Diagnostic and clinical observation on the infectious bronchitis virus strain Q1 in Italy

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Keywords
Broiler, CK/CH/LDL97I/97, Infectious bronchitis, Proventricolitis, Q1 variant.

Summary
This paper describes the diagnostic and clinical observations of an infectious bronchitis virus (IBV) variant, referred to as Q1, in clinically ill chickens in Italy. This IBV variant was described for the first time in 1998 in China. In the autumn of 2011 it caused a small-scale epidemic in non-vaccinated meat chickens in farms located in Northern Italy. The disease was characterized by increased mortality, kidney lesions and proventriculitis. Histopatological observations confirmed the nephritis and described an unusual erosive/necrotic proventriculitis with infiltration of lymphocytes, plasma cells and heterophils, as well as fibroplasia in the lamina propria. Despite these findings and the isolation of the Q1 IB virus directly from proventricular tissue, further studies are necessary to confirm the role of this IBV strain in the development of proventricular lesions. Phylogenetic analysis revealed that all the IBV isolates were very similar and probably had a common origin. The IBV Q1 variant appears to be now endemic in the North of Italy and at times it is detected in vaccinated backyard and commercial broiler farms. The importance of continuous monitoring in controlling the spread of known or emerging IBV variants is underlined.

Descrizione di focolai di bronchite infettiva sostenuta dal ceppo Q1 recentemente segnalato in Italia

Parole chiave
Bronchite infettiva, CK/CH/LDL97I/97, Pollo da carne, Proventricolite, Variante Q1.

Riassunto
L'articolo concerne la descrizione clinica e la diagnosi dei focolai sostenuti dalla variante Q1 del virus della bronchite infettiva aviare (IBV). Tale variante, descritta per la prima volta in Cina nel 1998, non era mai stata segnalata al di fuori del continente asiatico. Tuttavia, tra luglio e settembre 2011, in alcuni allevamenti di polli da carne (*Hubbard naked neck*) destinati al mercato dell'Italia settentrionale è stato rilevato un insolito aumento della mortalità (4,1-9,8%), preceduto da sintomatologia respiratoria caratterizzata da rantoli e scolo nasale. All'esame anatomopatologico sono stati rilevati congestione, edema polmonare, tracheite catarrale e aerosacculite fibrinosa. I reni degli animali deceduti sono risultati pallidi e globosi con depositi di urati intraparenchimatosi e, nei casi più gravi, sulle sierose viscerali. Anche l'apparato gastroenterico è risultato coinvolto con un ispessimento del proventricolo e congestione dello sbocco ghiandolare. L'esame istologico ha confermato la nefrite e ha evidenziato una grave proventricolite di tipo necrotico-erosiva con infiltrazione di linfociti, plasmacellule ed eterofili. L'isolamento di IBV Q1 direttamente dai proventricoli degli animali deceduti fa attribuire all'infezione virale un ruolo primario nella genesi della proventricolite. Ulteriori studi sull'argomento si rendono necessari visto che i dati presenti in letteratura risultano contrastanti. L'analisi filogenetica ha caratterizzato i virus isolati dai diversi allevamenti come appartenenti ad un unico cluster. L'elevata omologia genetica dei ceppi isolati e le connessioni epidemiologiche tra i vari allevamenti colpiti fanno ipotizzare un'origine comune dei focolai. La variante Q1, rilevata negli allevamenti del Nord Italia vaccinati contro la bronchite infettiva, suggerisce che IBV sia diventata endemica nel nostro Paese.

Introduction

Infectious bronchitis (IB) is an acute and highly contagious disease of affecting chickens. The viral agent of IB, a group 3 Coronavirus, is a single-stranded RNA virus characterised by rapid spread and a notable capacity to modify its genome both by spontaneous mutation and genetic recombination (6). The continuous emergence of new variants can threaten intensive poultry production despite the global availability and application of vaccination. The existence of undetected reservoirs for these viruses, which may include wild birds and/or domestic poultry in developing countries, has been suggested by Cavanagh and Gelb (7). The rapidly increasing intensive poultry production occurring in these days in the developing countries, associated with little knowledge of local infectious bronchitis virus (IBV) circulation and, consequently, the inadequate application of vaccines could act as a source of new IB variants (8). For example, an IB variant virus, firstly described in China and called QX, spread rapidly through Europe becoming one of the most economically damaging viruses of poultry of the last decade (8, 29). Indeed several new IBV variants have been emerging in developing country (9, 17, 18). At the same time, other viruses seemed to have disappeared for long time following their first description and only recently re-emerged in different Asian countries. This is the case of the Q1 IBV, also known as CK/CH/LDL97I/97 and described in this paper. The virus was initially detected in China and only after several years its presence has been reported in North Italy (1, 31). The reason why some strains spread readily at a global level while others remain more local is unknown. Indeed, little is known even on how these viruses spread between neighbouring countries.

In the Summer of 2011, an unexpected occurrence of the Q1 IB virus was reported in a diseased poultry flock in the North-East of Italy (26). Subsequently a small-scale IB epidemic caused by this IB variant was observed in the same region.

This paper aims to follow up the clinical and pathological features of first naturally occurring infection with IBV Q1 in Italian meat chickens.

Materials and methods

Case history

Between mid-July and the beginning of September 2011, 7 commercial broiler farms located in the North-East of Italy noticed the onset of non-specific clinical signs, such as depression, ruffled feathers and poor growth, sometimes associated with nasal discharge and rales. Total mortality was high, ranging from 4.1 to 9.8%. The affected subjects were 4 to 39 day old Hubbard naked neck chickens supplied by a hatchery located in the North-West of Italy and destined for sale to backyard flocks. Birds had been vaccinated at 1 day of age against Newcastle disease. At the same age, chicks were routinely vaccinated with IBV H120 strain, but vaccination was suspended from July the 4th to August the 13th, as the results of an independent decision taken by the hatchery.

Bacteriology and histopathology

Carcasses (n = 83) belonging to 12 flocks from 7 farms were submitted for necropsy. Samples from the pericardium, liver, brain, spleen, and air sacs of birds showing lesions indicating colisepticaemia (such as pericarditis, perihepatitis or airsacculitis) were plated on both Blood and Eosin-Methylene Blue agar plates. The plates were incubated at 37±1°C for 24-48 hrs under aerobic conditions. Swabs collected from air sacs and pericardium were plated on Columbia sheep blood agar in the presence of a *Staphylococcus hycus* helper culture and plates were incubated in 5% to 10% carbon dioxide for 18 to 48 hrs at 37°C. Identification of isolated strains was achieved by biochemical tests.

On the basis of the reported clinical signs and observed lesions, the presence of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* was also investigated in each farm by polymerase chain reaction (PCR) according to the protocol developed by Garcia *et al*. (10), from different organs namely: lungs, heart, larynx and joint (n = 15).

Samples of organs that were apparently more affected, such as the proventriculus and kidneys, were immediately fixed in 10% neutral buffered formalin for histological examination. After 24 hrs of fixation, samples were processed, paraffin embedded, stained with haematoxylin and eosin (HE) and observed by standard light microscopy.

Virology

Organs (kidneys, lungs, trachea, proventriculus, cecal tonsils) were homogenized with sterile quartz and inoculated in 9-11 day old Specific Pathogen-Free (SPF) embryonated fowl eggs for virus isolation according to standard procedures (11). Both 3 and 7-day-long passages were performed and allantoic fluids were analyzed by Real Time Reverse Transcriptase PCR (RT-PCR) following the protocol described below.

Viral RNA was extracted from target organs (kidney, lungs, trachea, proventriculus and cecal

tonsils) and RT-PCR targeting the conserved 5'- untranslated region of the viral genome was carried out to screen for the presence of the IBV genome. Four positive samples were processed by RT nested-PCR using primers annealing to a conserved region of the spike protein 1 (S1), as described by Jones *et al.* (14). The spike protein 1 (S1) gene fragment (approximately 320 nucleotides) of 6 out of 7 isolates (namely: farms 2, 3, 4, 5, 6 and 7) was sequenced. The virus isolated from farm 1 was selected as representative of the study group and processed by one-step RT-PCR following an in-house protocol (primers sequence available upon request), which made the generation of the complete S1 protein sequence (1493 nucleotides) possible. The generated PCR products were processed and sequenced in a 16 capillary ABI 3130 XL Genetic Analyzer (Applied Biosystems, Padova, Italy) genetic analyzed. Virus sequences of the S1 gene fragment were aligned and compared with representative sequences of different IBV variants available in GenBank. The phylogenetic tree was generated using Bayesian methods available in the MrBayes v.3.1.2 software (MrBayes, opensource software, available at http://mrbayes.sourceforge.net/index.php); nucleotide diversity was calculated using the Maximum Composite Likelihood method implemented in the MEGA4 software package (23, 25).

Gross lesions were occasionally observed on birds carcasses, which suggested the presence of viral diseases other than IBV. The organs were subsequently tested by real-time RT-PCR for Newcastle Disease (n = 12), Avian influenza (n = 12) and Infectious Bursal disease (n = 5) according to published protocols (5, 19, 22).

Serology

Sera collected from infected farms were analyzed by the haemagglutination inhibition (HI) test according to standard protocols (11). Homologous antigens were produced using the Q1 isolate obtained from farm 1. In a nutshell, virus was inoculated in 9-11 day old SPF embryonated fowl eggs and incubated for 72 hrs. Eggs were chilled and allantoic fluid harvested. This was then centrifuged for 92 min at 20,000*g* and the supernatant discarded; the remaining antigen was re-diluted with a ratio of 1:100 in phospholipase and incubated for 3 hrs at room temperature.

Haemagglutination inhibition was performed against classical IBV variants: M41; 793/B; 624/I; IT02, D274, QX and with the new Q1 antigen. In addition, sera were collected also from the parents of the affected chicks and analyzed in order to assess Q1 IBV antibody level.

Results

Post-mortem examination

Lesions of the respiratory, urinary and digestive apparatus were observed during the necropsy procedure. Lesions of the respiratory organs included congestion and oedema of the lungs and airsacculitis with deposition of fibrinous exudates on air sacs, while lesions of the urinary apparatus ranged from pale and swollen kidneys to urate crystal deposition in the kidney parenchyma and in the ureters and visceral uricosis (Figure 1). Fibrinous exudates were also seen on the heart and liver. Lesions of the digestive tract were confined to the proventriculus and consisted of the thickening of the wall, in some cases associated with proventricular congestion at the point of emergence of the glandular ducts (*papilla*) (Figure 2). The simultaneous involvement of all 3 apparati was not observed in all chickens. Nonetheless, at least 2 apparati per each group of chickens submitted to our laboratories for post-mortem investigations were affected.

Bacteriology and histopathology: the bacteriological examination of swabs collected from birds showing

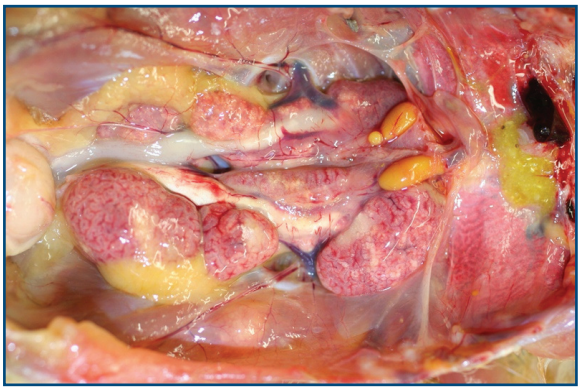

Figure 1. *Kidney, chicken.* Swollen kidneys with massive urate crystal deposition in the kidney parenchyma and in the ureters.

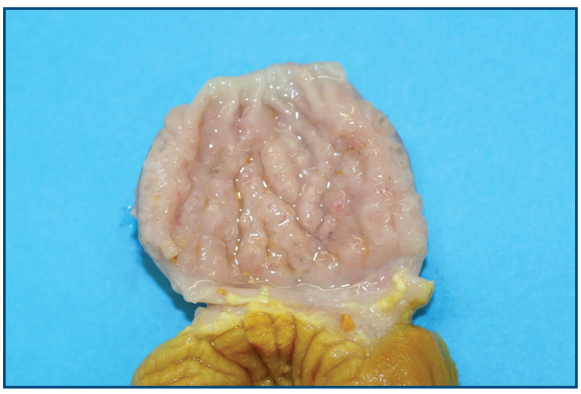

Figure 2. *Proventriculus, chicken.* Thickening of proventricular wall with prominence of *papilla*.

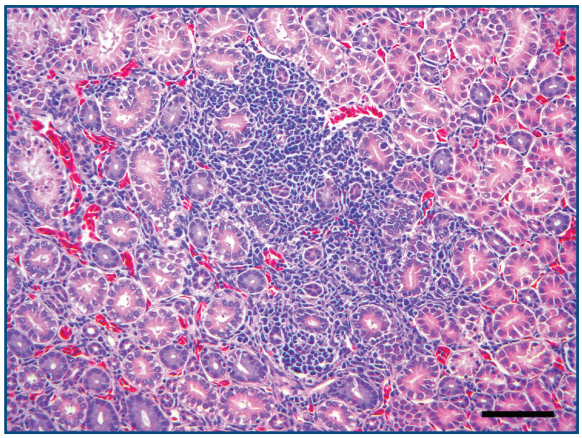

Figure 3. *Kidney, chicken.* Interstitial infiltration of lymphocytes and plasma cells in the renal cortex is evident. Hematoxylin and eosin stain. Bar = 50 µm.

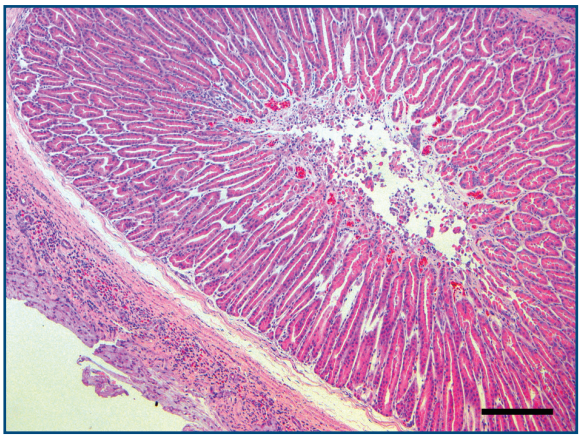

Figure 4. *Proventriculus, chicken.* Diffuse necrosis of superficial and glandular epithelium, associated with infiltration of heterophils lymphocytes and plasmacells in the lamina propria. Hematoxylin and eosin stain. Bar = 100 µm.

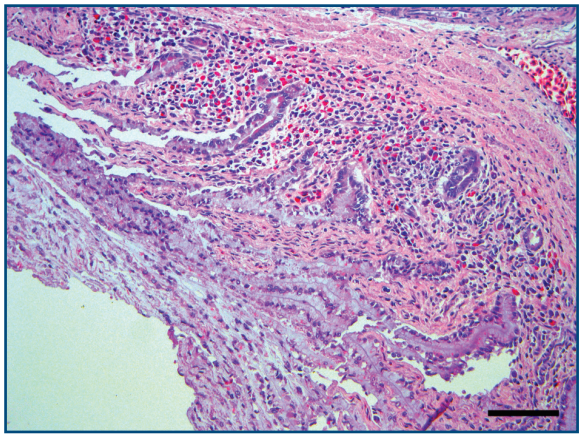

Figure 5. *Proventriculus, chicken.* Diffuse epithelial necrosis, associated with infiltration of heterophils lymphocytes and plasmacells in the lamina propria. Hematoxylin and eosin stain. Bar = 50 µm.

airsacculitis, pericarditis and perihepatitis yielded pure cultures of *Escherichia coli*. All the samples analysed for *M. gallisepticum* and *M. synoviae* were negative.

By histological examination, mild multifocal infiltration of plasma cells and lymphocytes was observed in the interstitium of the renal cortex (Figure 3). The proventriculus was affected by mild to severe proventriculitis, characterized by multifocal erosion and necrosis of the tunica mucosa and glandular epithelium, associated with infiltration of lymphocytes, plasma cells and heterophils, as well as fibroplasia in the lamina propria (Figure 4 and Figure 5).

Virology

Infectious bronchitis virus' RNA was detected by real-time RT-PCR in the majority of the organs tested, while no positive results were observed for the other viral diseases tested: Avian Influenza, Newcastle Disease and Infectious Bursal Disease.

Six out of the 7 viral sequences (those from farm 1, 2, 3, 4, 6 and 7) were identical and showed a percentage of identity of 99.2% when compared with the IBV virus of farm 5. For this reason, only the S1 sequences of viruses from farm 1 and farm 5 were included in the phylogenetic tree (Genbank accession number JQ290229 and JQ419754, respectively). The virus from farm 1 showed a nucleotide identity of 100% with the Q1 strain (Genbank accession number AF286302) and 99.2% with the strain CK/CH/LDL97I/97 (Genbank accession number DQ068701). The S1 sequence of the virus from farm 5 showed an identity of 98.3% with the CK/CH/LDL97I/97 strain (Figure 6). Furthermore, close similarity was observed between the viruses under study and the 624/I IBV variant (97.5% and 96.6% with farm 1 and 5, respectively).

Virus isolation was achieved in all organs (lungs, kidneys, trachea, proventriculus) and in most cases after only one passage in SPF embryonated chicken eggs. Typical lesions including dwarfed, haemorrhagic and curled embryos in positive fowl eggs were observed. Virus isolation was achieved from 5 out of 7 real-time RT-PCR positive periventricular tissue coming from 3 different infected farms. The IBV isolate from farm 1 was used as reference antigen for the HI test.

Serology

The HI test performed on serum samples collected from sick birds confirmed the presence of specific antibodies against Q1 IB variant. Specific HI titres against Q1 were generally 2 to 4 logs greater than those against classical IB virus variants (i.e. M41 and 793B). Cross reactions were observed with other IB variants, mainly with 624/I and D274 (Figure 7).

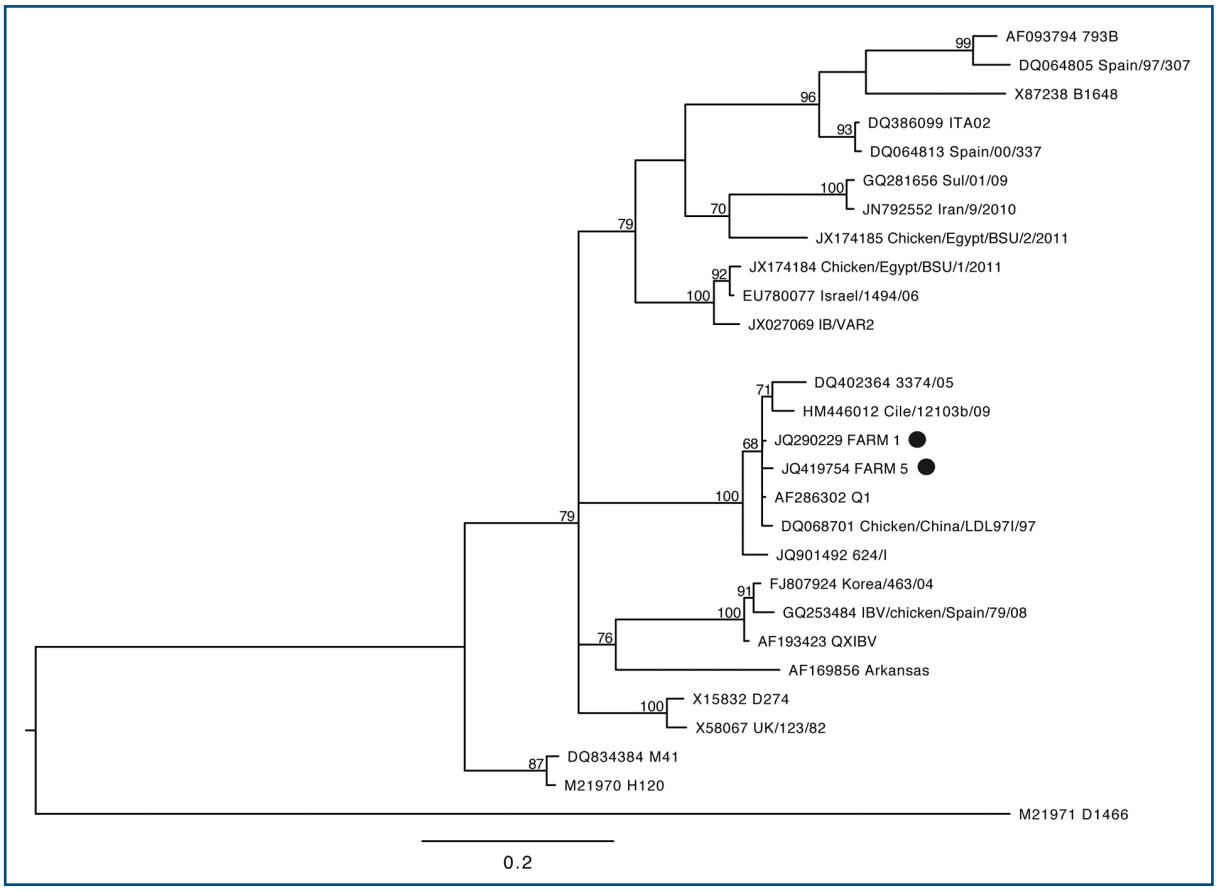

Figure 6. *Phylogenetic tree constructed by Bayesian analysis of the S1 gene fragment of IB viruses.* The viruses under examination are highlighted with a black circle in the tree. Posterior probabilities of the clades are indicated above the nodes. The scale bar indicates the number of nucleotide substitutions per site.

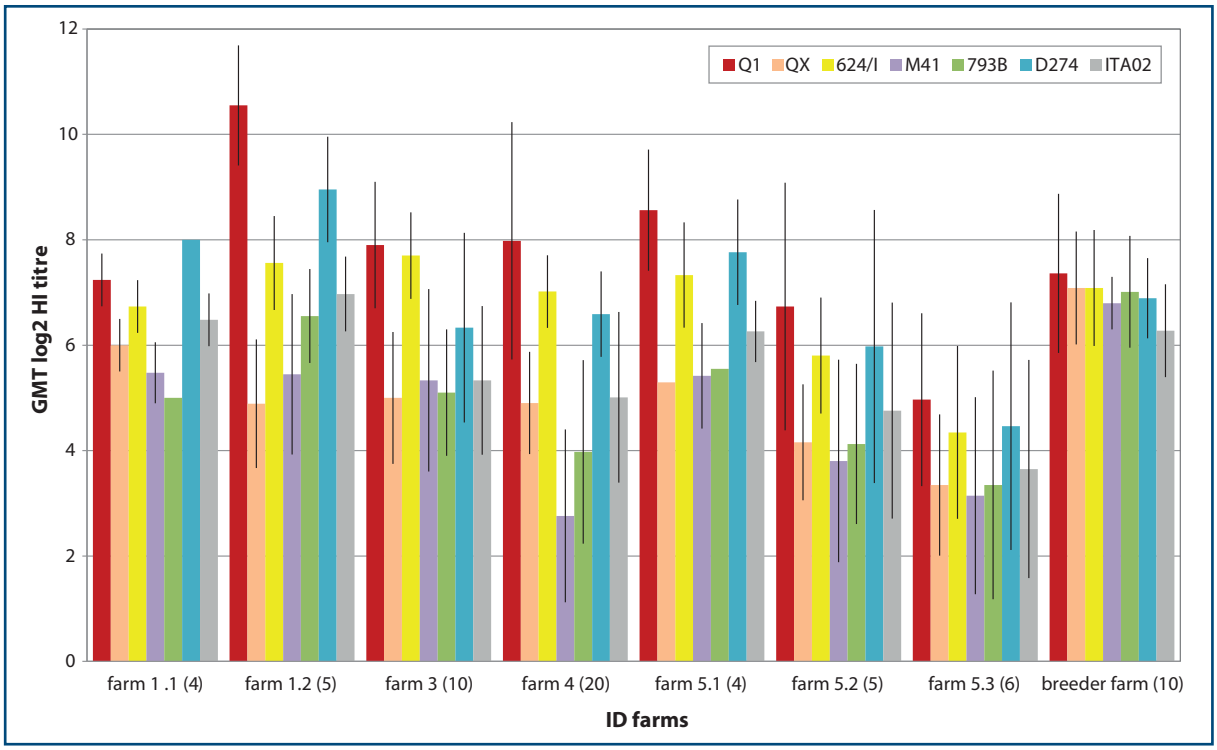

Figure 7. *GMT (geometric mean titer) of the log2 haemagglutination inhibition test titers of Q1 infected farms and breeder farm tested.* Vertical bars refer to standard deviations. Number of sera tested per farm is reported in brackets.

In serum samples collected from the broiler's breeder flock, the HI titres were high against all the IB antigens tested without great differences among the different IBV strains. This was expected, since this flock had undergone standard breeder vaccination for IB.

Discussion

Infectious bronchitis virus generally causes significant economic losses, primarily due to a reduced productivity rather than to an increased mortality rate. Nevertheless, the outcome of infection can vary depending on the breed of chickens, the presence and adequacy of vaccination, and the concomitant infection with other pathogens (6, 8, 20). In this case report a combination of factors such as the *E. coli* co-infection, the lack of protection given by a temporary suspension of IBV vaccination and, perhaps, the high susceptibility of the naked neck broiler chicken breed may have led to the onset of a small epidemic of IBV of the Q1 variant, characterized by a significant increase in mortality (ranging from 4 to nearly 10%). It is known that IB infection predisposes birds to *E. coli* infection, resulting in increased mortality (6). This was also our experience, in that *E. coli* was detected in samples from the majority of the tested organs, including lungs, air sacs, brain, spleen and liver of the birds submitted to the laboratory. The high mortality rate reported was probably due to the combined action of the two pathogens, IBV Q1 and *E. coli*.

In the reported outbreak, the main lesions did not differ from those caused by classical IBV strains, such as tracheitis, lung congestion, nephritis, except for those of the proventricular tissue which were frequently observed here, barring a few exceptions (26). Marked thickening of the proventricular wall and congestion of glandular ducts have always been associated with positive real time RT-PCR for IBV RNA. Notably, virus isolation analysis performed directly on this organ confirmed the presence of Q1 IBV in the proventricular tissue. The IBV Q1 variant, together with 2 other similar IBV strains (J2 and T3), was first identified in the proventricular tissue of 25-to 70-day-old layers suffering from the so called 'avian disease associated with proventriculus'. Samples were collected between 1996 and 1998 in China (31); Yu *et al*. isolated the newly detected Q1 variant and the disease was experimentally reproduced in SPF chickens. During the experiment, infected SPF chickens showed severe proventriculitis associated with respiratory and enteric signs, whereas no kidney lesions were reported either in the field or in experimentally infected birds. Very recently, this IBV variant, also called CK/CH/LDL97I/97, has re-emerged in vaccinated broiler flocks in China and in the Middle East showing tracheitis, nephritis but no proventriculitis (1, 17). Another IBV variant (QX) of worldwide significance, originating from China, has been primarily reported in association with proventriculitis (29). However, in all the relevant reports, included this one, it is almost impossible to unequivocally demonstrate the role of this IBV strain in the genesis of the proventriculitis, as well, factors other than IBV infection which may be the cause e of proventricular lesions have not been ruled out emphatically. Furthermore, the pathogenicity and tissue tropism of the IBV Q1 strain may change under different field conditions and therefore requires further characterization (12, 16, 31).

From a phylogenetic point of view, the Italian Q1 clustered separately from the classical IBV variants, appearing to have the closest relationship with the 624/I strain. All the viruses isolated during the outbreaks described herein showed a very high homology rate, suggesting a common origin due to a single introduction. A field study showed that all the affected farms were served by the same hatchery located in the North-West of Italy. This hatchery normally buys eggs from a single broiler breeder. However in July 2011 the hatchery purchased an additional batch of eggs from France. At the same time, for commercial reasons, the vaccination against IBV routinely performed (H120 strain by eye drop at one day) was interrupted for nearly 40 days. Additional investigations performed on the broiler parent flock showed no IB virus circulation (data not shown), and high levels of antibodies against IBV induced by multiple vaccinations. Considering all these data, it seems that the virus probably originated from the hatchery or was carried by the lorry used to move one day old chicks to the farms and, due to the lack of protection given by IBV vaccination, it was able to spread.

Very little is known on the protection against this variant induced by the available commercial vaccines. A consistent decrease in Q1 positive farms (Table I) after the re-establishment of vaccination with the H120 strain in the hatchery was observed. Since no other IB vaccines were used by the farmer, we may assume a certain level of cross-protection between H120 and Q1 variant. Nevertheless, at present the IBV Q1 variant appears to be established in the Northen Italy (data not shown), as it is detected, although not frequently, from vaccinated broilers. Further monitoring of the spread of this IB strain in Italy and of the efficacy of vaccination in controlling this variant is therefore highly recommended.

The way in which the Q1 virus may possibly have reached Italy remains unclear. As shown by a recent viral survey performed by Tosi *et al*. (28) between 2007-2010, in which 368 IB viruses were characterized by RT-PCR or sequencing, it emerged

Table I. *Summary of field data and laboratory results of Q1 positive farms.*

ID farm	Flock number	Age of chickens at submmission (days)	Total mortality (%)	Gross pathology	Main histopathological reports	Other patohgens found*
farm 1	1	16	4.5	Fibrinous exudates on heart, liver and air sacs Severe lung congestion Thickening of proventricular wall and congestion at the point of emergence of the glandular ducts Multiple necrotic foci in pancreas Catarrhal enteritis with petechial haemorrhages on the intestinal wall Pale and enlarged kidneys	Erosive proventriculitis Urate crystal deposition in kidneys	reovirus† and coccidia
	2	28	4.3	Thickening of proventricular wall Congestion of kidneys Catarrhal enteritis with petechial haemorrhages on the intestinal wall Bursa slightly enlarged	n.d.	reovirus†
farm 2	1	16	9.8	Lung congestion Fibrinous exudates on heart, liver and air sacs Thickening of proventricular wall and congestion at the point of emergence of the glandular ducts Pale and enlarged liver Pale and enlarged kidneys	Necrotic proventriculitis Necrotic hepatitis Urate crystal deposition in kidney	no
farm 3	1	19	4.5	Lung congestion Fibrinous exudates on air sacs Severe urate deposition in kidneys	n.d.	no
farm 4	1	12 and 28	5.6	Lung congestion Massive fibrinous exudates on heart and air sacs Thickening of proventricular wall Severe enlarged and congested kidney	Erosive proventriculitis Severe nephritis with urate crystal deposition	no
	2	25	4.1	Massive fibrinous exudates on heart and air sacs Enlarged hocks with accumulation of fibrinous exudates	n.d.	no
	3	4	4.5	Thickening of proventricular wall Severe urate deposition in kidneys with visceral uricosi	Erosive and necrotic proventiculitis	no
farm 5	1	21 and 25	4.9	Fibrinous exudates on air sacs Thickening of proventricular wall and congestion at the point of emergence of the glandular ducts Catarrhal enteritis Bursa slightly enlarged	n.d.	no
	2	39	4.8	Thickening of proventricular wall Enlarged liver Catarrhal enteritis Enlarged and congested bursa Swollen kidneys	Severe necrotic proventriculitis	no
farm 6	1	21	5.0	Congestion of lung and trachea Fibrinous exudates on air sacs and liver Pale pancreas Kidney congestion and urate depositions	Nephritis Erosive proventiculitis	adenovirus‡
	2	27	4.1	Congestion of trachea Thickening of proventricular wall Pale pancreas Enlarged and pale kidneys	Erosive proventriculitis	coccidia
farm 7	1	20	4.5	Fibrinous exudates on heart, liver and air sacs Enlarged and pale kidneys	n.d.	coccidia

n.d.= not done; **Escherichia coli* was detected in all tested farms; †Reovirus was isolated in cell cultures from pancreas; ‡Adenovirus was isolated in cell culture from lungs.

that the detected IB variants belonged mostly to the 793/B, M41, QX and IT02 variants with sporadic detection of D274, 624/I and B1648 (28). It is noteworthy that thre are no records of the Q1 variant in Italy prior 2011. Apparently, this IBV variant had disappeared from the field for a long time, despite several subsequent field surveillances performed in China and in neighbouring countries it had never been detected until recently, when it was detected again in China, Iran, Jordan, Saudi Arabia and Europe (1, 12, 13, 17, 21, 26). Apart from 793/B and, more recently, QX, IBV variants are rarely reported to spread from one continent to another (6, 27, 30). Diffusion via commercial routes or by wild birds has been suggested, since increasing evidence proves that IBV has a host range not limited to poultry (6, 8, 30). Whatever its origin, the case report herein described highlights the importance of monitoring programs to control the spread of known or emerging IBV variants.

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