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Wild birds as biological indicators of environmental pollution: biotyping and antimicrobial resistance patterns of *Escherichia coli* isolated from Audouin's gulls (*Larus audouinii*) living in the Bay of Gallipoli (Italy)

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ABSTRACT

E. coli biotyping and antimicrobial susceptibility tests were performed on 48 cloacal swabs collected from a population of Audouin's gulls (*Larus audouinii*) living in the Bay of Gallipoli (Lecce, Italy). The aim was to assess the pathogenic potential of the strains the gulls carry and shed into the environment and to gain a better understanding of the microbial pollution of the area they live in. Only one *E. coli* strain was rhamnose - (Rh) (biotype 8). All the other strains were Rh + with a prevalence of biotypes 30 and 31. Overall, the antimicrobial susceptibility tests showed a low level of antibiotic resistance. The findings suggest that the gull population does not have a heavy impact on the microbial nature of the Bay of Gallipoli and that the area does not present a high level of microbiological pollution.

Key Words: *E. coli*, Biotyping, Gulls, Antibiotic resistance.

RIASSUNTO

GLI UCCELLI SELVATICI COME INDICATORI BIOLOGICI DELL'INQUINAMENTO AMBIENTALE: BIOTIPIZZAZIONE E ANTIBIOTICO-RESISTENZA DI *E. COLI* ISOLATI DA GABBIANI CORSI (*LARUS AUDOUINII*) STANZIALI NELLA BAIA DI GALLIPOLI (LECCE)

In questo lavoro n. 48 stipti di *E. coli* isolati da tamponi cloacali eseguiti su esemplari di Gabbiano Corso (*Larus audouinii*), facenti parte di una colonia stanziale nella baia di Gallipoli (Lecce), sono stati biotipizzati e sottoposti al test di sensibilità agli antibiotici al fine di valutare la potenzialità patogena degli stipti veicolati ed eliminati nell'ambiente dagli animali ed ottenere informazioni sulla qualità microbiologica dell'ambiente in cui essi vivono. Un solo stipte ha manifestato una risposta negativa al test del ramnosio (Rh) (Biotipo 8). Tutti gli altri appartenevano a biotipi Rh + con una netta prevalenza dei biotipi 30 e 31. I test di sensibilità agli antibiotici hanno evidenziato una resistenza mediamente scarsa. Questi risultati sembrano dimostrare il modesto impatto esercitato dalla popolazione di gabbiano corso sulla qualità microbiologica dell'ambiente della baia di Gallipoli ed evidenziare lo scarso grado di inquinamento microbiologico dell'area.

Parole chiave: *Escherichia coli*, Biotipizzazione, Gabbiano corso, Antibiotico resistenza.

Table 1. Antibiotic susceptibility tests of n.48 *E. coli* strains isolated from cloacal swabs of Audouin's gulls.

Antimicrobial agents	Tested strains	Susceptible strains		Intermediate susceptibility strains		Resistant strains	
		n.	%	n.	%	n.	%
Gentamicin	47	47	100	0	0.0	0	0.0
Amikacin	31	31	100	0	0.0	0	0.0
Tetraciline	48	23	47.9	14	29.2	11	22.9
Enrofloxacin	46	42	91.3	2	4.3	2	4.3
Flumequine	46	41	89.1	1	2.2	4	8.7
Trimethoprim/Sulphametoxazole	48	44	91.7	0	0.0	4	8.3
Amoxicillin	48	33	68.8	2	4.2	13	27.1
Apramycin	48	44	91.7	4	8.3	0	0.0
Difloxacin	48	45	93.8	1	2.1	2	4.2
Nalidixic ac.	48	39	81.3	2	4.2	7	14.6
Neomycin	48	32	66.7	16	33.3	0	0.0
Colistin	21	20	95.2	1	4.8	0	0.0

Introduction

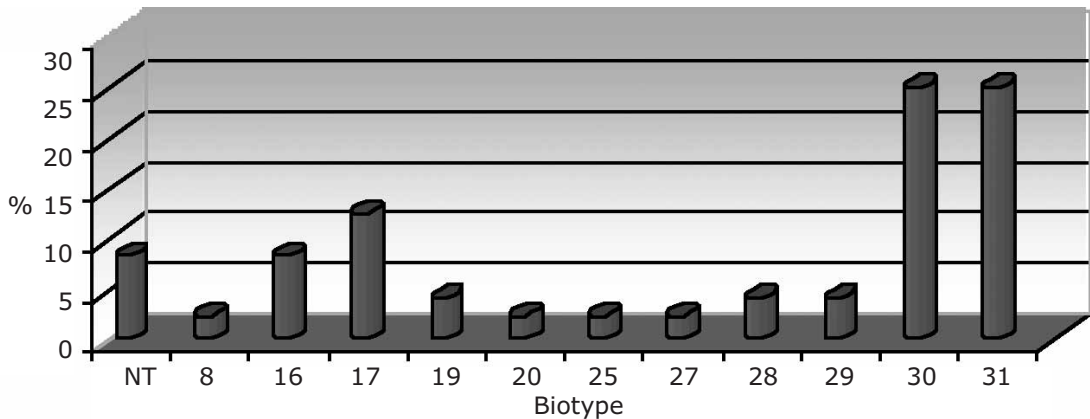
Gulls are considered to be important indicators of the level of pollution of their habitats. Their feces often contain enteric bacteria (*E. coli*, *Salmonella spp.*, *Aeromonas spp.*, *Campylobacter spp.*) that are potentially pathogenic for humans (Levesque *et al.*, 1993). It is generally thought that gull feces contribute to the bacterial pollution of superficial waters and coastal areas where the birds live (Fogarty *et al.*, 2003). *E. coli* is considered a quality indicator sentinel for coastal waters. Therefore, the characterization of *E. coli* can provide useful information on the quality of an environment and assess the potential repercussions of microbial pollution on public health. Amongst the methods used to differentiate *E. coli* strains, biotyping is a technique which is simple to perform and provides an indication of the pathogenicity of the strains analyzed (Camguilhem and Milon, 1989). Biotyping is based on the strains' ability to ferment 5 different sugars (dulcitol, D-raffinose, L-

rhamnose, sorbose, and saccharose). Evaluation of a strain's sensitivity to antibiotics is another useful way to differentiate *E. coli* given that strains with a greater pathogenic potential present resistance to a wide range of antibiotics (Krumperman, 1983; Harwood *et al.*, 2000). In this study *E. coli* strains isolated from Audouin's gulls (*Larus audouinii*) living in the Bay of Gallipoli (Lecce, Italy), were biotyped and tested for their antibiotic susceptibility to evaluate the pathogenic potential of the strains the gulls carry and shed into the environment and the related public health risks implied. .

Material and methods

Source of *E. coli* strains

Forty-eight *E. coli* strains isolated from cloacal swabs of Audouin's gulls (*Larus audouinii*), living in a permanent colony in Gallipoli Bay (Lecce, Italy), were analyzed. The birds were sampled during the 2002 spring ringing program. The samples were put into Amies medium immediately after they were col-

Figure 1. Biotype of *E. coli* isolated from Audouin's gulls.

NT: not typed

lected and sent under refrigerated conditions to the laboratory where they were cultured on Agar Mac Conkey (Oxoid) at 37°C for 24h. All the suspect bacterial colonies were isolated on nutrient agar and confirmed to be *E. coli* by the API 20E Test (bioMérieux). Each isolated *E. coli* strain was cultured in Brucella Broth supplemented with 20% glycerol and kept in cryovials at -80°C until the next tests were performed.

Biotyping

Biotyping was performed according to the method described by Camguilhem and Milon (1989).

Antibiotic susceptibility testing

Laboratory trials were performed in accordance with the principles described in the standard method of the *National Committee for Clinical Laboratory Standards* (NCCLS, 2002), using the following antimicrobial agents: gentamicin (GM10), amikacin (AN30), tetracycline (TE30), enrofloxacin (ENR5), flumequine (AR30), trimethoprim/sulphamethoxazole (SXT), amoxicillin (AMX 25), apramycin (APR30), difloxacin (DFX10), nalidixic acid (NA30), neomycin (N30), colistin (CL50).

Results and discussion

Based on the biotyping results (Fig. 1) only one *E. coli* strain was rhamnose - (Rh) (biotype:

8) while all the other strains were Rh +. Biotypes 30 and 31 were clearly prevalent.

Antimicrobial susceptibility tests showed a low resistance in all the strains. All the tested strains were susceptible to gentamicin and amikacin, while more than 90% of them were sensitive to colistin, difloxacin, apramycin, trimethoprim/sulphamethoxazole and enrofloxacin (Table 1); 54.16% of the strains showed no resistance to any of the tested drugs. Multi-resistance was present in very few strains with, 29.16% of the strains presenting resistance to only one antibiotic and 6.5% to 2 and 3 drugs. Only two strains showed a broader range of multi-resistance involving 6 antimicrobial agents

Conclusions

Gulls can play an important role in the spread of potentially pathogenic agents along humid coastal areas; their feces, abounding in *E. coli*, may contribute to the pollution of superficial waters as well as bathing places, often attended by human beings (Whitman and Nevers, 2003). The findings of our study suggest that gulls may carry a wide diversity of *E. coli* types. Nevertheless, the *E. coli* strains isolated were almost exclusively Rh + and this is indicative of a low pathogenic potential. Confirmation of these results by the currently ongoing molecular typing investigations would

prove that the gulls living in the Bay of Gallipoli are not the main cause for microbiological pollution of the area. The results of the antibiotic susceptibility tests seem to substantiate this assumption since the isolated strains were highly susceptible to drugs active against Gram- bacteria and showed a very restricted multi-resistance range, which in coliform bacteria is linked to plasmidic transmission. The peculiar diet of Audouin's gulls, which unlike other gull species do not visit rubbish dumps and prefer to feed on fish , may have concurred to the results reported . Detection of resistance to a narrow range of antibiotics coupled with the fact that these birds are mainly exposed to bacterial groups polluting the Gallipoli coastline and coastal waters, would suggest that there is not a high level of microbial pollution in the area populated by Audouin's gulls.

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Survey of *Campylobacter jejuni* and *Campylobacter coli* in different taxa and ecological guilds of migratory birds

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ABSTRACT

A total of 169 faecal samples were collected from migrating birds, belonging to the Order of *Passeriformes*, in Campania region in order to isolate *Campylobacter* spp.

Campylobacter spp. were isolated from 39 of the 169 birds examined (23.1%). Among these 36 were identified as *C. jejuni* and the remaining strains were identified as *Campylobacter coli*. Given the high isolation rates wild birds could be considered natural reservoir of infection.

Key Words: *Campylobacter jejuni*, *Campylobacter coli*, Migratory birds, Taxa, Ecological guilds.

RIASSUNTO

PRESENZA DI *CAMPYLOBACTER JEJUNI* E *CAMPYLOBACTER COLI* IN DIFFERENTI TAXA E NICCHIE ECOLOGICHE DI UCCELLI MIGRATORI

Sono stati effettuati 169 campioni fecali da uccelli migratori appartenenti all'Ordine *Passeriformes* nella regione Campania per la ricerca di *Campylobacter* spp. *Campylobacter* spp. è stato isolato da 39 dei 169 esemplari esaminati (23,1%). Tra questi, 36 sono stati identificati come *C. jejuni* mentre i rimanenti come *C. coli*. L'alta percentuale di isolamento confermerebbe gli uccelli selvatici quali reservoir naturali di infezione.

Parole chiave: *Campylobacter jejuni*, *Campylobacter coli*, Uccelli migratori, Taxa, Nicchie ecologiche.

Introduction

Campylobacter jejuni is regarded as the most common cause of foodborne bacterial enteritis in humans and have been isolated from a variety of ecological sources, including wild and domestic animals, foods, and the environment.

The main sources of infection of human campylobacteriosis are considered to be the consumption

of undercooked poultry meat, eggs, etc. (Chuma *et al.*, 2000). *C. jejuni* contamination of poultry meat during processing has been well documented. It is also well known that broilers on growing farms are often infected with *C. jejuni* or *C. coli* (Chuma *et al.*, 2000). However, the epidemiology of these infections to broilers is little known.

Gregory *et al.* (1997) reported that no specific contamination source could be identified but it

seemed to be found among any potential elements outside of broiler houses like rodents and wild birds. For decades wild birds have been considered natural vertebrate reservoirs of *Campylobacter* spp. (Waldeström *et al.*, 2002) and are frequently mentioned as possible vectors for transmission to poultry and humans. Due to their great mobility, wild-living birds may function as effective spreaders of disease through fecal contamination of pastures, forage, and surface waters (Kapperud and Rosef, 1983).

The aim of present study is to carry out a monitoring on the presence of thermotolerant *Campylobacter* in resident and migratory wild birds of Campania region and obtain more information about the role played by wild birds in spreading and maintaining of these agents in nature.

Material and methods

This study was carried out from February 2005 to April 2005 attending to a monitoring and ringing program of migratory birds at Migration Study Stations located in "Parco Regionale del Matese" in Campania region.

A total of 169 faecal samples were collected from birds belonging to the Order of Passeriformes. Passerines were trapped with mist-nets. With reference to the size of the birds, faecal samples or cloacal swabs were collected and placed in Amies Transport Medium (Oxoid Ltd., Basingstoke, Hampshire, UK).

Isolation and identification of *Campylobacter*

was carry out according to Chuma *et al.* (2000) procedure.

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

Results and discussion

Campylobacter spp. were isolated from 39 of the 169 birds examined. The mean prevalence of *Campylobacter* was 23.1% for all tested birds but varied from 0 to 46.3% between species. Among these isolates, 36 were identified as *C. jejuni* and the remaining strains were identified as *Campylobacter coli*.

The prevalence of *Campylobacter* spp. is heterogeneous also in different ecological guilds. No *Campylobacter* spp. was isolated in granivores or aerial insectivores. In contrast, most guilds that forage at ground level (*E. rubecula*, *T. merula*, *L. megarhynchos*), and arboreal and herbaceous insectivores (*S. atricapilla*, *S. melanocephala*, *S. rubetra*, *C. cetti*) showed high prevalence rates of *Campylobacter* spp (Table 2).

We compared prevalence rates to distance of migration for all species with the birds divided into two groups. The first group was made up of long-distance migrants (birds migrating to Africa, the Middle East, or Asia), while the second group was made up of short-distance migrants (birds migrating to different parts of Europe). Among long distance migrants 17 out of 99 birds (17.2%), representing 4 species (*Sylvia atricapilla*, *Sylvia*

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')
<i>C. coli/C.jejuni</i>	400	cadF2B (cadF) cadR1B	TTGAAGGTAATTTAGATATG CTAATACCTAAAGTTGAAAC
<i>C. coli</i>	894	COL 1 (ceuE) COL 2	ATGAAAAAATATTTAGTTTTTGCA ATTTTATTATTTGTAGCAGCG
<i>C. jejuni</i>	160	C-1 (undefined gene) C-4	CAAATAAAGTTAGAGGTAGAATGT GGATAAGCACTAGCTAGCTGAT

Table 2. Bird tested and prevalence of *Campylobacter* spp.

Family and species	Guild ^a	N. of birds tested	N. of positive		% of positivity per bird species
			<i>C.jejuni</i>	<i>C.coli</i>	
<i>Sylviidae</i>					
<i>Sylvia atricapilla</i>	AI	24	9/24	1/24	41.6
<i>Sylvia cantillans</i>	EI	11	-	-	-
<i>Sylvia melanocephala</i>	EI	8	3/8	-	37.5
<i>Sylvia communis</i>	EI	7	-	-	-
<i>Sylvia borin</i>	AI	2	-	-	-
<i>Phylloscopus sibilatrix</i>	AI	2	-	-	-
<i>Phylloscopus trochilus</i>	AI	2	-	-	-
<i>Hippolais icterina</i>	AI	1	-	-	-
<i>Phylloscopus collybita</i>	AI	1	-	-	-
<i>Muscicapidae</i>					
<i>Erithacus rubecula</i>	GFI	41	17/41	2/41	46.3
<i>Luscinia megarhynchos</i>	GFI	10	3/10	-	30
<i>Turdus merula</i>	GFI	10	2/10	-	20
<i>Ficedula hypoleuca</i>	I	9	-	-	-
<i>Saxicola rubetra</i>	EI	8	1/8	-	12.5
<i>Cettia cetti</i>	EI	4	1/4	-	25
<i>Turdus philomelos</i>	GFI	3	-	-	-
<i>Muscicapa striata</i>	I	2	-	-	-
<i>Phoenicurus phoenicurus</i>	AI	1	-	-	-
<i>Oenanthe oenanthe</i>	GFI	1	-	-	-
<i>Ficedula albicollis</i>	I	1	-	-	-
<i>Fringillidae</i>					
<i>Serinus serinus</i>	GFG	1	-	-	-
<i>Carduelis chloris</i>	GFG	1	-	-	-
<i>Passeridae</i>					
<i>Prunella modularis</i>	GFG	2	-	-	-
<i>Passer hispaniolensis</i>	GFG	2	-	-	-
<i>Paridae</i>					
<i>Parus major</i>	AI	6	-	-	-
<i>Parus caeruleus</i>	AI	1	-	-	-
<i>Hirundinidae</i>					
<i>Hirundo rustica</i>	I	8	-	-	-

^a Guild: AI: arboreal insectivores; EI: herbaceous insectivores; GFI: ground-foraging invertebrate feeders; I: aerial insectivores; GFG: ground-foraging granivores.

melanocephala, *Luscinia megarhynchos*, *Saxicola rubetra*), tested positive for *Campylobacter* spp. In contrast, out of 70 tested birds, 22 (31.5%) of the short-distance migrants, representing 3 species (*Erithacus rubecula*, *Turdus merula*, *Cettia cetti*), were positive.

Conclusions

In accordance with literature (Waldeström *et al.*, 2002), we found a high prevalence of *Campylobacter* spp. in migrating birds (23.1%). However, the distribution of *Campylobacter* among bird taxa and guilds was very heterogeneous. Certain bird taxa, representing 2 families (*Sylviidae* and *Muscicapidae*), showed high prevalences, e.g., *S. atricapilla* (41.6%), *E. rubecula* (46.3%), *S. melanocephala* (37.5%), *C. cetti* (25%), *T. merula* (20%), while others did not. The prevalence of *Campylobacter* spp. was highly influenced by feeding habits. In some ecological guilds, e.g., most types of aerial insectivores and granivores, *Campylobacter* spp. were never isolated. In contrast, in other guilds, such as ground-foraging invertebrate feeders and arboreal and herbaceous insectivores guilds, prevalence was found to be high.

Furthermore, in this study and in several others, high prevalences of *Campylobacter*, were found in apparently healthy birds (Kapperud and Rosef, 1983). High isolation rates could be interpreted as evidence for a coexistence between *Campylobacter* species and their bird hosts, which could be considered natural reservoir of infection. In addition, migration is a great stress for birds and resistance to infection, typical of wild avian species, might be decreased and the level of bacteremia might increase. Consequently, the shedding rate of pathogens increase, resulting in contamination of water and soil with faeces and in dissemination and survival of the agent in a new environment.

We do not know if the *Campylobacter* isolates found in this study are transmissible to humans or domesticated animals, but there might nevertheless be some epidemiological considerations. Given the occurrence of *C. jejuni* and *C. coli* in bird species capable of long-distance migration, many bird species could potentially act as vectors in

long-distance transmission of these pathogens to domesticated animals or humans. Finally, given the diversity of habitats occupied by different bird species and the resulting possibility of different species being exposed to *Campylobacter* spp. from different sources, we believe that this question deserves further investigation.

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Typing of *Campylobacter jejuni* and *C. coli* isolated from laying hens during the production cycle

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ABSTRACT

C. jejuni and *C. coli* isolated on three layer farms during the production cycle were typed by PCR-RFLP flagellin gene profiling. Three typical restriction patterns were detected for *C. coli* (H, O, R), 5 for *C. jejuni* (I, P, Q, T, S) and 8 were common for both. The D profile was present in all three flocks. C fla-type was detected on farms A and B. The results of the study suggest that some genotypes tend to prevail and persist more than others on farms and in certain geographic areas.

Key Words: *C. jejuni*, *C. coli*, PCR-RFLP typing, Laying hens.

RIASSUNTO

TIPIZZAZIONE DI *CAMPYLOBACTER JEJUNI* E *C. COLI* ISOLATI DA GALLINE OVAIOLE DURANTE IL CICLO DI PRODUZIONE

C. jejuni e *C. coli* isolati in tre allevamenti di galline ovaiole durante il ciclo di allevamento sono stati tipizzati mediante PCR-RFLP della flagellina. In totale sono stati distinti 3 pattern di restrizione (H, O, R) tipici di *C. coli*, 5 di *C. jejuni* (I, P, Q, T, S) e 8 comuni alle due specie (A, B, C, D, E, F, L, M). Il profilo D è stato evidenziato in tutti e tre i gruppi monitorati durante il ciclo di produzione. Anche il fla-tipo C manifestava un comportamento analogo negli allevamenti A e B. I risultati delle ricerche consentono di ipotizzare che alcuni tipi genetici tendono a prevalere ed a permanere nel tempo più di altri nell'allevamento e in una determinata area geografica.

Parole chiave: *C. jejuni*, *C. coli*, Tipizzazione PCR-RFLP, Galline ovaiole.

Introduction

Campylobacter coli and *C. jejuni* are recognized as two of the main causes for human foodborne poisoning. These bacteria are often transmitted through poultry. Several serotypes and biotypes can be distinguished using phenotypical methods such as serotyping and biotyping (Lior, 1984) although these methods are difficult to perform and sometimes fail to identify the strains. The

availability of molecular typing techniques has spurred new epidemiological investigations into this field. A fla PCR-RFLP, which is one of the most common gene profiling techniques, has proved to be helpful in discriminating *Campylobacter* strains during field studies (Petersen and Newell, 2001). In the United States, PCR-RFLP is the method of choice for human campylobacteriosis inspection and surveillance plans. Surveys based on *Campylobacter* molecular features have shown

that a wide range of bacterial types exist on layer farms. In this investigation, the dynamic behaviour of *C. coli* and *C. jejuni* strains isolated during the table egg production cycle on layer farms was studied by A flagellin PCR-RFLP.

Material and methods

Investigation were performed on three table egg production layer farms in the Apulia region. On all three farms, only one group of animals was housed in the sheds. There was no contact among the farms and the laying hens had been bought from different northern or southern Italian suppliers. Faecal samples were obtained with cloacal swabs, starting from the day the birds were housed and then collected every three months over the course of one year. Overall 48 cloacal swabs were obtained from farm A and 24 from farms B and C since the bird capacity of farm A was double that of farms B and C. The swabs were obtained from randomly chosen subjects. A total of 6 sampling sessions were carried out on farm A and 5 on farms B and C. All the samples were immediately put into tubes containing a selective enrichment broth (*Campylobacter enrichment broth*, OXOID) and sent to the laboratory under refrigerated conditions within a few hours. Methods described elsewhere (Camarda *et al.*, 2000) were used to isolate *Campylobacter* strains. Species identification was done using Multiplex PCR (Denis *et al.*, 1999). Each isolated *Campylobacter* strain was cultured in Brucella Broth (Biolife) supplemented with 20% glycerol and kept in cryovials at -80°C until the next tests were performed.

Molecular typing: flagellin PCR-RFLP. Overall 130 of the 183 isolated *Campylobacter* strains were genotyped following the standard methods provided by *Campynet European Network for the harmonisation and standardisation of molecular typing methods for Campylobacter* (<http://campynet.vetinst.dk>). Each RFLP profile was assigned a numerical value on the grounds of the obtained restriction patterns.

Results and discussion

Bacteriological analyses of specimens from newly arrived chicks were always negative in

agreement with previous studies carried out on hatching chicks (Di Modugno *et al.*, 1997). Starting from the second sampling the analyses were positive and positivity persisted throughout the egg production cycle. A total of 183 *Campylobacter* strains were isolated, broken down as follows: 83 on farm A, 41 on farm B, and 59 on farm C. *Campylobacter coli* was the most common species on the three farms (60.66% of the isolated strains) while detection of *C. jejuni* was less frequent (39.34%). Molecular typing (PCR-RFLP) distinguished 16 restriction profiles. Three patterns (H, O, R) were characteristic of *C. coli*, 5 (I, P, Q, T, S) were exclusively detected in *C. jejuni*, while 8 (A, B, C, D, E, F, L, M) were present in both species. Some of these restriction patterns were detected only on one farm while others were common to more than one. In the *C. coli* strains isolated on farms A and B, C *fla*-type prevailed over the others while *C. coli* F type was often found on farm C. In the *C. jejuni* strains, B, S and D were the most frequently detected *fla*-types on farms A, B and C, respectively. The trends measured during the production cycle indicated that some molecular types tended to prevail and persist longer than others. The D type, detected both in *C. jejuni* and *C. coli* strains, was always present in all the tested groups during the production cycle. *Fla*-type C was also always present on farms A and B. Some molecular types (H, O, P, Q, R, S, T) were only detected once and then never again.

Conclusions

Evaluation of the degree of contamination of the flocks broadly confirmed our expectations. Contamination of the birds increased proportionally to their age (Di Modugno *et al.*, 1997) through to the day of slaughtering when the contamination rates were almost 100%. All the newborn chicks were *Campylobacter* - free and this was in agreement with previous studies that reported that thermophilic *Campylobacter* was consistently absent in newly hatched chicks and during the perinatal phase (Di Modugno *et al.*, 1997). Examination of the obtained *fla A* profiles depicted the prevalence of some molecular types on each farm. These types were present in the groups during the production cycle with different rates. Other strains with a dif-

ferent molecular type joined the prevailing *Campylobacter* population during the breeding cycle and this is consistent with the fact that different *Campylobacter* molecular types can coexist in a same group of animals (Camarda *et al.*, 2000). It is likely that the egg production cycle, which may last up to 18 months, favours colonization with different genotypes. Moreover, the occurrence of new molecular types in the infecting *Campylobacter* population may be connected with the flagellin gene mutations detected by enzymatic restriction. The use of PCR-RFLP to study multiple farms over a relatively long period showed that specific genotypes (C, D) were present and persisted in all the flocks tested during the production cycle. These findings suggest that some genotypes may prevail over others in certain geographical regions. Epidemiological studies carried out in Great Britain (Shreeve *et al.*, 2002) seem to support this assumption. It is likely that the capacity of some *Campylobacter* clonal groups to infect animals and the environment may be related to their ability to reach and contaminate poultry products. Some *Campylobacter jejuni* genotypes that infect the broiler gut during the production cycle are also detected on the carcasses after slaughtering. Further studies will focus on whether there is a relation between the genetic patterns of these strains and the ones often associated with human illness.

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Serotypes of *E. coli* isolated from avian species in Lombardia and Emilia Romagna (North Italy)

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ABSTRACT

In this paper we report the results of n.105 *E. coli* strains serotyping, isolated during the period 2000-2004 in Lombardia and Emilia Romagna (North Italy) from avian species (poultry and turkeys), starting from cloacal swabs. The most frequently identified serogroup was O78 both in poultry and turkeys, with a large prevalence over the other detected serogroups. Remarkable was the non typeable percentage among the examined strains, datum which is in accordance with our and other authors' previous studies.

Key Words: *E. coli*, Serotyping, Poultry, Turkey, O78.

RIASSUNTO

SIEROTIPI DI *E. COLI* ISOLATI DA SPECIE AVIARIE IN LOMBARDIA ED EMILIA ROMAGNA

Obiettivo di questo studio era procedere alla sierotipizzazione di ceppi di E. coli isolati da polli e tacchini, allevati nelle regioni Lombardia ed Emilia Romagna, sia in ambiente intensivo che rurale. I ceppi batterici, isolati da tamponi cloacali, sono stati saggiati con n.37 differenti antisieri O; i risultati ottenuti hanno permesso di mettere in luce una larga prevalenza del sierogruppo O78 sugli altri, in entrambe le specie testate. Degno di nota è il riscontro relativo al numero dei ceppi non tipizzati, dato che concorda con quanto già evidenziato in nostri studi precedenti e confermato anche da risultanze sperimentali di altri autori.

Parole chiave: Escherichia coli, Sierotipizzazione, Pollo, Tacchino, O 78.

Introduction

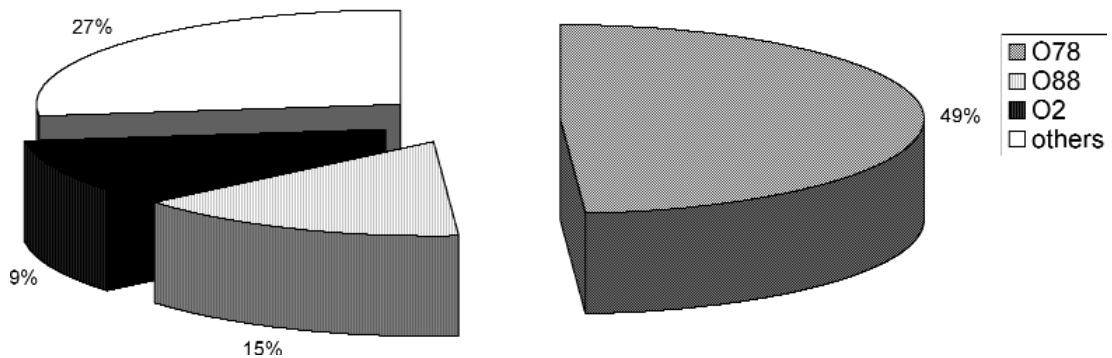
Avian pathogenic *Escherichia coli* (APEC) cause aerosacculitis, polyserositis, septicaemia and other mainly extraintestinal diseases in poultry, turkeys and other avian species. APEC are found in the intestinal microflora of healthy birds and most of the diseases associated with them are secondary to environmental and host predisposing factors (Dho-Moulin and Fairbrother, 1999).

The study consisted in the serotyping of n.105 *E. coli* strains, isolated during the period 2000-

2004 in Lombardia and Emilia Romagna (North Italy) from poultry and turkeys, reared both in rural and intensive units. These animals represented an heterogeneous picture of the avian population bred in Lombardia and Emilia-Romagna.

E. coli strains were isolated from cloacal swabs at one of the n.17 Provincial Diagnostic Sections of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Brescia (IZSLER), using standard bacteriological methods. Further examination for the characterization of the isolated strains i.e. determination of serogroup,

Figure 1. Results of O antigens' detection in poultry.



was performed at IZSLER "Specialized Bacteriology Department".

Material and methods

The adopted serotyping technique was the one used in Lugo's *E. coli* Reference Laboratory – Spain (Blanco and Blanco, 1993), opportunely adapted according to our laboratory procedures. All the strains were in advance confirmed as *E. coli* by the Enterotube II Roche Test; then, cultivated on Trypticase Soy Agar (TSA), Mac Conkey Agar and Trypticase Soy Broth (TSB) by successive steps. Each broth culture, developed by only one strain of *E. coli*, was heated in autoclave for 1 hour at 100 °C and doubled with 0.5% phenolic physiological solution (PPS). Serotyping was car-

ried out using a battery of monospecific antisera towards n.37 different somatic O antigens (O1, O2, O4, O6, O8, O9, O10, O11, O15, O18, O20, O21, O22, O26, O45, O49, O64, O73, O75, O78, O83, O86, O88, O101, O103, O109, O111, O115, O128, O132, O138, O139, O141, O147, O149, O153, O157). These antisera were selected according to national and international literature (Blanco and Blanco, 1993; Farina *et al.*, 1996) and to the present diffusion of O serotypes among domestic animal species in Italy (Farina *et al.*, 1996).

A part of each broth culture (50 µl) was put in contact with the same volume of each antiserum in U bottom polystyrene microtitre plates and incubated for 24 hours at 37 °C in a moist box to cause a slow sero-agglutination (SAL). The broth culture (50 µl) was also put in contact with the same volume of PPS to estimate the auto-aggluti-

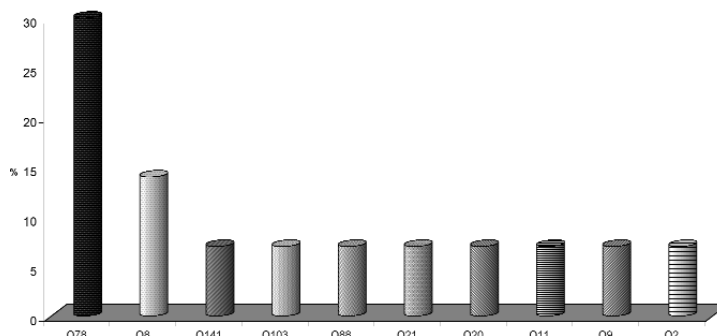
Figure 2. Serogroups results for *E. coli* isolates from turkeys.

Table 1. Detection of *E. coli* O antigens during the period 2000-2004.

Avian species	Examined strains	O antigens	
		Typed	Non typeable
Poultry	65	33	32
Turkeys	40	14	26
Total	105	47	58

nating power of each strain. Negative reactions were indicated by a sharp point, whereas positive reactions by a carpet. The *E. coli* strain was considered non typeable when agglutination was observed in four or more microwells. In addition, titration was carried out if agglutination was observed towards 2 or 3 antisera. The titre was determined by SAL using 50 µl of the broth culture and 6 base dilutions of the positive antisera and corresponded to the highest positive dilution.

Results and discussion

Figures 1 and 2 schematically report the results of O antigens' detection carried out in poultry and turkeys during last five years (2000-2004).

Poultry: O78 (49%), O88 (15%) and O2 (9%) were the serogroups most frequently detected, with a large prevalence over the other serogroups (O157, O149, O141, O111, O103, O101, O64, O22, O8), which made up 27% of the isolates.

Turkeys: of the n. 40 *E. coli* isolates examined, 30% belonged to serogroup O78, 14% to serogroup O8; O141, O103, O88, O21, O20, O11, O9 and O2 were the other identified serogroups, which represented the 56% of the all isolates.

A large number of isolates was untypeable, 49.2% and 65% in poultry and turkeys respectively. The comprehensive results of serotyping for the considered avian species are reported in table 1.

Conclusions

In previous reports other Authors indicated a predominance of O78, O88, O8 and O2 serogroups (Gross, 1994; Blanco *et al.*, 1998); in our study O78 was the most frequently recovered serogroup; this datum is in accordance with previous demonstra-

tions (Cloud *et al.*, 1985; Blanco *et al.*, 1998). Concerning the other *E. coli* serogroups identified in this study, they have already been reported from cases of avian colibacillosis (Barnes and Gross, 1997).

We have to consider that many pathogenic isolates do not belong to these identified serogroups, and they are commonly designated as "untypeable"; this results in difficulties in identifying APEC strains in veterinary laboratories.

However, at the moment the characterization of isolated strains, constitutes an indicative suggestion of *E. coli* serogroups mostly diffused in Lombardia and Emilia Romagna (North Italy) regions.

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Protection of chickens vaccinated with different schemes including the 4/91 IBV vaccine strain against field IBV strain Italy 02: preliminary results

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ABSTRACT

The ability of different vaccine programmes (including the 4/91 vaccine strain) to protect against field infectious bronchitis virus (IBV) strain Italy 02 was investigated using specific pathogen free (SPF) chickens. Protection, as measured by assessing ciliary activity of the tracheal epithelium following challenge, was excellent with all vaccine schedule used in this trial. The data provided by this study also indicates that vaccination programmes induced adequate protection against both challenges at 36 and at 56 days of age.

Key Words: Infectious bronchitis virus (IBV), Vaccination, Specific pathogen free (SPF) chickens, Italy 02 IBV strain.

RIASSUNTO

PROTEZIONE DI POLLI VACCINATI CON DIFFERENTI PROGRAMMI VACCINALI NEI CONFRONTI DEL CEPPLO DI CAMPO IT-02 DEL VIRUS DELLA BRONCHITE INFETTIVA: RISULTATI PRELIMINARI.

E' stata condotta una prova sperimentale al fine di valutare la protezione indotta da differenti programmi vaccinali (comprendenti anche il ceppo vaccinale 4/91) nei confronti dell'infezione sostenuta dal ceppo di bronchite infettiva aviaria denominato Italy 02. Il livello di protezione è stato calcolato attraverso la valutazione della ciliostasi osservata su colture d'organo (anelli tracheali). Sono stati rilevati buoni indici di protezione in tutti i programmi vaccinali impiegati nella prova. Inoltre, tale protezione è stata osservata in entrambe le età utilizzate per il challenge con il virus di campo (36 e 56 giorni).

Parole chiave: Virus della bronchite infettiva aviaria (IBV), Vaccinazione, Polli SPF, Ceppo It-02 dell'IBV.

Introduction

1999. An IBV strain isolated in Italy for the first time at the IZSLER laboratory in Forlì on SPF eggs from a broiler flock with respiratory disease. The strain was named 4682/FO.

2000. The cooperation work between the lab IZSLER in Forlì and "All Russian Research Institute for Animal Health (ARRIAH) – Russia" starts. Dr. Vladimir Drygin made the sequence analysis on 10 IBV strains isolated from IB outbreaks in Italy by the IZSLER Forlì lab during the year 1999. He renamed all the IBV tested strains

from It-01 to It-10. The IBV strains It-02, IT-04 and It-08 were found to be genetically different from the other strains tested.

2002. It-02 sequence published on NCBI-BLAST (accession number AJ457137). 2004. It-02 is widespread in Europe. It is the predominant genotype in the UK, but also in Germany, France, Spain, The Netherlands it is causing problems (Worthington et al., 2004).

The aim of the present study is to investigate the efficacy of different IB vaccine programs against an It-02 challenge in SPF chickens reared in isolators.

Material and methods

Experimental design: the experimental design is presented in Table 1. Three groups (A, B, C) of 20 specific pathogen free (SPF) chicks, 1 day old and housed in separate negative pressure isolators, were vaccinated by the oculonasal route (o.n.) with 10^3 EID₅₀ of the Nobilis IB Ma5 vaccine. At 1 day old, the group B was vaccinated with 103.6 EID₅₀ of Nobilis IB 4/91 too. At 2 weeks of age, the groups A and C were vaccinated o.n. with $10^{3.6}$ EID₅₀ of the Nobilis IB 4/91 vaccine. Two unvaccinated groups (D, E) were taken as control.

At 5 weeks of age, the control group D that had received no vaccinations and groups A and B vaccinated with Ma5 and 4/91, received a very strong challenge by oculonasale route with $10^{7.5}$ EID₅₀ of the Italian isolate It-02 strain (4682/FO) assayed in tracheal organ cultures. The fourth vaccinated group C was challenged with the same strain at the same concentration at 56 days of age. The fifth group E, served as the not vaccinated unchallenged control group.

At 4 and 7 days post-challenge, 4 chicks of each group was suppressed. The tracheas were removed and examined for ciliary activity as reported by Cavanagh *et al.* (1997). Each one of 10 tracheal rings prepared from each trachea was examined by low-power microscopy and ciliary activity scored as follows (Cook *et al.*, 1999): 0 - all cilia beating; 1 - 75% beating; 2 - 50% beating; 3 - 25% beating; 4 0% beating (100% ciliostasis).

This gives a maximum ciliostasis score of 40

for each trachea. A chick is intended as protected if the ciliostasis score for trachea is less than 20. For each group, the protection score was calculated according to the following formula:

$$\left(1 - \frac{\text{mean ciliostasis score for vaccinated/infected group}}{\text{mean ciliostasis score for challenged controls}}\right) \times 100$$

The higher the score, the better the level of protection provided by the vaccination programme.

Laboratory investigations: in all groups the serology at the 2nd, at the 4th and at the 9th week of age has been assessed testing sera for IB antibodies by Hemagglutination-inhibition (HI) and ELISA tests. 20 chicks per group were tested for HI using a M41Antigen and 793/B antigen (made by Central Veterinary Laboratory - Weybridge UK) and an Italy 02 antigen (made by IZSLER Brescia). The Elisa test has been performed with a commercial ELISA test (Synbiotics®). IBV isolation have been performed on all groups 7 days after challenge as in Table 2 using SPF eggs inoculated via the allantoic sac. Reverse transcriptase-polymerase chain reaction (RT-PCR) and nested PCR (Cavanagh *et al.*, 1999) have been performed on all groups 7 days after challenge as in Table 2.

Results

The results of the ciliostasis test and of the protection index are shown in Table 3 and Table 4.

Table 1. Experimental design.

Group	1 d	14 d	36 d challenge	56 d challenge
A (vaccinated)	Ma5*	IB 4-91*	Italy 02**	
B (vaccinated)	Ma5+4/91*	-	Italy 02**	
C (vaccinated)	Ma5*	IB 4-91*		Italy 02**
D (control: no vaccinated - challenged)	-	-	Italy 02**	
E (control no vaccinated - no challenged)	-	-	-	

*IBV vaccine strains used for the vaccination; ** IBV field strain used for the challenge.

Table 2. Experimental design and analysis.

Age	Groups and treatments				
	A	B	C	D	E
0	Ma5*	Ma5+4/91*	Ma5*	-	-
14	4/91* Serology	Serology	4/91* Serology	Serology	Serology
28	Serology	Serology	Serology	Serology	Serology
36	It-02** Challenge	It-02** Challenge	-	It-02** Challenge	-
40	TOC	TOC	-	TOC	-
43	TOC Virology	TOC Virology	-	TOC Virology	TOC
56	-	-	It-02** Challenge	-	-
60	-	-	TOC	-	TOC
62	Serology Virology	Serology Virology	TOC Serology Virology	Serology Virology	Serology Virology

*IBV vaccine strains used for the vaccination

** IBV field strain used for the challenge

TOC: tracheal organ culture

Serology

The HI test showed significant M41 titers 2 weeks after the vaccination in all groups, titers that declined 2 weeks later at 28 days, but rocketed at 62 days.

The HI test with 793/B antigen showed low antibody levels in the first two samples but a strong growth in the last one.

The HI serological data with It-02 antigen show an increase in all three sampling points (Figures 1, 2, and 3).

The Elisa test results are shown in the Fig 4. Group A showed an antibody peak at 28 days, after the revaccination with 4/91, but a decrease at 62 days. Group B showed a light increase which remained constant.

Group C showed a strong increase after the challenge, as did group D.

Virology

The virological tests performed were: virus isolation on SPF eggs, RT-PCR and type-specific nested PCR. The results are shown in Table 5. The virus isolation performed 7 days after the challenge was positive and the nested PCR identified the Italy 02 strain in the entire test. The isolations performed 26 days after challenge (62 days) were negative.

Discussion

Ciliostasis test

The results of the ciliostasis test show that all the vaccination schemes applied gave excellent

Table 3. Tracheal organ culture (TOC) score.

Group	Age of challenge	Average score 4 days post challenge	Average score 7 days post challenge
A (1d Ma5+14d 4/91)	36 days	2.5	3
B (1d Ma5+4/91)	36 days	3	4
C (1d Ma5+14d 4/91)	56 days	1.5	2.7
D Control infected	36 days	29.2	32.7
E Control non infected	-	0.2	0.2

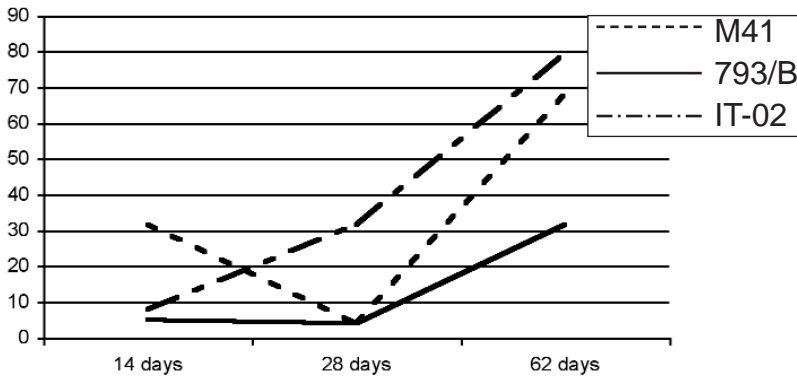
Table 4. Protection Index.

Group	Vaccination schedule	It-02	Protection index (4 days post challenge)	Protection index (7 days post challenge)
A	Ma5 at 1 day 4/91 at 14 days	36 days	92.35	90.82
B	Ma5+4/91 at 1 day	36 days	90.82	87.76
C	Ma5 at 1 day 4/91 at 14 days	56 days	95.41	91.74

Table 5. Virological data.

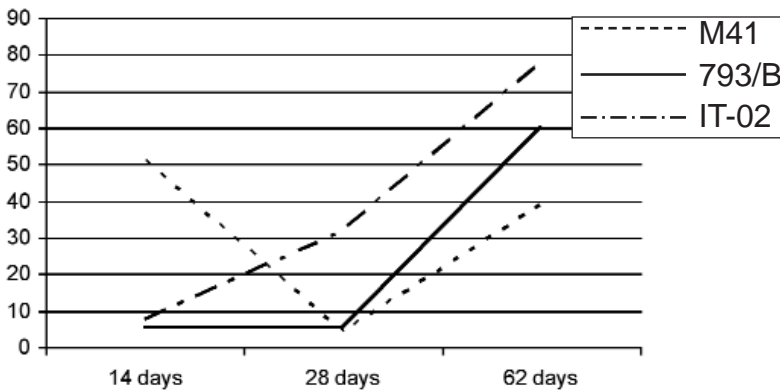
Group	43 days	62 days
A	Isolation PCR = It-02	neg neg
B	Isolation PCR = It-02	neg neg
C	Not done	Isolation PCR = It-02
D	Isolation PCR = It-02	neg neg

Figure 1. HI test Group A.



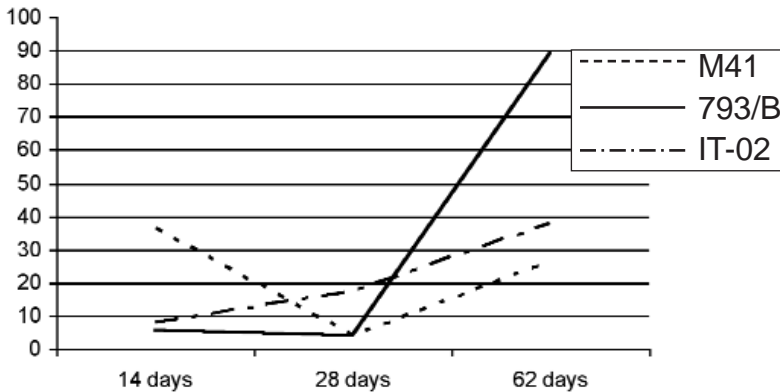
M41, 793/B, IT-02: IBV antigen used in HI test

Figure 2. HI test Group B.



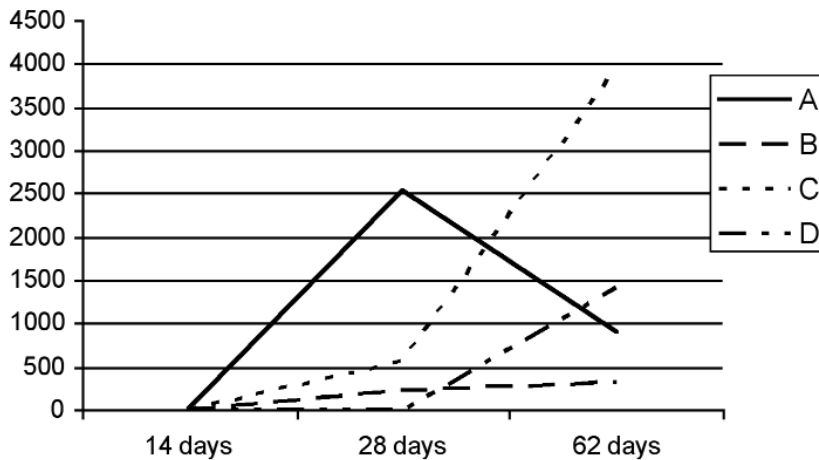
M41, 793/B, IT-02: IBV antigen used in HI test

Figure 3. HI test Group C.



M41, 793/B, IT-02: IBV antigen used in HI test

Figure 4. Elisa test per group.



A,B,C,D: groups of animals used in the trial

protection against the challenge with Italy 02, whereas the chicks in the challenged control group were not protected.

Protection index

The results of the protection index were equally very good. These data indicate a very good protection both in the early infection at 36 days of age and in the late one at 56 days in older birds.

Serology

HI test

The HI It-02 test shows a constant increase in all groups that could be caused by an antigenic cross reactions against both the M41 and the 793/B IBV strains. It would be interesting to investigate the real genetic relationships of the It-02 with the above-mentioned. As reported by Jackwood *et al.* (2005) the serotype of the IBV causing the disease must first be determined so that the birds can be properly vaccinated. The HI It-02 test show a constant increase in all groups that could mean an antigenic cross reactions against both M41 and 793/B IBV strains. Accordingly to the concept of Jakwood *et al.* this could explain the cross protection we proved in the present paper.

It could be interesting to investigate the real genetic relationship with these strains and with all

the other main IBV strains in the world. This study is in progress.

After the challenge all the groups showed an increase in the specific HI tests. It is important to underline that in group C (late challenge at 56 days) the mean titers against 793/B at the last sampling (62 days) were significantly higher than in other groups.

ELISA

It is well known that the ELISA test is not specific and indicates only a general response against IB.

Group A: we had an increase after the 2 vaccinations, but a decrease after the challenge. The explanation could be that the birds were well protected at day 35 of the challenge and the virus had therefore been blocked in the trachea by a strong local immunity without the possibility to penetrate further into the organism.

Group B: we ascertained a non-significant titre increase. The explanation could be the same as that applied to group A. The birds were well protected at day 35 of the challenge and the virus had therefore been blocked in the trachea by a strong local immunity without the possibility to penetrate further into the organism. This could be due to the fact that the group received a fourfold

vaccination (1 drop of Ma5 + IB 4-91 in each eye and 1 drop of Ma5 + IB 4-91 in each nostril).

Group C: there was a strong increase, probably due to the late challenge at 56 days. Comparing these data with the TOC and the protection index results, it appears that the birds are well protected against the disease, but not against the infection. The wild virus was able to pass through the organism and to stimulate the immune system.

Virology

Both PCR and reisolation of the virus were positive for the IBV strain Italy 02. This is probably due to the very high concentration (more than 1000 times higher than the standard one fixed by the European Pharmacopoeia) of the challenge virus. Nevertheless, the birds were not only well protected against the disease but, according to the different vaccination schemes, perhaps also against the infection.

This hypothesis should be confirmed through immune histochemical tests.

Conclusions

Based only on genomic data, the 4682/FO (or It-02) IBV strain is present in Italy since 1999.

A correlation between genotype and serotype is hardly possible. The study is in progress.

All vaccination programs included in this laboratory trial induced protection against It-02 challenges both at 36 and 56 days of age.

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Survey on circulation of infectious bronchitis virus strains in Northern Italy

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ABSTRACT

Infectious Bronchitis (IB) still causes significant health problems in the poultry industry with high economic impact. The presence of several serotypes of IB and the emergence of novel ones must be monitored in order to take appropriate action and to adapt the vaccination programmes to the prevalent serotypes. In order to establish which serotypes are circulating in densely populated poultry area (DPPA) of Northern Italy, a surveillance programme has been undertaken during 2004 and 2005. The results of this surveillance programme show the active circulation of 793-B, IT-02, the introduction of a novel strain, known as QXIBV, originally identified in China and the re-emergence of previously circulating serotype as B1648.

Key Words: Infectious Bronchitis, Broiler, QXIBV.

RIASSUNTO

INDAGINE SULLA CIRCOLAZIONE DEI VIRUS DELLA BRONCHITE INFETTIVA AVIARIA IN NORD ITALIA

La Bronchite Infettiva (IB) rappresenta ancora oggi uno dei principali problemi sanitari per l'allevamento avicolo intensivo. Allo scopo di approfondire le conoscenze sulla sua diffusione e sui sierotipi circolanti nelle aree densamente popolate (DPPA) del Veneto e Lombardia, dal 2004 è stato eseguito un piano di sorveglianza basato sull'introduzione di animali sentinella in allevamenti con sospetto di malattia e il monitoraggio degli allevamenti colpiti in precedenza. Il piano di monitoraggio ha confermato la significativa circolazione di sierotipi quali il 793-B e l'IT-02 ed ha evidenziato l'introduzione di un nuovo sierotipo precedentemente isolato in Cina e noto come QXIBV e il riemergere della variante nefropatogena belga B1648, la cui presenza in questa area non veniva segnalata da alcuni anni.

Parole chiave: Bronchite Infettiva, Broiler, QXIBV.

Introduction

Infectious Bronchitis (IB) still causes significant health problems in the poultry industry with a high economic impact due to the virulence of some strains and the costs of vaccinations as a control measure for this disease. The presence of several serotypes of IB and the emergence of

novel ones must be monitored in order to take appropriate action and to adapt the vaccination programmes to the prevalent serotypes.

In order to establish which serotypes are circulating in Northern Italy a surveillance programme has been undertaken during 2004 and 2005. The area involved in the monitoring programme was a densely populated poultry area

(DPPA) which rears 30% of the Italian poultry production.

Material and methods

The surveillance programme was carried out monitoring flocks through the introduction of sentinel birds. SPF (Specific Pathogen Free) chickens were introduced in to farms for a period of 7-10 days as sentinels. Tissue samples, collected from sentinels or sick birds (trachea, lung, kidney and cecal tonsils) were directly analysed in RT-PCR and submitted for virological investigations. The tissue homogenates were inoculated into the allantoic cavity of 9-to-11-day-old embryonated SPF eggs (Gelb *et al.*, 1998). Following a maximum of four blind passages, the allantoic fluid was harvested and examined by negative contrast electron microscopy for the presence of coronavirus particles (Hayat, 1985). In order to characterise the strains, viral RNA was extracted from the organs and from positive allantoic fluid. The strains 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 187 samples were collected from 60 farms.

Results and discussion

43/60 broiler and chicken backyard farms monitored were positive for IBV (Table 1). Of these, 20 were positive for IT-02, 13 for 793-B, 1 for B1648 and 8 for QXIBV. The presence of IT-02 was often associated with urolithiasis and enteric form, while 793-B was associated with mild respiratory form. QXIBV has been isolated only in the Veneto from 3 backyard flocks and from 5 broiler flocks. In

these flocks the presence of this strain was associated with urolithiasis and mild increased mortality. The signs were observed in young birds of 3-5 weeks of age. The chickens showed depression, ruffled feathers, wet dropping and decreased growth. The mortality rates were often only 1-2 % more than the standard conditions and the recovery occurred in 2-3 weeks, in some case the severity of the disease was higher and the performance of birds was significantly affected. In the outbreak of B1648 a nephritic syndrome was observed. Furthermore another B1648 strain was isolated in young birds of a pheasant flock with high mortality rates and severe kidney lesions.

These results show an active circulation of at least 4 different serotypes of IBV in Northern Italy. IT-02 appears to be the most prevalent strain and is also widespread in other European countries such as The Netherlands and England. Serotype 793-B is still present, although it is unclear whether the isolates are field or vaccine strains. B1648 (Belgium nephropatogenic strain) seems to have re-emerged after some year of non appearance. QXIBV is a strain of novel introduction and it has never been described before in Italy.

Infectious Bronchitis appears to be an evolving disease which still causes important economic losses. The most important epidemiological aspects of IB that must be focused are the emergence of novel serotypes and the re-emerging of old ones. This provides evidence of the importance of monitoring programmes as an effective tool in following the evolution of the disease in selected areas.

Conclusions

In reference to the circulation of QXIBV in Northern Italy, there are some questions to be

Table 1. IBV serotypes and related syntomatology.

IBV serotype	N. of positive/total of positive	Sintomatology
793/B	13/43	Respiratory form
ITA-02	20/43	Urolithiasis and enteric form
QXIBV	8/43	Urolithiasis
B1648	2/43	Nephritic form

answered. Little is known about the pathogenic characteristics and antigenic properties of this isolate, originally obtained in China and described also in Germany, Holland, Belgium and France (Worthing *et al.*, 2005). In Italy the presence of this serotype was associated with nephritis and increased mortality in broiler flocks while the Netherlands isolate was associated with “silent” hens in layer flocks. How this strain has reached Europe and whether it has been due to one or multiple introduction from Asia is unclear. The introduction of this Chinese strain indicates that avian viruses present in the Far East may reach backyard and industrial poultry population of European countries with important health consequences. With regard to the possibility that QXIBV could become predominant in a naïve population it is important to evaluate which vaccines could be cross-protective.

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CASE REPORT

Ceruminous otitis in native chicken breeders belonging to *Robusta Lionata* breed

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ABSTRACT

At the beginning of 2005, an outbreak of ceruminous otitis has been observed in a breeders flock belonging to the *Robusta Lionata* breed, a native chicken breed reared in a Centre for the valorisation and conservation of native poultry breeds. The disease caused a high morbidity rate (90% of birds), whereas the mortality affected only 10% of the birds. The death of the birds was preceded by clinical signs, such as worsening of the ceruminous otitis with abundant secretion of bad-smelling cerumen, lack of appetite and depression. The otitis externa, mainly bilateral, was the only evident finding at necropsy. Laboratory examinations showed only the presence of *Mycoplasma synoviae*, either by isolation on culture media or by PCR, on ear and choana samples. *Aspergillus fumigatus* was detected only in one bird. In this paper, the Authors report for the first time on an outbreak of ceruminous otitis in native chicken breeds and point out that the only pathogenic agent isolated from the birds has been *Mycoplasma synoviae*.

Key Words: Native chickens, Otitis, *Mycoplasma* spp.

RIASSUNTO

FOCOLAIO DI OTITE CERUMINOSA IN POLLAME DA RIPRODUZIONE DI RAZZA ROBUSTA LIONATA

Agli inizi del 2005 è stato osservato un focolaio di otite ceruminosa in un gruppo di riproduttori di Robusta Lionata allevati in un Centro per la valorizzazione e la conservazione delle razze avicole del Veneto. La patologia si è manifestata con un'alta morbilità (90% dei capi), mentre la mortalità ha interessato solo il 10% degli effettivi ed è stata preceduta dal progressivo aggravarsi dell'otite con abbondante secrezione di cerume maleodorante, inappetenza ed abbattimento. L'esame necroscopico ha evidenziato solo la presenza di otite ceruminosa, in genere bilaterale. Gli esami di laboratorio hanno evidenziato la sola presenza di Mycoplasma synoviae, sia con metodo culturale classico sia mediante PCR, in campioni prelevati dall'orecchio e dalle coane. Solo in un soggetto è stato isolato anche Aspergillus fumigatus. Con questo lavoro gli AA. intendono segnalare un episodio di otite ceruminosa mai descritta in bibliografia ed evidenziare il fatto che l'unico agente patogeno riscontrato in tutti i soggetti è stato Mycoplasma synoviae.

Parole chiave: : Razze avicole autoctone, Otite, *Mycoplasma* spp.

Introduction

In the last years many programs for the valorisation and protection of native chicken breeds

have been implemented in Italy. In the '50s native chicken breeds were commonly reared in our countryside, but after the industrialization of poultry production in the '70s and '80s, they

have undergone to a rapid decrease.

Only recently, following the work of many regional Agencies and Universities, some endangered chicken breeds have been reintroduced on our territories.

Health status of these native chicken breeds is interesting to investigate in order to obtain an outline of the diseases that can affect every breed and that may be very different to those of intensive reared chickens, either for the pathogenesis or for the morbidity and mortality rates.

At the beginning of 2005, an outbreak of ceruminous otitis has been observed in a breeders flock belonging to the *Robusta Lionata* breed, a native chicken breed reared in a Centre for the valorisation and conservation of native poultry breeds (Veneto Region, Northern Italy).

Robusta Lionata is a native breed reared in the Veneto Region, with a high rate of egg production, but mainly reared for meat production. The adults are distinguished by a tawny-coloured feathering, a black tail with greenish tints and dark colouration of the wings. Feet and skin are yellow-coloured. The standard weight of cocks is about 4 kg, whereas the hens weight up to 3 kg (Fracanzani, 1985; Zanon *et al.*, 2001).

Material and methods

Birds

The outbreak occurred in a breeding Centre working for the conservation of native chicken breeds in the Veneto Region. Chicken breeders belonging to *Robusta Lionata* breed, placed in flocks of 40 birds, were reared in open paddocks of about 400 square metres. Other than *Robusta Lionata*, in the Centre various other chicken breeds were reared, such as *Ermellinata di Rovigo*, *Pèpoi*, etc. About a thousand of birds were housed in the Centre.

The otitis affected a flock of male breeders at the end of the reproductive season, late in the spring.

The disease caused a high morbidity rate (90% of birds), whereas the mortality affected only 10% of the birds. The death of the birds was preceded by clinical signs, such as worsening of the ceruminous otitis with abundant secretion of bad-smelling cerumen, lack of appetite and depression.

Post-mortem examination

Necropsy was performed on few dead birds. The birds showed a good feathering, absence of external parasites and weight loss. The otitis externa, mainly bilateral and with different degrees of severity, was the only evident finding at necropsy.

Bacteriological examination

A bacteriological screening on swabs from the ear canals was carried out by direct streaking and following enrichment on Blood Agar (with 5% sheep blood) and MacConkey Agar incubated under aerobic, anaerobic and microaerophilic conditions. The bacteriological examination was performed on brain and liver samples of dead birds, as well.

Furthermore, the presence of mycoplasmas was investigated either by classical isolation techniques or by PCR on swabs collected from ear and choana.

Mycological examination

Direct streaking on Sabouraud Agar and following incubation at 37°C for 12 days was carried out from ear swabs.

Finally, cerumen samples were examined by stereomicroscopy for the possible presence of parasites.

Results and discussion

Cases of otitis externa have never been reported in chickens, therefore it is very difficult to explain our findings and determine the true cause of the disease.

Gross examination of bird carcasses showed only the presence of ceruminous otitis, usually bilateral; oedema on wattles or sinusitis were never detected.

Bacteriological examination performed on brain and liver samples showed no signs of bacterial growth, while the material collected from the ear canals yielded an aspecific polymicrobism, such as *Bacillus* spp., *E.coli* and *Corynebacterium* spp. *Pasteurella multocida* was never isolated. *Mycoplasma synoviae* was detected either by isolation on culture media or by PCR on both ear and choana samples.

Aspergillus fumigatus was detected only in one bird, whereas stereomicroscopy showed neither parasites nor foreign bodies.

Conclusions

In this paper, we report on an outbreak of ceruminous otitis in local chicken breeds and we point out that the only pathogenic agent isolated from the birds has been *Mycoplasma synoviae*. Nevertheless, it is unlikely that *M. synoviae* was the primary cause or a predisposing agent of the disease, because of its weak invasiveness through the stratified epithelium of ear canal. The presence of *M. synoviae* in loco could be ascribed to the deposition of environmental bacterial aerosol in non-SPF birds.

Certainly, one of the predisposing factors of the otitis is the characteristic anatomical conformation of the ears of this chicken breed, that is abundant presence of feathers which obstruct entirely the external ear opening.

Attempted systemic antimicrobial therapy (oxytetracycline, amoxicillin and tylosin) and topical medication (iodine solutions) of affected birds have yielded only a transitory, but never a complete recovery.

Cases of otitis have been reported in other animals, such as cattle and swine (Morita *et al.*, 1995; Duarte *et al.*, 2004; Lamm *et al.*, 2004), where mycoplasmas have been isolated and considered the primary agents of the disease.

Nevertheless, this condition has to be taken into account in breeding selection programmes, especially inbreeding of commercial broiler lines.

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CASE REPORT

Caecal coccidiosis in commercial male turkeys

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ABSTRACT

An outbreak of coccidiosis with high mortality is reported in 30-day-old commercial turkeys. Grossly, a severe typhlitis with a large fibrino-necrotic core was present. Large numbers of oocysts were observed in caecal smears. The location and the severity of the lesions and the oocyst morphology were strongly suggestive of *Eimeria adenoeides* infection. This species has already been reported in turkey flocks in Italy, but it is rarely responsible for clinical coccidiosis and severe lesions with high mortality. Other caecal parasitic infections are considered in differential diagnosis.

Key Words: Turkey, Typhlitis, Coccidiosis, *Eimeria adenoeides*

RIASSUNTO

SEGNALAZIONE DI UN CASO DI COCCIDIOSI CIECALE IN TACCHINI COMMERCIALI

Viene descritto un episodio morboso di elevata mortalità in tacchinotti di 30 giorni con grave tiflite e voluminosi stampi necrotico-fibrinosi lumenali nei quali erano presenti oocisti in elevato numero. Le dimensioni delle oocisti, la sede e la gravità delle lesioni sono indicative di un'infezione da *Eimeria adenoeides*. Questa specie è già stata segnalata in allevamenti italiani, ma non è comune il suo coinvolgimento in episodi di tale gravità. Viene discussa la diagnosi differenziale con altre tifliti parassitarie.

Parole chiave: Tacchino, Tiflite, Coccidiosi, *Eimeria adenoeides*.

Introduction

Coccidial infections in turkeys are sustained by seven *Eimeria* species (*E. adenoeides*, *E. gallopavonis*, *E. meleagritidis*, *E. dispersa*, *E. innocua*, *E. meleagris* and *E. subrotunda*) (McDougald, 2003). The most pathogenic species are *E. meleagritidis*, *E. adenoeides* and *E. gallopavonis* and they have been recently included in the guidelines for evaluating efficacy of anticoccidials in poultry (Holdsworth *et al.*, 2004). Turkey coccidiosis is commonly regarded as a minor economical issue that affects birds younger

than 6-8 weeks (McDougald, 2003). These infections are widely present but rarely associated with clinical signs (Trees, 2002) and disease outbreaks remain poorly characterized. However coccidia can interact with bacteria, viruses and mycotoxins often exacerbating a number of enteric diseases (Witlock *et al.*, 1984; Norton *et al.*, 1993; Droual *et al.*, 1994). Consequently, anticoccidials are routinely used in turkeys to prevent reductions in the weight gain and feed conversion. The aim of this paper is to describe a field case of caecal coccidiosis recently observed in a commercial turkey flock in Northern Italy.

Material and methods

In October 2004 an outbreak of disease characterized by high mortality and enteric disorders was reported in a commercial turkey farm located in Northern Italy. This farm housed 13,000 male birds in four houses and the enteric syndrome affected only one of them with a flock of 4000 birds. Monensin was used as coccidiostat in the feed. Five 30-day-old male turkeys were submitted for necropsy. Parasitological examination was carried out on caecal contents and mucosal scrapings. Samples of duodenum, caecum, pancreas, spleen, bursa of Fabricius, thymus and heart were collected in 10% buffered formalin for histopathology. Formalin-fixed tissues were embedded in paraffin wax, sectioned at 4 μm , and stained with Haematoxylin and Eosin. Digital micrographs of the caecal fresh smears were acquired (20x mag) and morphometric analysis (length and width) of 50 oocysts was performed by means of an image analysis software (Image Pro.Plus 5[®]). The average length and width and the shape index (length/width) were calculated.

Results and discussion

The outbreak of disease involving one of the four turkey houses was characterized by enteric disorders and mortality starting from the 4th week of age. A first attempt in controlling the syndrome with antibiotics (amoxicillin, colistin and flumequine) was unsuccessful. Histomoniasis was then suspected as field necropsy examinations of dead turkeys revealed severe caecal lesions in most of the birds. At slaughter time (140 days) the mortality in the affected flock reached 26% with an average weight of 18.8 Kg. The total mortality on the farm and the average slaughter weight were respectively 17% and 19.3 Kg with a feed conversion of 2.50.

The submitted turkeys were in poor condition and at necropsy the most striking feature was a moderate to severe typhlitis. The caeca were distended, thin-walled and contained a large grayish-white fibrino-necrotic core. A mild catarrhal enteritis was also present. The spleens appeared mildly smaller than normal and no liver lesion was present. The microscopic examination of

mucosal scrapings of affected caeca revealed a small number of ellipsoidal oocysts. Conversely, a huge number of oocysts was ascertained in the smears of the caecal fibrino-necrotic cores. The oocysts appeared uniform in shape and size. The average oocyst dimensions were 24.5 x 16.1 μm and the average shape index was 1.53. These data are consistent with the morphologic characteristics of *E. adenoides* (McDougald, 2003).

Histologically, a mild, chronic, catarrhal enteritis was detectable. The caecal lesions varied from a mild to moderate, multifocal sloughing of the villus epithelium with intact crypts to a diffuse disruption of the caecal mucosa. In the luminal exudate, large bacterial aggregates characterized by a mixed morphology were evident in association with parasites in different stages of development. Sections from spleens of 2 birds showed mild to moderate lymphocytic depletion, as well as one section from the bursa of Fabricius. Pancreas, thymus and heart were normal.

Enteric disorders, increased mortality and poor flock performances are commonly observed in commercial turkeys in association with a number of diseases. In the present case the early suspected bacterial involvement was excluded since antibiotics had failed to control the outbreak. The nature and the severity of the caecal lesions in the dead birds led the veterinary practitioners to suspect histomoniasis. However, the myriad of oocysts ascertained in the fibrino-necrotic caecal cores of the turkeys submitted to our laboratory was diagnostic of clinical coccidiosis. Histomoniasis was ruled out as no liver lesions were present and no histomonads were evident in the caecal smears and in the histologic sections. Moreover, microscopic examination revealed the caecal epithelium to be mostly affected and the lamina propria showed only a mild inflammatory involvement, whereas in histomoniasis it is usually severely affected and filled with numerous protozoa.

As for the etiology of this outbreak of coccidiosis, we assume that the unique involvement of the caeca, the severity of the lesion and the morphology of the oocysts are strongly suggestive of *E. adenoides*. However, *E. meleagridis* and *E. gallopavonis* have been considered in differential diagnosis. Although the former replicates in the caeca it is regarded as non-pathogenic. *E. gallopavonis* is

more pathogenic, its oocysts closely resemble those of *E. adenoides* but it affects primarily the lower ileum adjacent to the ileocaecal junction and caecal lesions can very rarely occur (McDougald, 2003; Holdsworth *et al.*, 2004). Moreover *E. gallopavonis* does not yet appeared to be recognized in Europe (Trees, 2002).

Eimeria spp. infections have already been reported in turkey commercial flocks in Italy (Grilli *et al.*, 2001). Neither enteric signs nor intestinal lesions were present in the monitored flocks though the presence of *E. adenoides* was suspected in the caecal content of 4-to-8-week-old birds from 3 of the 4 flocks examined. In the present case, however, the amount of oocysts was dramatically higher. Moreover the infection should have been very precocious or its course very rapid as clinical signs and mortality started from the 4th week. We cannot explain why the other 3 flocks on the farm remained unaffected as well as the previous and the following broods did.

Conclusions

This case was the most severe outbreak of turkey coccidiosis occurred in our diagnostic routine. The caeca were the only affected intestinal tract and contained myriads of oocysts. Other parasites as *Histomonas* were undoubtedly excluded. Only *E. adenoides* can be culprit for such severe caecal lesions. The early age of the birds may have played an essential role in the occurrence of the disease.

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CASE REPORT

Outbreak of *Eimeria kofoidi* and *E. legionensis* coccidiosis in red-legged partridges (*Alectoris rufa*)

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ABSTRACT

An outbreak of coccidiosis occurred in red-legged partridges is reported. At the post-mortem examination the birds showed a mucous haemorrhagic enteritis, mostly in the duodenal intestinal tract. Direct microscopic examination of intestinal content revealed the presence of a high number of oocysts. After incubation, on the basis of the morphological features, two species of coccidia were identified: *Eimeria kofoidi* and *E. legionensis*.

Key Words: Coccidiosis, Red-legged partridge, *Alectoris rufa*, *Eimeria kofoidi*, *Eimeria legionensis*.

RIASSUNTO

FOCOLAIO DI COCCIDIOSI DA *EIMERIA KOFOIDI* ED *E. LEGIONENSIS* IN PERNICI ROSSE (*ALECTORIS RUF*A)

Viene riportato un focolaio di coccidiosi verificatosi in pernici rosse allevate in una azienda faunistico-venatoria italiana. All'esame necroscopico i soggetti colpiti presentavano una duodenite catarrale-emorragica. L'esame microscopico diretto del contenuto intestinale metteva in evidenza la presenza di numerose oocisti che, dopo incubazione, sono state identificate su base morfologica come appartenenti a due specie distinte: *Eimeria kofoidi* ed *E. legionensis*.

Parole chiave: Coccidiosi, Pernice rossa, *Alectoris rufa*, *Eimeria kofoidi*, *Eimeria legionensis*.

Introduction

Game birds, including pheasants and partridges, are widely reared for releasing into the wild. Intensive farming of these species is frequently performed in pens and this could favour the diffusion of parasitic diseases. Coccidiosis, familiar to the poultry farmer, are prevalent on game farms too (Reck and McQuiston, 1994), however little literature is available on its real diffusion in game birds and on the involved coccidia species. In order to give a contribution to the knowledge of this parasitosis, we

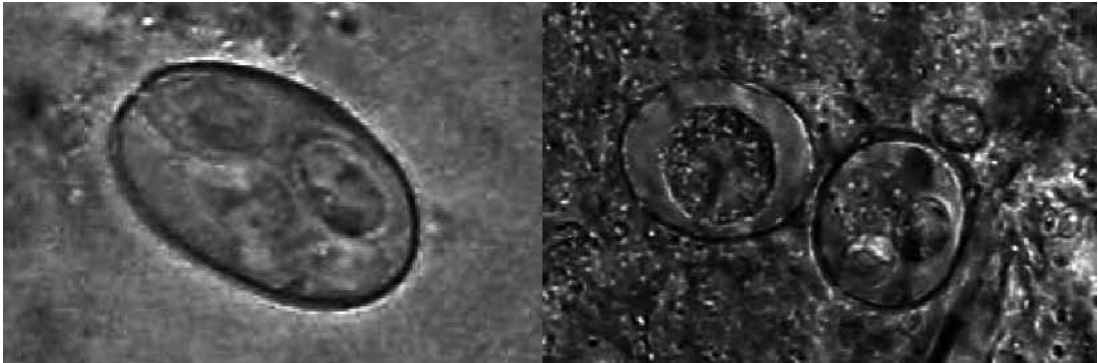
report an outbreak of coccidiosis occurred in red-legged partridges in Emilia Romagna region (Italy).

Material and methods

Case history

The outbreak occurred during the 2003 breeding season in a game farm producing pheasants (*Phasianus colchicus colchicus*), red-legged partridges (*Alectoris rufa*) and grey partridges (*Perdix perdix*) only from own breeders in order to release them in the farm's sor-

Figure 1. Oocysts of *E. legionensis* (a) and *E. kofoidi* (b).



bars=10 μ m

rounding area. Six different hatches of red-legged and grey partridges were obtained by artificial incubation. After each hatch, mixed and homogeneous groups were formed and housed on wood shavings in small pens previously cleaned and sanitised with caustic soda and dry heat. Drinkable water was usually used to water the birds except a short period during the breeding of the two first hatches when rain-water, collected from roofs in a basin, was used. Mortality occurred only in the two first hatches and affected exclusively the red-legged partridges. Never grey partridges have been affected. In the first hatch, composed by 74 red-legged partridges and 86 grey partridges, the mortality lasted 10 days starting from the 31st day of life and involved the 52.1% of the red-legged partridges. In the second outbreak mortality lasted from the 26th to the 31st day of age involving the 46.5% of red-legged partridges (53 birds out of 114). After the second hatch no mortality cases occurred. The affected birds were depressed and showed ruffled feathers 24 to 36 hours before death.

Post mortem examination and coproculture

Dead birds underwent necropsy and post mortem macroscopic lesions were recorded.

Intestinal contents of 4 partridges were removed and emulsified with 2% potassium dichromate and vermiculite and placed in a shallow layer in Petri dishes. The plates were kept at 26 + 2 °C, periodically humidified with mineral water, and examined at intervals for two weeks.

The oocysts were extracted by water suspension, filtration and centrifugation; they were fixed in 10% formalin, then observed with light microscope and measured with the "camera lucida".

Results and discussion

At the post-mortem examination red-legged partridges showed a mucous-haemorrhagic enteritis mostly in the duodenal intestinal tract. Direct microscopic examination of intestinal content revealed the presence of a high number of oocysts. After incubation, about half of the oocysts were sporulated and were morphologically distinguished in two groups:

- 1) rounded oocysts with smooth and thin wall, measuring 16-19 x 14-16 μ m (17.44 by 14.91 μ m in average, shape index 1.16). Almost half of these were non sporulated, with a granular spherical mass of 10 μ m centrally placed. When sporulated, the sporocyst were oval and measured 8.5 X 5.5 μ m, with a granular sporocyst residuum (Figure 1);
- 2) elliptic oocysts, measuring 19-24 x 13-16 μ m (21.97 by 15.34 μ m in average, shape index 1.43). A few of these were not sporulated, with central spherical sporont. Micropyle was present as a light hollow. The wall was smooth and thin (> 1 μ m). Most of the oocysts were sporulated: no oocyst residuum was observed; the sporocysts were almond-shaped and measured 9.73 x 5.56 μ m in average; Stieda bodies were present, sporocyst residuum was not evident (Figure 1).

On the basis of these morphological features,

we assumed the occurrence of a mixed infestation, and the two species of coccidia were identified as *Eimeria kofoidi* (Yakimoff and Matikaschwili, 1936) and *E. legionensis* (Cordero and Pla, 1966), respectively.

In literature, surveys of the partridges' coccidia are rare, and often there isn't agreement regarding the species reported and their intestinal localizations. In birds belonging to the *Alectoris* genus, different coccidia have been reported: *E. alectoriae*, *E. kofoidi*, *E. caucasica*, *E. gonzalescascrotoi*, *E. padulensis*, *E. phasiani*, *E. procera*, *E. legionensis*, *E. tenella* and *E. coturnicis* (Lizcano and Romero, 1972; Levine, 1988; Cordero and Hidalgo, 1999). *E. kofoidi* was described both in *A. graeca* and in *A. rufa*, in the terminal portion of ileum and in caecum (Lizcano and Romero, 1972), and in *Perdix perdix* (Pellerdy, 1974). *E. legionensis* was described for the first time in Spain, in the caecum of *A. rufa* (Cordero and Pla, 1966), and afterwards it was also found in *A. graeca*, where it caused a severe outbreak of coccidiosis with a catarrhal-inflammatory change of the small intestine (Pellerdy, 1974). Furthermore experimental infection of grey partridges with *E. legionensis* caused a significant weight decrease and severe diarrhoea with enteritis in ileum as well as thiphilitis; the oocysts production was low, but no mortality occurred (Vanparijs *et al.*, 1991).

Conclusions

It is not clear if the partridges' coccidia are host specific and which tract of intestine they affect. With regard to the first issue we did not observe any clinical sign or mortality in the grey partridges housed together with the affected red-legged partridges. We are not in a position to give any new

information to better understand the coccidia localisation, due to the fact that coproculture was carried out on the whole intestinal content, without distinguishing the different tracts. However the macroscopic lesions were mostly in the duodenum.

It remains to be clarified how the infection has been introduced. The only management difference occurred between the affected and health hatches was the use of rainwater instead of tap water for watering the birds. Therefore we hypothesize a possible faecal contamination of the rainwater by wild red-legged partridges free living in the farm area.

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