New insights on Avian orthoreovirus and Chicken astrovirus co-infection in an Italian broiler flock: preliminary biomolecular and pathological results

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Summary
Common pathogens of intensive poultry farms, either parasitic or bacterial, such as *Coccidia* or *Salmonella,* are well known and strictly controlled by veterinary management. This case study reports an unusual case of runting stunting syndrome (RSS) observed on a Sicilian poultry farm of broiler chickens during 2019. The investigation was carried out on five chickens which present delayed in body weight and growth performance. Animals showed also difficulty in deambulation and diarrhea. At necropsy, intestinal lesions were detected in three of the five clinical cases. Gut samples were collected and analyzed to identify potential pathogens responsible for the RSS. Presence of viruses was detected by using quantitative reverse transcription PCR (RT-qPCR), while selected tissues were fixed and embedded in paraffin wax according to routine procedures. All histological sections were stained with hematoxylin-eosin. RT-qPCR successfully detected both Chicken astrovirus (CAstV) and Avian orthoreovirus (ARV). Histology evidenced severe specific lesions on the intestinal mucosa in liver and kidneys. Chicken astrovirus and Avian orthoreovirus RNA was also detected in cecal tonsils, kidney and liver, thus implying their possible primary role in inducing the disease. Further studies are needed to evaluate the role of other possible factors (low biosecurity measures, e.g.) and, most of all, the consequences in terms of economic losses and animal health impairment.

Introduction

The intensive raising process of broiler chickens sometimes can facilitate the spread of opportunistic pathogens, such as bacteria, parasites and viruses, which could increase the mortality rate (FAO 2013). Most of the pathogens are spread throughout different vectors and may persist in the farm environment for long time (Rosenberger 2012). Indeed, after a fattening cycle, bacteria and parasites can survive in indoor sheds even after cleaning and disinfection procedures are applied. Conversely, viruses are more susceptible to environment condition and chemical disinfection although sometimes, they can survive even for long time after the disinfection (Guy 1998, Otto *et al.* 2006). In this report we focused our attention on two viruses linked to modern avian farming: Avian orthoreovirus (ARV) and Chicken astrovirus (CAstV).

The Avian orthoreovirus (ARV) was firstly isolated from a chicken with arthritis (Fahey and Crawley 1954). It is one of the most widespread avian viruses causing clinical disease in poultry farming, with subsequent economic losses to total meat production (Rosenberger 1989). In poultry flocks, the ARV infection causes arthritis and tenosynovitis (Heide 1977), immunodepression (Kibenge *et al.* 1987), enteric symptoms (Dutta and Pomeroy 1967) and so called "runting-stunting syndrome" (Goodwin *et al.* 1993). This infection is typically characterized by high morbidity and mortality, poor feed efficiency and delayed growth (Dobson and Glisson 1992). ARV belongs to the *Reoviridae* family and it is constituted by a segmented dsRNA genome (Spandidos and Graham 1976).

The Chicken astrovirus (CAstV) is an emerging disease and the most recently identified member

of avian astroviruses. CAstV belongs to the *Astroviridae* family and is characterized by a ssRNA genome. Like other astroviruses, it is a small round shape, non-enveloped virus (Méndez *et al.* 2013). Astroviruses, generally, cause enteric infection and occur in many animal species including humans (Smyth 2017). Historically, these viruses have been named according to the target species e.g. turkey astrovirus (TAstV), but cross infections among different host have been identified, such as CAstV isolated in turkey (Pantin-Jackwood *et al.* 2006). According to the International Committee on Taxonomy, it's possible to distinguish three species, namely Avastrovirus 1, 2 and 3 (Smyth 2017). CAstV is an enteric pathogen that often infects birds, either through horizontal transmission by fecal-oral route and also by vertical transmission from naïve in-lay parent bird. Therefore, chicks may hatch shedding high levels of virus. In comparison to other viruses, the lack of an envelope confers CAstV a higher resistance to lipid solvents, and its maintenance in the environment could be also related to Arthropoda which act as vectors (Rosenberger 2010).

Previous studies suggest that concomitant enteric viral infections may increase the severity of clinical signs in turkeys and in broiler chickens (Spackman *et al.* 2010, Reynolds *et al.* 1987, Bon-Sang *et al.* 2013). However, viral enteric outbreaks, especially those caused by CAstV and ARV in broilers, have never been reported in Southern Italy so far.

Case report

In a commercial farm, a total of five male chickens Ross 308, 46 days old, were selected during the last day of fattening cycle, in order to measure growth performance. In a large shed, previously disinfected and kept empty for 15 day after the previous fattening cycle, a total of 8,000 chickens from the same hatching were reared from the beginning of March to the end of April. Chicks were vaccinated against Infectious Bursal disease virus (IBVD), Infectious bronchitis (IB) (793b, H120) and Marek's diseases in the hatchery. A coccidiostat (nicarbazin, 40 ppm, and Narasin, 50 ppm) was added to the feeds. No antibiotics were added to diets and to water during raising process. Three out of five showed delay in growth, immature appearance if related with their age, and typical clinical signs of suffering such as reluctancy to move, fatigue, traces of diarrhea in the cloacal region and swelling of the infraorbital sinuses. The body weight of these three animals was significantly lower (about 60% less) if compared to weight of the other two chickens (Table I). Carcasses inspection showed hemorrhagic enteritis, hepatic necrosis and swelling of infraorbital sinuses, furthermore an abnormal size of kidney and liver, almost double in size, in one of the three chicks compared to the organs of the others two birds. Loss of weight was clearly visible in the muscular breast which were significantly underweight. Moreover, in the carcasses were evident the hemorrhagic lesions with necrotic areas in liver and signs of hemorrhagic enteritis in intestinal tracts (Figure 1). Representative portion of organs (intestine, liver, kidney and cecal tonsils), fecal content, tracheal and cloacal swabs were collected for laboratory investigation. At the end of this cycle, the mortality rate reached 3%. A peak of 1.7% was reached in the first two weeks of raising cycle. There was no visible changes in litter and differences in feed/water intake as the performance were measured only at the end of the cycle.

Microbiological screening was performed on the cloacal swabs and from liver and kidney. Samples were directly sowed on Columbia agar containing 5% sheep blood (Oxoid Limited, Hampshire, UK), McConkey agar (Oxoid Limited, Hampshire, UK) and inoculated aerobically and anaerobically at 37 °C up to 48 h; subsequently, suspected colonies were purified and identified by Vitek 2 (Biomerieux, France). At the same time, 10 grams of intestine were homogenized through stomacher in 90 ml of Tryptone Soya Broth and incubated aerobically for 24 h at 37 °C. After incubation, 1 ml of enriched broth was transferred to modified semi-solid rappaport-vassiliadis agar and incubated for 48 h at 40 °C. Presumptive *Salmonella* colonies were transferred on xylose lysine desoxychloate agar and brilliant green agar[1]. For all other bacteria, including fastidious species, samples of duodenum and caecum were inoculated directly into TSA medium, modified brilliant green medium and Columbia III w/5% SB medium (BD Diagnostic, Diagnostic Systems, Sparks, MD, USA) and incubated at 37 °C for 72 h. Microbiological investigation did not evidence the presence of pathogenic bacteria.

A parasitological examination has been carried out by direct mount technique through preparation of smears obtained either from each fecal sample (collected from the cloaca) or from intestinal material sampled from three sections of the

Table I. *Final body weight (BW) and presence of Chicken astrovirus (CAstV), Avian orthoreovirus (ARV) and Avian metapneumovirus (aMPV).*

Chick	BW (kg)	aMPV	CAstV	ARV
1	3.439	X	X	X
2	1.243	X	V	V
3	1.350	X	V	V
4	1.268	X	V	V
5	3.502	X	X	X

X = Absence; V = Presence.

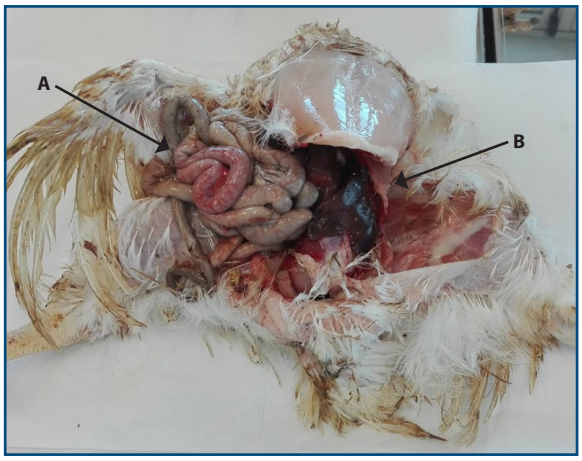

Figure 1. *Necropsy of a broiler infected by astrovirus (A = Hemorragic enteritis; B = Liver necrosis).*

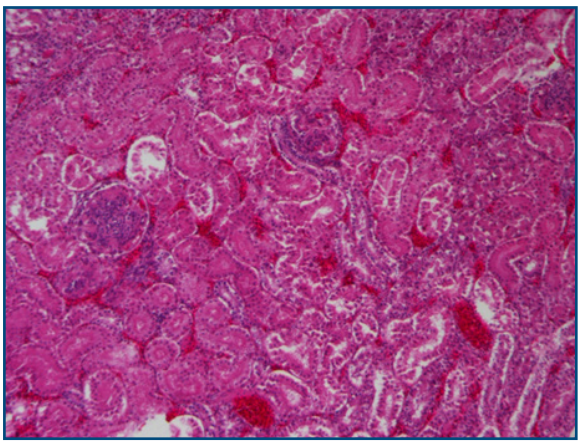

Figure 2. *Broiler: interstitial nephritis, Haematoxylin & Eosin, 20X.*

intestine (duodenum, jejunum and caecum). Fecal material collected from the intestine were then processed through a saturated sugar (density 1.27 g/ml) flotation technique, by plating the supernatant phase on smears and observed by direct light microscopy at 100X up to 400X magnification in order to identify eggs or eventually other stages of Cestode, Nematoda or Coccidia according to Quinn and colleagues (Quinn *et al.* 1980).

Cecal tonsils from the suspected clinical cases were used for molecular investigation in order to detect: Chicken astrovirus (CAstV), and Avian orthoreovirus (ARV) RNA. Tracheal swabs were also screened for Avian metapneumovirus (aMPV).

Total RNA and DNA were extracted directly from cecal tonsils, liver and kidney tissues and tracheal swabs using a DNA/RNA purification kit (Kylt, Germany) according to the manufacturer's instruction. For the PCR, commercial kits were used: aMPV A&B, Chicken astrovirus RT-qPCR, Avian reovirus RT-qPCR (Kylt, Germany). Each reaction consisted in 4 µl of total RNA, 10 µl of 2x RT-qPCR-Mix and 6 µl of Detection-Mix, as reported in the manufacture's instruction. The multiplex RRT-PCR was performed on a LineGene 9600 (Bioer, Hangzhou, China) according to the following conditions: 1 cycle at 50 °C for 10 min; 1 cycle of 95 °C for 1 min and 42 cycles at 95 °C for 10 sec and 60 °C for 1 minutes. Molecular screening confirmed the suspicion of viral mixed infection which affected the three broilers (number 2, 3 and 4). All the tissues belonged from the five chickens resulted positive for ARV and CAstV while all five animals were negative for Avian metapneumovirus (aMPV A&B) (Table I).

[1] ISO 6579-1. 2017. Microbiology of the food chain. Horizontal method for the detection, enumeration and serotyping of *Salmonella*. Available online https://www.iso.org/standard/56712.html (accessed on 22 May 2019).

Lesions collected from liver, spleen, kidneys and intestinal tract of duodenum, jejunum and ileum, were fixed in 10% buffered formalin, embedded in paraffine vax and routinely processed. Sections with a thickness of 2.5 microns were obtained and stained with hematoxylin and eosin (HE) for morphological examination. Histology examination showed an inflammatory condition of the liver with focal areas of monocyte infiltration and focal areas of degeneration; in the kidney (Figure 2) multifocal lympho-granulocyte infiltrates located in the interstitium associated to tubular necrosis were observed, while in the intestinal mucosa diffuse lymphocyte infiltrates with fusion of the villi were also detected (Figure 3). All the lesions could be associated to CastV and ARV infection, which may cause a degeneration of the internal structure of tissues reducing the absorption of nutrient at the intestine level and contemporary renal failure and reduced filtering capacity in kidney.

Conclusions

This study confirms the importance of intestinal inflammation (enteritis) during CAstV and ARV coinfection, both considered responsible for the runting stunting syndrome (RSS). Historically, CAstV and ARV have been associated with malabsorption syndrome of broiler chickens, but our study proves that these viruses, well known as opportunistic pathogen, are responsible for more aggressive syndromes. It's clear that clinically infected chicks show hatches small in size and white-colored, sometimes showing a delay in growth, and a general appearance resembling similar to younger chick with immature feathering, yellow color and reduced size of the beak (Rosenberger 2012). Common symptoms also include enteritis and diarrhea, leg weakness and irregular feathering (Kouwenhoven *et al.* 1978). While CAstV is predominantly an enteric virus, it is also recognized as infecting organs through the

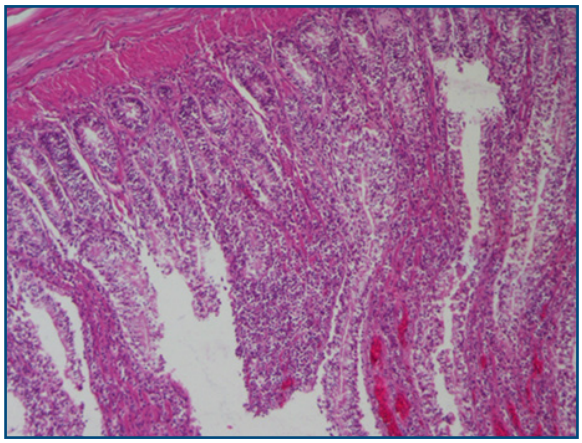

Figure 3. *Broiler: monocytic enteritis, Haematoxylin & Eosin, 20X.*

enteric tract, including the liver and kidneys (Bulbule *et al.* 2013). In our case, the presence virus DNA copies and infiltrate in liver and in the kidney showed that the two viruses, with a predominantly enteric tropism, may colonize and induce imbalances and so far pathological lesions in different body organs; in the kidney they affect the filtering function of the glomeruli; in the liver they cause malfunction due to diffuse tissue degeneration. In the intestine the worst damage was reported, in terms of a disorganized structure of villi that appeared confluent/fused with a diffuse lymphocytic infiltrate following the infection (Kang *et al.* 2018). These findings are consistent with a report that described enteritis simultaneously occurring with astrovirus infection (Koci *et al.* 2003). The initial suspicion of a viral infection was confirmed by the biomolecular examination, while the investigation for the other typical poultry pathogens (bacteria or parasites) were negative. Histopathological examination confirmed a severe and diffuse enteritis and massive monocyte infiltration in liver and kidney. These syndromes are strictly connected with intestinal disease, sometimes associated with tenosynovitis, that was not observed in this outbreak. Intestinal lesions reported in this study characterized by severe villous atrophy and crypt hyperplasia, appear to be significantly more severe if compared to previous reports and related to the direct damage of CastV, without any involvement of bacterial secondary infection (Guy 1998). This difference is related to a concomitant infection with ARV that induces more severe clinical signs as reported by Spackman and colleagues (Spackman *et al.* 2010) and Bog-San Koo and colleagues (Bog-San Koo *et al.* 2013). Concomitant infection of both enteric viruses may booster the pathogenicity of the syndromes and, in this case, it also causes lesions on kidney and liver, uncommon findings for infections with these kinds of viruses. When singularly detected, CastV and ARV are considered as opportunistic pathogens which may affect the curve of growth of chicks and their body weight (Hauck *et al.* 2016), but according to this report we have to consider the risk of mixed infection and the related risk of an enhancement of pathogenicity which may imply significant economical losses if the percentage of infected chicks increase significantly. The difference in live weight between sick and healthy broilers was relevant, even three time less than normal body weight at equal age. The loss of production could have a negative impact on farm incomes, therefore, as for all the other poultry pathogens, it is really important to avoid circulation of these viruses in the farm environment, improving all of those procedures to maintain high level of biosafety. This preliminary data may offer new perspectives for industrial poultry farming in Sicily, underlining the importance of applying "*ad hoc*" biosafety programs and represent an original report on lesions caused by these viruses and their extra intestinal spread to other organs.

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