in these B-vaccinated chicks showed a rise in *C. perfringens* already from the third week, when oocyst output was maximum – much higher than in the groups given vaccine A (Figure 1). The lesion score for vaccine B was not very different from A, except for the constant finding of lesions due to *E. tenella*, unlike in the report by Rois *et al.* (2002) in similar settings.

Histological lesions in the various segments of the intestine were correlated to the lesion scores, subjects B showing more marked cecal lesions, typical of *E. tenella* infection.

The productive performances of the two groups were in line with the specific literature for this cross. Male and female broilers given vaccine A were slaughtered respectively at 56.7 and 40.85 days, at average weights of 3.719 kg and 1.676 kg, with feed conversion ratio (FCR) 1.87 and 1.74, and mortality 4.05% and 2.46%. Male and female broilers given vaccine B were slaughtered respectively at 52 and 40.3 days, when average weights were 3.244 and 1.676 kg, FCR 2.00 and 1.81, and mortality 6.25 % and 1.78%.

The European Efficiency Index for broilers was 336,54 for group A males and 229,98 for females, and respectively 292,43 and 225,68 in group B.

Conclusions

Attenuated anticoccidial vaccines are the only alternatives to the drug prophylaxis that has for decades permitted the expansion of poultry breeding. These vaccines have been used increasingly in Italy in recent years. Commercial products are safe but must obviously be coupled with good breeding practice for maximum efficiency. The abolition of growth promoter additives, which controlled the anaerobic intestinal flora, particularly *C. perfringens* (Waldenstedt *et al.*, 1999), calls for care in selecting the right vaccine.

In this comparison both vaccines effectively controlled coccidiosis in a field setting. Vaccine A, given the same breeding conditions and feed, seemed to interact less on the chicken's intestinal microbial flora, without inducing NE, meaning there was less need for specific antibiotics. Zootechnical indices were better in Group A, especially for the males.

REFERENCES

ANONYMOUS, 2002. Determinazione di un metodo per la diagnosi in campo della disbatteriosi intestinale avicola. Ed. Eli Lilly Italia, Sesto Fiorentino (FI), Italy.

CHAPMAN, H.D., CHERRY, T.E., DANFORTH, H.D., RICHARDS, G., SHIRLEY, M.W., WILLIAMS, R.B., 2002. Sustainable coccidiosis control in poultry production: the role of live vaccines. Int. J. Parasitol. 32:617-629.

JOHNSON, J., REID, W.M., 1970. Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experimental with chickens. Exp. Parasitol. 28:30-36.

ROIS, O., RATAJ, Z., KRAPEZ, A.V., DOVC, U., CAJAVEC, S., 2002. Evaluation of safety of the anticoccidial vaccine Livacox® T in broiler chickens in experimental conditions. Praxis Vet. 50(3):231-238.

SHIRLEY, M.W., 1989. Development of a live attenuated vaccine against coccidiosis of poultry. Parasite Immunol. 11:117-124.

SHIRLEY, M.W., BEDRNÍK, P., 1997. Live attenuated vaccines against avian coccidiosis: success with precocious and egg-adapted lines of Eimeria. Parasitol. Today 13:481-484.

WALDENSTEDT, L., LUNDEN, A., ELWINGER, K., THEBO, P., UGGLA, A., 1999. Comparison between a live, attenuated anticoccidial vaccine and an anticoccidial ionophore, on performance of broilers raised with or without a growth promoter, in an initially *Eimeria*-free environment. Acta Vet. Scand. 40:11-21.

WILLIAMS, R.B., ANDREWS, S.J., 2001. The origins and biological significance of the coccidial lesions that occur in chickens vaccinated with a live attenuated anticoccidial vaccine. Avian Pathol. 30:215-220.

WILLIAMS, R.B., GOBBI, L., 2002. Comparison of an attenuated anticoccidial vaccine and an anticoccidial drug programme in commercial broiler chickens in Italy. Avian Pathol. 31:253-265.

WILLIAMS, R.B., MARSHALL, R.N., LA RAGIONE, R.M., CATCHPOLE, J., 2003. A new method for the experimental production of necrotic enteritis and its use for studies on the relationships between necrotic enteritis. coccidiosis and anticoccidial vaccination of chickens. Parasitol. Res. 90:19-26.

Epidemiological study on circulation of Infectious Bronchitis Virus strains in North Eastern Italy

Maria Serena Beato, Calogero Terregino, Roberta De Nardi, Anna Toffan, Giovanni Cattoli, Ilaria Capua

Istituto Zooprofilattico Sperimentale delle Venezie. Legnaro (PD), Italy

Corresponding author: Dr. Calogero Terregino. Istituto Zooprofilattico Sperimentale delle Venezie, Virology Dept. Viale dell'Università 10, 35020 Legnaro (PD), Italy – Tel. +39 049 8084389 – Fax: +39 049 8084360 – E-mail: virologia@izsvenezie.it

ABSTRACT

Infectious Bronchitis (IB) is still a major health problem in the poultry industry, as it is endemic in probably all countries which raise chickens. Previous investigations have shown that several IB variants are present in the Italian poultry industry. In order to establish which serotypes are circulating in the Veneto and Lombardia regions an investigation was carried out during 2004 in broiler and egg-layer farms. A total of 101 samples were collected from 29 farms with 16 IBV isolations. Of these, seven isolates were identified as being of the IT-02 serotype and six as 793-B on the basis of nucleotide sequencing.

Key Words: Chicken, Infectious Bronchitis, Broiler, North Italy, Epidemiology.

RIASSUNTO

INDAGINE EPIDEMIOLOGICA SULLA CIRCOLAZIONE DEI VIRUS DELLA BRONCHITE INFETTIVA AVIARIA IN VENETO

La Bronchite Infettiva (IB) rappresenta ancora oggi uno dei principali problemi sanitari per il pollame laddove l'allevamento ha carattere intensivo. Si è dato inizio ad un'indagine conoscitiva sulla epidemiologia della IB in Veneto, successivamente allargata ad alcune province della Lombardia. Dal gennaio 2004 sono stati monitorati 29 allevamenti, per un totale di 101 campioni esaminati, di cui 16 sono risultati positivi per IBV. Sette isolati sono stati identificati come sierotipo IT-02 e sei come 793-B.

Parole chiave: *Pollo, Bronchite infettiva, Broiler, Nord Italia, Epidemiologia.*

Introduction

Infectious Bronchitis (IB) is still a major health problem in the poultry industry, as it is endemic in probably all countries which raise chickens. IB is a severe systemic disease resulting in a variable morbidity depending on the virulence and tissue tropism of the strain. The clinical disease, is generally characterized by respiratory signs and nephrosis in broilers and drop in egg production in egg layers and broiler breeders. IB is caused by avian Coronavirus, an enveloped single stranded, positive-sense RNA with a large spike glicoprotein (S) that is responsible of the host antibody response. IBV may be classified in serotypes that can be characterized by serological and molecular biology techniques. Previous investigations have shown that several IB variants are present in the Italian poultry industry. Among these, some have been isolated primarily in Italy, such as variant 624-I (Capua *et al.*, 1999), while others originate from other European countries such as 793-B and B-

1648. Between 2000 and 2003 no surveys on IB were performed in the densely populated poultry areas of north-eastern Italy, although clinical signs indicative of this infection were observed. In order to establish which serotypes are circulating in the Veneto and Lombardia regions an investigation was carried out during 2004 in broiler and egg-layer farms in collaboration with veterinarians of farms where the study has been carried out.

Material and methods

The study has had as target the broiler and egg layer farms located in North-eastern Italy (Veneto and Lombardia regions) . In order to improve isolation rates, SPF (Specific Pathogen Free) chickens were introduced in the farms for a period of 7-10 days as sentinels. Tissue samples, collected from sentinels (trachea, lung, kidney and cecal tonsils) were submitted for virological investigations. The tissue homogenates were inoculated into the allantoic cavity of eggs from 9- to 11-day-old embryonated SPF fowl (Gelb *et al.*, 1998), following an overnight incubation at 4°C with PBS antibiotic solution. Following a minimum of two and a maximum of four blind passages, embryos were observed to detect typical IBV lesions (dwarfing, incomplete feathering pattern and stunting) and the allantoic fluid was harvested and examined by negative contrast electron microscopy for the presence of coronavirus particles (Hyatt, 1986). In order to characterise the isolates, viral RNA was extracted from the infectious allantoic fluid. The isolates were analysed in RT-PCR, with specific primers for the S1-gene (Adzhar *et al.*, 1996), in order to generate a complementary DNA (cDNA). This cDNA, was sequenced (Keeler *et al.*, 1998), and isolates were typed on the basis of the sequence. A total of 101 samples were collected from 29 farms.

Results and discussion

The results of the investigation indicate that IBV strains are actively circulating in broiler and layer farms of North-eastern Italy. In fact sixteen IBV were isolated from 29 farms: namely serotypes IT-02 and 793-B as shown in (Table 1). In farms where serotype 793-B prevailed respiratory forms were seen more often. In one of these, the respiratory form was associated to neprhosis. Enteric disorders were observed in farms where serotype IT-02 circulated. For three isolates obtained on three different farms, molecular characterisation is in progress.

The results presented above indicate that regardless of the geographical origin of the isolate strains, 793-B and IT-02 are present in Veneto and Lombardia regions. The findings of the present study indicate that strain 793-B is still present in Italy (Catelli and Lavazza, 2000; Tosi, 2001). Although evidence of the presence of this virus has been collected throughout the years, it is not possible to establish how many isolates are pathogenic field strains and how many are vaccine strains. In fact vaccination against this variant has been practised extensively with live attenuated vaccines, and currently there is no tool available to differentiate field from vaccine strains.

What appears of great interest is the significant number of isolations of strain IT-02. This strain appears to be widespread in broiler farms. Since at present there is no information on the pathogenicity and antigenicity of this novel variant, challenge experiments and cross protection studies should be performed in order to evaluate on one hand the pathogenicity of this variant, and on the other the possibility of using licensed products to protect birds against the clinical condition caused by this variant.

Conclusions

The results of this investigation indicate that a comprehensive knowledge on the serotypes of Infectious Bronchitis Virus circulating in particular areas is essential to plan and possibly update an appropriate vaccination strategy against this disease.

REFERENCES

ADZHAR, A., SHAW, K., BRITTON, P., CAVANAGH, D., 1996. Universal oligonucleotides for detection of infectious bronchitis virus by the polymerase chain reaction Avian Pathol. 25:817-836.

CAPUA, I., MINTA, Z., KARPINSKA, E., MAWDITT, K., BRITTON, P., CAVANAGH, D., COUGH, R.E., 1999. Co-circulation of four serotypes of infectious bronchitis virus (793-B, 624-I, B1648 and Massachustts).

Table 1. Province of isolation (in Italy), strains isolated lesions and clinical signs observed in the field.

Province	Type of farm and age of birds	Molecular characterization of isolate	Lesions and clinical signs
Verona	Broiler – 36 days	IBV serotype 793-B*	Mild respiratory form
Rovigo	Broiler – 36 days	IBV serotype IT-02	Not reported
Cremona	Broiler - 45 days	IBV serotype IT-02	Not reported
Mantova	Broiler - 60 days	IBV serotype 793-B	Respiratory form
Verona	Broiler - 53 days	IBV	Not reported
Padova	Broiler - 60 days	IBV	Respiratory form
Pordenone	Layers - 20 weeks	IBV	Respiratory form, depigmentation of the eggs' shell
Brescia	Broiler - 42 days	IBV serotype 793-B	Respiratory form, kidney lesions, growth problem, mortality
Verona	Broiler - 35 days	IBV serotype IT-02	Kidney lesions, enteric disorders,mortality
Verona	Broiler - 45 days	IBV serotype IT-02	Enteric forms
Cremona	Broiler - 53 days	IBV serotype IT-02	Respiratory and enteric forms
Padova	Broiler - 50 days	IBV serotype 793-B	Mild respiratory form
Padova	Broiler - 43 days	IBV serotype IT-02	Mild respiratory form
Vicenza	Broiler - 51 days	IBV serotype IT-02	Mild respiratory form
Brescia	Broiler - 45 days	IBV serotype 793B*	Not reported
Mantova	Broiler - 50 days	IBV serotype 793-B*	Mild respiratory form

** Farms where a live attenuated vaccine serotype 793/B was administered*

Avian Pathol. 28:587-592.

Catelli, E., Lavazza, A., 2000. Relazione sullo stato sanitario dell'allevamento avicolo nel 2000. Selez. Vet. 11:963-970.

Gelb, J., Jackwood, M.W., 1998. Infectious Bronchitis. In: Isolation and identification of avian pathogens, 4th ed. American Association of Avian Pathologists, Tallahassee, FL, USA, pp 169-174.

Hyatt, A.M., 1986. Basic techniques for transmission electron microscopy. Academic Press, Inc., San Diego, CA, USA.

Keeler, C.L., Reed, K.L., Nix, W.A., Gelb, J., 1998. Serotype identification of avian infectious bronchitis virus (IBV) by RT-PCR of the peplomer (S-1) gene. Avian Dis. 42:275-284.

Tosi, G., 2001. Relazione sullo stato sanitario dell'allevamento avicolo nel 2001. Large Anim. Rev. 7(6):37-40.

Typing of Poultry Influenza Virus (H5 and H7) by Reverse Transcription-Polymerase Chain Reaction

Paola Massi, Giovanni Tosi, Paolo Cordioli,
Francesca Paganelli, Cesare Bonacina

Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna. Forlì, Italy

Corresponding author: Dr. Paola Massi. Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Sezione di Forlì. Via Marchini 1, 47100 Forlì, Italy – Tel. +39 0543 721533 – Fax: +39 0543 721533 – Email: paolamassi@bs.izs.it

ABSTRACT

The ability of the influenza *Orthomixovirus* to undergo to continually antigenically changes that can affect its pathogenicity and its diffusion, explains the growing seriousness of this disease and the recent epizoozies in various parts of the world. There have been 15 HA and 9 NA type A sub-types of the influenza virus identified all of which are present in birds. Until now the very virulent avian influenza viruses identified were all included to the H5 and H7 sub-types.
We here show that is possible to identify the H5 and H7 sub-types with reverse transcription-polymerase chain reaction (RT-PCR) by using a set of specific primers for each HA sub-type. The RT-PCR is a quick and sensitive method of identifying the HA sub-types of the influenza virus directly from homogenised organs.

Key Words: Poultry, Avian influenza virus, Hemagglutinine, Reverse transcription-polymerase chain reaction (RT-PCR).

RIASSUNTO

TIPIZZAZIONE DEI VIRUS INFLUENZALI AVIARI (H5 E H7) TRAMITE RT-PCR

La capacità dell'Orthomixovirus dell'influenza di andare incontro a continue variazioni antigeniche, determinanti la patogenicità e la diffusibilità, spiega l'accresciuta importanza di questa malattia e le recenti epizoozie segnalate in varie parti del mondo.
Nel virus influenzale di tipo A sono stati identificati 15 sottotipi di HA e 9 NA, tutti presenti negli uccelli. Finora i virus influenzali aviari che hanno presentato caratteri d'elevata virulenza sono tutti appartenenti ai sottotipi H5 e H7.
E' possibile identificare i due sottotipi H5 e H7 tramite una reverse transcription-polymerase chain reaction (RT-PCR) *utilizzando un set di primers specifici per ogni sottotipo HA.*
La RT-PCR si presenta come un rapido e sensibile strumento per l'identificazione dei sottotipi HA del virus influenzale direttamente da omogenato d'organi.

Parole chiave: *Pollame, Virus influenzale aviare, Emoagglutinina,* RT-PCR.

Introduction

Influenza was first described by Hippocrates in 412 B.C., and the miniscule virus has continually mutated throughout the centuries, spreading the disease right up to the latest H5N1 epidemics which hit Asia in 2003 and 2004. The influenza virus is part of the Orthomyxoviridae family (genus) and is divided into three genus: A, B and C.

The A type viruses have essentially avian origins and only occasionally do they jump the species barrier to affect other animals or man. Recently, knowledge of the virus, its pathogenesis and prevention, have greatly increased but unfortunately this has not allowed us to avoid great (economic) losses due to recurrent epizoozies from highly viru-

lent strains in different parts of the world.

The virus contains 8 separate segments of RNA, which codify genes of at least 10 different proteins. This unusual genetic structure explains why rearrangement of the segments occurs so frequently. Exchange of genetic segments can easily occur which can cause up to 256 different descendents.

The A type influenza strains are characterised by the structural changes of 2 glycoproteines; hemagglutinine (HA) and neuroaminidase (NA) which project from the surface of the viral particles. Sub-type sub-division is based on a combination of the 15 HA and 9 NA which have so far been identified, all of which are present in birds (Alexander, 1993; Easterday *et al.*, 1997).

Epidemics occur when one or the other of these proteins undergoes a mutation. The unforeseeable nature of the influenza virus comes from their ability to change the surface HA and NA proteins, thus escaping the immune system's vigilance.

If we consider the epidemiological polyedric aspects, the speed of the influenza diagnosis and therefore quick confirmation of the infection becomes particularly important. For these reasons, we can say that reverse transcription-polymerase chain reaction (RT-PCR) has given particularly encouraging results.

To date, all highly pathogenic stains that have been isolate are A influenza viruses of the H5 and H7 sub-types.

The molecular base for distinguishing the antigenic differences of the HA sub-type are based on the amino-acid sequence differences between 20% and 74% and so this percentage is reflected in the nucleotide sequence of the HA sub-type (Ming-Shiuh *et al.*, 2001).

These characteristics are fundamental for the typing of HA sub-types by RT-PCR as the PCR is determined by the difference in the nucleotide sequence. We have here evaluated a quick identification procedure to identity H5 and H7 subtypes of the influenza virus using RT-PCR.

Material and methods

Samples

For the study we used organs (tracheal exudates, lungs, cecal tonsils) in that previously the following avian influenza virus strains were isolated and identified: H7N1/99 chicken and H7N3/02 turkey. Moreover, starting from allantoic fluid, the following strains were also used: H5N2/73 England (turkey), H6N2/90 (turkey) and H9N2/86 (chicken).

Identification of the Type A influenza virus with RT-PCR

The viral RNA was extracted using the Rneasy® Minikit (Qiagen, Valencia, CA, USA) protocol. Once we had the RNA sample we proceeded to the retro-transcription using ProstarTM Kit (Stratagene®, USA). The retro-transcription product was then used directly in the PCR with the addition of a reagent and the tampon which are part of the Accuprime TaqPCRx DNA Polymerase (Invitrogen® USA). The research of the viral genome also includes the use of M52C and M253R primers (Fouchier *et al.*, 2000), which amplifies a region coding for the matrix protein of the type A influenza virus.

Typing H5 and H7 with RT-PCR

The cDNA obtained by retro-transcription was used for a further PCR according to above protocol. To differentiate the H5 and H7 subtypes, sets of specific primers for each sub-type in respect to

Table 1. Primer used for the HA-sub-typing of avian influenza viruses by RT-PCR.

Primer	Primer sequences	PCR product (bp)
H5-155F	5′ ACACATGCYCARGACATACT 3′	545
H5-699R	5′ CTYTGRTTYAGTGTTGATGT 3′	
H7-12F	5′ GGGATACAAAATGAAYACTC 3′	634
H7-645R	5′ CCATABARYYTRGTCTGYTC 3′	

Table 2. Results of identification and subtyping.

Sample	AIV strain	Type	Subtype
Allantoic fluid	H5N2/73 Turkey England	A	H5 (545bp)
Allantoic fluid	H9N2/86 Chicken Italy	A	"
Allantoic fluid	H6N2/90 Turkey Italy	A	"
Organs	H7N1/99 Chicken Italy	A	H7 (634bp)
Organs	H7N3/02 Turkey Italy	A	"

their sequence and characteristics were used as triggers (Ming-Shiuh *et al.*, 2001) (Table 1). The reactions occurred in the following amplification profile: 95°C for 3 minutes, (hot start) followed by 35 cycles of three steps: 95°C for 30 seconds (denaturation), 55°C for 40 seconds (annealing), 72°C for 40 seconds (extension), at the end of cycle there was an additional 10 minutes at 72°C for any possible extensions. The amplified product then underwent an electrophoresis course in agarose gel at 1% with ethidium bromide and was visualised using a UV trans-illuminator.

Results and discussion

Results about avian influenza virus (AIV) identification and subtyping are shown in table 2. Given the variable nature of the influenza virus, it is almost certain that it can escape immune surveillance, thus making it difficult to keep this disease under control.

The particular characteristics of AIV make quick identification and characterisation of the antigenic variants indispensable to be able to intervene as quickly as possible in the more convenient way.

Furthermore, early identification if the new A type influenza virus subtypes, which could be possible causes of pandemics, must be one of the main objectives to insure that vaccines include the immunological characteristics of the virus prevalently in circulation. RT-PCR response perfectly to the requirements of identifying the H5 and H7 subtypes both for its precision and its speed (48 hours).

Conclusions

RT-PCR technique could be a valid test in support of classical diagnosis at the same time being an efficient control technique of poultry breeding. It has been shown to be a sensitive and specific method both using homogenised organs and liquid allantoid.

REFERENCES

ALEXANDER, D.J., 1993. Orthomyxovirus infection. In: J.B. McFerran and M.S. McNulty (eds.) Virus Infections of birds. Elsevier Science Publishers B.V., Amsterdam, The Netherlands, pp 287-316.

EASTERDAY, B.C., HINSHAW, V.S., HALVORSON, D.A., 1997. Influenza. In: B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald, L.M. Saif (eds.) Diseases of Poultry. 10th ed. Iowa State University Press, Ames, Iowa, USA, pp 583-605.

FOUCHIER, R.A.M., BESTEBROER, S.H., VAN DE KEMP, L., RIMMELZWAAN, G.F., OSTERHAUS, A.D., 2000. Detection of Influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene. J. Clin. Microbiol. 38:4096-4101.

MING-SHIUH, L., POA-CHUN, C., JUI-HUNG, S., MING-CHU, C., HAPPY, K.S., 2001. Identification and subtyping of avian influenza viruses by reverse transcriptio-PCR. J. Virol. Meth. 97:13-22.

Prevalence of *Campylobacter jejuni* in poultry breeder flocks

Lucia Francesca Menna[1], Gianluca Matteoli[1], Marzia Fontanella[1], Alessandra Cuomo[1], Antonio De Paola[2], Tiziana Pepe[3], Isolina Di Marco[3], Ludovico Dipineto[1]

[1]Dipartimento di Patologia e Sanità Animale. Università di Napoli "Federico II", Italy
[2]Arena Holding Spa. Bojano (CB), Italy
[3]Dipartimento di Scienze Zootecniche ed Ispezione degli Alimenti. Università di Napoli "Federico II", Italy

Corresponding author: Prof. Lucia Francesca Menna. Dipartimento di Patologia e Sanità Animale. Facoltà di Medicina Veterinaria. Università di Napoli "Federico II". Via Delpino 1, 80137 Napoli, Italy – Tel. +39 081 451802 Fax: +39 081 5091993 – Email: fioretti@unina.it

ABSTRACT

The aim of this work is to present the preliminary results of a study about the prevalence of *Campylobacter jejuni* in poultry breeder flocks. It was examined three different breeder flocks of Bojano in Molise region.
A total of 360 cloacal swabs and 80 enviromental swabs was collected. Of the 3 flocks studied, 6.9% tested were positive for *Campylobacter spp.* The most-prevalent isolated species is *C. jejuni* (8.2%). Only 3 of the 360 cloacal swabs samples examined were associated with *C. coli*. The environmental swabs resulted negative. This results confirms again that poultry is a reservoir of this germ.

Key Words: Campylobacter jejuni, Prevalence, Breeder flocks.

RIASSUNTO

INDAGINE SULLA PREVALENZA DI *CAMPYLOBACTER JEJUNI* IN GRUPPI DI RIPRODUTTORI AVICOLI

Il presente lavoro si propone di illustrare i risultati preliminari di uno studio volto a valutare la prevalenza di Campylobacter jejuni *in gruppi di riproduttori. Sono stati esaminati 3 gruppi di riproduttori ubicati a Bojano in Molise. Dei tre gruppi valutati, il 6,9% risultava positivo a* Campylobacter spp. C. jejuni *era la principale specie isolata (8,2%). Solo 3 dei 360 tamponi cloacali esaminati era associata a* C. coli. *I tamponi ambientali risultavano negativi. Tali risultati confermano ancora una volta il ruolo del pollame come reservoir di questo microrganismo.*

Parole chiave: Campylobacter jejuni*, Prevalenza, Riproduttori.*

Introduction

Campylobacter jejuni is the leading cause of bacterial foodborne illnesses in human medicine (Newell and Fearnley, 2003). The vast majority of human campylobacteriosis cases primarily result from consumption of undercooked poultry or other foods cross-contaminated with raw poultry meat during food preparation. However, other risk factors besides poultry such as contact with house pets, or consumption of raw milk, untreated water, and undercooked beef or pork have also been linked to human infections (Corry and Atabay, 2001). As poultry is considered a major reservoir for human campylobacteriosis, reduction or elimination of poultry contamination with *C. jejuni*

would greatly reduce the risk of *Campylobacter* for public health. Although numerous farm-based studies have been conducted in the past decades, the sources of flock infection, modes of transmission, and the host and environmental factors affecting the spread of *Campylobacter* on poultry farms are still poorly understood (Sahin *et al.*, 2002). Potential sources of flock infection include used litter, untreated drinking water, other farm animals, domestic pets, wildlife species, house flies, insects, farm equipment and workers, and transport vehicles (Newell and Fearnley, 2003). However, none of these suspected sources has been conclusively identified as the formal source of infection for broilers farms. Despite these observations, vertical transmission of *C. jejuni* is still questionable because live *Campylobacter* have not detected in the eggs of commercial breeders, young hatchlings or hatcheries under natural conditions. Therefore the exact role of vertical transmission in introducing *Campylobacter* to broiler flocks remains unclear (Sahin *et al.*, 2003).

An estimated 2.1-2.5 million cases of human campylobacteriosis, characterized by watery and/or bloody diarrhoea, occur annually in the United States, exceeding the cases of salmonellosis (Friedman *et al.*, 2000). The reported incidence of *Campylobacter* infection in Europe is estimated to be 1000-2300 cases per 100.000 (Padungton and Kaneene, 2003).

The aim of this work is to present the results of a study about the prevalence of *Campylobacter jejuni* in poultry breeder flocks.

Material and methods

This study was conducted during the period October 2003/July 2004 in the Arena breeders-farm of Bojano in Molise region.

Samples collection

It was examined three different breeder flocks respectively named A, B and C. The amount of population for each breeder flocks was 11.500, 6000, 12.000, respectively for flock A, flock B and flock C. Each flock was visited five times. The first visit occurred during cleaning and disinfection procedures before placing the chicks. The second visit took place at one day of age, the third visit at 4 weeks of age, the fourth at 20 weeks of age and the last visit took place at 30 weeks. During every visit 30 cloacal swabs samples and 10 environmental samples (wall, water, litter, feed) was collected.

Isolation and identification procedure

The samples were added to Campylobacter Selective Enrichment broth (Oxoid) and incubated at 42°C for 24 h under microaerophilic conditions. Then, each sample was streaked onto Campylobacter blood free selective agar base - Modified CCDA Preston (Oxoid) plates. Plates were incubated at 42°C under microaerophilic conditions for 48 h. Therefore, the isolates was streaked onto blood-agar plates and incubated at 42°C for 24 h. Isolates were identified using a commercial identification method (API Campy, bioMérieux).

Table 1. PCR primers for *C. jejuni* and *C. coli* employed in the multiplex PCR.

Species targeted	Product size (bp)	Primer name (target gene)	Sequence (5′ – 3′)
C. coli/C. jejuni	400	cadF2B (cadF)	TTGAAGGTAATTTAGATATG
		cadR1B	CTAATACCTAAAGTTGAAAC
C. coli	894	COL 1 (ceuE)	ATGAAAAAATATTTAGTTTTTGCA
		COL 2	ATTTTATTATTTGTAGCAGCG
C. jejuni	160	C-1 (?)	CAAATAAAGTTAGAGGTAGAATGT
		C-2	GGATAAGCACTAGCTAGCTGAT

Table 2. Percentage of positivity from cloacal swabs (30/flock).

Age of breeder flocks	Flocks (amount of population)		
	Flock A (11,500)	Flock B (6000)	Flock C (12,000)
1 day	0%	0%	0%
4 weeks	0%	0%	0%
20 weeks	50%	3.3%	10%
30 weeks	40%	3.3%	3.3%

Multiplex PCR

A multiplex PCR assay was carried out to all isolates in accordance with the Cloak and Fratamico procedure (Cloak and Fratamico, 2002). The primers employed in this assay are shown in table 1.

Results and discussion

Of the 3 flocks studied, 6,9% tested were positive for *Campylobacter spp.* (table 2). The most-prevalent isolated species was *C. jejuni* (8,2%). Only 3 of the 360 cloacal swabs samples examined were associated with *C. coli*. The environmental swabs resulted negative.

The results of this study shows a low prevalence of *Campylobacter* in the breeder flocks examined. This results confirms again that poultry is a reservoir of this germ.

Conclusions

The literature suggests that standard biosecurity procedures are inadequate for the maintenance of flock negativity (Newell and Fearnley, 2003). This is a consequence of high exposure, low dose, and rapid bird-to-bird transmission rates. Nevertheless, stringent biosecurity may either delay positivity or reduce the number of flocks that become positive. However, it is generally considered that adequate biosecurity procedures are difficult to sustain in the farm environment (Pattison, 2001). For example, routine procedures such as the effective use of hygiene barriers, hand washing, and boot disinfection may be readily undertaken under normal conditions, but during emergencies, such as fan failure, such procedures may be ignored. Well-designed and well-located farms, the development of appropriate standard operating procedures to minimize risk factors, staff education, and incentives to maintain biosecurity at the highest level would all contribute to the reduction of flock positivity.

We would like to thank Mrs. Fortuna Pisa for her technical collaboration.

REFERENCES

CLOAK, O.M., FRATAMICO, P.M., 2002. A multiplex polymerase chain reaction for the differentiation of *Campylobacter jejuni* and *Campylobacter coli* from a swine processing facility and characterization of isolates by pulsed-field gel electrophoresis and antibiotic resistance profiles. J. Food. Protect. 65:266-73.

CORRY, J.E., ATABAY, H.I., 2001. Poultry as a source of Campylobacter and related organisms. J. Appl. Microbiol. 90:96-114.

FRIEDMAN, C.R., NEIMANN, J., WEGENER, H.C., TAUXE, R.V., 2000. Epidemiology of *C. jejuni* infections in the United States and other industrialized nations. In: I. Nachamkin and M.J. Blaser (eds.) Campylobacter. 2nd ed. ASM Press, Washington, DC, USA, pp 121-138.

NEWELL, D.G., FEARNLEY, C., 2003. Sources of *Campylobacter* colonization in broiler chickens. Appl. Environ. Microb. 69:4343-4351.

PADUNGTON, P., KANEENE, J.B., 2003. *Campylobacter spp* in human, chickens, pigs and their antimicrobial resistance. J. Vet. Med. Sci. 65(2):161-170.

PATTISON, M., 2001. Practical intervention strategies for campylobacter. J. Appl. Microbiol. 90:121S-125S.

SAHIN, O., KOBALKA, P., ZHANG, Q., 2003. Detection and survival of Campylobacter in chicken eggs. J. Appl. Microbiol. 95:1070-1079.

SAHIN, O., MORISHITA, T.Y., ZHANG, Q., 2002. *Campylobacter* colonization in poultry: sources of infection and modes of transmission. Anim. Health Res. Rev. 3(2):95-105.

Evaluation of an egg yolk enzyme-linked immunosorbent assay antibody test and its use to assess the prevalence of *Mycoplasma gallisepticum* infection in laying hens in Italy

Giovanni Tosi, Roberto Leonelli, Marco Tamba, Roberto Calabrese

Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna. Forlì, Italy

Corresponding author: Dr. Giovanni Tosi. Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna. Via Marchini 1, 47100 Forlì, Italy – Tel. +39 0543 721533 – Fax: +39 0543 721533 – Email: gbattista.tosi@bs.izs.it

ABSTRACT

The prevalence of *Mycoplasma gallisepticum* infection in commercial layers was established by the presence of antibodies in eggs. Saline-extracted yolks were used with a commercial enzyme-linked immunosorbent assay kit. For the prevalence study, yolks from 30 eggs were obtained from each of 66 flocks coming from 36 layer farms. The prevalence of egg antibodies to *Mycoplasma gallisepticum* was 33.3% in single-age farms and 77.8% in multi-age farms. In 27 flocks, antibody titers were compared with results obtained from blood samples taken in the same flock and in the same period and analyzed with the same kit. This study has confirmed that egg yolk enzyme-linked immunosorbent assay antibody test is a suitable and practical approach for assessing the flock prevalence of *Mycoplasma gallisepticum* infection in layer hens.

Key Words: Layer hens, *Mycoplasma gallisepticum*, ELISA test, Egg yolk.

RIASSUNTO

APPLICAZIONE DI UN TEST IMMUNOENZIMATICO PER LA DETERMINAZIONE DEGLI ANTICORPI NEL TUORLO E SUO IMPIEGO NELLA VALUTAZIONE DELLA PREVALENZA DELL'INFEZIONE DA *MYCOPLASMA GALLISEPTICUM* NEGLI ALLEVAMENTI DI GALLINE OVAIOLE DA CONSUMO IN ITALIA

La prevalenza dell'infezione da Mycoplasma gallisepticum *in allevamenti di galline ovaiole da consumo è stata determinata attraverso la valutazione del titolo anticorpale nel tuorlo (dopo estrazione in soluzione salina) mediante un test immunoenzimatico commerciale. Per lo studio di prevalenza sono stati esaminati campioni di 30 uova provenienti ciascuno da 66 gruppi di galline ovaiole appartenenti a 36 aziende. In 27 gruppi i titoli anticorpali rilevati nel tuorlo sono stati confrontati con quelli riscontrati in analoghe campionature di sangue. La prevalenza di anticorpi nel tuorlo nei confronti di* Mycoplasma gallisepticum *è stata del 33,3% in allevamenti costituiti da un'unica unità produttiva e del 77,8% in allevamenti multi-età. Lo studio ha inoltre confermato che la determinazione del titolo anticorpale nel tuorlo nei confronti di* Mycoplasma gallisepticum *fornisce risultati sovrapponibili a quelli ottenuti dalla medesima ricerca eseguita in campioni di sangue. Rispetto a quest'ultima, inoltre, fornisce alcuni vantaggi di ordine pratico.*

Parole chiave: *Galline ovaiole,* Mycoplasma gallisepticum*, Test ELISA, tuorlo.*

Introduction

Mycoplasma gallisepticum (MG) is an important pathogen of poultry worldwide. MG infection is considered an important problem in broilers, breeders and commercial layers. Economic losses in the poultry industry caused by this infection are significant. In breeders and layers the disease causes a 10% to 20% decrease in egg production (nearly fewer 16 eggs per hen) and a 5% to 10% increase in embryo mortality. In addition MG cause respiratory disease commonly complicated by other agents such as *Escherichia coli*. The mortality can be low in uncomplicated disease but may reach 15-

20% in complicated outbreaks. Few data are available about the prevalence of MG infection in commercial layers in Italy. In routine work detection of MG infection may be achieved by detecting antibodies against MG in the host organism. For this purpose various tests are used including serum plate agglutination (SPA), haemagglutination-inhibition (HI) and enzyme-linked immunosorbent assay (ELISA). Several ELISA kits are available. This test is more specific than SPA and more sensitive than HI. In addition, ELISA test is used for testing of yolk samples.

The aims of this study were the following: 1) to evaluate a commercial ELISA kit when used to measure MG antibodies in eggs. 2) To study the prevalence of MG infection in layer farms located in different parts of Italy.

Material and methods

ELISA test

The ProFLOK® MG ELISA kit (Synbiotics corporation®) was used in this study according to the manufacturer's instructions. The absorbance was read at 405 nm on a Biorad 550 microplate reader (BIORAD®). In order to be valid the mean negative control absorbance should be below 0.200 and the corrected positive control value range should be between 0.250 and 0.900. The serum/positive ratio (SP) was calculated as: (mean of test sample – mean of negative control) / (mean of positive control – mean of negative control). An MG titer was calculated by the following suggested equation: Log_{10} titer= (1.464 X Log_{10} SP) + 3.197. Yolk samples were pre-diluted (1:10) using phospate buffer solution (PBS). According to the manufacturer's instructions the SP and the ELISA titer was interpreted using the following value ranges: SP less than 0.200 (titer range 0)= negative; SP 0.200 to 0.599 (titer range 149 to 743)= probable or not conclusively; SP greater or equal to 0.600 (titer 744 or greater)= positive. A flock was considered positive when antibody titer was greater than 744 in more than 10% of the tested samples. In multi-age farms, a farm was considered positive when one or more production unit (flock) resulted positive.

Prevalence study

Thirty eggs/flock were collected. In some flocks blood samples were also obtained. For every flock, anamnestic data including age, production type (cage or floor farms), location of the farm, number of animal housed and clinical signs were registered.

Statistical analysis

The geometric mean titers and the standard deviations for every sample of yolk and blood were calculated. Comparison between serum and yolk titers were made by a *t*-test.

Results and discussion

Prevalence study

During the period November 2003 – January 2004, 36 commercial layer farms located in 7 different Italian regions were monitored (Table 1). Overall, eggs from 66 flocks were tested. In 27 flocks blood samples were also examined. Approximately 2,200,000 of housed layers were involved in this study. 33 of 66 flocks tested resulted positive. The correlation between MG antibody *status* of the flock and the observation of clinical

Table 1. Geographical distribution of tested farms in Italy.

Region	Single-age farms	Multi-age farms	Total
Emilia Romagna	5	11	16
Veneto	3	5	8
Lombardia	5	1	6
Marche	3	0	3
Toscana	0	1	1
Piemonte	1	0	1
Campania	1	0	1

Table 2. Correlation between MG antibody *status* and clinical signs.

MG antibody status / clinical signs	n. flocks
MG+ / clinical signs +	14
MG+ / clinical signs -	19
MG- / clinical signs +	8
MG- / clinical signs -	25
Total	66

signs related to MG infection (respiratory signs and/or drop in egg production and/or decreased egg shell quality) is summarized in table 2. 14 of 18 multi-age farms resulted positive for MG antibodies giving a prevalence of 77.8%. In single-age farms the prevalence was 33.3%.

Comparison between yolk and serum titers

Comparison between blood and yolk titers were made in 27 flocks. Descriptive statistics are reported in table 3. For all tested flocks no significant differences in level of antibodies were detected between antibody titers in layer serum and these obtained in egg yolk (t=0.947; d.f.=1013; p=0.344) (Figure 1).

Conclusions

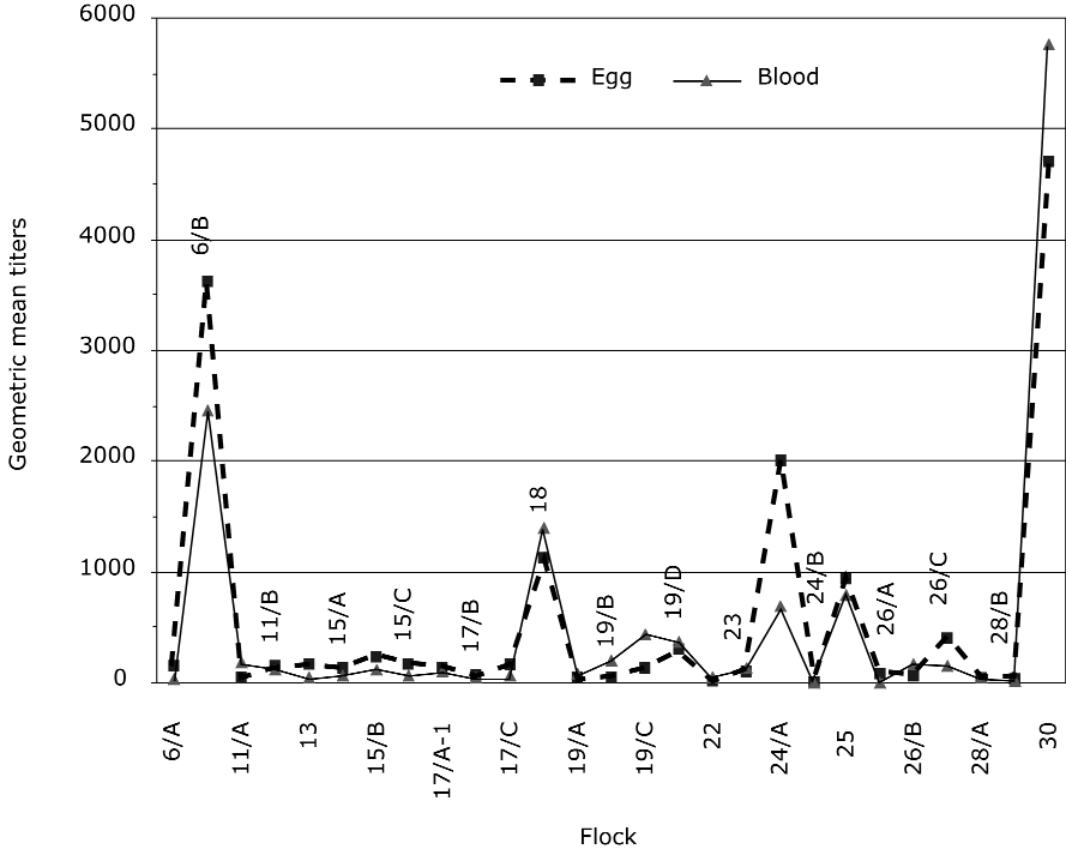
The findings from the prevalence study indicated that MG infection was higher in multi-age farms. MG infection persists in the flock indefinitely and the chickens may shed the organism intermittently, especially following a period of stress. As MG free pullets are brought onto the multi-age complex, they are exposed to MG infection at the onset of egg production. This cycle of spread continues in a complex with new flock introductions (Levisohn *et al.*, 2000). In many cases detection of MG antibodies were not associated to clinical signs. A possible explanation could be the variability of clinical symptoms of MG between species. Infected adult chickens may show no outward signs if the infection is uncomplicated (Stipkovits *et al.*, 1996).

Previous studies indicates that chloroform extraction of the egg yolks is the more suitable method to obtain a greater distinction between positive and negative samples (Mohammed *et al.*, 1986; Hagan *et al.*, 2004). A possible explanation of the benefits of this method of extraction is that chloroform removes the lipids from the samples that otherwise interfere with the binding of antibodies to the antigen. However in our study no significant differences were observed between antibody titers obtained in blood samples e these detected in egg yolk after saline extraction. Testing eggs for routine screening of MG infection avoids the expense of blood sampling, the need for trained staff and subjecting the birds to the stress of being handled. In addition, sampling personnel can spread infection from farm to farm. Finally, eggs are easy to collect and inexpensive.

Table 3. Descriptive statistics.

Statistical data	Egg yolk	Blood
Number of tested samples	810	205
Geometric mean	156.8	137.3
Arithmetic mean	664.1	734.7
Median	132	115
SD	1310.9	1613.9
Min.	1	1
Max.	7762	9333

Figure 1. Comparison between geometric mean titers obtained in blood and in egg yolk samples.



The authors thank the technicians of the laboratory of Serology, Istituto Zooprofilattico di Forlì (Sara Turchi, Stefano Forlivesi and Leonardo Leoni) involved in this study. Thank also to all the vets who submitted the samples used in the study.

REFERENCES

Hagan, J.C., Ashton, N.J., Bradbury, J.M., Kenton, L.M., 2004. Evaluation of an egg yolk enzyme-linked immunosorbent assay antibody test and its use to assess the prevalence of *Mycoplasma synoviae* in UK laying hens. Avian Pathol. 33:93-97.

Levisohn, S., Kleven S.H., 2000. Avian mycoplasmosis *(Mycoplasma gallisepticum)*. Rev. Sci. Tech. OIE. 19:425-442.

Mohammed, H.O., Yamamoto, R., Carpenter, T.E., Ortmayer, H.B., 1986. Comparison of egg yolk and serum for the detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* antibodies by enzyme-linked immunosorbent assay. Avian Dis. 30:398-407.

Stipkovits, L., Kempf, I., 1996. Mycoplasmosis in poultry. Rev. Sci. Tech. OIE. 15:1495-1525.

Plasma corticosterone levels in laying hens from three different housing systems: preliminary results

Maria Pia Franciosini, Claudio Canali, Patrizia Casagrande Proietti, Omar Tarhuni, Elena Fringuelli, Giampaolo Asdrubali

Dipartimento di Scienze Biopatologiche Veterinarie. Università di Perugia, Italy

Corresponding author: Dr. Maria Pia Franciosini. Dipartimento di Scienze Biopatologiche Veterinarie. Facoltà di Medicina Veterinaria, Università di Perugia. Via S. Costanzo 4, 06123 Perugia, Italy – Tel. +39 075 5857768 – Fax: +39 075 5857738 – Email: mpfra@unipg.it

ABSTRACT

Adrenocortical and thyroidal hormones are physiological indicators of various forms of stress in the fowl. In order to establish possible variations in corticosterone levels, blood samples were collected from ISA Brown hens reared in three different housing systems (cage, floor and organic way). Results showed that corticosterone concentrations were highest in caged hens, intermediate in organic reared hens and lowest in floor reared hens. It could be assumed that in the last one system birds have an adequate space in controlled environment that permits them to satisfy, though partially, their behavioural needs without the presence of different chronic stress factors acting in the other systems.

Key Words: Laying hen, Housing systems, Corticosterone, Plasma.

RIASSUNTO

LIVELLI DI CORTICOSTERONE PLASMATICO IN GALLINE OVAIOLE IN TRE DIFFERENTI SISTEMI DI ALLEVAMENTO: RISULTATI PRELIMINARI

Sono state valutate possibili variazioni nei livelli di corticosterone in galline allevate in gabbia, a terra e seguendo il metodo biologico. I livelli più alti di corticosterone si sono riscontrati in gabbia mentre quelli più bassi a terra. Una situazione intermedia era ascrivibile al gruppo allevato biologicamente. E' verosimile ipotizzare che l'allevamento a terra, fornendo alle galline un adeguato spazio, permetta di soddisfare alcune loro esigenze comportamentali senza la presenza di fattori di stress cronico che agiscono sugli altri allevamenti.

Parole chiave: *Galline ovaiole, Sistemi di allevamento, Corticosterone, Plasma*

Introduction

Conditions of intensive farming often deprive animals of stimuli important for the general welfare. This is a potential cause of stress and in a number of cases the lack of stimuli and or space has been associated with behavioural and endocrinological changes indicative of stress and reduced well being. Adrenocortical and thyroidal hormones are physiological indicators of various forms of stress in the fowl (Edens and Siegel, 1975). Elevated plasma corticosterone levels have been associated with thirst, hunger or heat stress in laying hens (Beuving and Vonder, 1978). Management conditions also have been shown to influence plasma corticosterone levels in laying hens (Koelkebeck and Cain, 1984; Mashaly *et al.*, 1984; Cheng and Muir, 2004). The aim of this work was to determine whether or not there are plasma corticosterone variations in laying hens reared in three housing systems (cages, floor and organic way).

Material and methods

Investigations were performed in a farm consisting of 32,000 commercial ISA Brown hens, housed at 16 weeks of age in three different housing systems: 26,000 hens (A) were located in battery (4 birds per 50 x 50 cm sized cages), 3000 (10 per square/meters) were reared on floor (B) in conventional house and 3000 in organic(C) way according to Council Regulation (EC) N.1804/99. The birds reared in floor and in cages were fed on layers mash and a similar photoperiod was provided in both systems by supplementary electric lighting (16L:8D). Blood samples were collected monthly from 20 birds from each management systems. The first blood sampling was performed when the birds were 30 weeks old. Extreme care was taken to minimize handling stress and to randomize treatment samplings. Time from initial bird contact through sample collection was monitored. The corticosterone concentration was measured by Radioimmunoassay using the commercial kit (Gamma-B^{125}I-Corticosterone RIA, PANTEC Torino). The data were examined for statistical differences by analysis of variance using the variance to one way and t Student's test.

Results and discussion

The results of this study, reported in table 1 indicated that plasma corticosterone concentrations were highest in caged hens, (26.4 ng/ml) intermediate in organic (25.3 ng/ml) hens and lowest in floor-reared hens (21.5 ng/ml). If plasma corticosterone status of birds may provide a measure of welfare of birds in each system, it could be speculated that in the present study the floor-reared hens were exposed to fewer stress factors than those in caged, with the organic rearing occupying an intermediate position. It is to underline that this study was performed when the social interactions into the cages, often responsible for the increase of plasma corticosterone (Mashaly *et al.*, 1982) were established from time. Previous studies (Compton *et al.*, 1981) reported that a decrease in space could chronically elevate corticosterone levels in response to alteration in "personal space", particularly when the space allowance was below 400 cm^2/birds. It is also known that the intensive farming conditions often deprive animals of access to stimuli that may be of significance for the performance of the normal behavioural needs (dustbathing, lack of nests); this could be a potential cause of a chronic stress. However this conclusion should be considered with caution since the discrepancy of results was observed in different studies. Koelkebeck and Cain (1984) indeed reported the lowest mean plasma corticosterone levels occurred among hens in cage and the highest on litter, indicating that the social relationship within a large group may act as a greater stress factor than conditions found in small groups reared in cage.

Data obtained from organic hens (Table 1) showed that mean corticosterone levels were higher then those reported in hens reared on floor, though the organic system should be the best in satisfying the animal welfare. The results related to corticosterone plasma levels in organic hens also showed significant differences among the three blood samplings. On April, indeed, we observed an increase of corticosterone, likely explained by the fact that in this month the hens started to come out and many uncontrolled agents could act as chronic stress factors (prey presence, a number of visual and olfactory stimulation). The corticosterone reduction detected in the last sampling (20.97 ng/ml) may be sign of establishing of an increasing "environmental confidence" from the hens.

In our study the lowest corticosterone levels were detected in floor reared hens. It seems reasonable to speculate that in this rearing system birds have an adequate space allowance in controlled environment that permits them to satisfy, though partially, their behavioural needs without the presence of various stress factors acting in the other systems. The time of blood sampling collection should not have any influence on the corticosterone concentration since blood was taken within 1 min. in each bird from all systems. Craig and Craig (1985) showed that delays of 2 and 3 min. after catching have little, if any, effect on basal plasma corticosterone concentration.

Table 1. Mean plasma corticosterone levels and standard deviation in differently housed hens in relation to the date of sampling.

Sampling date		Cage (a)		Floor (b)		Organic (c)		Significance
		Mean	SD	Mean	SD	Mean	SD	
March '04	Corticosterone	27.25	5.11	20.04	5.63	25.91	5.40	*a-b* P<0.01 *b-c* P<0.01
	Temp. °C	22-23		20		20		*a-c* ns
April '04	Corticosterone	25.69	7.94	22.63	5.91	28.91	6.90	*a-b* ns *b-c* P<0.01
	Temp. °C	22-23		22		23		*a-c* ns
May '04	Corticosterone	25.71	5.59	21.73	5.11	20.97	6.90	*a-b* P<0.05 *b-c* ns
	Temp. °C	22-23		24		24		*a-c.* P<0.05
Total	Corticosterone	26.4	6.3	21.5	5.6	25.3	7.2	*a-b* P<0.01 *b-c* P<0.01 *a-c* ns

ns: not significant

Conclusions

Further investigations are necessary to establish if plasma corticosterone can be an useful measure of long term stress or welfare in hens since the physiological level of the "stress" hormone in the hens is not still well defined and there are difficulties with the interpretation of circulating hormone concentrations due to diurnal patterns and to the sampling procedures. It may be corrected in concluding that other parameters, analogously to other species (Barone *et al.*, 2003), as practical estimates of welfare should be considered in different housing conditions, particularly in organic farming, where the corticosterone response may be dependent on a complex interaction with a number of other environmental variables difficult to identify.

REFERENCES

BARONE, A., CANALI, C., DIVERIO, S., FEDERICI, C., PELLICCIA, C., 2003. The effects of farming system and slaughtering on rabbit welfare. Page 238 in Proc. 10th Int. Sym. UFAW, Edinburgh, UK.

BEUVING, G., VONDER, G.M.A., 1978. Effect of stressing factors on corticosterone levels in the plasma of laying hens. Gen. Comp. Endocr. 35:153-159.

CHENG, H.W, MUIR, W.M., 2004. Chronic social stress differentially regulates neuroendocrine responses in laying hens: II. Genetic basis of adrenal responses under three different social conditions. Psychoneuroendocrinology. 29:961-971.

COMPTON, M.M., VAN KREY, H.P., RUSZLER, P.L., GWASDAUSKAS F.C., 1981. The effect of claw removal and cage design on the production performance, gonadal steroids and stress response in caged laying hens. Poultry Sci. 60:2127-2135.

CRAIG, J.V., CRAIG, J.A., 1985. Corticosteroid level in White Leghorn hens as affected by handling, laying-house environment, and genetic stock. Poultry Sci. 64:809-816.

EDENS, F.W., SIEGEL, H.S., 1975. Adrenal responses in high and low ACTH response lines of chickens during acute heat stress. Gen. Comp. Endoc. 25:64-73.

KOELEBECK, K.W., CAIN, J.R., 1984. Performance, behaviour, plasma corticosterone and economic returns of laying hens in several managements alternatives. Poultry Sci. 63:2123-2131.

MASHALY, M.M., WEBB, M.L., ROUSH, W.B., 1982. Adrenal gland response of laying hens to different cage densities. Poultry Sci. 61:1506 (Abstr.).

MASHALY, M.M., WEBB, M.L., YOUTZ, S.L., ROUSH, W.B., GRAVES, H.B., 1984. Changes in serum corticosterone concentration of laying hens as a response to increased population density. Poultry Sci. 63:2271-2274.

Antimicrobial susceptibility of *Salmonella* spp. strains isolated from Layer Hens in Campania Region from 2000 to 2003

Ludovico Dipineto, Claudia Scarpetta, Mariarosaria Calabria, Mariangela Sensale, Antonio Baiano, Lucia Francesca Menna, Alessandro Fioretti

Dipartimento di Patologia e Sanità Animale. Università di Napoli Federico II, Italy

Corresponding author: Prof. Alessandro Fioretti. Dipartimento di Patologia e Sanità Animale. Facoltà di Medicina Veterinaria, Università di Napoli Federico II. Via Delpino 1, 80137 Napoli, Italy – Tel. +39 081 451802 Fax: +39 081 5091993 – Email: fioretti@unina.it

ABSTRACT

The aim of this study was to determine the antimicrobial resistance in 60 Salmonella strains (*S. enteritidis, S. thyphimurium, S. gallinarum*) isolated from layer hens in Campania region from 2000 to 2003. *S. gallinarum* showed resistance against ciprofloxacin and enrofloxacin, in contrast, *S. enteritidis* and *S. typhimurium* were fully susceptibile. In all of isolates high levels of resistance were observed for neomycin, gentamicin and oxytetracycline. Also, one significant observation was that all of the isolates showed full susceptibility to Sulphamethoxazole/Trimethoprime. These results suggest importance to restrict the use of antibiotics in layers hens flocks in order to reduce the selection and spread of multiresistant strains.

Key Words: Layer Hens, Antimicrobial resistance, *Salmonella*, Campania region.

RIASSUNTO

SENSIBILITÀ AGLI ANTIBIOTICI DI CEPPI DI SALMONELLA SPP. ISOLATI DA GALLINE OVAIOLE IN CAMPANIA NEL TRIENNIO 2000/2003

*Scopo del presente lavoro è stato quello di testare la sensibilità antibiotica di 60 ceppi di Salmonella (*S. enteritidis, S. typhimurium, S. gallinarum*) isolati da galline ovaiole nel periodo compreso tra il 2000 e il 2003.* S. gallinarum *mostrava resistenza nei confronti di ciprofloxacina ed enrofloxacina (rispettivamente 15% e 23%), al contrario di* S. enteritidis *e* S. typhimurium *che manifestavano una completa sensibilità. Tutti i sierotipi valutati presentavano alte percentuali di resistenza nei confronti di neomicina, gentamicina e ossitetraciclina. Nei confronti dei sulfamidici i ceppi testati presentavano resistenza nulla. Tali risultati suggeriscono un uso più moderato e mirato degli antibiotici negli allevamenti in modo da ridurre la selezione e diffusione di ceppi multiresistenti.*

Parole chiave: *Galline ovaiole, Antibiotico-resistenza,* Salmonella*, Campania.*

Introduction

Antimicrobial resistance is the capacity of bacteria to survive exposure to a defined concentration of an antimicrobial substance. It is the natural response of bacterium to defend itself against the effects of an antibiotic. The development of antimicrobial resistance is an ecological phenomenon. Any antibiotic use, whether in humans, animals or plants/environment may lead to resistance (OIE, 2003).

The extensive use of antibiotics, not only in human and veterinary medicine, but also in livestock production for disease prevention or as growth-promoting feed additives, has led to a serious increase in, and spread of, multiple antibiotic-resistant bacteria (Cruchaga *et al.*, 2001).

All this caused considerable problems to

approach prophylactics and therapeutics plans versus various bacterial pathologies.

Salmonellosis has a particular role in avian medicine whether host-specific serotypes (*S. gallinarum, S. pullorum*) or non-host specific serotypes (*S. enteritidis, S. typhimurium*) implicated in foodborne zoonoses.

Within the routine control programmes carried out in the poultry farms organized from the Avian Pathology Section of the Dipartimento di Patologia e Sanità Animale in University of Bologna, Italy; the Salmonella strains isolated showed an increase of antibiotic resistance pattern.

The aim of this study was to determine the antimicrobial resistance in Salmonella strains (*S. enteritidis, S. thyphimurium, S. gallinarum*) isolated from layer hens in Campania region from 2000 to 2003.

Material and methods

Sample collection

From January 2000 to November 2003 a total of 60 Salmonella strains were isolated from layer hens flocks, respectively belonged to *S. gallinarum, S. enteritidis, S. typhimurium*. The strains collected were 20 for each serotype.

Isolation and identification procedure

The Salmonella isolation procedures were carried out following the WHO standard methods (WHO, 1994). All the strains were serotyped at National Reference Centre for Salmonella (Istituto Zooprofilattico Sperimentale delle Venezie, Padova - Italy).

Antimicrobial susceptibility tests

Antimicrobial susceptibility profiles of the isolates were determined by the disk diffusion method according to the NCCLS guidelines (National Committee for Clinical Laboratory Standards, 2002). The antimicrobial agents (Oxoid) tested and corresponding concentration were as follows: Ciprofloxacin 5μg (C), Enrofloxacin 5μg (E), Flumequine 30μg (F), Nalidixic acid 30μg (NA), Apramycin 15μg (AP), Amoxicillin 10μg (A), Neomycin 30μg (N), Gentamicin 10μg (G), Oxytetracycline 30μg (O), Sulphamethoxazole/Trimethoprime 25μg (S/T).

The diameters of the inhibition zone for the interpretation of resistance and susceptibility were those recommended by the NCCLS (National Commitee for Clinical Laboratory, 2002). Results were scored as susceptible, moderately susceptible or resistant according to NCCLS criteria (2002). The reference strain used was *Escherichia coli* ATCC 25 922.

Results and discussion

As seen from Table 1, resistance of *S. gallinarum* was significantly higher than other two serotypes examined. In particular, *S. gallinarum* showed resistance against two fluoroquinolone (ciprofloxacin and enrofloxacin, respectively 15% and 23%), in contrast, *S. enteritidis* and *S. typhimurium* were fully susceptibile. In all of isolates high levels of resistance were observed for neomycin, gentamicin and oxytetracycline. It was also found that *S. enteritidis* and *S. gallinarum* were resistant to apramycin (33.3% and 38.5% respectively) and *S. gallinarum* was resistant

Table 1. Antimicrobial resistance of 60 *Salmonella spp.* isolates.

Serotype	n.	Antimicrobial resistance (%) against antimicrobial agents tested									
		C	E	NA	AP	A	N	G	F	S/T	O
S. enteritidis	20	0	0	6	33.3	0	55.5	38.8	5.5	0	33.3
S. typhimurium	20	0	0	13	0	0	12.5	25	12.5	0	50
S. gallinarum	20	15	23	15	38.5	23.1	53.8	55.3	23.1	0	46.1

C=Ciprofloxacin, E=Enrofloxacin, F=Flumequine, NA=Nalidixic acid, AP=Apramycin, A=Amoxicillin, N=Neomycin, G=Gentamicin, O=Oxytetracycline, S/T=Sulphamethoxazole/Trimethoprime

to amoxicillin too (23.1%). In conclusion, one significant observation was that all of isolates showed full susceptibility to Sulphamethoxazole/Trimethoprime.

An increase in the incidence of antibiotic resistance in Salmonella isolated from humans and animals related to exhaustive application of antibiotics in both groups has been documented worldwide (Chruchaga *et al.*, 2001). Recently, Lee *et al.* (Lee *et al.*, 2003) reported, in an antimicrobial susceptibility test against 258 isolates of *S. gallinarum*, a reduced susceptibility to ampicillin (13.0%), gentamicin (43.4%), kanamycin (69.6%), enrofloxacin (6.5%), ciprofloxacin (10.9%), norfloxacin (52.5%) and ofloxacin (82.6%). A study on antimicrobial-resistant Salmonella enterica serovars isolated from chickens in Spain showed high percentage of resistance to chloramphenicol (44.6%), ampicillin (34.8%) and tetracycline (33.9%) (Hernandez *et al.*, 2002). Jones *et al.* (2002) reported *S. typhimurium* strains resistant to ampicillin, sulphonamides, streptomycin, chloramphenicol and tetracyclines as well as *S. typhimurium* isolated from poultry resistant to nalidixic acid (Jones *et al.*, 2002).

Fluoroquinolones resistance was rarely found among Salmonella species until Heisig reported *S. typhimurium* serovar *Copenhagen* from cattle was highly resistant to ciprofloxacin (Heisig, 1993).

Conclusions

An important finding is the antimicrobial resistance observed in *S. gallinarum* against fluoroquinolones (ciprofloxacin and enrofloxacin). High percentage of resistance observed in *S. typhimurium* and *S .enteritidis* for neomycin, gentamycin and oxytetracycline demonstrate improper use of these antibiotics in the control of avian salmonellosis, particularly in metaphylactic sense.

These results confirm importance to restrict the use of antibiotics in layers hens flocks in order to reduce the selection and spread of multiresistant strains and underlines the need for integrated surveillance systems of antibiotic resistance that consider isolates not only from human disease but also from the animal reservoirs and the food vehicles.

We would like to thank Dr. Antonia Ricci, National Reference Centre for Salmonella - Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy, for the serotyping of our isolates and Mrs. Fortuna Pisa for her technical collaboration.

REFERENCES

CRUCHAGA, S., ECHEITA, A., ALADUENA, A., GARCIA-PENA, J., FRIAS, N., USERA, M.A., 2001. Antimicrobial resistance in salmonellae from humans, food and animals in Spain in 1998. J. Antimicrob. Chemoth. 47:315-321.

HEISIG, P., 1993. High-level fluoroquinolone resistance in a Salmonella typhimurium isolate due to alterations in both gyrA and gyrB genes. J. Antimicrob. Chemoth. 32:367-377.

HERNANDEZ, T., RODRIGUEZ-ALVAREZ, C., AREVALO, M.P., TORRES, A., SIERRA, A., ARIAS, A., 2002. Antimicrobial-resistant Salmonella enterica serovars isolated from chickens in Spain. J. Chemotherapy. 14:346-350.

JONES, Y.E., CHAPPELL, S., MCLAREN, I.M., DAVIES, R.H., WRAY, C., 2002. Antimicrobial resistance in Salmonella isolated from animals and their environment in England and Wales from 1988 to 1999. Vet. Rec. 150(21):649-654.

LEE, Y.J., KIM, K.S., KWON, Y.K., TAK, R.B., 2003. Biochemical characteristics and antimicrobials susceptibility of *Salmonella gallinarum* isolated in Korea. J. Vet. Sci. 4(2):161-166.

NATIONAL COMMITEE FOR CLINICAL LABORATORY STANDARDS, 2002. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, second edition: approved standard M31-A2. NCCLS, Wayne, PA, USA.

OFFICE INTERNATIONAL DES ÉPIZOOTIES, 2003. OIE International Standards on Antimicrobial Resistance. OIE, Paris, France.

WORLD HEALTH ORGANIZATION, 1994. Guidelines on detection and monitoring of Salmonella infected poultry flocks with particular reference to Salmonella enteritidis. C. Wray and R.H. Davies eds.,WHO Veterinary Public Health Unit, Geneva, Switzerland.

Field trials with the use of a live attenuated temperature-sensitive vaccine for the control of *Mycoplasma gallisepticum* infection in meat-type turkeys

Enrico Alessandri[1], Paola Massi[2], Francesca Paganelli[2], Francesco Prandini[3], Mario Saita[4]

[1] Gruppo PAI. Malo (VC), Italy
[2] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna. Forlì, Italy
[3] Merial Italia S.p.A. Assago (MI), Italy
[4] Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria. Università di Milano, Italy

Corresponding author: Dr. Francesco Prandini. MERIAL Italia S.p.A.. Milanofiori Strada 6 Palazzo E/5, 20090 Assago (MI), Italy – Tel. +39 02 577661 – Fax: +39 02 57766307 – Email: Francesco.prandini@merial.com

ABSTRACT

Mycoplasma gallisepticum (MG) continues to be an important pathogen of poultry, causing significant production losses in many parts of the world. Eradication is the preferred method of control but it could result impractical after the organism has been introduced in an area with high density of poultry farms. TS-11®, a temperature-sensitive live attenuated MG vaccine, is currently utilized in several countries for the control of MG infections in commercial layers and broiler breeders. In the present field trial, conducted in an industrial meat-turkey farm (belonging to an integrated company), previously affected by severe MG infections, the ability of TS-11® in effectively colonizing the upper respiratory tract in a turkey flock was evaluated ("TS-11®" flock). A second flock grown in an adjacent pen of the same farm was vaccinated with an inactivated MG vaccine ("Inactivated" flock). Polymerase Chain Reaction (PCR) and Random Amplified Polymorphic DNA (RAPD) analysis were applied for the detection and differentiation of TS-11® from other MG strains possibly present in the same flocks, such as the field strains and the 6/85 live vaccine strain currently utilized in commercial layers in Italy. PCR-RAPD results achieved in the "TS-11®" flock were compared with those of a flock of turkey grown in the same farm but vaccinated with an inactivated MG vaccine. Encouraging results were achieved by means of PCR-RAPD detection of TS-11® from all of the samples up to eight weeks post vaccination, whereas it was never detected in the "Inactivated" flock. Moreover, the field strain was never detected in the "TS-11®" flock but in the "Inactivated" one it was detected either 5 and 8 weeks after the vaccination. The aggregate production data of the two flocks resulted significantly improved when compared to the performance of the previous flocks grown in the same farm and similar to the production standard of the integrated company.

Key Words: Turkeys, *Mycoplasma gallisepticum*, Live vaccine, Polymerase Chain Reaction (PCR), Random Amplified Polymorphic DNA (RAPD).

RIASSUNTO

PROVE DI CAMPO NELL'IMPIEGO DI UN VACCINO VIVO ATTENUATO TERMO-SENSIBILE PER IL CONTROLLO DELL'INFEZIONE DA *MYCOPLASMA GALLISEPTICUM* IN TACCHINI DA CARNE

Mycoplasma gallisepticum *(MG) continua ad essere un importante agente patogeno del pollame, dove provoca gravi perdite produttive in diverse aree del mondo. L'eradicazione rappresenta il miglior metodo per il controllo di MG, tuttavia può risultare difficilmente praticabile una volta che l'infezione è stata introdotta in un'area caratterizzata da elevata concentrazione di allevamenti avicoli. TS-11®, un vaccino MG vivo attenuato termo-sensibile, viene attualmente impiegato in diversi Paesi del mondo per il controllo delle infezioni da MG nelle galline ovaiole commerciali e nei riproduttori pesanti. Nella prova di campo, condotta in un allevamento commerciale di tacchini da carne (appartenente ad un gruppo avicolo integrato), colpito nei cicli produttivi precedenti da gravi infezioni MG, è stata valutata la capacità di TS-11® di colonizzare efficacemente le vie respiratorie superiori (gruppo "TS-11®"). Un secondo gruppo allevato in un*

capannone adiacente nella stessa azienda è stato vaccinato con un vaccino inattivato (gruppo "Inattivato"). Le tecniche PCR e RAPD sono state applicate per l'individuazione e la differenziazione di TS-11® da altri ceppi di MG eventualmente presenti negli stessi gruppi, quali i ceppi di campo ed il vaccino vivo 6/85 attualmente utilizzato nelle ovaiole commerciali in Italia. I risultati dei test PCR-RAPD ottenuti nel gruppo "TS-11®" sono stati comparati con quelli del gruppo "Inattivato", allevato in un capannone adiacente, nella stesso allevamento. Sono stati così ottenuti risultati incoraggianti, infatti TS-11® è stato individuato sistematicamente su tutti i campioni prelevati tra le 2 e le 8 settimane dopo la vaccinazione mentre non è mai stato identificato nei tamponi prelevati dal gruppo "Inattivato". Inoltre, il ceppo di campo non è mai stato evidenziato nel gruppo "TS-11®" mentre nel gruppo "Inattivato" i tamponi tracheali sono risultati positivi rispettivamente a 5 e 8 settimane post-vaccinazione. I dati produttivi complessivi sono risultati significativamente migliori rispetto alle performance dei cicli precedenti allevati nella stessa azienda ed in linea con lo standard produttivo del gruppo avicolo integrato.

Parole chiave: *Tacchino,* Mycoplasma gallisepticum*, Vaccino vivo,* PCR, RAPD.

Introduction

Mycoplasma gallisepticum (MG) plays an important role as pathogen of poultry, causing significant economic losses, particularly in the areas where higher is the concentration of industrial farms. In meat-type turkeys infected with MG, severe respiratory syndromes occur with losses due to increased mortality rate and condemnation of carcasses, poor performance and increased medication costs.

Strategies to minimize if not eliminate the impact of MG infections can include eradication programmes, specific antimicrobial treatments and immunization with live or inactivated vaccines.

TS-11® is a temperature-sensitive live attenuated MG vaccine, currently utilized in Italy and many other Countries for the control of MG infections in commercial layers. The vaccination of broiler chicken breeders prevented the infection by field MG in their tracheas and infraorbital sinuses and in the vitelline membrane of their embryos (Barbour *et al.*, 2000).

In spite of its use on a large scale in commercial layers in the United States, it was never isolated from field turkeys (Kleven *et al.*, 2000).

In a vaccination trial conducted by Kleven (2000), administration of TS-11® to meat turkeys resulted safe and there was some protection detected against lesions in the upper respiratory tract, thus indicating a potential use in certain conditions. During the last years, in some densely poultry populated areas of Northern Italy, an increasing number of severe MG outbreaks, occurred consecutively in the same industrial turkey farms; either individual and mass antimicrobial treatments were not able to control the clinical disease. The promiscuity of the staff and the equipment utilized in the farms of the same area was probably critical in perpetuating the infection, in spite of an all in-all out policy applied in every single farm.

The main purpose of the present trial, conducted in one of the farms, (belonging to an integrated company), affected by MG infections in the previous cycles, was to examine the ability of TS-11® in colonizing in the mucosa of the upper respiratory tract of turkey and to evaluate the persistency and its possible effect in inducing protection against field MG strains.

PCR and RAPD analysis (Paganelli *et al.*, 2003) applied on tracheal swabs drawn at regular intervals from turkey flocks, enabled the detection and the genetic differentiation of TS-11® from the field strains.

Material and methods

MG vaccines

TS-11® (Merial Italia S.p.A.), a frozen temperature-sensitive live attenuated MG vaccine (Whithear *et al.*, 1990), was stored at –20°C then thawed in a water bath at 30 to 35°C just prior to the administration; each bird of the first flock ("TS-11®") received one dose, containing at least $10^{7.4}$ Colour-Changing Units (CCU) in 50 microlitres, applied by ocular instillation.

One dose (0.5 ml) of a commercial inactivated oil emulsified MG vaccine was administered subcutaneously to the second flock ("Inactivated") in the same farm.

MG serological tests

Rapid plate agglutination test (RPAT) for MG antibodies was carried out with a commercial antigen.

A commercial Enzyme-Linked Immuno-Sorbent Assay (ELISA) test kit was used to detect MG antibodies.

DNA extraction and MG identification

The Polymerase Chain Reaction (PCR) protocol was adapted from the procedure described by Lauermann *et al.* (2003) and performed on DNA after extraction from tracheal swabs (Paganelli *et al.*, 2003). Each sample (100-2000 ng/µl) was treated with PCR reagents included in Accuprime TaqPCRx DNA Polymerase (Invitrogen®,USA). We used primers "forward" MG-F+ and primers "reverse" MG-R (Table 1). DNA amplification is performed in a thermocycler (TpersonalTM, Biometra, Germany). The optimized PCR program was as follows: 5 minutes at 94°C (hot start), 35 cycles with 3 steps: 94°C for 30 sec. (denaturation), 55°C for 30 sec. (annealing), 72°C for 1 minute (extension); finally 1 minute at 72°C for final extension. PCR products were separated using electrophoresis on a 1,7% agarose gel containing ethidium bromide for 30 minutes at 80V. MG amplicons were visualized with an ultraviolet transilluminator along with control samples (positive and negative) and PCR marker GeneRuler 100bp DNA ladder (Fermentans®, Lithuania). When MG positive, amplified product presented a 185bp fragment (Paganelli *et al.*, 2002).

RAPD analysis

A further PCR on extracted DNA was performed using a commercial kit (Ready-to go RAPD Analysis – Pharmacia Biotech®, Piscataway, USA) containing a set of primers. Primer 6 was used for the first reaction. A combination of primers 3 and 4 was used for the second reaction (Table 1) (Paganelli *et al.*, 2003). RAPD was performed in a thermocycler (TpersonalTM, Biometra®, Germany). RAPD program was as follows: 5 minutes at 95°C (hot start), 45 cycles with 3 steps: 95°C for 1 minute, 36°C for 1 minute, 72°C for 2 minutes; finally 1 cycle at 4°C. Each reaction included the vaccine strains TS-11 and 6-85.

RAPD products were separated using electrophoresis on a 2% agarose gel containing ethidium bromide for 30 minutes at 80V. MG amplicons (185 bp) were visualized with an ultraviolet transilluminator, comparing *patterns* each other, along with marker GeneRuler 100bp DNA ladder (Fermentans®, Lithuania) (Paganelli *et al.*, 2003).

Field trial design

8500 day-old turkey toms were divided in two flocks of the same size and housed in adjacent pens in the same farm (belonging to an integrated company), previously undertaken to cleaning, disinfection and downtime. Before vaccination 15 swabs were randomly taken from trachea and choanal cleft respectively and 20 blood samples were randomly drawn from the turkeys of each flock to exclude any MG positivity. At three weeks of age "TS-11®" flock was eye-drop vaccinated while "Inactivated" flock was vaccinated subcutaneously. At the age of 5, 8, 11 and 15 weeks respectively, from each of the two flocks were randomly drawn 15 blood samples and 15 tracheal swabs; from every set of 15 swabs 3 pools were obtained and tested by PCR-RAPD.

Due to practical reasons, the production parameters of the two flocks could not be registered separately but the aggregate data were compared with the correspondent data of the previous production cycle.

Table 1. Sequence of RAPD primers.

MG-F	5′ GAGCTAATCTGTAAAGTTGGTC 3′
MG-R	5′ GCTTCCTTGCGGTTAGCAAC 3′
RAPD 3	5′-d [GTAGACCCGT]- 3′
RAPD 4	5′-d [AAGAGCCCGT]- 3′
RAPD 6	5′-d [CCCGTCAGCA]- 3′

Results and discussion

Clinical monitoring detected no reaction after TS-11® vaccination and no considerable treatments were needed during the whole production cycle.

The results of PCR and RAPD monitoring on pooled tracheal swabs are summarized in Table 2. Table 3 shows the serological results. In Table 4 the aggregate performance of the two flocks are compared to the results of the previous cycle and to the production standard of the Integrated Company.

PCR and RAPD analysis enabled the identification of TS-11® in the trachea of turkeys up to 8 weeks after the vaccination. The live vaccine strain was detected only in the TS-11® flock whereas the field infection was demonstrated only in the "Inactivated" flock. Serological results showed a late response in the TS-11® flock, more evident with the RPA Test; however, a possible effect of the field infection could not be ruled out even if not detected by PCR-RAPD techniques. To the knowledge of the authors, data on serological response of turkeys vaccinated with TS-11® are not reported in the relevant literature, yet in a trial conducted in an isolation room, no serological response was detected (unpublished data). In any case the protection against MG seems non correlated with serological titres. Production results of the two flocks, although were not registered separately, showed a significant improvement of all the parameters when compared to those of the previous cycle grown in the same farm and were similar to the production standard of the integrated company.

Table 2. Results of PCR and RAPD tests applied on pools of tracheal swabs drawn at different ages from TS-11® and Inactivated flocks.

Age	3 weeks		5 weeks		8 weeks		11 weeks		15 weeks	
Test	PCR	RAPD	PCR	RAPD	PCR	RAPD	PCR	RAPD	PCR	RAPD
TS-11®	Neg.	-	Pos. 3/3	TS-11®	Pos. 3/3	TS-11®	Pos. 3/3	TS-11®	Neg.	-
Inactivated	Neg.	-	Neg.	-	Pos. 1/3	'Field' MG	Pos. 2/3	'Field' MG	Neg.	-

Table 3. Percentage of turkeys sero-positive to MG at different ages in TS-11® and Inactivated flocks.

Age	3 weeks		5 weeks		8 weeks		11 weeks		15 weeks	
Test	RPAT	ELISA	RPAT	ELISA	RPAT	ELISA	RPAT	ELISA	RPAT	ELISA
TS-11®	0	-	0	0	0	0	100	60	100	0
Inactivated	0	-	0	0	0	80	27	87	100	53

RPAT: Rapid plate agglutination test.

Table 4. Performance compared the previous cycle and to the Company production standard.

Parameters	Age days	Mortality %	Avg. body weight Kg	Feed Conversion	Medication costs Euro/kg
Field trial	137	8.82	18.7	2.57	0.017
Previous cycle	134	13.88	16.0	2.62	0.076
Company standard	135	9.50	18.5	2.56	0.025

Conclusions

The data of PCR and RAPD analysis, even if achieved only by a field trial, enable to suppose the ability of infection of TS-11® in the upper respiratory tract of turkey and the possibility of a protection against the field infection. It is fair to consider that the traditional microbiological techniques make it possible to isolate MG strains with the capability of replication whereas the PCR test can only detect the bacterial genome; however, the recovery of TS-11® repeated during a long time interval would give evidence of its ability in the tracheal colonization and persistence.

Good production performance in spite of the field infection in the farm, support the hypothesis of the protective effect of this live Mg vaccine.

Further trials are needed to confirm the results achieved, before considering the possibility of utilization of TS-11® on a large scale as an additional tool for the control of MG infections in turkeys.

The authors would like to thank the technical staff of the Sezione Diagnostica di Forlì (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna) *for the valuable support.*

REFERENCES

BARBOUR, E.K., HAMADEH, S.K. EID, A., 2000. Infection and immunity in broiler chicken breeders vaccinated with a temperature-sensitive mutant of *Mycoplasma gallisepticum* and impact on performance of offspring. Poultry Sci. 79:1730-1735.

KLEVEN, S.H., 2000. Mycoplasma update. Poultry Informed Professional. 42:1-4.

KLEVEN, S.H., GARCIA, M L., TONGRUI BEITING, V., HALL, B., 2000. Centralized laboratory effort dedicated to the archiving and characterization (fingerprinting) of pathogenic species of avian Mycoplasma. Final Report to U.S. Poultry and Egg Association. U.S. Poultry and Egg Association ed., Tucker, GA, USA.

LAUERMAN, L.H., HOER, F.J., SHARPTON, A.R., SHAN, S.M., SATEN, V.L., 1993. Development and application of a polymerase chain reaction assay for *Mycoplasma synoviae*. Avian Dis. 37:829-834.

PAGANELLI, F., FIORENTINI, L., LEONELLI, R., 2003. Utilizzo della random amplified polymorphic DNA (RAPD) per differenziare ceppi di *Mycoplasma gallisepticum* vaccinali da ceppi di campo. Large Anim. Rev. 9(6):95-96.

PAGANELLI, F., MASSI, P., TOSI, G., 2002. Applicazione del metodo polymerase chain reaction alla diagnosi di *Mycoplasma gallisepticum* e di Mycoplasma synoviae. Large Anim. Rev. 8(6):103-104.

WHITHEAR, K.G., SOERIPTO HARRIGAN, K.E., GHIOCAS, E., 1990. Immunogenicity of a temperature-sensitive mutant *Mycoplasma gallisepticum* vaccine. Aust. Vet. J. 67:168-174.

Serological evidences showing the involvement of free-living pheasants in the influenza ecology

Maria Alessandra De Marco[1], Laura Campitelli[2], Mauro Delogu[3], Elisabetta Raffini[4], Emanuela Foni[5], Livia di Trani[6], Michele Scaffidi[3], Isabella Donatelli[2]

[1] Istituto Nazionale per la Fauna Selvatica "A. Ghigi". Ozzano Emilia (BO), Italy
[2] Dipartimento di Malattie Infettive, Parassitarie e Immunomediate. Istituto Superiore di Sanità, Roma, Italy
[3] Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale. Università di Bologna, Italy
[4] Istituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia Romagna. Lugo (RA), Italy
[5] Istituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia Romagna. Parma, Italy
[6] Dipartimento di Sanità Animale e Alimentare. Istituto Superiore di Sanità, Roma, Italy

Corresponding author: Dr. Maria Alessandra De Marco. Istituto Nazionale per la Fauna Selvatica "A. Ghigi". Via Ca' Fornacetta 9, 40064 Ozzano dell'Emilia (BO), Italy – Tel. +39 051 6512244 – Fax: +39 051 796628 – Email: infs.adem@iperbole.bologna.it

ABSTRACT

From 1995 to 2002, 219 sera were collected in Northern Italy from wild pheasants, in order to establish the possible involvement of these *Galliformes* birds in the influenza ecology. A serological survey for avian influenza viruses (AIVs) was carried out by ELISA test in order to detect type A influenza antibodies. The overall seroprevalence was 12.3%, with yearly values ranging from 0% to 42.5%. No antibodies against either H5 or H7 AIV subtypes were found by hemagglutination-inhibition test. Data from 16 recaptured birds, among 113 animals banded for individual identification, showed seroconversions in 2 pheasants. Our results indicate AIV circulation in free-living pheasants; the involvement of this land-based bird species in influenza ecology is discussed.

Key Words: Pheasant, Avian influenza, Influenza ecology, Serological survey.

RIASSUNTO

DIMOSTRAZIONE SU BASE SIEROLOGIA DEL COINVOLGIMENTO DI FAGIANI A VITA LIBERA NELL'ECOLOGIA DELL'INFLUENZA

Nel periodo compreso tra il 1995 e il 2002, 196 fagiani a vita libera sono stati catturati in un'area protetta dell'Italia Settentrionale, situata in Emilia-Romagna. Le catture, durante le quali 113 fagiani sono stati inanellati per un'identificazione individuale e quindi rilasciati nell'area di studio, sono avvenute durante cinque periodi invernali di cui 4 consecutivi e hanno consentito di raccogliere 219 emosieri. I campioni ottenuti sono stati sottoposti ad esami sierologici per valutare: i) il possibile coinvolgimento di una popolazione di fagiani a vita libera caratterizzata da un'elevata densità di animali sul territorio, nell'ecologia dei virus influenzali di tipo A; ii) l'eventuale circolazione tra questi volatili di virus influenzali appartenenti ai sottotipi H5 o H7. La ricerca è stata eseguita esaminando i campioni di siero tramite una metodica ELISA in grado di evidenziare anticorpi nei confronti della nucleoproteina dei virus influenzali di tipo A (NP-ELISA): la sieroprevalenza totale era uguale al 12,3%, mentre i risultati osservati annualmente variavano da una percentuale pari allo 0% (durante il 1995) a un valore massimo del 42,5% (durante il 2001). I campioni risultati positivi alla metodica NP-ELISA sono stati testati mediante la prova di inibizione dell'emoagglutinazione (IEA) effettuata impiegando come antigeni 5 ceppi di virus influenzali a bassa patogenicità, isolati in Italia e appartenenti ai sottotipi H5N2, H5N3, H5N9, H7N1, H7N3. Tutti i campioni esaminati mediante IEA sono risultati sieronegativi per i sottotipi H5 e H7.
L'esame degli emosieri ottenuti da 16 fagiani inanellati e catturati più di una volta, hanno consentito di dimostrare in 2

soggetti sieroconversioni verso i virus influenzali di tipo A, indice di una recente infezione legata alla contemporanea circolazione virale nell'area di studio.
Dai risultati della presente indagine si evince come nella popolazione naturale di fagiani esaminata siano circolati, per i quattro anni consecutivi del campionamento, virus influenzali di tipo A e come questi non appartenessero ai sottotipi H5 e H7, responsabili delle recenti epidemie italiane del pollame. L'esistenza di zone umide nell'area di studio costituisce una possibile interfaccia ecologica tra specie aviarie serbatoio naturale dei virus influenzali (uccelli acquatici) e fagiani che utilizzandone l'acqua per l'abbeverata condividono questa probabile fonte di infezione. Ulteriori indagini consentiranno di approfondire il ruolo epidemiologico di questo galliforme selvatico nell'ecologia dei virus influenzali.

Parole chiave: *Fagiano, Influenza aviare, Ecologia dell'influenza, Indagine sierologica*

Introduction

Land-based birds belonging to the *Galliformes* Order include species, such as turkey, chicken, and quail, that are highly susceptible to avian influenza viruses (AIVs) primarily harboured in wild aquatic birds (Webster *et al.*, 1992). In addition to heavy economic losses due to influenza epidemics in poultry, important public health implications could arise from AIV circulation in land-based birds, recently indicated as a potential source of pandemic strains (Perez *et al.*, 2003).

AIV infections have been described in Italy in reared pheasants *(Phasianus colchicus)* both as limited outbreaks (Rinaldi *et al.*, 1967) or associated to severe poultry epidemics (Capua *et al.*, 2003). Although sporadic isolations of AIVs have been reported in free-living pheasants (Romváry *et al.*, 1976), the epidemiological role played by wild populations of this *Galliformes* species is not well understood, to date.

Aims of this serological survey, carried out on wild pheasants trapped in northern Italy, were: i) to establish the occurrence of type A influenza infection; ii) possibly, to detect the circulation of H5 and H7 AIV subtypes.

Material and methods

Sampling

Free-living pheasants were monitored on an estate (about 35 hectares) located in a protected lowland area (Bologna province, Emilia Romagna region). The number of birds occupying the study area ranged from 150 to 40 in autumn and spring, respectively. From 1995 to 2002 a total of 196 pheasants were trapped; 113 of them were banded for individual identification then released into the wild. Overall, 219 sera were collected (Table 1) including 23 recapture samples (Table 2). Serum samples were stored at –20°C until tested. Bird sex and age were recorded whenever possible. Phenotypic characteristics allowed sex determination. The age of pheasants was determined as described (Cattadori *et al.*, 1997): juveniles were birds hatched during the last breeding season, adults were birds hatched any year before the last breeding season.

Serological test

Sera were assayed for antibodies against influenza A virus nucleoprotein using an ELISA test (NP-ELISA) performed as described (De Marco *et al.*, 2003b).

Available NP-ELISA positive sera were assayed as described (De Marco *et al.*, 2004) by hemagglutination-inhibition (HI) test, in order to detect antibodies against 5 different Italian strains belonging to both H5 and H7 subtypes of AIVs (Table 1).

Recapture data (Table 2) were analysed in order to evidence a significant increase in antibody titres (De Marco *et al.*, 2003b).

Statistical analysis

Chi-square test was performed in order to test non-random associations between the overall seroprevalences and: i) pheasant age; ii) pheasant sex. The significance level was set at a $P<0.05$.

Results and discussion

As shown in Table 1, the overall NP-ELISA antibody frequency to avian influenza viruses (AIVs), including data from 23 recaptures, was 12.3% (27/219). Pheasants seropositive for influenza A viruses were found in 4 of the 5 sampling periods, and the prevalence of sera found positive

Table 1. Serological results for antibodies against avian influenza viruses in 196 free-living pheasants trapped in a protected area of the Emilia Romagna region (Northern Italy). Seroprevalences were calculated on overall sera sampled, including 23 sera obtained from 16 recaptured bird.

Sampling period	NP-ELISA prevalence % (positive/tested sera)	HI antibody frequencies (positive/tested sera) calculated on NP-ELISA positive sera against the following AIV subtypes(*)				
		H5N2	H5N3	H5N9	H7N1	H7N3
Mar. 1995	0 (0/34)	nd	nd	nd	nd	nd
Jan. 1999/Feb. 1999	3.8 (2/53)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)
Feb. 2000/Mar. 2000	5.6 (3/54)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)
Jan. 2001/Mar. 2001	42.5 (17/40)	0 (0/15)	0 (0/16)	0 (0/16)	0 (0/15)	0 (0/16)
Jan. 2002	13.2 (5/38)	0 (0/4)	0 (0/5)	0 (0/5)	0 (0/4)	0 (0/5)
Total 1995/2002	12.3 (27/219)	0 (0/23)	0 (0/25)	0 (0/25)	0 (0/23)	0 (0/25)

(*) Low-pathogenic AIV strains used: A/mallard/Italy/80/93(H5N2); A/mallard/Italy/208/00(H5N3); A/chicken/Italy/9097/97(H5N9); A/turkey/Italy/6423-1/99(H7N1); A/mallard/Italy/33/01(H7N3); nd = not done.

Table 2. Serological data for antibodies against influenza A viruses obtained from 16 free-living pheasants trapped between 1995 and 2002 in the study area, and recaptured once or twice.

Bird n.	Bird age^	1st capture time (NP-ELISA result)	1st recapture time (NP-ELISA result)	2nd recapture time (NP-ELISA result)
1	Ad	14/03/95 (-)	02/02/00 (-)	nd
2	Ad	03/02/99 (-)	22/02/00 (-)	02/03/00 (-)
3	Ad	04/02/99 (-)	22/02/00 (-)	29/02/00 (+)^^
4	Ad	09/02/00 (-)	18/02/00 (-)	24/02/00 (-)
5	Ad	09/02/00 (-)	14/02/00 (-)	29/02/00 (+)^^
6	Juv	11/02/00 (-)	15/02/00 (-)	nd
7	Un	11/02/00 (-)	22/02/00 (-)	01/03/00
8	Ad	11/02/00 (-)	28/01/02 (-)	nd
9	Ad	22/02/00 (-)	24/02/00 (-)	29/02/00 (-)
10	Ad	23/02/00 (-)	29/02/00 (-)	nd
11	Ad	23/02/00 (-)	08/02/01 (-)	nd
12	Juv	23/02/00 (-)	01/03/00 (-)	nd
13	Juv	24/02/00 (-)	29/01/02 (-)	nd
14	Ad	29/02/00 (-)	06/02/01 (-)	28/01/02 (-)
15	Ad	13/02/01 (-)	27/02/01 (-)	nd
16	Un	01/03/01 (+)^^	06/03/01 (+)^^	nd

Ad: adult; Juv: juvenile; Un: unknown; (+) positive; (-) negative; nd: not done;
^: determined during the 1st capture; ^^:positive antibody titre = 8.

ranged from 0% (in 1995) to 42.5% (in 2001). Samples were not collected during 1996, 1997 and 1998, thus it is not possible to establish precisely the first occurrence of AIV infection, however seropositive birds were constantly found between 1999 and 2002. No sex-related differences were found whereas the age-related NP-ELISA seroprevalences resulted significantly higher in the juvenile birds, compared to the adult ones, thus suggesting a higher susceptibility of juvenile pheasants that congregate in post-breeding periods to AIV transmission.

No H5 and H7 positive sera were found by HI assay, performed using 5 different low-pathogenic AIV strains recently isolated in Italy from both wild and domestic avian species (Table 1).

Among 16 birds captured more than once, seroconversion for type A influenza viruses (Table 2, data evidenced with^^) was observed in pheasants n. 3 and n. 5, indicating that AIVs circulated in the study area during the winter 2000.

Conclusions

Our findings indicate the occurrence of avian influenza virus (AIV) infection in free-living pheasants. In general wild birds are potentially exposed to AIVs perpetuated by natural reservoirs, in particular the pheasants examined in the present study drank from small ponds located in the study area and occasionally used by migrating ducks. Faecal contamination of waters could represent an ecological interface between primary hosts of AIVs and other susceptible bird species (Webster *et al.*, 1992).

The high population density characterising the study area could facilitate the virus circulation; indeed the data we observed are in contrast with seronegative results obtained from pheasants living in protected sites characterised by a lower population density (De Marco *et al.*, 2003a).

Fortunately, our results indicate that neither H5 nor H7 subtypes of AIV, recently involved in Italian poultry epidemics (Capua *et al.*, 2003; Campitelli *et al.*, 2004), circulated within the examined bird population. Further surveillance studies will enable us to acquire information to better understand the dynamics of influenza infection in pheasants, a land-based bird species potentially implicated in the interspecies transmission of AIVs harboured in natural reservoirs (Wood *et al.*, 1985; Perez *et al.*, 2003).

Thanks are due to: Dr. Fausto Marzadori, and Mr. Paolo Tozzoli (IZS della Lombardia e dell'Emilia-Romagna, Lugo); the Province of Bologna, Mr. Gino Rossi and other game management personnel; Mr. Valter Trocchi (Istituto Nazionale per la Fauna Selvatica, Ozzano Emilia).

REFERENCES

Campitelli, L., Mogavero, E., De Marco, M.A., Delogu, M., Puzelli, S., Frezza, F., Facchini, M., Chiapponi, C., Foni, E., Cordioli, P., Webby, R., Barigazzi, G., Webster, R.G., Donatelli, I., 2004. Interspecies transmission of an H7N3 influenza virus from wild birds to intensively reared domestic poultry in Italy. Virology. 323:24-36.

Capua, I., Marangon, S., Dalla Pozza, M., Terregino, C., Cattoli, G., 2003. Avian Influenza in Italy 1997-2001. Avian Dis. 47:839-843.

Cattadori, I., Gatti, P., Toso, S., 1997. Fagiani naturali e di allevamento: confronto fra alcuni parametri biometrici. In: M. Spagnesi, S. Toso, and P. Genovesi (eds.) Proc. 3rd Nat. Congr. of Game Biologists. Ric. Biol. Selvaggina. 27 (Suppl.):423-430.

De Marco, M. A., Campitelli, L., Foni, E., Raffini, E., Barigazzi, G., Delogu, M., Guberti, V., Di Trani, L., Tollis, M., Donatelli, I., 2004. Influenza surveillance in birds in Italian wetlands (1992-1998): is there a host restricted circulation of influenza viruses in sympatric ducks and coots? Vet. Microbiol. 98:197-208.

De Marco, M.A., Foni, E., Campitelli, L., Raffini, E., Delogu, M., Donatelli, I., 2003a. Long-term monitoring for avian influenza viruses in wild bird species in Italy. Vet. Res. Commun. 27(Suppl. 1):107-114.

De Marco, M.A., Foni, E., Campitelli, L., Raffini, E., Di Trani, L., Delogu, M., Guberti, V., Barigazzi, G., Donatelli, I., 2003b. Circulation of influenza viruses in wild waterfowl wintering in Italy during the 1993-1999 period: evidence of virus shedding and seroconversion in wild ducks. Avian Dis. 47:861-866.

Perez, D.R., Webby, R. J., Hoffmann, E., Webster, R.G., 2003. Land-based birds as potential disseminators of avian/mammalian reassortant influenza A viruses. Avian Dis. 47:1114-1117.

Rinaldi, A., Nardelli, L., Pereira, H.G., Mandelli, G.C., Gandolfi, R., Cervio, G., 1967. Focolaio di Influenza A nel Fagiano (*Phasianus colchicus* L.). Proc. 21st Nat. Congr. SISVet, Senigallia, Italy, 21:867-872.

ROMVÁRY, J., MÉSZÁROS, J., TANYI, J., RÓSZA, J., FÁBIÁN, L., 1976. Influenza infectedness of captured and shot wild birds on north-eastern and south-eastern parts of Hungary. Acta Vet. Acad. Sci. H. 26:363-368.

WEBSTER, R.G., BEAN, W.J., GORMAN, O. T., CHAMBERS T. M., KAWAOKA, Y., 1992. Evolution and ecology of Influenza A viruses. Microbiol. Rev. 56:152-179.

WOOD, J.M., WEBSTER, R.G., NETTLES, V.F., 1985. Host range of A/chicken/Pennsylvania/83 (H5N2) influenza virus. Avian Dis. 29:198-207.

Preliminary results of an influenza surveillance in wild birds, game birds, domestic ducks and geese in North Eastern Italy

Roberta De Nardi[1], CalogeroTerregino[1], Giovanni Cattoli[1], Anna Toffan[1], Maria Serena Beato[1], Vittorio Guberti[2], Mara Scremin[2]

[1] Istituto Zooprofilattico Sperimentale delle Venezie. Legnaro (PD), Italy
[2] Istituto Nazionale per la Fauna Selvatica "A. Ghigi". Ozzano Emilia (BO), Italy

Corresponding author: Dr. Calogero Terregino. Istituto Zooprofilattico Sperimentale delle Venezie. Virology Dept. Viale dell'Università 10, 35020 Legnaro (PD), Italy – Tel. +39 049 8084389 – Fax: +39 049 8084360 – Email: virologia@izsvenezie.it

ABSTRACT

Following the avian influenza (AI) epidemics which occurred in Italy between 1997 and 2003, a surveillance program, funded by the Italian Ministry of Health was implemented. Among the tasks of this surveillance program was an investigation on wild and domestic birds to asses circulation of avian influenza viruses in their natural reservoirs. In this study we collected samples from migratory wild birds *(Anseriformes* and *Charadriiformes)*, from national and importated game fowls, and from 7 backyard farms of geese and ducks. Cloacal swabs were screened by means of real-time RT-PCR (RRT-PCR) and/or directly processed for attempted virus isolation in embryonated fowl's SPF eggs and blood samples for presence of antibodies against avian influenza viruses. Avian influenza viruses were only obtained from migratory waterfowls belonging to the family *Anseriformes*, and not from domestic waterfowls or game birds. This study confirms that the risk of introduction of novel influenza viruses in densely populated areas of poultry farms in Veneto is linked to migratory wild birds and in particular from birds belonging to the family *Anseriformes*.

Key Words: Avian influenza, Wild birds, Game birds, Ducks, Geese.

RIASSUNTO

INDAGINE SUL RISCHIO DI INTRODUZIONE NEL POLLAME DOMESTICO DI VIRUS DELL'INFLUENZA AVIARIA DA POSSIBILI RESERVOIR IN VENETO

In questi ultimi anni l'allevamento avicolo veneto è stato gravemente colpito da alcune ondate epidemiche di influenza aviaria (AI) responsabili di ingenti danni economici diretti ed indiretti al patrimonio avicolo nazionale. Lo scopo della presente indagine è stato quello di verificare la presenza, nei principali serbatoi naturali, di Influenzavirus A. La ricerca si è svolta mediante l'analisi di campioni prelevati da uccelli acquatici selvatici (anatidi e limicoli), da selvaggina di allevamento di provenienza nazionale ed estera e da allevamenti rurali di anatre ed oche. I risultati indicano che nel nord-est dell'Italia, come nel resto del mondo, gli anatidi selvatici migratori rivestono un ruolo estremamente importante nell'epidemiologia dell'AI. Nessun virus influenzale è stato isolato dai limicoli, ulteriori indagini potranno chiarire in futuro il reale ruolo di questi uccelli in Italia. Tutti gli allevamenti di selvaggina e di anatidi monitorati sono risultati negativi.

Parole chiave: *Influenza aviaria, Uccelli selvatici, Selvaggina, Anatre, Oche.*

Introduction

In previous years Italy was affected by several waves of avian influenza (AI) caused by type A Influenza viruses, thus determining heavy economic losses for the poultry industry and devastating consequences for the social community (Marangon *et al.*, 2003). The epidemics occurred primarily in the densely populated poultry areas (DPPA) of Veneto and Lombardia regions.

Waterfowls, particularly *Anseriformes* (Alexander *et al.*, 2000) and *Charadriiformes* (Stallknecht *et al.*, 1988) are believed to be natural hosts and reservoirs of influenza A viruses. During recent H5N1 outbreaks in Eastern Asia the presence of H5N1 viruses in dead migratory birds suggests that wild bird populations may be involved in spreading Highly Pathogenic Avian Influenza (HPAI). In fact the timing and distribution of H5N1 infection in poultry in China in 2001 coincides with the general period of winter bird migration from northern to southern part of the country (Li *et al.*, 2004). In Europe, Anseriformes coming from north-eastern region use wetlands located in Veneto as a wintering site. *Charadriiformes* originating from the same areas, but with different habitats, use Italian wetlands as foddering sites before they leave for Africa. Thus, several species congregate in Italian wetlands in autumn, and this represents an ideal ecological situation for the perpetuation of avian influenza viruses (AIV). On the contrary, in winter there are fewer species but the numbers of birds present in the wetlands are more significant than those which can be found in the previous season. In addition, the majority of birds which are present in the wetlands are juveniles, born during the previous spring and theoretically highly susceptible to AIV.

Aim of the present study was to evaluate the circulation of AIV in wild birds, game birds, domestic ducks and geese in Veneto region (north eastern Italy).

Material and methods

During 2003, 742 cloacal swabs and 309 serum samples were collected. Of these, 560 cloacal swabs were collected from 9 different species of migratory wild *Anseriformes* and from one species of *Charadriiformes*, *(Calidris alpina)* present in wetlands area of Veneto region. The remaining swabs were collected in 12 game farms rearing birds of national and imported origin and from 7 backyard farms rearing geese and ducks.

Serum samples were tested by means of the haemagglutination inhibition test (HI) and by agar gel immunodiffusion test (AGID) as described in European Union Directive 92/40/CEE (CEC, 1992). The species and samples tested are illustrated in Table 1.

Cloacal swabs from migratory birds were screened by means of a real-time RT-PCR (RRT-PCR) (Cattoli *et al.*, 2004), and the positive samples were subsequently processed for virus isolation.

Cloacal swabs, diluted in phosphate-buffered saline with antibiotics, were inoculated into the allantoic cavity of 9-day-old embryonating specific pathogen free (SPF) eggs for attempted virus isolation according to EU Directive 92/40. Haemagglutinating isolates were identified by means of the haemagglutination-inhibition test, and if shown to be influenza isolates, were fully characterised by means of the neuraminidase inhibition (NI) test.

Results and discussion

All serum samples resulted negative for antibodies to avian influenza. 18 out 560 cloacal swabs (9 mallards, 1 teal, 4 pintails, 1 wigeons, and 3 shovellers) tested were positive for type A avian influenza in RRT-PCR. Following virus isolation attempts, two isolates were obtained, an H1N1 subtype, was obtained from a male mallard and an H10N4 subtype was obtained from a female pintail.

All cloacal swabs collected from game birds and rural geese and ducks were negative for AIV (Table 1).

The results of the present investigation support previous studies which indicate that migratory waterfowls represent a source of infection for domestic birds. In an investigation carried out between 1992 to 1998 in which a total of 802 cloacal swabs were collected from migratory and resident waterfowl on the west coast of Italy, 22 isolates obtained (18 H1N1, 1 H3N8, 1 H5N2 and 2 H10N8).

In USA birds belonging to the order *Charadriiformes* are considered one of main reservoirs of AIVs (Stallknecht *et al.*, 1988). As part of the investigation we collected 82 cloacal swabs from dunlins *(Calidris alpina)* in order to obtain information on the role of *Charadriiformes* as reservoirs of AI viruses in Italy, since this information is currently lacking. No isolates were obtained from the samples collected.

Table 1. Results of serological and virological tests.

	Species	N. samples collected		Results of laboratory tests		
		Cloacal swabs	Sera	RRT-PCR (n. positive/n. samples tested)	Virus isolation (n. positive/ n. samples tested)	Serological tests (HI, AGID) (n. positive/ n. samples tested)
Game farms	Pheasant *(Phasianus colchicus)*	60	219	nd	0/60	0/219
	Partridge *(Perdix perdix)*	10	45	nd	0/10	0/45
	Rock Partridge *(Alectoris greca)*	0	5	nd	0/5	0/5
Ducks and Geese farms	Domestic Duck *(A. platyrhynchos var. domestica)*	62	0	nd	0/62	nd
	Muschovy Duck *(Cairina moscata)*	35	25	nd	0/35	0/25
	Mallard *(Anas platyrhynchos)*	5	5	nd	0/5	0/5
	Goose *(Anser anser var. domestica)*	10	10	nd	0/10	0/10
Wild Birds	Mallard *(Anas platyrhynchos)*	206	0	9/206	1/9	nd
	Teal *(Anas crecca)*	27	0	1/27	0/27	nd
	Pintail *(Anas acuta)*	115	0	4/115	1/115	nd
	Wigeon *(Anas penelope)*	92	0	1/92	0/92	nd
	Coot *(Fulica atra)*	2	0	0/2	0/2	nd
	Gadwall *(Anas strepera)*	6	0	0/6	0/6	nd
	Shoveler *(Anas clypeata)*	23	0	3/23	0/23	nd
	Pochard *(Aythya furina)*	5	0	0/5	0/5	nd
	Tufted Duck *(Aythya fuligula)*	2	0	0/2	0/2	nd

nd=not done

The discrepancy between the number of samples which resulted positive at the RRT-PCR and the virus isolation attempts, probably lies in the greater sensitivity of the molecular test (Foni *et al.*, 2002; Cattoli *et al.*, 2004) compared to virus isolation.

Conclusions

In conclusion, the results of this study emphasise the continuous need to monitor wild bird population in order to gain more knowledge on the ecology of AI infections.

REFERENCES

ALEXANDER, D.J., 2000. A review of avian influenza in different bird species. Vet. Microbiol. 74:3-13.

CATTOLI, G., DRAGO, A., MANIERO, S., TOFFAN, A., BERTOLI, E., FASSINA, S., TERREGINO, C., ROBBI, C., VICENZONI, G., CAPUA, I., 2004. Comparison of three rapid detection systems for type A influenza virus on tracheal swabs of experimentally and naturally infected birds. Avian Pathol. 33:432-437.

EUROPEAN COMMISSION, 1992. Council Directive of 19 May 1992 introducing Community measures for the control of avian influenza, 92/40/EEC. In: Official Journal, L 167, 19.05.1992, pp 1-15.

FONI, E., CHIAPPONI, C., LORI, D., DE MARCO, M.A., DELOGU, M., RAFFINI, E., MASSI, P., 2002. Applicazione di RT-PCR e metodiche tradizionali nella ricerca di virus influenzale di tipo A in corso di infezione sperimentale in anatre. Large Anim. Rev. 8(6):99-100.

LI, K.S., GUAN, Y., WANG, J., SMITH, G.J., XU, K.M., DUAN, L., RAHARDJO, A.P., PUTHAVATHANA, P., BURANATHAI, C., NGUYEN, T.D., ESTOEPANGESTIE, A.T., CHAISINGH, A., AUEWARAKUL, P., LONG, H.T., HANH, N.T., WEBBY, R.J., POON, L.L., CHEN, H., SHORTRIDGE, K.F., YUEN, K.Y., WEBSTER, R.G., PEIRIS, J.S., 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature. 430:209-213.

MARANGON, S., BORTOLOTTI, L., CAPUA, I., BETTIO, M., DALLA POZZA, M., 2003. Low-pathogenicity avian influenza (LPAI) in Italy (2000-01): epidemiology and control. Avian Dis. 47:1006-1009.

STALLKNECHT, D.E., SHANE, M., 1988. Host range of avian influenza virus in free-living birds. Vet. Res. Commun. 12:125-141.

The alterations of plumage of parasitic origin

Mario Principato, Federica Lisi, Iolanda Moretta, Nada Samra, Francesco Puccetti

Dipartimento di Scienze Biopatologiche Veterinarie. Università di Perugia, Italy

Corresponding author: Prof. Mario Principato. Dipartimento di Scienze Biopatologiche Veterinarie. Facoltà di Medicina Veterinaria, Università di Perugia. Via S. Costanzo 4, 06100 Perugia, Italy – Tel. +39 075 5857741 – Fax: +39 075 5857743 – Email: drprincipato@email.it

ABSTRACT

Described herein are the main lesions to the plumage caused by insects and mites, both on the vane or the calamus of feathers. Practical data are given, aimed to make a correct differential diagnosis. *Mallophaga* cut the barbs of feathers, whereas *dermestidae* can cut also the rachis. Mites make holes in the vane of feathers and sometimes they stick the barbs the ones to the others or they attack the calamus both inside and by digging tunnels in the outside wall of the calamus causing the fall of feathers.

Key Words: Insects, Mites, Plumage, Calamus, Vane.

RIASSUNTO

ALTERAZIONI DEL PIUMAGGIO DI ORIGINE PARASSITARIA

Vengono descritte le principali lesioni al piumaggio prodotte da insetti ed acari, sia sulla parte vessillare delle penne, che sul calamo, fornendo elementi concreti per una corretta diagnosi differenziale. I mallofagi tagliano le barbe delle penne, mentre i dermestidi possono reciderne anche il rachide. Gli acari forano la parte vessillare delle penne e talora ne appiccicano le barbe, oppure attaccano il calamo sia internamente che scavando tunnel nella sua parete esterna, causando la caduta delle penne.

Parole chiave: *Insetti, Acari, Piumaggio, Calamo, Vessillo.*

Introduction

Arhropods can interact with fowls damaging their plumage, breaking, perforating and also causing its loss. Some attack preferably the calamus, some others the vane of the feathers. The lesions reported are almost always well distinguishable to the naked eye or by the aid of a stereomicroscope, but it is not always easy to find out the arthropod that causes them. To this aim, the main kinds of lesions of feathers occurred to our observation during the past ten years were selected in order to make it easy to effect a differential diagnosis and quickly to reveal the agent causing the pathology.

Material and methods

A number of 520 fowls was examined belonging to the families *Struthionidae, Turnicidae, Phasianidae, Anatidae, Psittacidae, Columbidae; Passeriformes* of the families *Cinclidae, Troglodytidae, Sturnidae, Estrildidae, Fringillidae, Corvidae, Ploceidae, Turdidae, Alaudidae, Hirundinidae, Motacillidae, Sylviidae and Paridae.*

The macroscopic exam of plumage was carried out on them, by a stereomicroscope and at the same time the isolation of all the arthropods present was effected by using micro-needles and thin-pointed pincers. The feathers damaged and the

arthropods isolated were kept in 80% alcohol, whereas some samples were clarified in warm lactic acid and mounted on slide in Berlese's solution to be identified.

To circumscribe the field of our research, in this study some mites causing mange and other causing indirectly the loss of feathers without lesions observable macroscopically were excluded.

Results and discussion

The arthropods identified as agents causing evident lesions of plumage belonged to two classes: *Insecta* and *Acarina*. In the former, two orders were recorded of particular interest for the plumage: *Mallophaga* and *Coleoptera*; in the latter, the order *Actinedida (=Prostigmata)* and *Acaridida (=Astigmata)* (Table 1).

The insects are a cause mainly of macroscopic lesions on the vane of feathers, which can weaken and break, whereas mites cause less evident lesions both to the feather barb and to the calamus, determining in some cases its loss. Among insects, the first to be isolated are *Mallophaga* belonging to the suborders *Amblycera* and *Ischnocera*. All of them have a strong masticatory mouth apparatus, but unable to cut the rachis of bigger feathers, such as the flight feathers (remiges - primaries, secondaries and tertials) or the tail feathers (rectrices). On the contrary they can cut the dorsal and pectoral small feathers and the feathers of sides under the wings. Their action on the barb is linear and very precise. The feather appears cut horizontally and each barb is cut singularly. When *Mallophaga* are very young, they cut the barbules and make small holes close to

Table 1. Classification and main characters of insects and mites affecting plumage.

Parasite	Localization	Lesion	Degree of seriousness
Mallophaga	Mainly underwing coverts, dorsal and pectoral feathers	Young *Mallophaga*: cut the barbules and make small holes ranged horizontally Adult Mallophaga: lean cut the barbs horizontally causing breaking of feather	Seriousness in proportion with the number of parasites
Coleoptera Dermestidae	Mainly tail feathers and flight feathers	They clench barbs in groups. They can cut also the rachis, breaking the feather	They cause the depreciation of birds
Feather vane mites: *Pterolichidae, Analgidae. Proctophyllodidae, Dermoglyphidae* and others	Flight feathers (primaries)	They shift or cut the barbules producing small holes ranged irregularly	They cause the loss of impermeability of feathers
Internal calamus mites: *Syringophilidae*	Inside the calamus	They cause inflammation of feather papilla	They cause the fall of feathers, above all of the upperwing medial and lesser coverts
External calamus mite: *Mesoknemidokoptes laevis*	Outside wall of calamus	They min externally the calamus producing some cortical tunnels	They cause the fall of the wing feathers
Mites: *Cheyletidae Ornithocheyletia*	Basis of head feathers, above all around the eyes and auditory meatus	They stick the feathers with sericeous secretions	Itch. The hearing is reduced, when the mite is localized in the auditory meatus
Mites: *Harpyrhynchidae*	At the basis of periocular feathers and around the auditory meatus	They lay their eggs on the barbs, which become yellowish	Severe itch

each other, without cutting the barbs completely.

Other insects, instead, less known under this aspect than *Mallophaga*, are the *Coleoptera* of family *Dermestidae, Dermestes* genus. Those insects are able to attack the alive animals' plumage, if the feathers are dirty of faeces or of feedstuff. This occurs frequently, for instance, in intensive pheasant farms, where the animals are often kept to sleep on the ground, even near carcasses of new-born pullets or close to broken eggs. Those materials attract *Coleoptera* adults and larvae of *Dermestes* genus, that, by their masticatory mouth, stronger and bigger than *Mallophaga*'s one, can break sleeping animals' feather barbs, by clenching them in groups. This determines a typical bend in the place of the cut, which is the differential element in comparison with lesions caused by *Mallophaga*. Besides the *Dermestidae* can break the rachis of smaller feathers and inlay the one of the bigger feathers. Typical lesion caused by *Dermestidae* beetles is the one at the level of pheasants'tail, which, under the action of *Dermestidae* at the end breaks with a consequent depreciation of the birds.

Among the mites, a distinction is to be made between those localizing in the feather vane and those localizing at the level of calamus. Among the formers are *Astigmata* mites, belonging to the suborder *Psoroptidia* with many families, such as *Pterolichidae, Analgidae, Proctophyllodidae, Dermoglyphidae* and others. Many species of those families live on barbs, near the rachis in the internal part of feathers, often at the level of primaries (the localization varies depending on the species). All these mites cannot cut the barbs, but can shift or cut off the barbules, producing small holes hardly visible if the feather is held up against the light. The holes are very small and scattered and in this they are different from those caused by young *Mallophaga* that, generally, producing holes on the same line. The second group of mites we observed localizes, instead, at the level of feather calamus. They belong either to *Actinedida*, prostigmates of the suborder *Eleutherengona*, family *Syringophilidae*, either to the above mentioned order of *Astigmata Psoroptidia*, family *Knemidokoptidae*. Mites of family *Syringophilidae* localize in the internal part of calamus of feathers, whereas *Mesoknemidokoptes laevis*, a species of family *Knemidokoptidae*, mines externally the calamus by producing some cortical tunnels.

Both of them, but mainly the mites of family *Syringophilidae*, cause the inflammation of feather papilla and its consequent fall. There can be itch and the animal can tear it by itself.

Finally, we observed another group of mites localizing at the basis of feathers and among the barbs. They are Prostigmata mites belonging to the suborder *Eleutherengona*, of the family *Cheyletidae*. Eggs of this mite are kept inside silky cobwebs spun from substance secreted by female mites. Feathers appear sticky and barbs are attached like a web. Mites belonging to the suborder *Eleutherengona*, family *Harpyrhynchidae*, lay their eggs on the barbs at the basis of feathers, above all the periocular ones and around the auditory meatus in *Passeriformes*. In this case the feather has a typical look and white-orange colour.

Conclusions

The main lesions by arthropods to feathers are certainly those caused by *Mallophaga* (Atyeo *et al.*, 1966; Post, 1981; Wheeler *et al.*, 1989) whereas those caused by typical mites of the feathers (*Astigmata* mites of the suborder *Psoroptidia*) are less evident. In *Passeriformes* lesions to feathers by *Harpyrhynchus* and *Syringophilus* are frequently recorded (Clark, 1964; Principato *et al.*, 1992, 1995). Lesions caused by *Ornithochyletia* and *Mesoknemidokoptes* result to be more rare (Krantz, 1978; Principato *et al.*, 1987, 1995). On the contrary it is frequent to observe the attack of plumage by *Dermestidae*, but it is caused by the presence of organic rests and poor hygiene of the farms (Théodoridèd, 1949). A differential diagnosis may be easy if one takes into account not only the morphology of lesions, but also their place and the host's species.

A treatment with parasiticides is not always successful, as it is in the case of Syringophilosis, and anyhow a possible treatment must be necessarily carried out considering the role of the environment as well (for instance: *Dermestidae* or contagion with feathers fallen on the ground) in the upset of the pathology.

REFERENCES

ATYEO, W.T., BRAASCH N.L., 1966. The feather mite genus *Proctophyllodes (Sarcoptiformes: Proctophyllodidae)*. Bull. Univ. Nebraska State Mus. 5:1-354.

CLARK, G.M., 1964. The acarine genus *Syringophilus* in North American birds. Acarologia. 6(1):77-92.

KRANTZ, G.W., 1978. A manual of Acarology. 2nd ed. O.S.U. Book Stores Inc., Corvallis, OR, USA.

POST W., 1981. The prevalence of some ectoparasites diseases, and abnormalities in the yellow-shouldered blackbird. J. Field Ornithol. 52(1):16-22.

PRINCIPATO, M., COLETTI, M., TACCONI, G., 1987. Studio sull'acarofauna dei volatili. Il ruolo degli acari negli stati patologici aspecifici. Isolamento di nuove specie patogene. Summa. 4:229-237.

PRINCIPATO, M., FRANCIOSINI, M.P., DEL ROSSI, E., 1995. Rogna deplumante della tortora da *Mesoknemidokoptes laevis* Railliet, 1885 (Acari: *Knemidokoptidae*): un acaro nuovo per l'Italia. Zootecnica International. 17(2):123-126.

PRINCIPATO, M., GIRELLONI, V., COLETTI, M., SACCONI, G., 1992. Arpirinchiasi dei volatili: osservazioni su un raro caso di acariasi cistica in *Coccothraustes coccothraustes (Passeriformes: Fringillidae)*. Zootecnica International. 14(2):29-33.

PRINCIPATO, M., SACCONI, G., LIBERTI, L., 1995. Siringofilosi dei volatili: un'acariosi poco conosciuta. Zootecnica International. 17(2):127-130.

THÉODORIDÈD, J., 1949. Les coléoptères nuisibles aux animaux domestiques. Ann Parasit. 24:116-123.

WHEELER, T.A., THRELFALL, W., 1989. Synopsis of the parasites of vertebrates of Canada: ectoparasites of birds. Alberta Agriculture, Animal Health Division, Edmonton, Alberta, Canada.

CASE REPORT

Detection of Caliciviruses in young pheasants *(Phasianus colchicus)* with enteritis in Italy

Anna Toffan, Luca Bano, Francesco Montesi, Maria Serena Beato, Roberta De Nardi, Calogero Terregino, Ilaria Capua

Istituto Zooprofilattico Sperimentale delle Venezie. Legnaro (PD), Italy

Corresponding author: Dr. Anna Toffan. Istituto Zooprofilattico delle Venezie. Laboratorio di Microscopia Elettronica. Viale dell'Università 10, 35020 Legnaro (PD), Italy – Tel. +39 049 8084389 – Fax: +39 049 8084360 Email: microscopia@izsvenezie.it

ABSTRACT

During June 2004 a severe enteritis was reported in a farm of 21-28 day old pheasants reared in intensive conditions in North-Eastern Italy. Mortality in the flock had reached 25%. Virological investigations on cell culture of the gut content yielded reoviruses while electron microscopy examination revealed viral particles morphologically related to calicivirus in association with parvovirus-like and rod shaped virus-like particles.

Key Words: Pheasant, Enteritis, Calicivirus.

RIASSUNTO

INFEZIONE DA CALICIVIRUS ASSOCIATA AD UNA SINDROME ENTERICA IN GIOVANI FAGIANI *(PHASIANUS COLCHICUS)*

Il presente lavoro descrive un grave episodio di sindrome enterica osservato in un gruppo di fagiani di allevamento di 21-28 giorni d'età. Oltre all'isolamento di reovirus dal contenuto intestinale, gli esami di microscopia elettronica hanno permesso di evidenziare la presenza di particelle virali morfologicamente riferibili a calicivirus associate a parvovirus-like e rod shaped virus-like particles. Dalla letteratura consultata questo risulta essere il secondo caso di enterite del fagiano associato a infezione da calicivirus, ed in considerazione della grave sintomatologia osservata sarebbe auspicabile un approfondimento della casistica per chiarire il ruolo di questo patogeno nel determinismo delle enteriti dei volatili in generale e del fagiano in particolare.

Parole chiave: *Fagiano, Enterite, Calicivirus.*

Introduction

Viral enteritis is commonly reported in young birds, and among these, game birds appear to be highly susceptible particularly if reared in intensive farming conditions. Young pheasants *(Phasianus colchicus)* suffer this pathologic condition with mortality rates ranging between 3% to 30% (Gough *et al.*, 1985). Most viral enteric problems in pheasants are caused by rotavirus (Gough *et al.*, 1985; Reynolds *et al.*, 1986) while parvovirus-like can cause acute hepatitis (Gelmetti *et al.*, 1996). Calicivirus infection is commonly reported in enteric diseases in mammals (human, canine, bovine, porcine) (Murphy *et al.*, 1999), but are less frequently observed in birds (Gough *et al.*, 1992). The present paper reports of the results of laboratory investigations in a flock of pheasants

with acute enteritis, in which the examination of the gut content by electron microscopy resulted in the detection of viruses which are unusually found in this species.

Material and methods

In June 2004, 28 day-old dead pheasant chicks which had experienced severe enteritis were submitted for laboratory investigations. The birds originated from an industrial pheasant farm consisting of 70,000 birds located in northern Italy. Pheasants were kept in 5 sheds and reared on the ground. The clinical condition was characterized by depression, dehydration, severe enteritis and increased mortality rate. Between day 21 to day 28 of age the overall mortality rate reached 25% and then decreased to 3% per day.

The birds had been vaccinated for Newcastle disease and Marble Spleen disease and treated with ampicillin for a mild respiratory disease two weeks prior to the onset of the clinical condition.

Birds were necropsied and bacteriological (aerobic and anaerobic) and parassitological investigations were performed by routine methods. Intestinal contents were processed for attempted virus isolation in chicken embryo liver (CEL) cultures as described (Gough *et al.* 1988) and processed by negative contrast electron microscopy.

The contents of intestine were diluted in phosphate buffered saline (PBS) and centrifuged for 30 minutes at 4500 *g* for clarification. The supernatant obtained was filtered 0,22 μm and ultracentrifuged with Airfuge Beckman for 15 minutes on carbon-coated Formvar copper grids. Negative staining was finally performed using 2% sodium phosphotungstate. Examination was made using a Philips 208 S electron microscope.

Due to the gross findings, the liver was tested for micotoxin detection (aflatoxin B1,B2, G1,G2, 17-epossi-tricotecen and ocratoxin A) with a commercial ELISA kit and fixed in buffered formalin 20% for histological examination.

Results and discussion

On post-mortem examination cloacal pasting, haemorrhagic catarrhal enteritis, undigested food in the lumen, swelling of intestinal loops and thinning of intestinal wall were observed. The liver was enlarged and congested. The bone marrow also exhibited congestion. Blood appeared watery and the kidneys and gizzard exhibited haemorrhages.

Bacteriological and parassitological examinations did not yield any pathogens, except for rare coccidial oocistis, which were found in two birds. Ocratoxin A was detected in an irrelevant amount (4 ppb) in the liver.

Histological examination showed diffuse haemorrhages with necrosis and necrobiosis of hepatocytes, lymphocytic infiltration of the hepatic cords and intranuclear inclusion bodies in the hepatocytes. All these features are considered to be characteristic of viral infection.

Virus isolation attempts yielded reoviruses on first passage in CEL cultures.

Electron microscopy examination revealed three different particles. The most plentiful ones, measuring 30-35 nm, showed a typical cup-shaped depression of capsomeres arranged in icosahedral symmetry typical of Caliciviruses (Wyeth *et al.*, 1981) (Figure 1). The second smaller particles (18-20 nm of diameter) were morphologically related to Parvoviruses (Figure 1). In addition, elongated, striated particles measuring 50-60 nm in length by 18-20 nm in diameter (rod shaped virus-like particles) of unknown significance, but often reported in association with other intestinal viruses (Lavazza *et al.*, 1990) (Figure 2) were also detected. Since no samples of liver tissue were

Figure 1. Electron micrograph of caliciviruses in association with parvovirus-like in the intestinal contents of 28 day old pheasant chicks.

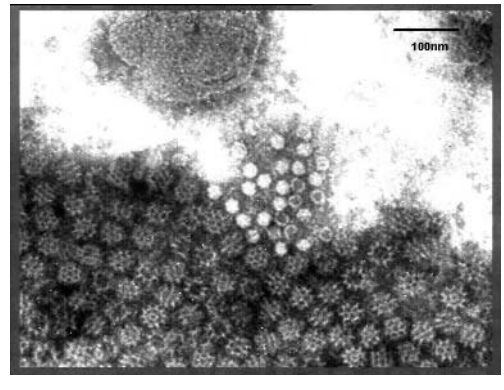
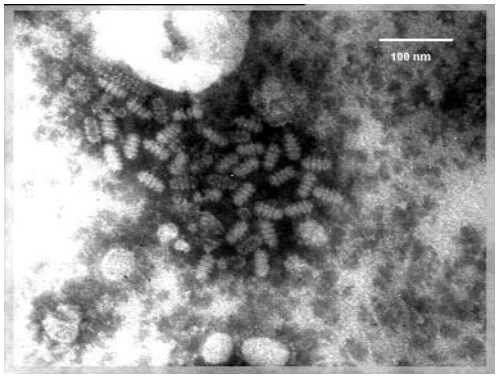


Figure 2. Electron micrograph of rod shaped virus-like particles in the intestinal contents of 28 day old pheasant chicks showing the typical striped aspect.



submitted for electron microscopy, it was not possible to establish whether this organ was also affected.

Conclusions

The present report confirms other findings (Gough, 1992; Gelmetti *et al.*, 1996) in which multiple viral infections are associated with enteritis in game birds. Although it appears extremely difficult to ascertain the role of each virus in the development of the clinical condition, the presence of a great amount of Calicivirus particles in the gut suggest that these viruses could be responsible for at least part of the intestinal lesions and related enteritis observed. Calicivirus infection of pheasants appears to be a rare finding, however, due to the severity of the clinical condition further studies should be carried out in order to establish the role of this virus in the development of enteritis in pheasants.

REFERENCES

GELMETTI, D., FABBI, M., SIRONI, G., GRILLI, G., LAVAZZA, A., 1996. Identification of parvovirus-like particles associated with three outbreaks of mortality in young pheasants *(Phasianus colchicus)*. J. Vet. Diagn. Invest. 8:108-112

GOUGH, R.E., WOOD, G.W., COLLINS, M.S., SPACKMAN, D., KEMP, J., GIBSON, L.A.C., 1985. Rotavirus infection in pheasant poults. Vet. Rec. 116:295.

GOUGH, R.E., ALEXANDER, D.J., COLLINS, M.S., LISTER, S.A., COX, W.J., 1988. Routine virus isolation or detection in the diagnosis of disease in birds. Avian Pathol. 17:893-907.

GOUGH, R.E., DRURY, S.E.D., BYGRAVE, A.C., MECHIE, S.C., 1992. Detection of calicivirus from pheasants with enteritis. Vet. Rec. 131:209-291.

LAVAZZA, A., PASCUCCI, S., GELMETTI, D., 1990. Rod shaped virus-like particles in intestinal contents of three avian species. Vet. Rec. 9:581.

MURPHY, F.A., GIBBS, E.P.J., HORZINEK, M.C., STUDDERT, M.J., 1999. *Caliciviridale*. In: Veterinary Virology. 3rd ed. Academic Press, San Diego, CA, USA. pp. 533-541.

REYNOLDS, D.L., THEIL, K.W., SAIF, Y.M., 1986. Demonstration of Rotavirus and Rotavirus-like virus in the intestinal contents of diarrheic pheasant chicks. Avian Dis. 31:376-379.

WYETH, P.J., CHETTLE, N.J., LABRAM, J., 1981. Avian calicivirus. Vet. Rec. 21:109-477.

CASE REPORT

Big liver and spleen disease in broiler breeders in Italy

Paola Massi, Giovanni Tosi, Daniela Bassi, Daniela Gelmetti, Antonio Lavazza, Guerino Lombardi, Gloria Torcoli

Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna. Forlì, Italy

Corresponding author: Dr. Paola Massi. Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna. Via Marchini 1, 47100 Forlì, Italy – Tel.+39 0543 721533 – Fax: +39 0543 721533 – Email: paolamassi@bs.izs.it

ABSTRACT

For the first time in Italy, we have reported two outbreaks resembling big liver and spleen disease in broiler breeder flocks. The combination of clinical signs and pathological findings and the laboratory investigation results appeared corrisponde to previously recorded outbreaks in other countries.

Key Words: Big liver and spleen disease, Broiler breeders, *Calicivirus*-like virus, Diagnosis.

RIASSUNTO

BIG LIVER AND SPLEEN DISEASE IN RIPRODUTTORI PESANTI IN ITALIA

Per la prima volta in Italia vengono descritti due casi riferibili alla sindrome denominata big liver and spleen disease *in gruppi di polli riproduttori pesanti. I segni clinici e le lesioni anatomo-patologiche osservate nonché i risultati delle indagini di laboratorio condotte sui gruppi colpiti appaiono sovrapponibili a quelle già descritte in passato in altri paesi.*

Parole chiave: *Pollo, Malattia del fegato e della milza grossi, Riproduttori pesanti,* Calicivirus*, Diagnosi.*

Introduction

Big liver and spleen disease (BLS) is an infectious, transmissible disease of uncertain, but probably viral, aetiology. It is characterized by sudden drop in egg production, increased mortality and enlargement of the liver and spleen of mature chickens, especially broiler breeders and, less commonly, egg layers. BLS was first recognized in Australia in 1980. The syndrome is considered economically important to the mayor integrated companies of broiler breeders in that country. Antibody has also been found in the United Kingdom and later in the USA. Moreover infection with the BLS agent has been associated with a condition known as "primary feather and drop syndrome" in the United States (Barnes, 1997). Until now there are no reports of this syndrome in Italy. This paper gives a report of the clinical, pathological and laboratory findings noted in two broiler breeder flocks affected with a disease related to BLS.

Material and methods

Carcasses and blood samples from two broiler breeder farms were submitted for diagnostic examination and laboratory investigation at the Istituto Zooprofilattico Sperimentale of Forlì. Anamnestic data were collected, post-mortem examinations were carried out on the carcasses and appropriate samples were taken for the following laboratory investigation.

Laboratory examinations

For bacteriological examinations samples of liver, spleen and ovary were inoculated on blood agar and Hektoen Enteric Agar and incubated aerobically at 37°C.

For virological examination, samples of liver, spleen, intestine, ovary, trachea, lung and brain were inoculated on Chicken Embryo Liver Cell Cultures (CEL) and Specific Pathogen Free (SPF) embryonated chicken eggs A Polymerase Chain Reaction (PCR) for group 2 avian adenovirus was also performed.

The following serological tests were performed on blood samples: 1) agar-gel diffusion precipitin (AGP) for group 1 avian adenovirus. 2) Enzyme-linked immunosorbent assay (ELISA) for group 2 avian adenovirus (Synbiotics®). 3) ELISA test for Human Epatitis E Virus (Nuclear Laser Medicine®).

For Electron Microscopy (EM) observation, samples of liver and spleen were frozen and thawed twice, the supernatant was harvested and centrifuged at 4,000 *g* for 20 min. and at 9300 *g* for 10 min. for clarification. The second supernatant (85 µl) was then ultracentrifuged in Airfuge Beckman for 15 min. at 21 psi (82,000 g). Grids were then stained using 2% sodium phosphotungstate (NaPt), pH 6.8, for 1.5 min., and observed with a TEM Philips CM10.

For histological examination, representative samples of spleen, liver, heart and kidney were fixed in 10% buffered formalin, dehydrated in ethanol, cleared in xylene and embedded in paraffin, according to standard techniques. Thin sections (4µm) were cut and stained with haematoxylin-eosin.

For genetic identification of avian HEV, reverse transcription polymerase chain reaction (RT-PCR) was run on liver homogenate sample that has been collected from a chicken which has been resulted positive by electron microscopy (EM). Viral RNA was extracted with guanidine/isothiocyanate technology (NucleoSpin RNA II, GTC/silice); total RNA was reverse-trancribed with avian HEV-specific primers FAHEVEpF/RAHEVEpR (Huang *et al.*, 2004) targeting ORF2 region of HEV genome (fragment lenght 372 bp). The parameters for each round of PCR included denaturation at 95°C for 6 min, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 50 sec at 58°C and extension for 1 min at 72°C, with a final incubation at 72°C for 7 min. Negative control has been included. Finally, PCR product has been analysed by gel-electrophoresis.

Results and discussion

During January 2004, 37 week-old Ross 508 broiler breeders from a farm of 17,000 animals housed in three flocks experienced a slightly increased mortality (+0.2%) in two flocks and a 2% drop in egg production for three weeks. A large number of chickens had a drop of feathers (resembling premature moulting) that persisted for several weeks after recovery from the disease. In the same period a similar syndrome was observed in another farm of 44 week-old Ross 508 broiler breeders of 24,000 animals. At necropsy, all the dead birds were in good condition. The most frequently found lesion was an enlarged spleen. The liver was also enlarged and friable. Other lesions included: cyanosis of the head, hydropericardium, pulmonary congestion and oedema, enteritis, ovarian regression and congestion, swollen kidneys. Microscopically the following lesions were recognized: congestion and oedema of the liver associated with multiple foci of necrosis and an increase of perivascular lymphoid tissue; widespread necrosis of lymphoid tissue and proliferation of macrophages in the spleen; *adipositas cordi* in the heart.; congestion and interstitial infiltration of lymphoid tissue in the kidneys. Bacteriologically a strain of *Escherichia coli* was isolated from the liver. Virological examinations (i.e. embryo egg inoculation, and PCR for group 2 adenovirus) were negative. By negative staining Electron Microscopy few viral isolated particles morphologically resembling calicivirus were observed in the liver and spleen. EM-positive liver sample turned out to be positive for avian HEV by RT-PCR, showing a 372 bp fragment by gel-electrophoresis.

Serological tests demonstrated reacting antibodies with group 1 and group 2 avian adenovirus. Furthermore, 3 of 15 blood samples collected from the first observed outbreak were positive with ELISA test for human hepatitis E virus antibodies.

Conclusions

Clinical signs, macroscopic and microscopic lesions, age and production type of the affected flocks were sufficient for a presumptive diagnosis of BLS. Other diseases that produce splenomegaly were excluded. Seroconversion toward group 2 avian adenovirus wasn't associated to other features of the avian adenovirus group 2 splenomegaly chicken disease (AAS). *Escherichia coli* strain recovered from affected birds is a consequence of BLS, but it is not involved in causing the disease (Barnes, 1997). To confirm the diagnosis of BLS, the electron microscopy observation, of few isolatd calicivirus-like particles in the liver and spleen, was very interesting as well as the RT-PCR results, the histological lesions and the detection of antibodies reacting with human hepatitis E virus. Recently, in the United States, a new virus, designated avian hepatitis E virus (avian HEV) was identified and characterized from chickens with hepatitis-splenomegaly syndrome (HS), a disease very similar to BLS described in Australia (Haqshenas *et al.*, 2001). So far, it is likely that BLS in Australia and the HS syndrome in North America are caused by variant strains of the same virus (Haqshenas *et al.*, 2002). Avian HEV is genetically related to but distinct from human and swine HEV (Huang *et al.*, 2004). The antigenic cross reactivity among avian and human HEVs was confirmed by ELISA (Huang *et al.*, 2002). HEVs (including human, swine and avian strains) were formerly classified as a member of the family *Caliciviridae*. But the lack of common features between HEVs and caliciviruses has led to the recent removal of HEV from this family (Berke *et al.*, 2000). In conclusion field and laboratory data reported in this work lead us to confirm the diagnosis of BLS in the broiler breeder flocks observed.

REFERENCES

BARNES, H.J., 1997. Big liver and spleen disease. In: B.W. Calnek (ed.) Diseases of Poultry, 10th ed. Iowa State University Press, Ames, Iowa, USA.

BERKE, T., MATSON, D.O., 2000. Reclassification of the Caliciviridae into distinct genera and exclusion of hepatitis E virus from the family on the basis of comparative phylogenetic analysis. Arch. Virol. 145:1421-1436.

HAQSHENAS, G., SHIVAPRASAD, H.L., WOOLCOCK, P.R., READ, D.H., MENG, X.J., 2001. Genetic identification and characterization of a novel virus related to human hepatitis-splenomegaly syndrome in the United States. J. Gen. Virol. 82:2449-2462.

HAQSHENAS, G., HUANG, F.F., FENAUX, M., GUENETTE, D.K., PIERSON, F.W., LARSEN, C.T., SHIVAPRASAD, H.L., TOTH, T.E., MENG, X.J., 2002. The putative capsid protein of the newly identified avian hepatitis E virus shares antigenic epitopes with that of swine and human hepatitis E viruses and chicken big liver and spleen disease virus. J. Gen. Virol. 83:2201-2209.

HUANG, F.F., HAQSHENAS, G., SHIVAPRASAD, H.L., GUENETTE, D.K., WOOLCOCK P.R., LARSEN, C.T., PIERSON, F.W., ELVINGER, F., TOTH T.E., MENG, X.J., 2002. Heterogeneity and Seroprevalence of a Newly Identified Avian Hepatitis E Virus from Chickens in the United States. J. Clin. Microbiol. 40:4197-4202.

HUANG, F.F., SUN, Z.F., EMERSON, S.U., PURCELL, R.H., SHIVAPRASAD, H.L., PIERSON, F.W., TOTH, T.E., MENG, X.J., 2004. Determination and analysis of the complete genomic sequence of avian hepatitis E virus (avian HEV) and attempts to infect rhesus monkeys with avian HEV. J. Gen. Virol. 85:1609-1618.

CASE REPORT

Sancassania berlesei (Michael, 1903): an opportunistic mite infesting litters in poultry farms causing dermatitis in humans and animals

Mario Principato, Federica Lisi, Iolanda Moretta, Nada Samra, Francesco Puccetti

Dipartimento di Scienze Biopatologiche Veterinarie. Università di Perugia, Italy

Corresponding author: Prof. Mario Principato. Dipartimento di Scienze Biopatologiche Veterinarie. Facoltà di Medicina Veterinaria, Università di Perugia. Via S.Costanzo 4, 06100 Perugia, Italy – Tel. +39 075 5857741 – Fax: +39 075 5857743 – Email: drprincipato@email.it

ABSTRACT

Reported herein are some cases of human dermatitis caused by *S. berlesei*, a mite coming from seriously infested poultry farms. It appears unable to determine traumatic lesions on human skin, but it causes itch and inflammation also at the level of mucosas. Besides this mite can be found accidentally also on reared fowls'wounds by peak.

Key Words: Mite, *Sancassania berlesei*, Dermatitis, Itch, Exam of home dusts.

RIASSUNTO

SANCASSANIA BERLESEI (MICHAEL, 1903): UN ACARO OPPORTUNISTA CONTAMINANTE LA LETTIERA DI ALLEVAMENTI AVICOLI, CAUSA DI DERMATITI NELL'UOMO E NEGLI ANIMALI

Vengono presentati alcuni episodi di dermatite umana provocati da Sancassania berlesei*, un acaro proveniente da allevamenti avicoli fortemente infestati. Questa specie non sembra in grado di determinare lesioni traumatiche sulla cute dell'uomo, ma, piuttosto, prurito e infiammazione, soprattutto a livello delle mucose dei genitali. Questo acaro si rinviene, occasionalmente, anche nelle ferite da beccata di volatili in allevamento.*

Parole chiave: *Acaro,* Sancassania berlesei*, Dermatite, Prurito, Esame delle polveri ambientali.*

Introduction

Sancassania berlesei, better known as *Caloglyphus berlesei*, is an environmental mite of zootechnic interest, for it develops both in dried feedstuff and in litters of big industrial poultry farms.

The species was described morphologically in 1903 by Michael and *Sancassania genus* in 1916 by Oudemans. The interest for this mite in veterinary medicine was reported for the first time in Italy by Principato *et al.* (1987; 1991a). Besides recording the presence of that mite both in dried feedstuff and inside the farms, they described some lesions on fowls from which *S. berlesei* was isolated.

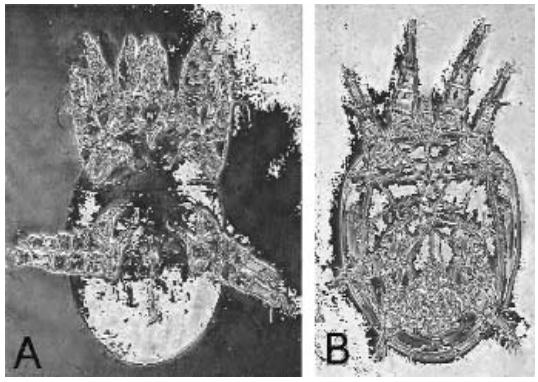
The hosts, where the mite was observed, were *Gallus gallus, Numida meleagris, Phasianus calchicus e Turdus merula.*

Crusty lesions were variously scattered on the hosts' skin, above all in periocular areas, around the beak, but the mites were observed also on the feathers.

The inflammation caused itch and in some samples, particularly infested, the symptom of diarrhoea was reported as well.

Afterwards Principato *et al.* (1991b; 1991c) supplied with a S.E.M. description of adults and hypopial deutonymphs (Figure 1) of this species and in 1992 they recorded its presence in some umbrian intensive chicken farms. Nowadays, *S. berlesei* seems to be widespread and it is recorded everywhere, sometimes even in dwellings. Reported herein are some cases of human and animal dermatitis referred to the presence of *S. berlesei*.

Figure 1. *Sancassania berlesei* adult (A) and Hypopial deutonymph (Hypopus) (B).



Material and methods

The cases of dermatitis herein described were recorded through our exams of environmental dusts carried out from 2000 to 2004 in dwellings, whose owners had frequent contact with conserved farinaceous food and feedstuff and besides that took care of rearing fowls, such as chickens, turkeys, pigeons and geese. A number of 76 exams of dust samples nearly all coming from rural chicken farms was examined, coming from those dwellings where symptoms of dermatitis and itch of indefinite cause were reported.

A number of 27 were recorded in spring-summer and 49 in autumn-winter. The diagnosis was made by examining both home dusts and the dust removed from the poultry houses. The exam was effected by flotation with a saturated solution of NaCl after filtering and precipitation in absolute ethyl alcohol.

Results and discussion

The direct exam of home dusts of owners of fowls revealed the presence of *S. berlesei* in 68% of dermatites observed in spring-summer (Figure 2) and 14% of the cases reported in autumn-winter (Figure 3). In all the cases in which *S. berlesei* was isolated in the houses, a great spread of that mite was recorded also in fowl runs with an average of mites of about n. 4000/g of dust in spring-summer and about n.1000/g of dust in autumn-winter. The number of hypopial deutonymphs increased about at half the cycle of rearing of animals. The dermatitis observed were in most cases itchy, though not continuously. The mites were frequently isolated in clothes and in underwear and itch occurred more frequently on patients' genitals, inguinal area, arms and head. In the most serious cases it was spread also on their trunk and neck. The dermatites observed appeared always as folliculitis complicated by scratching.

Mites could be isolated from some pets' skin. On dogs the inflammation of their skin of abdomen and of the internal part of tighs resulted accompanied by a strong stimulus to scratch themselves.

On *Passeriformes* and on *Psittaciformes* the tendency of animals to pluck their feathers was evident. In chickens S. berlesei was present mainly inside the wounds caused by peaks, in their feathers and periocular and cloacal areas.

In general in spring-summer a higher percentage of cases of dermatitis by *S. berlesei* was reported (72%) in comparison with the autumn-winter period (28%).

Conclusions

Although the cases of human dermatitis caused by *S. berlesei* from birds are very few, it is a fact that this mites can determine itch and allergy in humans and animals. The presence of the arthropod also in underwear, with the high frequency of itch in the genital and perigenital areas appears to be interesting. *S. berlesei* is a mite that colonizes, though accidentally, animals' wounds and mucosas and its presence also in women's mucosa of vulva and vagina cannot be excluded. In this case its sanitary interest is to be correlated to the presence of bacteria of which the mite is certainly a reservoir. The possibility for this mite to

Figure 2. Frequency of cases of human dermatitis caused by environmental mites in spring-summer in people dealing with poultry houses.

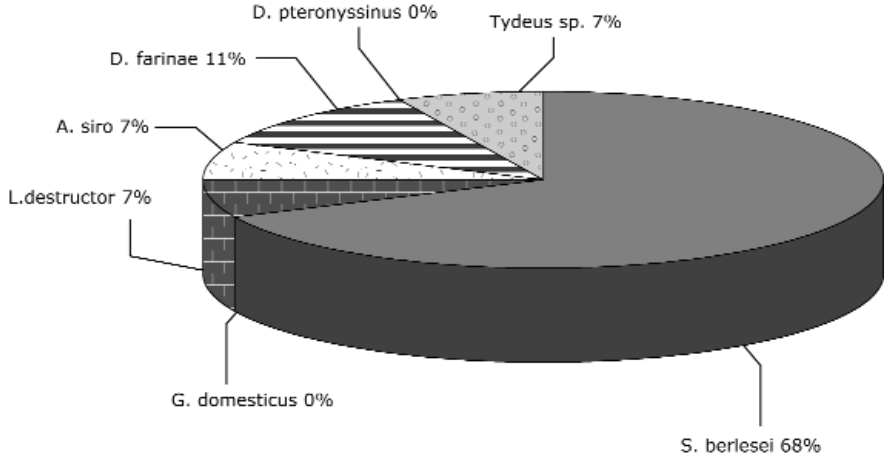
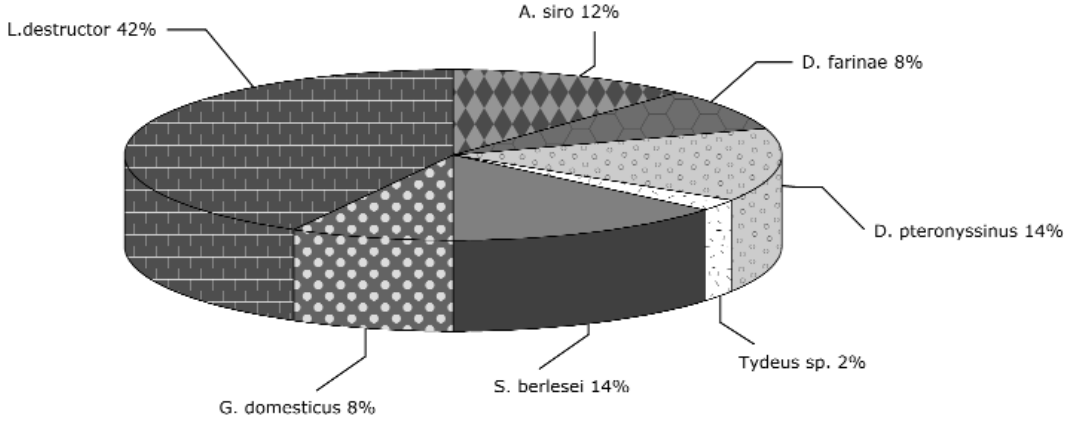


Figure 3. Frequency of human dermatitis caused by environmental mites in autumn-winter in people dealing with poultry houses.



transform itself in hypopes makes it possible the infestation from poultry houses to human dwellings with problems of allergy that can arise even some years after the contagion.

Since *S. berlesei* is a mite present in a lot of poultry farms and it is easily adaptable to any fowl run, it is necessary to contain its number through targeted treatments of litters, in order to avoid the chance of human contagion.

A useful note for a differential diagnosis is that, contrarily to the dermatitis caused by *Glycyphagus domesticus*, the one caused by *S. berlesei* appears mainly in springtime and in summer without strophuloid lesions.

REFERENCES

PRINCIPATO, M., COLETTI, M., SACCONI, G., 1987. Studio sull'acarofauna dei volatili. Il ruolo degli acari negli stati patologici aspecifici. Isolamento di nuove specie patogene. Summa. 4:229-237.

PRINCIPATO, M., GALLI, R., SANNIPOLI, C.G.T., 1991a. Observations on the presence of *Caloglyphus berlesei (Astigmata: Acaridae)* in the litter of some industrial poultry farms in Umbria. Zootecnica International. 13(2):40-45.

PRINCIPATO, M., GROSSI, M., POLIDORI, G.A., 1991b. Osservazioni al M.E.S. sulle forme invasive di *Caloglyphus berlesei (Acarina: Acaridae)*. pp 99-100 in Proc. 18th Nat. Congr. Microscopia Elettronica, Padova, Italy.

PRINCIPATO, M., ROSSODIVITA, M.E., GROSSI, M., POLIDORI, G.A., 1991c. Le ninfe ipopiali di *Caloglyphus berlesei (Michael) (Acarina: Acaridae)*: osservazioni al M.E.S. pp 95-96. Atti del 18° Congr. Naz. di M.E., 24-28 Sett. 1991. Padova, Italia.

CASE REPORT

Ascaridia galli: a report of erratic migration

Daniela Piergili Fioretti, Fabrizia Veronesi, Manuela Diaferia, Maria Pia Franciosini, Patrizia Casagrande Proietti

Dipartimento di Scienze Biopatologiche Veterinarie. Università di Perugia, Italy

Corresponding author: Prof. Daniela Piergili Fioretti. Dipartimento di Scienze Biopatologiche Veterinarie, Sezione di Parassitologia. Università di Perugia. Via S. Costanzo 4, 06126 Perugia, Italy – Tel. +39 075 5857740 Fax: +39 075 5857743 – Email: dpf@unipg.it

ABSTRACT

This paper describes a case of an unusual recovery of adult *Ascaridia galli* in hen's egg. Several data are available on this occurrence but it appears to be the first case described in Italy. The worm was identified as an adult female, 6.8 cm in length, with three trilobed lips, cervical narrow alae, oesophagus club-shaped without posterior bulb, vulva near the middle of body, with gravid uteri containing a large number of eggs. The presence of *Ascaridia galli* in hen's eggs cannot be considered as hazard for public health but may be cause of a potential consumer complaint. Moreover it is a sign of presence of ascaridiosis, parasitosis that still produces economic losses in modern poultry production system.

Key Words: Hen, *Ascaridia galli*, Erratic migration, Egg.

RIASSUNTO

ASCARIDIA GALLI: UN CASO DI MIGRAZIONE ERRATICA

Nel seguente lavoro viene descritto l'inconsueto ritrovamento nell'albume di un uovo commerciale di gallina di un esemplare adulto di Ascaridia galli*. Dalla bibliografia a nostra disposizione, relativa all'argomento, questa risulta essere la prima segnalazione effettuata nel nostro Paese.*

Il nematode isolato è stato identificato come femmina adulta di Ascaridia galli*, di 6,8 cm di lunghezza, morfologicamente caratterizzato da bocca trilabiata, esofago claviforme con assenza di bulbo posteriore, strette ali cefaliche, vulva situata nella parte mediana del corpo. L'esemplare inoltre era gravido con un utero contenente numerose e caratteristiche uova. Il riscontro di* Ascaridia galli *nelle uova di gallina, pur non rappresentando un rischio per la salute pubblica, è tuttavia fonte di estremo disagio per i consumatori. Recentemente l'affermarsi di sistemi di allevamento alternativi ha di nuovo reso attuale la presenza di questa parassitosi nel settore avicolo.*

Parole chiave: *Gallina ovaiola,* Ascaridia galli*, Migrazione erratica, Uovo.*

Introduction

Ascaridia galli, the largest and the most common helminth of the small intestine of chickens, is a parasite with a direct life cycle and poultry is infected by ingestion of embryonated eggs containing the second larval stage (L2). The infection is a direct consequence of faecal contamination of environment. The parasite completes its life cycle exclusively in the intestinal tract with a larval migratory phase into the enteric wall.

One of the most striking effects of infection is the occasional finding of this parasite in the hen's egg. Although several observations of this phenomenon have been made in literature (Akinyemi *et al.*, 1980; Omran, 1982; Manna, 1992), as far as we know, it is the first case reported in Italy.

Material and methods

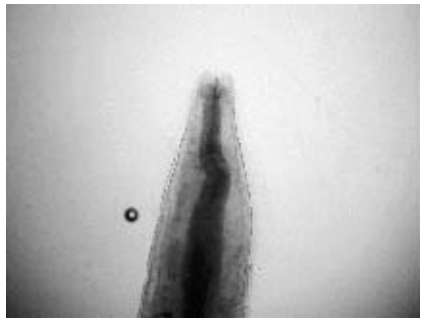
In this study a fresh chicken egg, with a white filiform structure referable to a round worm in the albumen (Figure 1), bought by a private consumer and coming from an Umbrian commercial farming, was submitted to our attention.

The worm isolated, washed in distilled water, kept in physiological solution for 2 hours at 40°C to help the extension, fixed in 70% alcohol for 24 hours, clarified in lactophenol of Amman for 10 hours, mounted on a microscope slide, was observed by light microscopy at 10x- 40x.

On the basis of morphometric characteristics (6,8 cm in lenght, three trilobed lips, cervical narrow alae, oesophagus club-shaped without posterior bulb (Figure 2), vulva near the middle of body, gravid uteri containing a large number of eggs, the worm was identified as an adult female of *Ascaridia galli* (Yamaguti, 1961).

Figure 1. An adult of *Ascaridia galli* in albumen of hen's egg.

Figure 2. Oesophagus club-shaped without posterior bulb.



Results and discussion

The floatation method with a sugar $NaNO_3$ solution allowed to evidence the presence in the albumen of typical *Ascaridia galli* eggs, oval in shape, with smooth shell and size of 75 x 30 _m.

It is possible that this occurrence is quite frequent but the common use of hard-boiled eggs could make unnoticeable the presence of these worms in the boiled egg albumen.

A primary localization in the genital apparatus of *Ascaridia galli* larva L_2 with subsequent maturation to aduld worm has to be excluded because the egg production is more quick than the time required for maturity of larva. In addition the adult worm in egg was gravid and *Ascaridia galli* is not an hermaphrodite nematode. Presumably the worms migrate up the oviduct via the intestinal wall and peritoneal cavity or via the cloaca with subsequent inclusion in the egg.

Although the presence of *Ascaridia galli* in hen's eggs cannot be considered as hazard for public health, it may be cause of potential consumers complaint.

This finding is noteworthy since it indirectly points out the problems related to ascaridiosis, that at present is playing an important role in the alternative rearing systems, such as the organic farming.

It is known that the most of the conventional farming could be shortly converted on the floor system on the basis of 74/99 CEE regulation.

Conclusions

Recent epidemiological studies carried out on different poultry breeding systems showed a *Ascaridia galli* prevalence of 63.9% in organic chickens and a prevalence of 41.9% in chickens reared in covered strawyard. The prevalence reported in breeders and in caged hens was respectively 37.5% and 5% (Permin *et al.*, 1999).

On the basis of these epidemiological data and of our report further investigations are suggested to obtain more information on the occurrence of Ascaridiosis in Umbria (Central Italy) in relation to diffusion of alternative housing systems in hens.

REFERENCES

AKINYEMI, J.O., OGUNJI, F.O., DIPEOLU, O.O., 1980. A case of adult *Ascaridia galli* in hen's egg. Int. J. Zoonoses. 7:171-172.

MANNA, B., 1992. A report of *Ascaridia galli* within the albumen of hen's eggs. Ind. J. Anim. Health. 31 (1):89.

OMRAN LAILA, A.M., 1982. *Ascaridia galli* (Shrank, 1788): an erratic parasite in a fowl's egg albumin. J. Egypt Soc. Parasitol. 12 (1):167-168.

PERMIN, A., BISGAARD, M., FRANDSEN, F., PEARMAN, M., KOLD, J., NANSEN, P., 1999. Prevalence of gastrointesrinal helminths in different poultry production system. Brit. Poultry Sci. 40:439-443.

YAMAGUTI, S., 1961. Systema Helminthum. 1st ed. Interscience, Publishers Inc., New York, NY, USA.

The Editor-in-Chief and the Guest Editors are indebted with the following reviewers for their assistance in refereeing papers and case reports submitted to the Scientific Committee of the Congress.

Camarda, A.; Catelli, E.; Fioretti, A.; Gallazzi, D.; Gavazzi, L.; Grilli, G.; Lavazza, A.; Moreno Martin, A.; Ortali, G.; Pascucci, S.; Piccirillo, A.; Stonfer, M.; Terregino, C.; Tosi, G.