
UNIVERSITA' DEGLI STUDI DI MILANO

Graduate School of Animal Health and Production: Science, Technology and Biotechnologies

Department of health, Animal Science and Food Safety

Ph.D Course in Biotechnologies applied to veterinary and animal sciences

(Class XXVIII)

Doctoral Thesis

# DEVELOPMENT OF INNOVATIVE DIAGNOSTIC PROTOCOLS TO BE APPLIED TO THE STUDY OF THE EPIDEMIOLOGY AND GENETICS OF AVIAN ROTAVIRUSES

(VET/05)

Ph.D Candidate: **Dr. Elena Canelli**

**R10354**

Tutor: **Prof. Lauretta Turin**

External tutor: **Dr. Antonio Lavazza**

Coordinator: **Prof. Fulvio Gandolfi**

Academic Year 2014-2015

In lovely memory of Dr. Paolo Cordioli

my guide and mentor

**INDEX**

## 1. ABSTRACT

Rotaviruses (RVs) are the major cause of acute gastroenteritis in infants and young animals of mammalian and avian species. In avian species, group A and D are represented with high frequency, while group F and G are sporadic. Enteric syndrome in young birds is a major concern to the poultry industry, causing severe economic losses. Monitoring rotavirus distribution in different avian species is crucial to uncover diversity among strains and to better understand RV ecology in the field.

The aims of this project were: i) to provide information on distribution of the different RV groups in avian species suffering enteritis in Italy; genetic diversity of RVs in these species; dynamics and timing of RV infection within flocks; and ii) to develop new and updated diagnostic protocols to be applied in the diagnostic routine and for research use.

The data collected during a period of nine years, indicate that the infection is widespread in the Italian production, with an average prevalence of 20% in commercial flocks and 15% in game-hunting species.

All the 117 samples analysed in the present study originated from poultry flocks that presented one or more clinical signs and lesions associated with enteric diseases. Clinical manifestations consisted mostly of diarrhoea, dehydration, anorexia, weight loss and increased mortality. By statistical analysis it was not observed any significant correlation between the general clinical signs/lesions or gastro-enteric lesions observed in the field and at post mortem examination with the presence of different AvRV groups or different combinations of groups, even with regard to the different species involved ($p>0.05$).

From the 117 samples tested by group-specific RT-PCRs, AvRV-D was detected in 107 (91.5%), AvRV-A in 70 (59.9%), AvRV-F in 61 (52.1%) and AvRV-G in 31 (26.5%). Only 17% of samples showed the presence of a single rotavirus group (AvRV-A or AvRV-D), but dual or multiple presence of rotaviruses of different AvRV groups was observed in the majority of samples. Group D is the predominant RV in Italy, representing the most commonly found RV in all the species investigated. The identification of several avian rotaviruses (AvRVs) belonging to group F and group G in partridge, pheasant and guinea fowl, suggests that the lack of specific detection methods could have underestimated the real diffusion of these two groups in the past.

The use of new group-specific RT-PCRs developed and performed on a set of clinical specimens tested positive for RVs by Electron Microscopy (EM), allowed the rapid genetic screening of AvRVs circulating within the avian rotavirus main groups. In this study, 175 complete sequences were obtained (17 of NSP4A, 20 of VP6A, 5 of VP4A, 35 of NSP4D, 34

of VP6D, 9 of VP4D, 17 of VP7D, 21 of VP6F and 13 of VP6G). No correlation between year of isolation nor avian species and the different RV-groups was observed.

Regarding results obtained from longitudinal studies in turkeys, the infection was already present in the first weeks of life; after 2/3 weeks a second infection outbreak happened in the same herds. Phylogenetic analysis of samples collected during the longitudinal study in turkey flocks showed that different RV-groups and different strains from the same group were present in the same flock, evidencing complex RV groups/strains patterns that modified in time. Results of chicken longitudinal study reinforced the hypothesis of a primary pathogenetic role of astrovirus-rotavirus infections in enteric sindrome during first weeks of life.

## 2. INTRODUCTION

Rotavirus (RV) is the major etiological agent of viral enteric disease in young individuals of several mammalian and avian species (Estes *et al.*, 1983; McNulty *et al.*, 1984; Estes & Cohen, 1989) and likely has a worldwide distribution.

### 2.1 Virus Structure

The fully infectious RV particle consists of 3 protein layers. By electron microscopy, this particle resembles wheels (lat. *rota*) (Figure 1), and this appearance has led to the name of Rotavirus for the genus (Flewett *et al.* 1974). Intact RV virion consists of two icosahedral capsid shells of approximately 50 and 70 nm in diameter (Guy 1998; McNulty 2003). Based on cryo-electron microscopy and image reconstruction data (Jayaram *et al.*, 2004), structure of icosahedral symmetry has been recognized: the single layered particle (SLP=core shell) is formed by 120 molecules of the viral protein 2 (VP2), arranged as 60 dimers in a T=1 symmetry (Figure 2). The core shell encloses the viral genome of 11 segments of dsRNA as well as the viral RNA dependent RNA polymerase (RdRp), VP1 and the capping enzyme, VP3. The viral core is surrounded by 260 trimers of VP6, which form the middle layer and constitute double-layered particles (DLPs).

Figure 1: Transmission Electron Microscope image of Rotavirus particles (Bar = 50 nm); (IZSLER archive)

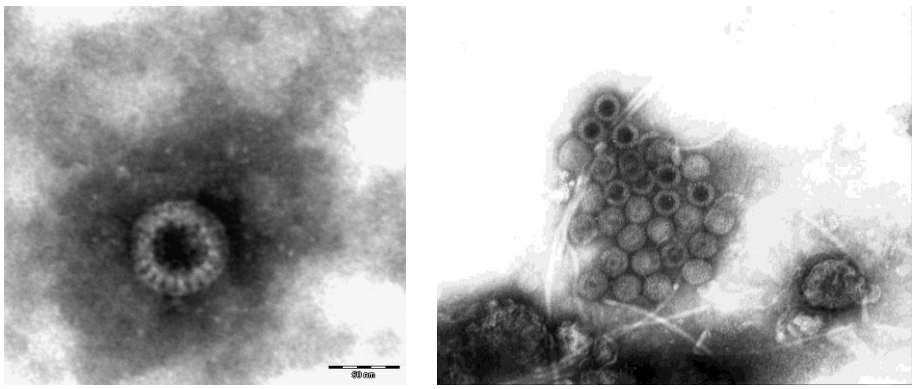
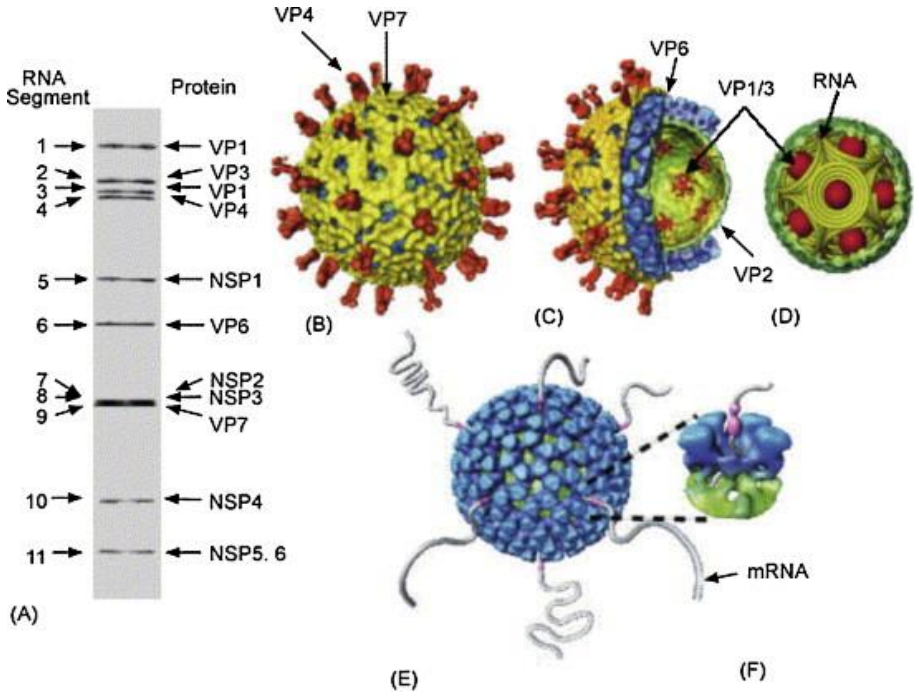


Figure 2: Aspects of rotavirus structure. (A) PAGE gel showing 11 dsRNA segments comprising the rotavirus (RVA) genome. The gene segments are numbered on the left and the proteins they encode are indicated on the right. (B) Cryo-EM reconstruction of the rotavirus triple-layered particle. The spike protein VP4 is colored in orange and the outermost VP7 layer in yellow. (C) A cutaway view of the rotavirus TLP showing the inner VP6 (blue) and

VP2 (green) layers and the transcriptional enzymes (in red) anchored to the inside of the VP2 layer at the fivefold axes. (D) Schematic depiction of genome organization in rotavirus. The genome segments are represented as inverted conical spirals surrounding the transcription enzymes (shown as red balls) inside the VP2 layer in green. (E and F) Model from Cryo-EM reconstruction of transcribing DLPs. The endogenous transcription results in the simultaneous release of the transcribed mRNAs from channels located at the fivefold vertices of the icosahedral DLP. From Jayaram *et al.* (2004)




## 2.2 Genome organization

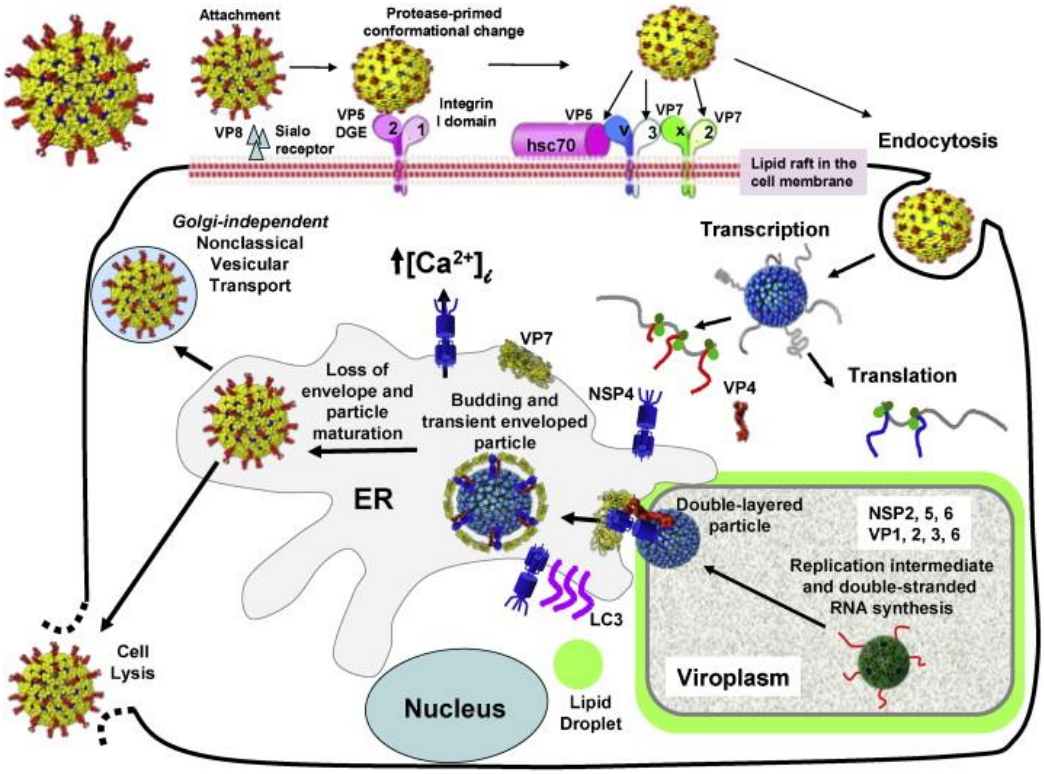
The RV genome consists of 11 segments of double-stranded (ds) RNA which encode 6 structural and 6 non-structural proteins. The genes are monocistronic, except for genome segment 11, which encodes two proteins. This dsRNA has a molecular weight of approximately 106 Da, and each RNA segment consists of open reading frame (ORF) that encodes viral proteins (Estes & Cohen 1989; Guy 1998). Viral genome encodes six structural (VP1-VP4, VP6, VP7) and non-structural proteins (NSP1-NSP6) each (Mori *et al.* 2002a), out of which 10 major polypeptides have been identified for their prominent roles (McNulty 2003). The protein VP2 forms the first layer, encompassing proteins VP1 and VP3, both of these together have a role in virus transcription. The VP6 protein (encoded by 6th gene segment) forms the second layer and the outermost protein layer is composed of structural proteins VP7 (encoded by 7/8/9th gene segments based on the strain) and spike protein VP4 (encoded by 4th gene segment). VP7 (denoted as 'G'-glycoprotein) and VP4 (denoted as 'P'-

protease sensitive protein) proteins are capable of generating neutralizing antibodies that protect birds from disease (Niture *et al.* 2010b). Also, VP4 can undergo proteolytic cleavage that further enhances infectivity of RVs (McNulty 2003). Among non-structural proteins (NSP), Avian RV (AvRV) NSP4- a viral enterotoxin, is known to have major differences in amino acids when compared to similar protein in mammalian RVs (Mori *et al.* 2002a; Kusumakar *et al.* 2010).

## 2.3 Replication cycle

Replication and assembly of RVs occurs in the cytoplasm of host cells and virus particles are commonly found within vacuoles. The RV replication cycle (Figure 3) includes the following steps: attachment, mediated by VP4 and VP7; penetration and un-coating; plus strand ssRNA (=mRNA) synthesis, mediated by VP1, VP3 and VP2; viroplasm formation, mediating RNA packaging, minus strand RNA synthesis (=RNA replication) and DLP formation; Virus particle maturation (to TLPs) and release (Desselberger, 2014).

Figure 3. The rotavirus replication cycle. Estes and Greenberg (2013)



## 2.4 Rotavirus evolution (Dhama *et al.*, 2015)

The evolution of RVs has been elucidated by widespread genome-wide RT-PCR genotyping supported by cDNA sequencing (Matthijnssens and Van Ranst, 2012). Several mechanisms were identified (Iturriza-Gomara *et al.*, 2003): frequent point mutations in all RNA segments, either sporadically occurring or sequentially accumulating (Iturriza-Gómara *et al.*, 2000, Ianiro *et al.*, 2013, Hemming and Vesikari, 2013a and De Grazia *et al.*, 2014); genome reassortment occurring in doubly infected individual cells and organisms in vivo (Iturriza-Gómara *et al.*, 2001), often involved in zoonotic transmission (Steyer *et al.*, 2008, Martella *et al.*, 2010, Todd *et al.*, 2010, Matthijnssens *et al.*, 2011b, Papp *et al.*, 2013, Mullick *et al.*, 2013, Soma *et al.*, 2013, Cowley *et al.*, 2013); genome rearrangements, consisting of partial duplications or deletions of nucleotide sequences of individual segments, a special form of recombination (Desselberger, 1996); true genome recombination involving several segments (Parra *et al.*, 2004, Phan *et al.*, 2007, Cao *et al.*, 2008, Martínez-Laso *et al.*, 2009, Donker *et al.*, 2011 and Jere *et al.*, 2011); several of the aforementioned mechanisms acting in combination. The main mechanisms appear to be point mutations that occur continuously due to the high error rate of the RV RdRp and genome reassortments. Animal RVs can also be directly transmitted to humans (Soma *et al.*, 2013 and Steyer *et al.*, 2013).

## 2.5 Classification

Rotaviruses constitute the genus Rotavirus, one of the 15 genera of *Reoviridae* family which is subdivided into the sub-families of the *Sedoreovirinae* (genera *Cardoreovirus, Mimoreovirus, Orbivirus, Phytoreovirus, Rotavirus, Seadornavirus*) and the *Spinareovirinae* (genera *Aquareovirus, Coltivirus, Cypovirus, Dinovernavirus, Fijivirus, Idnoreovirus, Mycoreovirus, Orthoreovirus, Oryzavirus*).

Classification of AvRVs was initially obtained by cross-immunofluorescence studies or polyacrylamide gel electrophoresis (PAGE) analysis of dsRNA segments (Guy 1998). Analyzed by PAGE, the genomic RNA segments cluster into four regions, I to IV. According to the distribution of segments in each region, the AvRV-A has a pattern of 5:1:3:2, RV-D has a pattern of 5:2:2:2, while mammalian RV-A show a pattern of 4:2:3:2, respectively (McNulty *et al.* 1981) (Figure 2). On the pattern of electrophoretic migration of the RVs genome segments at least 8 different groups, also termed species, are differentiated (termed RVA-RVH) (Matthijnssens *et al.*, 2012; Estes & Kapikian, 2007). The antigenicity of RV is determined by three major structural proteins: VP4, VP6 and VP7, with multiple serotypes recognized in each serogroup. According to the serological reactivity RVs share a group

(serogroup) antigen have historically been termed group A RV, the RVs which lack RV-A antigen are referred to as atypical RVs that belong to groups D, F, G and H (Guy 1998; McNulty 2003; Otto *et al.* 2012; Hemida 2013). RV-A is the most predominant across the world (Matthijnssens *et al.* 2011c). RV-D, RV-F and RV-G have been seen exclusively in poultry (Urasawa *et al.* 1992; Saif & Jiang 1994; Santos & Hoshino 2005; Kattoor *et al.* 2013a; Kattoor *et al.* 2013b). AvRV-A and D have been shown to predominate in sick individuals of several avian species, whereas AvRV-F and G have been occasionally reported (Otto *et al.*, 2006; Otto *et al.*, 2012; Kindler *et al.*, 2013; Beserra & Gregori, 2014).

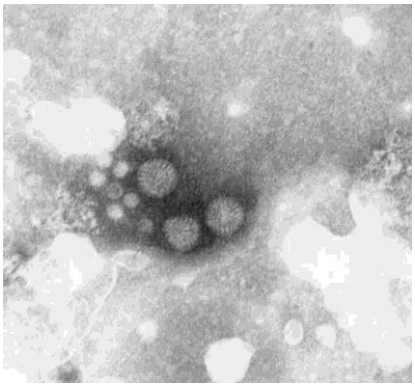
The RV-A species comprises at least 27 G types (according to the nucleotide (nt) sequence of VP7) and 37 P types (according to the nt sequence of VP4) (Matthijnssens *et al.*, 2011a and Rotavirus Classification Working Group, 2013). For G types, serotypes and genotypes are synonymous, e.g. G1, G2, etc. For P types, there are many more P genotypes than reference sera determining P serotypes: therefore, a double nomenclature has been introduced, e.g. P1A[8] designating the P serotype 1A and P genotype 8, etc. (Estes and Greenberg, 2013). A comprehensive, nt sequence-based classification comprising the complete genome has been introduced for RVAs, in which the VP7–VP4–VP6–VP1–VP2–VP3–NSP1–NSP2–NSP3–NSP4–NSP5/6 genotypes are identified and differentiated according to particular cut-off points of nt sequence identities (Matthijnssens *et al.*, 2008a, Matthijnssens *et al.*, 2008b, Matthijnssens *et al.*, 2011a and Maes *et al.*, 2009). Phylogenetic analysis placed RVs in two major clades consisting of rotavirus A/C/D/F and rotavirus B/G/H (Kindler *et al.* 2013). The AvRVs are antigenically related and morphologically identical to mammalian RVs (McNulty *et al.* 1978, 1979).

### 2.6 Epidemiology/Pathogenicity

The RV enteritis in poults and chickens has been reported from Europe, UK, USA, Argentina, Brazil, China, Russia, Bangladesh and India (Savita *et al.* 2008a; McNulty 2003; Kattoor *et al.* 2013a). Although Rotaviruses cause enteric diseases in mammals and birds, RVs are often detected in otherwise healthy flocks, particularly when sensitive molecular diagnostic assays are used. A retrospective look at cases involving poultry enteritis in California from 1993 to 2003 reveals that RVs were the most common viruses detected via EM during that time (Woolcocka P.R. and Shivaprasad H.L., 2008). In a similar study in Minnesota, turkey flocks diagnosed with Poult Enteritis Syndrome (PES) were determined by EM to be infected with RV 48% of the time. In another study on PES, 93% of PES cases studied were positive for RV by RT-PCR (Jindal *et al.*, 2009, 2010).

Virus associated gastrointestinal diseases have a tendency to preponderate mainly in young chicks (Dhama al., 2015). Other infectious agents convolute these diseases under field conditions. Many authors reported mixed disease of RV with other viruses, Reoviruses, Enteroviruses (Figure 4), Adenoviruses, Astroviruses (AsTVs), not only in chicken and turkey poults, but also in minor avian species (Andral *et al.* 1985 and Saif *et al.* 1985; Reynolds *et al.* 1987, Gough *et al.* 1990), and bacteria/protozoa, like *Cryptosporidium, Salmonella, E. coli*, *Enterococcus, Eimeria* spp (Yu *et al.* 2000; Jindal *et al.* 2009, 2010, Perry *et al.* 1991).

Figure 4: Transmission Electron Microscope image of Rotavirus and enterovirus-like virus particles; (IZSLER archive)



Symptoms occur due to prolific viral replication in intestinal epithelium, resulting in nutrient mal-absorption; finally affecting feed conversion ratio and inflicting severe economic losses to poultry industry (McNulty 2003; Villarreal *et al.* 2006). Presence of virus in fecal material and extreme resistance of viruses have paved way for a persistent presence of this disease in poultry environments. Many reports have shown that flocks of broilers and turkeys frequently experience simultaneous/sequential diseases with different RV groups (McNulty *et al.* 1984; Todd & McNulty 1986; Reynolds *et al.* 1987; Theil & Saif 1987) and mixed disease with other enteric pathogens.

Mammalian rotaviruses are known to have varying degrees of virulence, but evidence for any variance among AvRVs is limited to indirect evidence. Anyway, the major obstacle in controlling disease is attributed to high antigenic variation particularly due to antigenic shift (Iturriza-Gomara *et al.* 2004; Simmonds *et al.* 2008).

## 2.7 Host range

In poultry, rotavirus enteric infection occurs frequently in turkeys, chickens and pheasants, but only sporadically in guinea fowls, partridges, quails, pigeons, ducks, scoters and lovebirds (Takase *et al.*, 1986; Reynolds *et al.*, 1987; Gough *et al.*, 1990; Pascucci & Lavazza, 1994; Legrottaglie *et al.*, 1997; Otto *et al.*, 2012; Minamoto *et al.* 1988; Takehara *et al.* 1991; Pantin-Jackwood *et al.* 2007).

Although RV affects birds of all age groups, young birds (1-2 weeks) are most susceptible with high mortality (Dey 2003; Tamehiro *et al.* 2003; Islam *et al.* 2009; Yamamoto *et al.* 2011). Most of natural AvRV diseases occur during age of less than 6 weeks in turkeys, chickens, pheasants, partridges and ducks.

It has been suggested previously that RVs of avian species are separated from RVs of mammals early during the process of evolution (Dhama *et al.*, 2015). They have got more similarity to RVs of avian species than to RVs of mammals in terms of both genetic as well as antigenic properties.

## 2.8 Transmission

The resistance and the extreme stability permit the persistence of the virus in the environment (Brussow *et al.* 1992a; Rohwedder *et al.* 1995, 1997; Mori *et al.* 2001). The RVs can survive in waste for 2 to 6 months (Guy 1998; Boone and Gerba, 2007). No information is available of AvRv in feces, but by extrapolation from mammalian RV, environmental condition is likely to be persistent. Water as well as sewage and inanimate objects have been found to be rich sources of RVs detected in poultry sheds (Brussow *et al.* 1992a; Rohwedder *et al.* 1995, 1997; Mori *et al.* 2001; Savita *et al.* 2008a). Huge quantum of AvRVs is excreted via avian feces and horizontal transmission readily occurs by oral route or direct contact (McNulty 2003). There are no reports of vertical or egg transmission of RVs in flocks till to date (Dhama *et al.*, 2015), but RV detection in 3-day-old turkey poults prompted speculation that transmission occurs either in or on egg (Theil and Saif, 1987). No evidence is available for a carrier state of RVs in birds. Despins and Axtell, 1994 demonstrated darkling beetle larvae as a mechanical vector for turkey RVs.

Several investigators have reported natural cases in which inter-species transmission of AvRVs (RV 993/83), especially to bovines as well as experimental animals has been reported (Brussow *et al.* 1992a, 1992b; Mori *et al.* 2001, 2002a; Tamehiro *et al.* 2003; Ahmed & Ahmed 2006). Besides, there are also reports of mammalian RVs having the ability to get transmitted to avian species (Wani *et al.* 2003; Schumann *et al.* 2009).

## 2.9 Pathogenesis

The RVs present in environment gains entry into body through ingestion. The dual capsid protein coat makes virus very resistant to stomach pH and digestive enzymes in the gastrointestinal tract. After ingestion, replication commences, mainly in mature villus epithelium of small intestine (McNulty 1997, 2003). The outer capsid protein VP4 plays an important role in initiating a viral infection via attachment and entry, it gets cleaved into two fragments known as VP5 and VP8. The VP8 interacts with host receptor resulting in attachment and entry of virions into host cells (Dhama *et al.*, 2015). Specifically, RV invades epithelial cells especially at the top of intestinal villi, where vacuolization and epithelial loss can be observed, followed by crypt hyperplasia. Subsequent viral replication results in lysis of host intestinal cells, thereby impairing nutrient absorption (see also replication cycle chapter). AvRVs causes decreased glucose-stimulated sodium transport and net absorption of sodium, potassium, chloride and water resulting in rapid onset of severe, watery diarrhea with loss of electrolytes in feces (Hamilton & Gall 1982). After efficient multiplication of AvRV, progeny virions are excreted via feces within a period of 2 to 5 days post exposure (McNulty *et al.* 1983; Guy 1998). In birds, besides small intestine, viral multiplication has also been observed in colon and cecum (Lublin *et al.* 2004). Diarrhea occurs due to destruction of mature villous enterocytes and replacement by immature epithelial cells from crypts (Moon 1978). Diarrhea may also occur as a result of mal-absorption and mal-digestion. However, recently one of non-structural proteins, NSP4, has been attributed to a major cause of rotavirus-mediated disease pathogenesis. NSP4 has been shown to be an enterotoxin that is capable of causing secretory diarrhea (Kapikian *et al.* 2001; Dhama *et al.*, 2015).

In synthesis, disease mechanism main factors are: mal-adsorption following destruction of epithelium (Estes and Atmar, 2003), villus ischemia (Osborne *et al.*, 1991), the action of NSP4, a viral enterotoxin (Ball *et al.*, 1996, Greenberg and Estes, 2009), and also the activation of the enteric nervous system (Lundgren *et al.*, 2000).

## 2.10 Clinical signs

In commercial poultry, rotavirus-associated enteric disease may range from subclinical to severe forms. Diarrhea is the major clinical outcome, together with characteristic features as dehydration and anorexia, decreased feed absorption and subsequent reduced weight gain, unpaired flock uniformity (Figure 5), increased mortality (McNulty 1997; Guy 1998; McNulty 2003; Tamehiro *et al.* 2003; Otto *et al.* 2006). In young chickens, milder version of disease may be noticed that can lead to a more severe clinical manifestation in chickens of age

group between 12-21 days. This is mainly characterized by unrest and ingestion of litter, watery feces, wet litter, and severe diarrhea (Barnes 1997).

The disease is often complicated by concomitant and/or secondary infections with other pathogens, and is largely influenced by a reduced immune status of affected birds and inappropriate management procedures (Villareal *et al.*, 2006; Yegani & Korver, 2008; Spackman *et al.*, 2010). Similar to avian Reoviruses, it has been suggested that RVs may also cause runting and stunting syndrome in poultry (Otto *et al.* 2006). Co-infections of AvRVs with other enteric pathogens such as AsTVs and Coronaviruses (CoVs) have been recognized to cause enteritis in poultry flocks (Chandra *et al.* 2001). Such kind of mixed disease has been more intensely identified in broiler flocks.

Variations in virulence and severity of clinical signs associated with different rotavirus strains have been reported. The pathogenesis and clinical signs of group A rotavirus in birds has been well established (Pantin-Jackwood *et al.*2008; Schuman *et al.* 2009; Trojnar *et al.* 2009; Jindal *et al.*, 2010; Ursu *et al.* 2011). Prevalence of groups D, F and G RVs has only been described recently (Trojnar *et al.* 2010; Johne *et al.* 2011; Otto *et al.* 2012) . Otto *et al.* (2006) reported that group D rotavirus plays a major role in pathogenesis of runting and stunting syndrome (RSS) in flocks with severe villous atrophy. Variation in virulence might be due to the differences in virulence of RV strains or interaction of other infectious agents, environmental stress or management factors (McNulty 1997), but studies on pathogenesis and clinical signs in birds are lacking.

Figure 5: Symptoms of a Rotavirus infection (IZSLER images): A. Impaired flock uniformity; B. Diarrhea

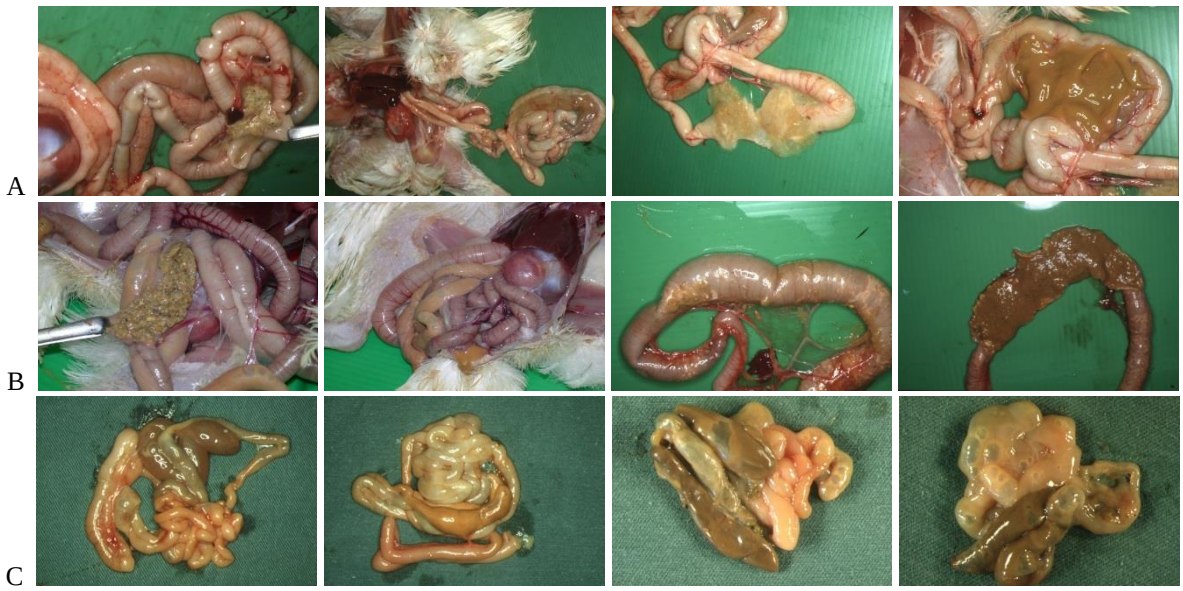
A  B 

2.11 Gross lesions and histopathology (Figure 6)

The major pathological lesions of RV enteritis in birds include whitish-transparent intestinal walls, enlarged gall bladder, and atrophy of the pancreas along with degeneration of bursa of Fabricius, rickets and proventriculitis (Lublin *et al.* 2004). Large amount of fluids and gas

could be seen in intestine and ceca. The carcass may be dehydrated, reveal stunting growth, pasted and inflamed vents, anemia due to vent pecking, litter in gizzard and inflammation of plantar surface of foot (Bergeland *et al.* 1977; Horrox 1980; McNulty *et al.* 1980, 1983; Yason *et al.* 1987; Shawky *et al.* 1993; Haynes *et al.* 1994). In some cases, hemorrhages are also noticed in caecal walls especially in pheasant chicks (Gough *et al.* 1990).

Figure 6: A Chicken enteritis; B Turkey enteritis; C Guinea fowl enteritis (IZSLER images)




Histopathology shows vacuolation of enterocytes, separation and desquamation of enterocytes from *lamina propria*, and infiltration of inflammatory cells in *lamina propria* (McNulty 2003). Generally, in RV infected birds, decrease in mean villous lengths as well as increase in crypt depths results in reduced villus to crypt ratios. Subsequent to this, morphometric changes are more pronounced in duodenum and jejunum than ileum (Hayhow & Saif 1993; Shawky *et al.* 1993; Yason *et al.* 1987). All these lesions are not pathognomonic for RV disease.

### 2.12 Immunity

Maternally derived antibodies against RV are passively transferred to the avian embryo through egg yolk. This antibody titer progressively decreases in serum and is undetectable at 3-4 weeks of age (Yason & Schat 1986a). Circulating maternally derived IgG protects the intestinal mucosa during first week of life against RV disease (Yason & Schat 1986a; Shawky *et al.* 1993). Evidence showed that IgG gets transferred from blood to the intestine. However,

maternally derived IgG could not be detected in intestinal washings of poults hatched from naturally infected hens (Shawky *et al.* 1994).

Older birds generally develop higher antibody titers and respond more quickly than younger birds. Information regarding development and duration of immunity to RVs following disease of birds are scarce. Rotavirus specific IgM, IgG and IgA were detected in serum where intestinal antibody response entirely consisted of IgA.

## 2.13 Diagnosis

The classical way to diagnose AvRV infection in the laboratory is to identify the virus in feces or intestinal contents by EM. It is a sensitive diagnostic approach that detects RVs of all serogroups (Theil *et al.* 1986c), but it is a costlier and cumbersome option.

Another methods is the direct detection of 11 different segments of RNA and their typical pattern of migration in PAGE performed via silver staining (Svensson *et al.* 1986). Compared to EM, PAGE is equally sensitive and it gives provisional information of the subgroup/s present (Guy 1998).

AvRv can be isolated on embryonated chicken eggs, in primary cell culture (chick embryo liver cells/chick kidney cells) or in continuous cell lines (MA104). The isolation is useful only for group A avian RVs, but it is not frequently used for diagnosis and it has been proven extremely difficult to cultivate other rotavirus serogroups in cell cultures (McNulty *et al.* 1984; Yason & Schat 1985; Rodriguez *et al.* 2004; Villarreal *et al.* 2006). Because infections with non-group A RV constitute the majority of infections in chicken and turkeys, virus isolation cannot be recommended as a diagnostic technique.

Detection of avian rotaviral antigens in tissues using fluorescent antibody (FA) and immune EM requires specific antisera. However, these procedures may be used to identify specific serogroups (Saif *et al.* 1985; Theil *et al.* 1986c).

Flocks could be checked for AvRV by group specific RV VP6-antigen using enzyme-linked immunosorbent assay (ELISA) or by immunohistochemistry (McNulty 2003; Lublin *et al.* 2004). Commercially available ELISAs are used for diagnosis of group A RVs in mammalian. However, no ELISAs are available to detect RVs of groups D, F and G so far.

Recently, a great deal of progress has been made in the development of molecular diagnostic assays for AvRVs. While considering molecular detection tools for RV, best option is highly rapid and sensitive reverse transcription polymerase chain reaction (RT-PCR) (Guy 1998; McNulty 2003; Otto *et al.* 2006; Schumann *et al.* 2009; Trojnar *et al.* 2009, 2010; Kattoor *et al.* 2014). Aside to detection, for molecular characterization of the AvRVs, genomic

variations are a big obstacle. Further characterization of VP7 and VP4 genes of AvRV isolates could give idea regarding additional serotypes that might exist in poultry environment (Hoshino and Kapikian 1996).

Serologic diagnosis of RV infection is difficult and not recommended; ELISA kits for RV-A antibodies are used to control SPF flocks.

## 2.14 Intervention strategy

### 2.14.1 *Management procedures*

For control of disease, secondary bacterial enteritis has to be kept under control through antimicrobial medication. In order to reduce environmental contamination and exposure of young birds to RVs, control should aim at ensuring thorough cleaning and disinfection of poultry houses (Dhama et al., 2015). Few published papers are available regarding susceptibility of avian RVs to chemical and physical inactivating agents. Glutaraldehyde had greater inactivating capacity against AvRV than sodium hypochlorite and iodophor disinfectants; RVs are also sensitive to phenol and formaldehyde (Minamoto & Yuki 1988). RVs are relatively heat-stable (Guy 1998). Strict biosecurity measures must be followed in order to prevent any chance of spread of disease from one flock to another one (Attoui *et al.* 2012). Frequent removal of litter and thorough cleaning of poultry house and equipment before restocking with a new flock could minimize the chance of disease.

### 2.14.2 *Vaccination*

It has been well proven that maternally derived antibodies have a paramount role in protection of intestinal mucosa against avian RV-A attack, especially during the initial few weeks of life (Shawky *et al.* 1993; 1994; Saif & Fernandez 1996). As a consequence, vaccines have to be developed and should be made commercially available. Till to date, as it is difficult to develop vaccines largely due to high antigenic variation of AvRVs and the fact that non-RV-A are difficult to grow in cell culture, such vaccines are not in practice.

The long-term persistence of a high antibody level in yolk and simplicity of generation of large amounts of chromatographically pure antibody preparations may open new ways for their employment as an effective strategy to defend AvRV diseases. The viral enterotoxin, NSP4 is another option for vaccines as the NSP4 antigenic structure is highly conserved among RVs and is a good candidate for vaccine development (Borgan *et al.* 2003). Detailed studies on immune response of birds to rotavirus disease are insufficient and research studies regarding vaccine aspects are lacking.

## 3. WORKING HYPOTHESIS AND AIMS OF THE Ph.D PROJECT

RVs are considered as emerging pathogens with potential to cause huge economic losses to the growing poultry industry (Jones *et al.* 1979; Theil *et al.* 1986a; Holland 1990; Barrios *et al.* 1991; Dodet *et al.* 1997; Tamehiro *et al.* 2003; Mcnulty 2003; Jackwood *et al.* 2007; Chauhan *et al.* 2008; Dhama *et al.* 2009; Jindal *et al.* 2012; Malik *et al.* 2012, 2013a). As a consequence, control of rotaviral diarrhea is a major concern in the poultry production sector, which can be achieved by vaccination and sanitation. However, appearance of multiple groups in RVs and high genetic diversity within groups hinders production of an effective vaccine against AvRVs (Borgan *et al.* 2003). **Monitoring rotavirus distribution in different avian species is crucial to uncover diversity among strains and to better understand RV ecology in the field and to obtain the best management solutions for solving the problem.**

Infection with AvRVs from different groups might explain the high variability of clinical signs and lesions associated with rotavirus enteritis in avian species (Otto *et al,* 2006). **Moreover, it is not clear if particular signs or symptoms are correlated with the infection from a specific group.**

Differently from mammalian rotaviruses, AvRVs have generally been paid little attention in spite of their wide diffusion among several bird species and their important role in term of economic and commercial impact. Although several studies have highlighted the presence of RV enteritis in different avian species worldwide, only a few epidemiological molecular studies, mostly limited to chickens and turkeys, investigated the distribution of the different groups of AvRVs (Otto *et al.*, 2012; Kindler *et al.*, 2013; Beserra *et al.*, 2014; Lavazza *et al.*, 2005). **The consequence is that we have very few and old data about infection prevalence, dynamics and epidemiological features of rotavirus strains both of industrial and game birds.**

Regarding diagnosis, in the past, electron microscopy with negative staining (nsEM) has aided considerably the detection of RVs in faecal samples, but it doesn't allow distinguishing between the different rotavirus groups. Grouping of rotaviruses on the basis of their electrophoretic RNA migration profile in polyacrylamide gel electrophoresis (RNA-PAGE) is hampered by lower sensitivity as compared to other molecular methods such as RT-PCR and could be complicated by the presence of unusual RNA migration patterns and the possible occurrence of genome rearrangements. **In general, screening diagnostic protocols to rapidly and correctly detect all circulating strains and new methods for a correct, punctual and more rapid grouping are needed.**

On the basis of the detected issues, this project aims at:

- giving accurate data on prevalence and strains characterization for commercial and game-hunting poultry flocks;
- giving data to better understand the dynamics and the timing of RV infection in avian species;
- giving answers to the epidemiological gap. In particular by the use of longitudinal studies associated to genetic studies, the project tried to give more contextual and accurate data about the infection dynamics in the flock;
- evaluating a possible correlation between specific symptoms and lesion severity with the infection with a specific group;
- developing modern and accurate diagnostic tools, in particular group-specific RT-PCR protocols to be applied to the diagnosis and characterization of AvRVs belonging to the main groups.

The articulation of the project into phases has been defined as follows:

1) samples/data collection from routine diagnostics (current and retrospective);

2) samples/data collection from longitudinal studies;

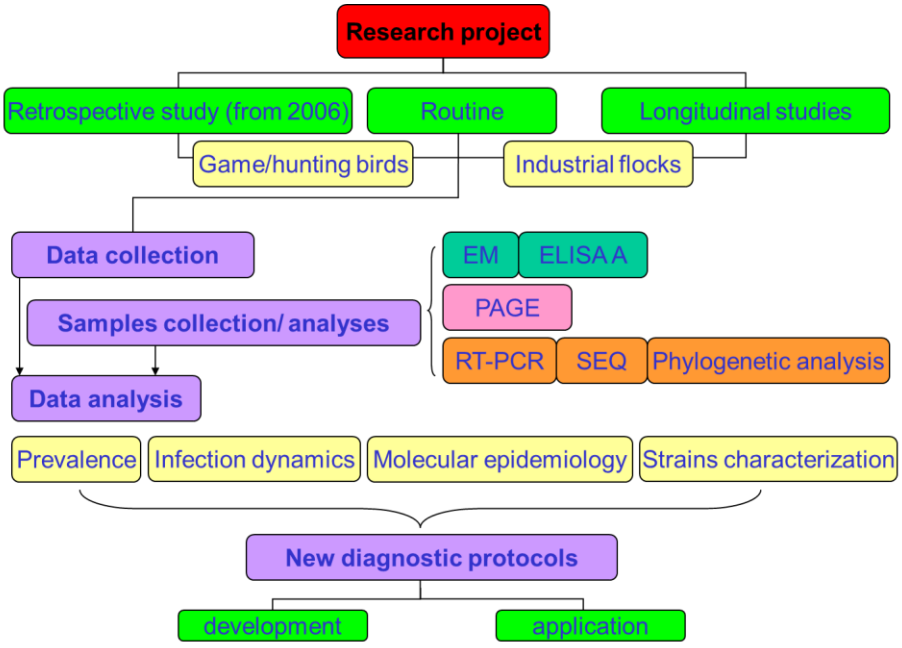
3) strains identification and characterization;

4) sequencing and phylogenetic analysis of the obtained sequences;

5) development of innovative diagnostic methods.

The project phases are summarized in Figure 7.

Figure 7. Schematic project phases



# 4. MATERIALS AND METHODS

## 4.1 Retrospective data collection/strains characterization

### 4.1.1 Prevalence and symptoms/pathological findings data

Data on the diffusion of RVs infection and on symptoms/pathological features associated to the infection in avian species from commercial and game-hunting species flocks were systematically collected starting from the internal data management program of the IZSLER (DarwIn) using the IZSLER data search engine (Bobj). Nine years (2006-2014) were included in the analysis.

### 4.1.2 Strains selection and characterization

#### 4.1.2.1 *Samples*

In order to identify and characterize the highest number of RVs strains related to the main avian species both in commercial and game-hunting fields, a selection of samples previously found positive for AvRV in nsEM in different years (n=117, Table 1 and 2) was further analysed. Samples belonged to different species: 76 chicken, 5 guinea fowl, 21 turkey, 10 pheasant, 5 partridge. The age of the birds varied from one to six weeks of age. The 117 selected samples included 108 pooled samples and 9 individual samples. Each pooled sample is composed by feces or intestinal contents of 2 to 30 birds (more frequently 5-10) that were chosen randomly within each flock.

Table 1: Samples grouped by collection year

| Year | Number |
|---|---|
| 2012 | 10 |
| 2011 | 13 |
| 2010 | 8 |
| 2009 | 16 |
| 2008 | 22 |
| 2007 | 25 |
| 2006 | 23 |
| **Total** | **117** |

Table 2: Samples grouped by internal number, species and nsEM results

| Year | Internal number | Specie | nsEM |
|---|---|---|---|
| 2006 | 166 | guinea fowl | rota+/- enterolike+ |
| | 930 | partridge | rota+ |
| | 1090 | partridge | rota+ |
| | 1354 | chicken | rota+/entero+ |
| | 1427 | turkey | rota ++++ |
| | 1573 | chicken | rota+/- |
| | 1595 | chicken | rota++ |
| | 1705 | chicken | rota+ |
| | 1914 | guinea fowl | rota++++ |
| | 1917 | turkey | rota+ |
| | 1959 | turkey | rota+ |
| | 194 | turkey | rota +++ fagi + |
| | 1972 | chicken | rota+++ |
| | 2087 | chicken | rota+++ |
| | 2138 | chicken | rota ++ |
| | 2198 | chicken | rota + |
| | 194 | turkey | rota ++++ |
| | 339 | chicken | rota+++++ |
| | 385 | chicken | rota +++ |
| | 516 | chicken | rota++ |
| | 515 | chicken | rota + |
| | 600 | chicken | rota +++++ |
| | 855 | chicken | rota++ |
| 2007 | 237 | chicken | rota ++ |
| | 612 | chicken | rota +++ enterolike + |
| | 774 | pheasant | rota ++++ |
| | 821 | turkey | rota +++ astro ++ |
| | 835 | chicken | rota +++ |
| | 981 | turkey | rota + |
| | 981 | guinea fowl | rota + |
| | 1235 | turkey | rota +/- |
| | 1238 | chicken | rota + |
| | 1339 | chicken | rota ++ |
| | 1832 | chicken | rota ++++ |
| | 276 | turkey | rota+ astro++ |
| | 1854 | chicken | rota +++ |
| | 1946 | chicken | rota +++ |
| | 1967 | chicken | rota ++ |
| | 2061 | chicken | rota + entero + |
| | 2068 | chicken | rota + |
| | 2127 | chicken | rota +++ |
| | 276 | turkey | rota+ astro+++ |
| | 279 | chicken | rota ++ fagi +++++ |
| | 435 | chicken | rota + |
| | 462 | chicken | rota + fagi |
| | 484 | turkey | rota ++ |
| | 543 | turkey | rota +++ |
| | 570 | chicken | rota ++ |
| 2008 | 20 | chicken | rota + |
| | 854 | chicken | rota + |
| | 882 | pheasant | rota ++ fagi ++ |

| | | | |
|---|---|---|---|
| | 886 | chicken | rota ++ |
| | 1220 | turkey | rota + fagi ++ |
| | 1239 | chicken | rota +++ |
| | 1277 | pheasant | rota + |
| | 1375 | pheasant | rota ++ |
| | 1676 | chicken | rota + |
| | 1793 | chicken | rota++ |
| | 1821 | chicken | rota + |
| | 451 | chicken | rota + |
| | 1873 | chicken | rota ++ |
| | 1872 | chicken | rota ++ |
| | 2196 | turkey | rota +++ |
| | 499 | chicken | rota + fagi + |
| | 581 | chicken | rota + |
| | 583 | chicken | rota + |
| | 692 | guinea fowl | rota + |
| | 729 | pheasant | rota +++ |
| | 793 | chicken | rota + fagi + |
| | 814 | chicken | rota +++ |
| 2009 | 252 | chicken | rota++ |
| | 743 | pheasant | rota++ |
| | 809 | pheasant | rota+ |
| | 1232 | guinea fowl | rota+ |
| | 1314 | chicken | rota+ |
| | 1451 | turkey | rota++ |
| | 1567 | turkey | rota+ |
| | 257 | chicken | rota+++ |
| | 371 | chicken | rota+++ |
| | 503 | chicken | rota++ |
| | 504 | chicken | rota+++ |
| | 521 | turkey | rota+++ |
| | 596 | chicken | rota++ |
| | 662 | chicken | rota++ |
| | 690 | chicken | rota+ |
| 2010 | 615 | chicken | rota + |
| | 691 | chicken | rota++ |
| | 308 | chicken | rota+ |
| | 824 | chicken | rota+ |
| | 956 | partridge | rota++ |
| | 574 | partridge | rota++ |
| | 1010 | pheasant | rota ++ |
| 2011 | 1035 | chicken | rota ++++ |
| | 1414 | chicken | rota + |
| | 580 | chicken | rota ++++ |
| | 745 | chicken | rota +/- |
| | 319 | chicken | enterolike ++ rota + |
| | 928 | chicken | rota ++++ |
| | 826 | turkey | rota ++ |
| | 387 | turkey | rota ++ |
| | 723 | partridge | rota+++ |
| | 864 | pheasant | rota ++ |
| | 318 | chicken | rota + |
| | 162 | chicken | rota ++ |
| | 1354 | chicken | rota + |

| | | | |
|---|---|---|---|
| 2012 | 568 | chicken | rota+++ |
| | 1359 | chicken | rota++ |
| | 612 | chicken | rota+++ |
| | 646 | pheasant | rota++ |
| | 660,1 | chicken | rota++ entero+++ |
| | 660,2 | chicken | rota+++ entero +++ |
| | 860 | chicken | rota++++ |
| | 1095 | turkey | rota+ astro++ |
| | 1238 | chicken | rota++ |
| | 1358 | chicken | rota++++ |

4.1.2.2 *Methods*

Samples positive by nsEM were further analysed by group A ELISA and by groups RT-PCRs. In addition, a further selection (n=58) from the 117 samples was analysed by RNA-PAGE.
Data on clinical signs and lesions at necropsy and the results of complementary laboratory investigation (microbiological examination), when available, were also recorded.

*Group A ELISA*

Positive samples were analyzed by an in-house sandwich ELISA test for group A rotaviruses (Lavazza A., 1989). The samples were diluted 1/10 (w/v) in PBS-A, centrifuged at 3000 rpm for 30 min and surnatants were used for the ELISA test.

*AvRVs RT-PCRs*

All 117 samples were subjected to viral RNA extraction from 140µl of each suspension using a commercial kit (QIAamp Viral RNA kit; *Qiagen*), following the manufacturer's instructions, and stored at -80°C until use. Extracted viral RNA was quantified by using Infiniter® 200 NanoQuant (*Tecan*) spectrophotometer and was subjected to four different group-specific RT-PCR assays, using AvRV-A and AvRV-D specific primers (Table 3) described by Otto *et al.* (2012) and reported in the table, and AvRV-F and AvRV-G primers newly designed as described below. RT-PCR assays were performed in a one-step format using the commercial QIAGEN OneStep RT-PCR kit (*Qiagen*). Briefly, 10µl of extracted RNA were mixed with 1.5µl of each primer (20µM), subjected to incubation at 98°C for 5 min to allow the denaturation of double-stranded viral RNA and cooled immediately on ice. Then 13µl of RNA template was added to 37µl of a RT-PCR-master mix containing 2µl of enzyme-mix, 2µl of dNTPs Mix (10mM each dNTP), 1× RT-PCR buffer (included in the kit) and $ddH_2O$ to a final volume of 50µl. The initial steps of the cycle program consisted of a cycle of reverse transcription at 50°C for 30 min followed by *Taq* polymerase activation at 95°C for 15 min. This was followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 1

min, extension at 72°C for 1 min. After a final elongation step at 72°C for 10 min, the amplification products were examined by electrophoresis in a 2% agarose gel, stained with ethidium bromide and visualized under UV light.

Table 3: Primers used for VP6 RT-PCR (Otto *et al.* 2012)

| Specificity | Name | Sequence (5'-3') | Position (nt)* | Amplicon size |
|---|---|---|---|---|
| Rotavirus group A | ARVA6-1F | CACCACGACTTATGCAGAGA | 709-728 | 493bp |
| | ARVA6-1R | CTCCGAATGGATGCTACTGT | 1201-1182 | |
| Rotavirus group D | ARVD6-1F | GCGACAACTGAGACAACTG | 1008-1026 | 186 bp |
| | ARVD6-1R | GGAAGCAGTTGTCATCAAC | 1193-1175 | |

* Nucleotide position on VP6 of RV-A strain 02V0002G3 (acc. number DQ096805) and D strain 05V0049 (acc. number GU733451)

*Primer design for complete AvRV-F and AvRV-G VP6 and for AvRV-A and AvRV-D NSP4,VP6,VP4*

Primers were selected by alignment of sequences from Gen Bank, by using Bioedit and designed by using Primer 3 program (Table 4). Primer sequences were reported in the Table 5. The sensitivity of the newly designed group-specific primers was determined by testing six replicates of 10-fold serial dilutions of PCR products from positive control samples, starting with an initial concentration of $10^8$ to a final concentration of $10^0$ gene copies/reaction. The specificity was determined by RT-PCR analysis of a panel of 19 isolates of avian viruses different from rotavirus. Selected avian rotavirus samples found to be positive by PAGE (Rotavirus A Partridge/Italy/930/2006, Rotavirus D Chicken/Italy/600/2006, Rotavirus F Partridge/Italy/956/2010 and Rotavirus G Partridge/Italy/956/2010) were partially sequenced on the VP6 gene by using primers designed in laboratory to confirm proper group assignment through BLAST search in NCBI GenBank database and used as positive control in group specific RT-PCR assays. The respective sequences were submitted to GenBank with the following accession numbers: KT073226, KT073227, KT073228 and KT073229.

A further selection of 36 samples (Table 6) was analysed by using these RT-PCRs and then sequenced.

Table 4: Access numbers of aligned sequences for the selection of primers for segments NSP4, VP4, VP6 and VP7 of group A and D, and of VP6 of F and G group

| GROUP A | | | | GROUP D | | | | GROUP F | GROUP G |
|---|---|---|---|---|---|---|---|---|---|
| NSP4 | VP6 | VP7 | VP4 | NSP4 | VP6 | VP7 | VP4 | VP6 | VP6 |
| AB009627 | EF687020 | AB080737 | AB009632 | GU733452 | JQ065735 | GU733451 | GU733445 | HQ403603 | HQ403604 |
| AB065285 | D16329 | AB080738 | EU486956 63 | | GU733448 | KC669408 | | | |
| AB065286 | D82980 | D82979 | JQ085405 | | HM060260 | KC669409 | | | |
| AB065287 | DQ096805 | EU486971 77 | JX204814 | | HM060261 | KC669410 | | | |
| AY062937 | DQ478589 | FJ169861 | JX204825 | | JN034682 | KC669411 | | | |
| EF204132 43 | EU486964 70 | FN393054 | KC962115 | | JN703463 | KC669412 | | | |
| EU400300 27 | FJ169858 | FN393055 | KC962116 | | JQ065734 | KC669413 | | | |
| FJ169862 | JN635503 | FN393056 | KC962117 | | JQ065736 | KC669414 | | | |
| FJ794426 65 | JQ085406 | JQ085407 | KC962118 | | | NC_014519 | | | |
| JF309130 38 | JX204815 | JX204816 | KC962119 | | | | | | |
| JN374833 39 | JX204826 | JX204827 | | | | | | | |
| JQ085408 | L13765 | KC962122 | | | | | | | |
| JX474765.1 | X98870 | KC962123 | | | | | | | |
| | X98871 | L01098 | | | | | | | |
| | X98872 | S58166 | | | | | | | |
| | | X56784 | | | | | | | |

Table 5: Primers used for the amplification of segments NSP4, VP4, VP6 and VP7 of AvRV-A and D and the segment of the VP6 of AvRV-F and G

| | | sequence (5'-3') | position (nt) | amplicon lenght |
|---|---|---|---|---|
| Avian RV-A | NSP4A_1 for | GAGAGAGCYCGTGYGGAAA | 21-39 | 636 |
| | **NSP4A_2 rev** | **CTYCWTGMCACACCCGATCA** | **657-638** | |
| Start | VP6A_5'UTR for | GGSYTTTWADRCGAAGTCTY | 1-20 | 741 |
| | **VP6A_IntB rev** | **GGWATAACYGCTCTYTGY** | **741-725** | |
| End | VP6A_IntA for | AACAYGTYGTACCADTRSGA | 651-670 | 699 |
| | **VP6A_3'UTR rev** | **GGTCACATCCTCTCACTATA** | **1350-1330** | |
| | VP7A_FOR | AGW AAT TYY CGT HTC TCA | 13 - 30 | 1035 |
| | **VP7A_REV** | **GAG TYT ATA CTC TRW RGT AAT** | **1048 - 1027** | |
| | VP4A_1FOR | TCGTATAYAAGACARCTTYTA | 20 - 40 | 894 |
| | **VP4A_1REV** | **CATAYGTRTAYTCATARTTCAT** | **914 - 892** | |
| | VP4A_M1_F | TGGAARGARRTRCRRTATAA | 781 - 800 | 932 |
| | **VP4A_2REV** | **CWGCRTCDGATARTTCTTC** | **1713 - 1693** | |
| | VP4A_3FOR | ATG TTT KCA ATG TTY TCW GG | 1576 - 1596 | 773 |
| | **VP4A_3REV** | **MATCCTCAYAGACAVCTT** | **2349 - 2332** | |
| Avian RV-D | NSP4D_5'UTR for | GGTTTTAAAATTTATTAGTTG | 1-21 | 765 |
| | **NSP4D_3'UTR rev** | **GGTCACAATTTATTAGTTTCC** | **765-745** | |
| Start | VP6D_5'UTR for | GGKTTTTAAATAGTAATCAA | 1-20 | 744 |
| | **VP6D_IntB rev** | **TTGRTTYCTRATCATACTYG** | **744-725** | |
| End | VP6D_IntA for | CTKTAYAAYTTRAGRACRCC | 652-671 | 702 |
| | **VP6D_3'UTR rev** | **GGTCAAAGATAATAGCTGCT** | **1354-1338** | |
| | VP7D_start | AAGGCATCTTAACCATATAG | 9 - 28 | 976 |
| | **VP7D_end** | **AACCACTCTGTAGATTCCA** | **985 - 967** | |
| | VP4D_start_F | ATCGTTATGGACTAGTTTGAA | 18 - 38 | 845 |
| | **VP4D_M1_R** | **TCTACTGAAACATCAGCATC** | **863 - 844** | |
| | VP4D_M1a _F | GCAAATATAAATATGCARATG | 814 - 834 | 984 |
| | **VP4D_M2b_R** | **ATGCYTTTGAYTTAGAGAAT** | **1798 - 1779** | |
| | VP4D_M2_F | GAATTTAATGCACTTTCACA | 1636 - 1655 | 676 |
| | **VP4D_end_R** | **ATTCTATCTGTTATGATAGG** | **2312 - 2293** | |
| Avian RV-F | VP6F_1for | CCAGATGTGGGAATCAAAT | 2 - 23 | 699 |
| | **VP6F_1rev** | **GTCATAAAGTTCCTGTTACGC** | **681 - 701** | |
| | VP6F_2 for | GCTTATAAAAGTCAATCAGTCG | 634 - 652 | 679 |
| | **VP6F_2rev** | **CGACTTGTATTGTAGCATCAG** | **1313 - 1293** | |
| Avian RV-G | VP6G_1for | AAAGAAATCTCCAACCTAGC | 3 - 22 | 699 |
| | **VP6G_1rev** | **CTGGTGCTGGAACATAGA** | **684 - 702** | |
| | VP6G_2for | GGAAGATGGTCTGGTAACAA | 576 - 595 | 691 |
| | **VP6G_2rev** | **GGGTCTTTATTTTATCAGTAAAC** | **1267 - 1245** | |

Table 6: Analysed samples grouped by species

| Specie | Number |
|---|---|
| Chicken | 14 |
| Turkey | 7 |
| Pheasant | 5 |
| Partridge | 5 |
| Guinea fowl | 5 |
| **Total** | **36** |

*Sequencing and phylogenetic analysis*

PCR products were purified using NucleoSpin Gel and PCR clean-up kits (*Macherey-Nagel)*, according to manufacturer's instructions. Nucleotide sequences were performed by Big Dye Terminator (*Applied Biosystems*) using the same primers used for RT-PCR. The phylogenetic dendogram was constructed by comparing RV sequences from various groups, including human and animal groups A-D RVs. Analysis was performed on the coding regions of the four segments. The alignment was performed using the CLUSTAL W method in the MEGALIGN module of the DNASTAR software package and phylogenetic trees were constructed using neighbor-joining method as implemented in the MEGA Vsoftware package. Bootstrap analysis in phylogenetic trees was performed with 1000 replicates and 111 random seeds.

*RNA-PAGE*

Fifty-eight out of the 117 samples positive for RVs by nsEM were further analyzed by RNA-PAGE. After ultracentrifugation of the samples on a 25% sucrose cushion, dsRNA was extracted using Trizol (following manufacturer instructions, *Invitrogen*), denatured and subjected to electrophoresis in 7.5% polyacrylamide slab gels at 150V for 16h. The migration patterns were visualized by SilverXpress® SilverStaining Kit (*Invitrogen*).

### 4.1.3 Statistical analysis

Correlation of general signs/lesions or gastro-enteric lesions observed in the field and at *post mortem* examination with the presence of specific RV groups or different combinations of RV groups, even with regard to the different species involved, was accomplished. The Chi-square test for independence with Monte-Carlo resampling approximation (Agresti, 2002), using a R software version 3.2.0 for statistical computing (http://www.r-project.org) was applied.

## 4.2 Longitudinal studies

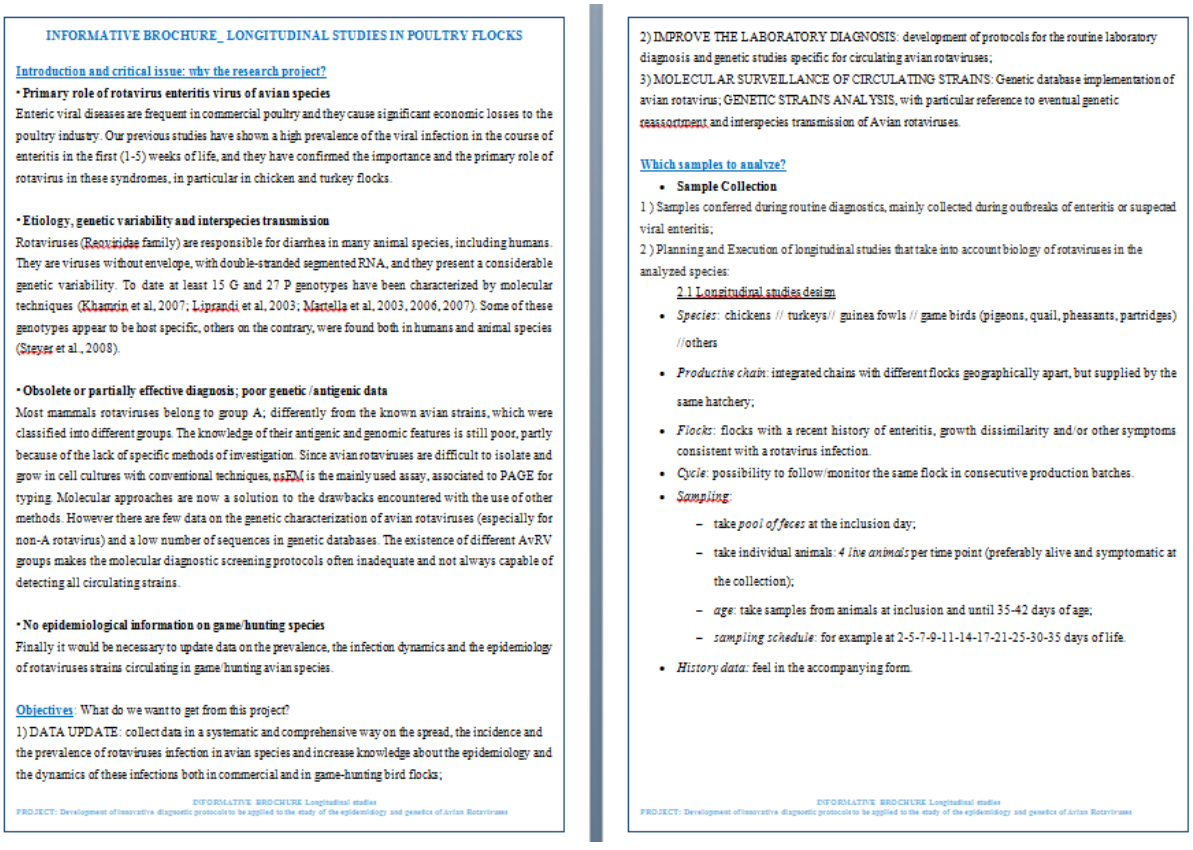
The study also aimed to define longitudinal studies that may be useful to better understand the dynamics and the timing of infection in avian species.

Therefore it was decided to: define the longitudinal study (sampling method, collection step, production/age groups), inform field veterinarians (distribution of information material, anamnestic info sheet), collect and analyze samples (nsEM, and, if positive, RV-A ELISA, RT-PCR for AstVs and RVs, and sequencing), analyze obtained results.

### 4.2.1 Study design

At the annual national meeting of avian pathology (52° SIPA Annual Meeting, April 11, 2013, Fiera di Forlì), given the presence of the majority of veterinarians involved in the avian production field, the study and the arrangements for collaboration in the collection of samples in longitudinal mode ("brochure", Figure 8) were presented. It was also illustrated the anamnestic info sheet accompanying samples ("info sheet", Figure 9).

Figure 8. Brochure explaining longitudinal studies intent



INFORMATIVE BROCHURE_ LONGITUDINAL STUDIES IN POULTRY FLOCKS

Introduction and critical issue: why the research project?

• **Primary role of rotavirus enteritis virus of avian species**

Enteric viral diseases are frequent in commercial poultry and they cause significant economic losses to the poultry industry. Our previous studies have shown a high prevalence of the viral infection in the course of enteritis in the first (1-5) weeks of life, and they have confirmed the importance and the primary role of rotavirus in these syndromes, in particular in chicken and turkey flocks.

• **Etiology, genetic variability and interspecies transmission**

Rotaviruses (Reoviridae family) are responsible for diarrhea in many animal species, including humans. They are viruses without envelope, with double-stranded segmented RNA, and they present a considerable genetic variability. To date at least 15 G and 27 P genotypes have been characterized by molecular techniques (Khamrin et al, 2007; Liprandi et al, 2003; Martella et al, 2003, 2006, 2007). Some of these genotypes appear to be host specific, others on the contrary, were found both in humans and animal species (Steyer et al., 2008).

• **Obsolete or partially effective diagnosis; poor genetic /antigenic data**

Most mammals rotaviruses belong to group A; differently from the known avian strains, which were classified into different groups. The knowledge of their antigenic and genomic features is still poor, partly because of the lack of specific methods of investigation. Since avian rotaviruses are difficult to isolate and grow in cell cultures with conventional techniques, nsEM is the mainly used assay, associated to PAGE for typing. Molecular approaches are now a solution to the drawbacks encountered with the use of other methods. However there are few data on the genetic characterization of avian rotaviruses (especially for non-A rotavirus) and a low number of sequences in genetic databases. The existence of different AvRV groups makes the molecular diagnostic screening protocols often inadequate and not always capable of detecting all circulating strains.

• **No epidemiological information on game/hunting species**

Finally it would be necessary to update data on the prevalence, the infection dynamics and the epidemiology of rotaviruses strains circulating in game/hunting avian species.

Objectives: What do we want to get from this project?

1) DATA UPDATE: collect data in a systematic and comprehensive way on the spread, the incidence and the prevalence of rotaviruses infection in avian species and increase knowledge about the epidemiology and the dynamics of these infections both in commercial and in game-hunting bird flocks;

INFORMATIVE BROCHURE Longitudinal studies
PROJECT: Development of innovative diagnostic protocols to be applied to the study of the epidemiology and genetics of Avian Rotaviruses

2) IMPROVE THE LABORATORY DIAGNOSIS: development of protocols for the routine laboratory diagnosis and genetic studies specific for circulating avian rotaviruses;

3) MOLECULAR SURVEILLANCE OF CIRCULATING STRAINS: Genetic database implementation of avian rotavirus; GENETIC STRAINS ANALYSIS, with particular reference to eventual genetic reassortment and interspecies transmission of Avian rotaviruses.

Which samples to analyze?

- **Sample Collection**

1 ) Samples conferred during routine diagnostics, mainly collected during outbreaks of enteritis or suspected viral enteritis;

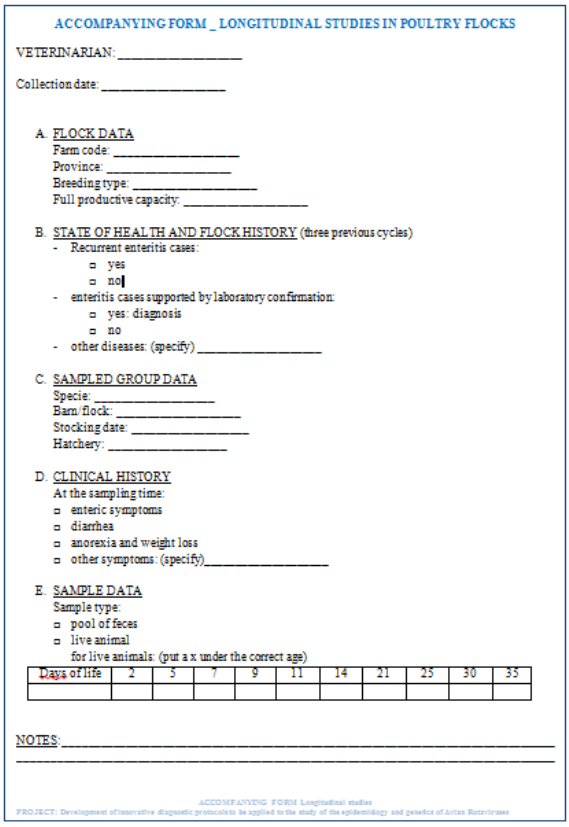
2 ) Planning and Execution of longitudinal studies that take into account biology of rotaviruses in the analyzed species:

2.1 Longitudinal studies design

- *Species*: chickens // turkeys// guinea fowls // game birds (pigeons, quail, pheasants, partridges) //others
- *Productive chain*: integrated chains with different flocks geographically apart, but supplied by the same hatchery;
- *Flocks*: flocks with a recent history of enteritis, growth dissimilarity and/or other symptoms consistent with a rotavirus infection.
- *Cycle*: possibility to follow/monitor the same flock in consecutive production batches.
- *Sampling*:
  - take *pool of feces* at the inclusion day;
  - take individual animals: *4 live animals* per time point (preferably alive and symptomatic at the collection);
  - *age*: take samples from animals at inclusion and until 35-42 days of age;
  - *sampling schedule*: for example at 2-5-7-9-11-14-17-21-25-30-35 days of life.
- *History data*: feel in the accompanying form.

INFORMATIVE BROCHURE Longitudinal studies
PROJECT: Development of innovative diagnostic protocols to be applied to the study of the epidemiology and genetics of Avian Rotaviruses

Figure 9: Info sheet (accompanying form) for samples conferred as longitudinal studies samples



ACCOMPANYING FORM _ LONGITUDINAL STUDIES IN POULTRY FLOCKS

VETERINARIAN: _____

Collection date: _____

A. FLOCK DATA
Farm code: _____
Province: _____
Breeding type: _____
Full productive capacity: _____

B. STATE OF HEALTH AND FLOCK HISTORY (three previous cycles)
- Recurrent enteritis cases:
  - ▫ yes
  - ▫ no
- enteritis cases supported by laboratory confirmation:
  - ▫ yes: diagnosis
  - ▫ no
- other diseases: (specify) _____

C. SAMPLED GROUP DATA
Specie: _____
Barn/flock: _____
Stocking date: _____
Hatchery: _____

D. CLINICAL HISTORY
At the sampling time:
- ▫ enteric symptoms
- ▫ diarrhea
- ▫ anorexia and weight loss
- ▫ other symptoms: (specify)_____

E. SAMPLE DATA
Sample type:
- ▫ pool of feces
- ▫ live animal

for live animals: (put a x under the correct age)

| Days of life | 2 | 5 | 7 | 9 | 11 | 14 | 21 | 25 | 30 | 35 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | |

NOTES: _____

ACCOMPANYING FORM Longitudinal studies
PROJECT: Development of innovative diagnostic protocols to be applied to the study of the epidemiology and genetics of Avian Rotaviruses

### 4.2.2 Inclusion criteria/sampling schedule

In order to select the flocks for the longitudinal sampling, some base selection criteria were lead down:

- integrated chains with different flocks geographically apart, but supplied by the same hatchery;
- flocks with a recent history of enteritis, growth dissimilarity and/or other symptoms consistent with a rotavirus infection;
- possibility to follow/monitor the same flock in consecutive production batches.

Two integrated production chains (chain 1: turkey industrial breeding; chain 2: broiler industrial breeding) were selected and included in the project.

*Chain 1*

Five flocks, all supplied by the same hatchery, were sampled. In all the flocks history of enteritis during the first weeks of life and /or productivity reduction associated with reduced growth, was reported. Biosecurity and health conditions were similar for all flocks included.

*Chain 2*

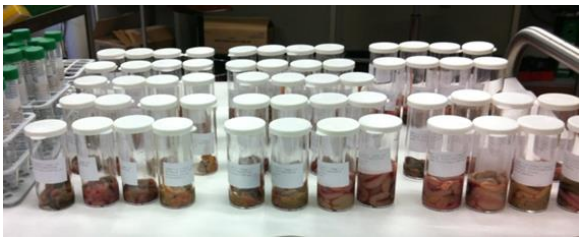
The study in this chain has been managed as a case-control study. In particular, the "case" included animals from the breeding flock with a history of enteritis and growth disparity, moreover the "control" includes animals of the same chain, but without obvious symptoms attributable to rotavirus infection.

For both chains, samples were weekly collected in a longitudinal way from inclusion to 35-42 days of age. The detailed schedule of sampling for the different flocks is reported in the Table 7. At every collection point, four chicks were collected (preferably alive and symptomatic at the collection) (Figure 10).

Table 7: Schedule of sampling for the different flocks

| **CHAIN 1** | **Number of sampling** | **Days** |
|---|---|---|
| Flock 1 | 5 times | 13,21,28,34,42 |
| Flock 2 | 5 times | 5,14,21,27,35 |
| Flock 3 | 6 times | 7,14,21,28,35,45 |
| Flock 4 | 4 times | 5,13,19,27 |
| Flock 5 | 6 times | 3,10,17,24,33,38 |
| **CHAIN 2** | | |
| Case Flock | 6 times | 8,12,16,20,24,28 |
| Control Flock | 8 times | 8,12,16,20,24,28,32,36 |

Figure 10: A. Samples consisting in 4 chicks per pack; B. Obtained single intestines from chicks conferred for longitudinal studies

A.  B. 

### 4.2.3 Methods

All samples were analysed by nsEM, RT-PCR for AstV and RT-PCR for VP6 of RV groups A, D, F and G. When positive for RV they were also sequenced. All the sequences were phylogenetic analysed (in-flock and intra-flocks analyses).

*Negative staining electron microscopy*

For nsEM, the samples were diluted 1/10 (w/v) in distilled water, double centrifuged at low speed (3,300g and 9,300 g for 30 min) to eliminate gross debris and then ultra-centrifuged at 130,000g, for 15 min in a Beckman Airfuge (Lavazza *et al*, 1990; Ntafis *et al.*, 2010). In this way the viral suspension was directly pelleted on grids formvar coated. The grids were negatively stained with 2% NaPT, pH 6.8 for 3 min and observed using a FEI Tecnai 12 BioTwin microscope operating at 85Kv at magnifications ranging from 19000-46000x. The identification of viral particles was made based on morphological and dimensional characteristics. Regarding turkeys samples, the assay used is the immune-nsEM. In particular, samples were incubated at 37°C for 60 minutes with a specific antiserum (convalescent serum). The positivity in nsEM was expressed as a semi-quantitative result (from 1+ to 5+, depending on the number of particles/groups observed per grids part).

*RT-PCRs*

Viral RNA was extracted from 140 µl of each suspension using QIAamp Viral RNA kit (*Qiagen*) or Trizol reagent (*Invitrogen*).

AstV RT-PCR: samples were analyzed using primers and protocol by Tang *et al.* 2005.

AvRVs RT-PCRs: extracted viral RNA was subjected to four different group-specific RT-PCR assays, using RV-A, RV-D VP6 specific primers, and RV-F and RV-G VP6 primers as described above.

*Sequencing and phylogenetic analysis*

PCR products were analyzed as described in the previous chapter.

# 5. RESULTS

## 5.1 Retrospective data collection/strains characterization

### 5.1.1 Prevalence data

A total of 1367 samples collected in Northern and Central Italy between 2006 and 2014 in the course of necropsy of birds showing clinical signs of enteric disease, from both poultry flocks (n=1177: chicken, turkey, guinea fowl) and game birds (n=190: pheasant, partridge) were conferred to the IZSLER EM laboratory in Brescia.

The data analysis showed that a total of 257 samples out of 1367 were found to be positive (19%) for rotavirus by nsEM (Figure 11).

Overall, the data indicate that the RV infection has an average prevalence of about 20% in commercial flocks (Table 8) and 15% in game-hunting bird ones (Table 9), when samples from birds with enteritis or with an history related to enteric viral infection were analyzed.

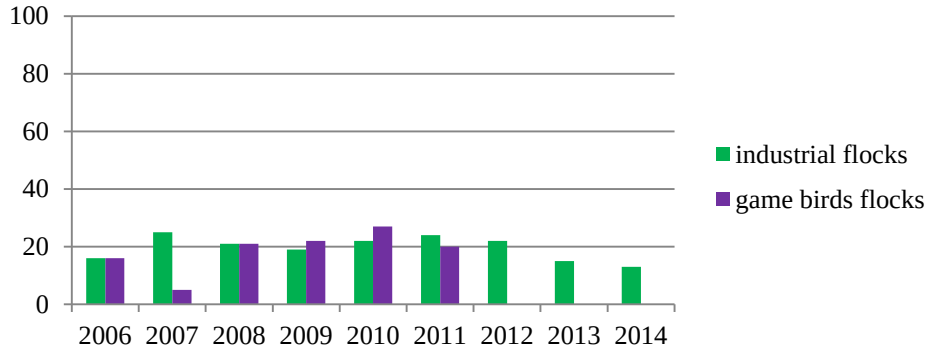
Table 8: Commercial flocks, samples from 2006 to 2014 (Jan-Jun): samples positive for RV

| Year | Examined samples: total | Rotavirus positive samples | % |
|---|---|---|---|
| 2006 | 172 | 26 | 16 |
| 2007 | 185 | 46 | 25 |
| 2008 | 181 | 38 | 21 |
| 2009 | 182 | 34 | 19 |
| 2010 | 67 | 15 | 22 |
| 2011 | 71 | 17 | 24 |
| 2012 | 111 | 24 | 22 |
| 2013 | 136 | 20 | 15 |
| 2014 (1-6) | 72 | 9 | 13 |
| Total | 1177 | 229 | 20% |

Table 9: Game-hunting flocks, 2006-2014 (Jan-Jun), samples positive for RV

| Year | Examined samples: total | Rotavirus positive samples | % |
|---|---|---|---|
| 2006 | 31 | 5 | 16 |
| 2007 | 44 | 2 | 5 |
| 2008 | 56 | 12 | 21 |
| 2009 | 22 | 5 | 22 |
| 2010 | 11 | 3 | 27 |
| 2011 | 5 | 1 | 20 |
| 2012 | 13 | 0 | 0 |
| 2013 | 8 | 0 | 0 |
| 2014 (1-6) | 0 | 0 | = |
| total | 190 | 28 | 15% |

Figure 11: Prevalence (%) per year, for industrial and game birds




5.1.2 Symptoms/pathological findings data

Signs and symptoms were also recorded and categorized (Table 10). On the basis of the systems involved the general signs and lesions observed in the birds under study were classified into six categories. Salient general and clinical features included diarrhea, dehydration, reduced feed intake together with anorexia and weight loss, nervous symptoms, starvation and cachexia, which resulted in increased mortality. Gastro-enteric lesions of different entity, alone or in association with concomitant lesions in other organ/systems, were observed in 95% of samples. Among these, sero-catarrhal enteritis was the most prevalent (35.6%), followed by the finding of watery and foamy gas contents in the large intestine in 14.8% and 13.7% of the cases, respectively (Table 11).

Table 10: Categorized general signs and lesions observed in the birds

| **A. General and B. Growth/locomotory signs and lesions** | **C. Gastro-enteric lesions** | **D. Respiratory lesions** | **E. Urogenital lesions** | **F. Hepatic lesions** | **H. Splenic lesions** |
|---|---|---|---|---|---|
| a1. increased mortality<br>a2. weight loss, feed intake reduction, anorexia<br>a3. dehydration<br>a4. nervous symptoms (including head tilt and *opistotonus*)<br>a5. sensory depression, starvation and cachexia<br>a6. uneven growth, stunting, runting, abnormal feathering<br>b1. birds huddling together near heat sources, leg problems, incoordination, lameness, muscle atrophy, proximal tibia-tarsus joint enlargement, loss of balance<br>b2. osteodystrophy, rickets, osteomyelitis, osteoporosis | c1. small gizzards, gastrointestinal tract containing little or no *ingesta*<br>c2. undigested feed in the intestinal contents and feces; impaction<br>c3. congestion of the gut wall<br>c4. distension of intestinal loops, pale thin-wall intestine<br>c5. edema and hyperplasia of the intestinal mucosa<br>c6. gizzard erosions/necrosis<br>c7. proventriculitis<br>c8. enlarged proventriculi<br>c9. sero-catharral enteritis<br>c10. cloacal pasting<br>c11. watery content<br>c12. yellow-orange content<br>c13. foamy gas content<br>c14. melena, blood stained feces | d1. catarrhal exudate in nasal cavities and sinus<br>d2. tracheal congestion<br>d3. air sacs opacity<br>d4. catarrhal aerosacculitis | e1. nephritis<br>e2. nephrosis, swollen kidneys<br>e3. pale kidneys with hemorrhages<br>e4. urates in the ureters | f1. liver enlargement<br>f2. liver degeneration<br>f3. liver necrosis<br>f4. edema of gallbladder wall | h1. spleen enlargement and congestion<br>h2. splenic hypoplasia<br>h3. perisplenitis |

Table 11: Gastro-enteric lesions (C-type lesions) observed at post mortem examination either alone or in association in the birds examined

| **Gastro-enteric lesions** | **% Samples** |
|---|---|
| Small gizzards, gastrointestinal tract containing little or no ingesta | 3.9 |
| Undigested feed in the intestinal contents and feces; impaction | 4.7 |
| Congestion of the gut wall | 0.4 |
| Distension of intestinal loops, pale thin-wall intestine | 6.8 |
| Edema and hyperplasia of the intestinal mucosa | 2.2 |
| Gizzard erosions/necrosis | 1.8 |
| Proventriculitis, enlarged proventriculi | 3.2 |
| Sero-catarrhal enteritis | 35.6 |
| Fibro-hemorrhagic enteritis | 2.9 |
| Cloacal pasting | 2.9 |
| Watery content | 14.8 |
| Yellow-orange content | 3.2 |
| Foamy gas content | 13.7 |
| Melena, blood stained feces | 3.9 |
| Total | 100.00 |

### 5.1.2 Strains selection and characterization

*EM results*

By direct nsEM examination, most of the 117 samples (81.2%) were positive only for RVs, whereas 18.8% showed simultaneous infection with a variety of other enteric viruses. In addition to phages, which were observed in 10.2% of samples from chickens, pheasants and turkeys, enterovirus-like viruses or small round fecal viruses (6.0%) were detected in chicken and guinea fowl samples, and Astroviruses (3.4%) were identified only in turkey samples (Table 12). Laboratory investigations also revealed the frequent presence of *Escherichia coli* and *Clostridium* spp, whereas C*ampylobacter s*pp and *Salmonella* spp, as well as parasites such as coccidia occurred only occasionally.

Table 12: Enteric viruses present in association with Rotavirus by using nsEM

| **Pathogen** | **Chicken** | **Guinea fowl** | **Partridge** | **Pheasant** | **Turkey** | **Total** |
|---|---|---|---|---|---|---|
| Rotavirus | 64 | 4 | 5 | 9 | 13 | 95 (81,20%) |
| Rotavirus + phages | 6 | 0 | 0 | 1 | 4 | 11 (9,40%) |
| Rotavirus + enterovirus-like virus | 6 | 0 | 0 | 0 | 0 | 6 (5,13%) |
| Rotavirus + astrovirus | 0 | 0 | 0 | 0 | 4 | 4 (3,42%) |
| Rotavirus + phages + enterovirus-like virus | 0 | 1 | 0 | 0 | 0 | 1 (0,85%) |
| **Total** | 76 | 5 | 5 | 10 | 21 | 117 (100%) |

*Group A ELISA*

Eight out of 117 samples resulted positive in RV-A ELISA (6.8%).

*AvRVs RT-PCRs*

Molecular detection of AvRV of different groups was performed by established group-specific RT-PCR assays. In all cases, corresponding group-specific positive control samples yielded a specific product in 2% agarose gel, as expected.

Sensitivity of our newly designed AvRV-F and -G group-specific primers in RT-PCR assays, tested using limiting dilutions of PCR products from positive control samples, showed detection limits equivalent to $10^4$ and to $10^3$ copies per reaction, respectively. The specificity of the newly designed AvRV-F and G group-specific primers was confirmed by the lack of amplification by RT-PCR of a panel of 19 isolates of avian viruses different from rotavirus.

From the 117 samples tested by group-specific RT-PCRs, AvRV-D was detected in 107 (91.5%), AvRV-A in 70 (59.9%), AvRV-F in 61 (52.1%) and AvRV-G in 31 (26.5%).

Only 20 samples (17.1%), showed the presence of a single rotavirus group. In this regard, AvRV-A and AvRV-D alone were detected in 4 and 16 samples (3.4% and 13.7%), respectively. Dual or multiple presence of rotaviruses of different AvRV groups was observed in 97 samples (83%), originating both from individual birds and from pooled samples (Table 12).

As summarized in Table 13, out of 117 samples tested, single infections were present in 20 samples (17%) and multiple infections were present in 97 samples (83%) with different patterns. In particular, the majority of samples belonging to the RV-A group were obtained from game birds and guinea fowls (40%), whereas the majority of samples belonging to RV-D group were derived from chicken and turkeys (48% and 55%, respectively). The presence of both RV-A and RV-D is prevalent in chicken (48%), but also evident in turkeys (20%).

Table 13: Distribution within the different avian rotavirus groups (A, D, F and G) detected by group-specific RT-PCR assays in different species

| **Species** | **A** | **D** | **AD[a]** | **DF[a]** | **AF[a]** | **DG[a]** | **AG[a]** | **ADF[a]** | **DFG[a]** | **ADG[a]** | **ADFG[a]** | **Tot** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Chicken | 1[b] | 10 | 21 | 9 | 3 | 2 | 0 | 12 | 11 | 2 | 5 | 76 |
| Turkey | 3 | 4 | 3 | 3 | 1 | 0 | 1 | 3 | 2 | 0 | 1 | 21 |
| Pheasant | 0 | 1 | 2 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 2 | 10 |
| Guinea fowl | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 5 |
| Partridge | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 5 |
| Total | 4 | 16 | 28 | 15 | 4 | 2 | 2 | 19 | 14 | 4 | 9 | 117 |
| **%** | **3.4** | **13.7** | **24** | **12.8** | **3.4** | **1.7** | **1.7** | **16.3** | **11.9** | **3.4** | **7.7** | **100** |

a Multiple presence in the samples of viruses belonging to the listed avian rotavirus groups

b Number of positive samples identified for the different avian rotavirus groups (percentage)

*Sequencing and phylogenetic analysis*

One-hundred and seventy five complete sequences in the following gene segments were performed: 17 of NSP4A, 20 of VP6A, 5 of VP4A, 35 of NSP4D, 34 of VP6D, 9 of VP4D, 17 of VP7D, 21 of VP6F and 13 of VP6G. Phylogenetic trees for VP4, VP6, VP7, NSP4 segments were constructed on the basis of the entire nucleotide sequences as shown in Figures 12 to 15 (the sequences generated in this study are marked in blue and GenBank's reference sequences are marked in black). No correlation between year of isolation or avian species and the different RV-groups were observed in all the analysed segments by phylogenetic analysis.

Figure 12: VP4 phylogenetic tree

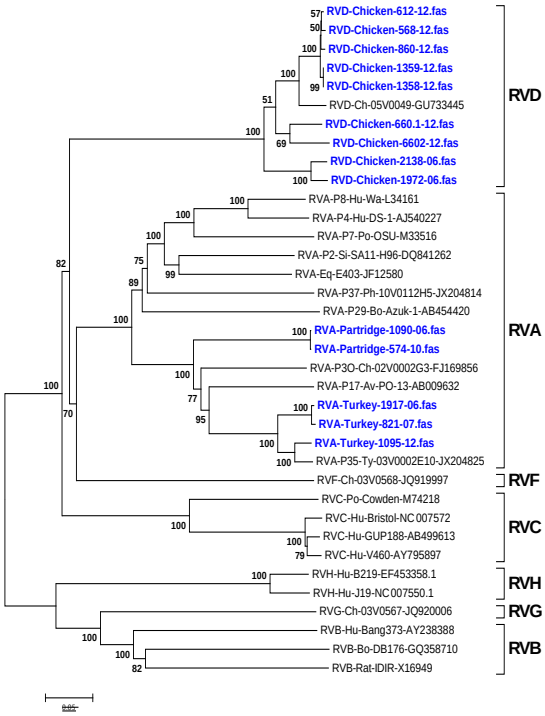



Figure 13: VP6 phylogenetic tree

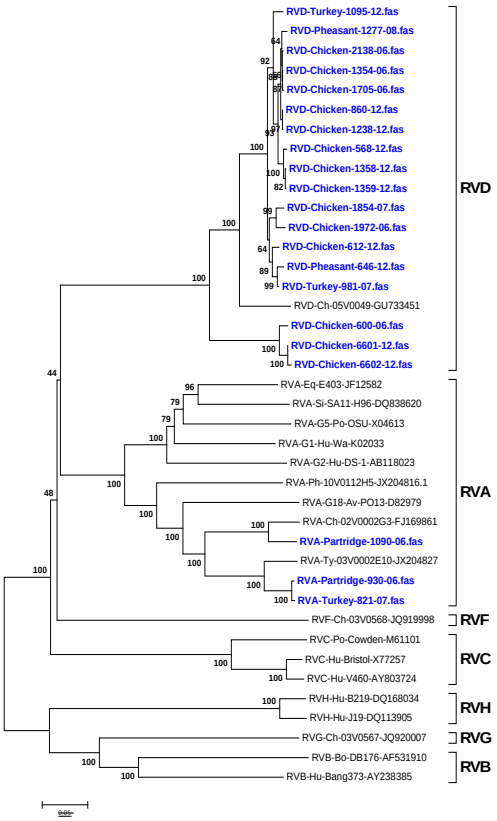


Figure 14: VP7 phylogenetic tree

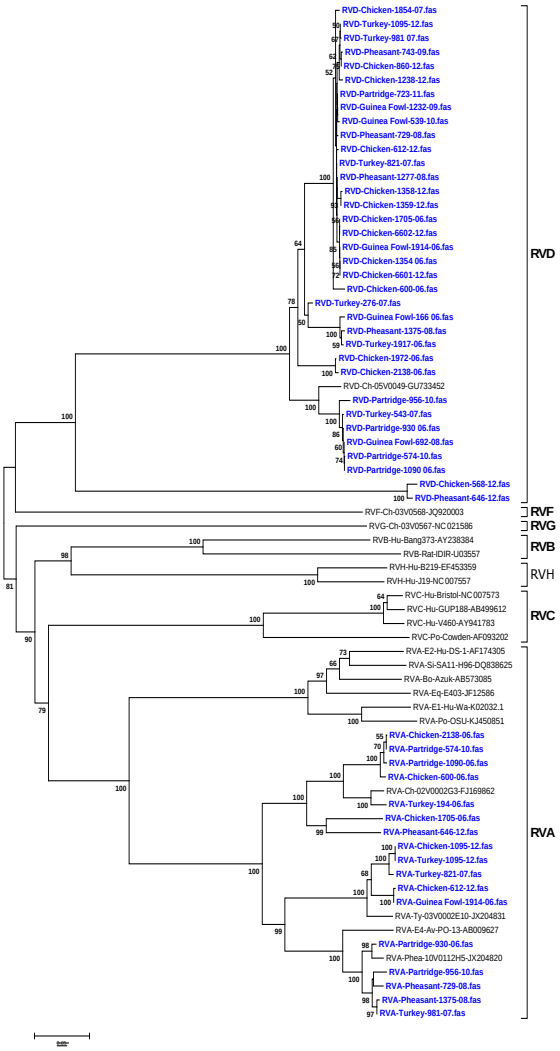
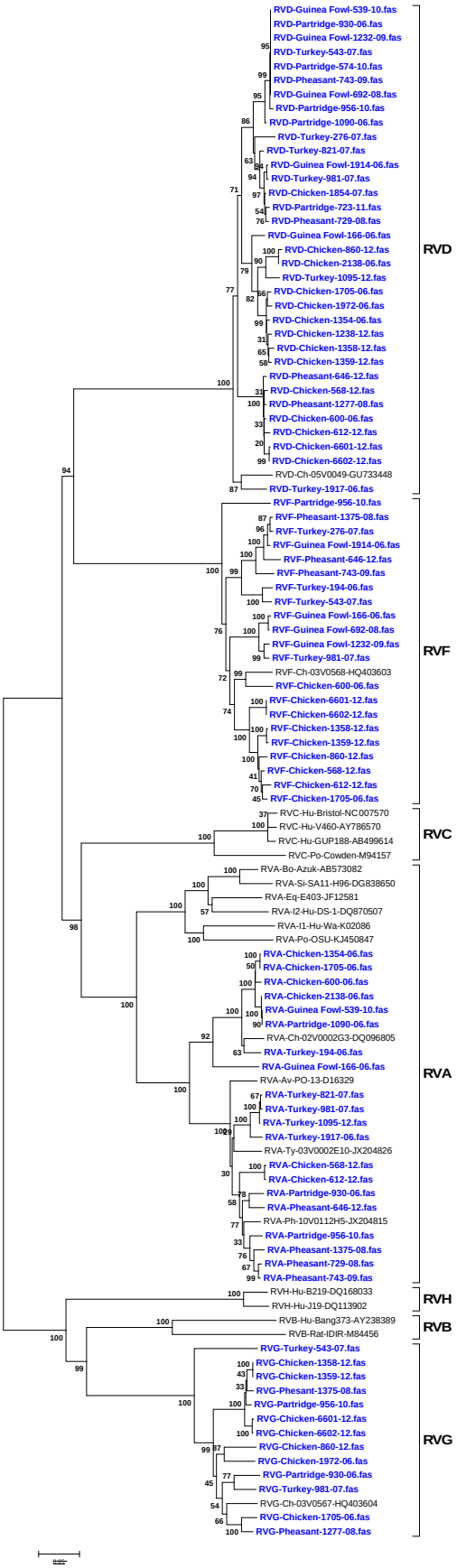



Figure 15: NSP4 phylogenetic tree



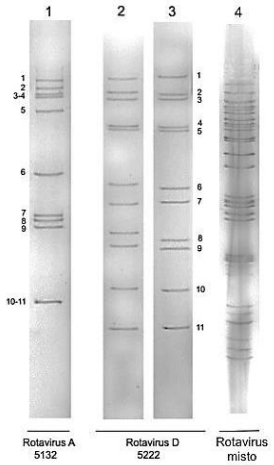
*RNA-PAGE*

Of the 58 samples selected for rotavirus RNA-PAGE characterization, AvRVs were detected in 20 samples (34.4%). Genomic segment migration profile analysis yielded typical rotavirus electrophoretic patterns consistent with group A or group D avian rotaviruses in 6 and 12 samples (5% and 10%), respectively (Table 14). Two samples revealed unusual RNA migration patterns with overlapping segment migration profiles (Figure 16).

Table 14: Migration RNA-PAGE patterns of analysed RV

| Year | Internal number | Specie | AvRV PCR | Electropherotype |
|---|---|---|---|---|
| 2012 | 646 | pheasant | A D F G | 5132 |
| | 660/2 | chicken | D F G | 5222 |
| | 660/1 | chicken | D F G | Mixed |
| | 860 | chicken | D F G | 5222 |
| 2010 | 574 | partridge | A D | 5222 |
| | 691 | chicken | A D F G | 5222 |
| | 956 | partridge | A D F G | Mixed |
| 2008 | 451 | chicken | A D F | 5222 |
| | 2196 | turkey | A D | 5132 |
| 2007 | 237 | chicken | D G F | 5222 |
| | 543 | turkey | D G F | 5222 |
| | 774 | pheasant | A G | 5132 |
| | 821 | turkey | A D | 5132 |
| | 1854 | chicken | D | 5222 |
| 2006 | 194 | turkey | A F | 5132 |
| | 516 | chicken | D G | 5222 |
| | 600 | chicken | A D F | 5222 |
| | 930 | partridge | A D G | 5132 |
| | 1972 | chicken | A D G | 5222 |
| | 2138 | chicken | A D | 5222 |

Figure 16: Representative image of the electrophoretic profile of the three samples of avian rotavirus, in position 1 is present the sample 774 of 2007 that has a profile 5132 typical of the group A avian, in position 2 and 3 the electrophoresis of the samples 1972 and 2138 of 2006 , respectively, which both show a 5222 typical profile of avian rotavirus group D, finally in position 4 is present the sample 956 of 2010, which presents a mixed electrophoretic profile



### 5.1.3 Statistical analysis

After the genetic characterization, the correlation of general signs/lesions or gastro-enteric lesions observed in the field and at *post mortem* examination with the presence of specific RV groups or different combinations of RV groups, even with regard to the different species involved, was accomplished.

By statistical analysis it could not be observed any significant correlation between the general clinical signs/lesions or gastro-enteric lesions observed in the field and at *post mortem* examination with the presence of different AvRV groups or different combinations of groups, even with regard to the different species involved ($p > 0.05$; Table 15).

Table 15: Categorized results based on clinical signs and lesions (type A-H, as described in Tab.10) by RV group

| **RV group** | **Clinical signs and lesions** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H |
| **A** | 1 | 0 | 4 | 0 | 0 | 1 | 1 | 1 |
| **AD** | 3 | 7 | 28 | 0 | 3 | 4 | 6 | 6 |
| **ADF** | 3 | 6 | 17 | 1 | 2 | 2 | 2 | 1 |
| **ADFG** | 0 | 2 | 9 | 1 | 0 | 1 | 1 | 1 |
| **ADG** | 1 | 0 | 4 | 0 | 0 | 0 | 0 | 0 |
| **AF** | 2 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| **AG** | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| **D** | 4 | 7 | 15 | 1 | 2 | 2 | 3 | 0 |
| **DF** | 3 | 4 | 15 | 0 | 0 | 1 | 3 | 2 |
| **DFG** | 2 | 7 | 12 | 1 | 2 | 4 | 2 | 1 |
| **DG** | 0 | 1 | 2 | 0 | 0 | 0 | 1 | 1 |
| $X^2 = 43$ | | | | | P-value= 0.995 | | | |

In order to assess if there was an effect between the type of lesions and the RT-PCR results, an Independence test has been used. This is a Chi-squared test with Monte-Carlo resampling approximation due to low (and null) frequencies in the cells. A p-value greater than 0.05 showed that the null hypothesis, that there is no effect between clinical signs and results, could not be rejected. The same test has been applied to the results grouped by species (Table 16). None of the species showed a dependence pattern among clinical signs and results.

Table 16: Result of clinical signs and lesions grouped by species

| **Species** | **$X^2$** | **Number of lesions** | **P-value** |
|---|---|---|---|
| Chicken | 41.6 | 166 | 0.97 |
| Turkey | 26.7 | 30 | 0.98 |
| Pheasant | 9.1 | 14 | 1 |
| Guinea Fowl | 4.1 | 9 | 1 |
| Partridge | 5 | 5 | 0.60 |

As a next step, all the lesions but A, B and C have been deleted from the dataset. The same tests have been applied in order to know if there was a difference in the results patterns due to presence of lesions A, B or C.

No effect has been assessed, neither in the total dataset (p-value=0.8), nor for stratification by species (p-values: Chicken=0.99, Turkey=0.47, Pheasant=0.94, Guinea Fowl=0.78, Partridge=0.80).

C-type lesions (gastro-enteric lesions) analysis

The Chi-squared test with Monte-Carlo resampling approximation had been applied to assess the dependence between different levels of C-type lesions and the results for the Rotavirus (Table 17).

Table 17: C-type signs and lesions (c1-c14, as categorized in Tab. 10) grouped by RV group

| **RV group** | **C-type lesions** | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 | C13 | C14 |
| **A** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 2 | 1 | 2 | 0 |
| **AD** | 2 | 7 | 1 | 7 | 2 | 2 | 4 | 23 | 5 | 0 | 8 | 2 | 9 | 3 |
| **ADF** | 2 | 0 | 0 | 5 | 0 | 0 | 2 | 16 | 1 | 2 | 5 | 2 | 6 | 1 |
| **ADFG** | 0 | 1 | 0 | 2 | 1 | 2 | 0 | 8 | 0 | 1 | 4 | 3 | 4 | 1 |
| **ADG** | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 4 | 0 | 0 | 1 | 0 | 0 | 0 |
| **AF** | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 1 | 2 | 0 | 1 | 0 |
| **AG** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 2 | 0 |
| **D** | 3 | 2 | 0 | 2 | 1 | 1 | 2 | 14 | 0 | 1 | 6 | 1 | 6 | 3 |
| **DF** | 2 | 1 | 0 | 0 | 2 | 0 | 0 | 12 | 0 | 2 | 9 | 0 | 6 | 0 |
| **DFG** | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 11 | 1 | 1 | 3 | 0 | 2 | 2 |
| **DG** | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 1 |
| $X^2$= 96.1 | | | | | P-value= 0.98 | | | | | | | | | |

A p-value greater than 0.05 showed that there is no evidence of any effect between different C-type lesions and results.

The same test had been applied to signs and lesions grouped by species (Table 18). P-values greater than 0.05 showed that different results are not due to differences in C-type lesions for any species.

Table 18: C-type clinical signs and lesions grouped by species

| **Species** | **$X^2$** | **Number of C-type lesions** | **P-value** |
|---|---|---|---|
| Chicken | 74.4 | 180 | 0.99 |
| Turkey | 29.7 | 45 | 0.99 |
| Pheasant | 28 | 26 | 0.99 |
| Guinea Fowl | 5.3 | 17 | 0.97 |
| Partridge | 4.1 | 10 | 0.96 |

## 5.2 Longitudinal studies

***Chain 1***

One-hundred and seventeen samples were collected from flocks of the chain 1. Of these, 95 samples (81,2%) resulted positive by EM and/or RT-PCR (Table 19). In the following tables (Tables 20-24) results of EM and RT-PCR for AsTV and AvRVs (A-D-F-G groups) of single samples of all the sampling points, grouped per flocks are shown.

Table 19: Samples collected from flocks of the chain 1, grouped by time of sampling and positivity for RV

| Flock | Age (d) | Samples (n) | | |
|---|---|---|---|---|
| | | neg | pos | tot |
| **1** | 13 | 0 | 7 | 7 |
| | 21 | 1 | 6 | 7 |
| | 28 | 2 | 3 | 5 |
| | 34 | 1 | 4 | 5 |
| | 42 | 0 | 5 | 5 |
| **2** | 5 | 0 | 7 | 7 |
| | 14 | 0 | 6 | 6 |
| | 21 | 2 | 3 | 5 |
| | 27 | 0 | 5 | 5 |
| | 35 | 0 | 4 | 4 |
| **3** | 7 | 1 | 0 | 1 |
| | 14 | 6 | 0 | 6 |
| | 21 | 0 | 4 | 4 |
| | 28 | 3 | 0 | 3 |
| | 35 | 4 | 0 | 4 |
| **5** | 3 | 2 | 5 | 7 |
| | 10 | 0 | 8 | 8 |
| | 17 | 0 | 8 | 8 |
| | 24 | 0 | 8 | 8 |
| | 33 | 0 | 7 | 77 |
| | 38 | 0 | 5 | 5 |
| | **Total** | **22** | **95** | **117** |

Table 20: Chain 1 – flock 1 results of EM, RT-PCRs grouped by age of sampling

| Age (d) | Sample | EM | AsTV RT-PCR | AvRV RT-PCR | | | |
|---|---|---|---|---|---|---|---|
| | | | | A | D | F | G |
| 13 | 1 | N | P | P | N | P | N |
| | 2 | | | P | N | N | N |
| | 3 | | | P | N | N | N |
| | 4 | RV + AsV ++ | P | P | P | P | P |
| | 5 | | | P | P | P | P |
| | 6 | | | P | P | P | P |
| 21 | 1 | N | P | N | P | N | N |
| | 2 | | | P | P | N | N |
| | 3 | | | N | N | N | N |
| | 4 | | | P | N | N | N |
| | 5 | | | P | N | N | N |
| | 6 | | | P | N | N | N |
| | 7 | | | N | P | N | N |
| 28 | 1 | N | N | N | N | N | N |
| | 2 | | | N | P | P | N |
| | 3 | | | N | P | N | N |
| | 4 | | | N | N | P | N |
| | 5 | | | N | N | N | N |
| 34 | 1 | RV +++++ AsV +++ | P | N | N | N | N |
| | 2 | | | P | P | N | N |
| | 3 | | | P | P | N | N |
| | 4 | | | N | P | N | N |
| | 5 | | | P | P | N | N |
| 42 | 1 | NEG | P | P | P | N | P |
| | 2 | | | P | P | N | P |
| | 3 | | | P | P | N | P |
| | 4 | | | P | P | N | P |
| | 5 | | | P | P | P | N |

Table 21: Chain 1 – flock 2 results of EM, RT-PCRs grouped by age of sampling

| Age (d) | Sample | EM | AsTV RT-PCR | AvRV RT-PCR | | | |
|---|---|---|---|---|---|---|---|
| | | | | A | D | F | G |
| 5 | 1 | RV ++ | P | P | N | N | N |
| | 2 | | | P | N | N | N |
| | 3 | | | P | N | P | N |
| | 4 | RV ++ AsV +++ | P | P | P | P | P |
| | 5 | | | P | N | N | P |
| | 6 | | | P | N | N | N |
| | 7 | | | P | N | N | N |
| 14 | 1 | N | P | P | N | N | N |
| | 2 | | | N | P | N | N |
| | 3 | | | P | P | N | N |
| | 4 | | | P | P | P | N |
| | 5 | | | P | P | N | N |
| | 6 | | | N | P | P | N |
| 21 | 1 | N | P | P | P | N | N |
| | 2 | | | N | P | N | N |
| | 3 | | | N | N | N | N |
| | 4 | | | N | P | N | N |
| | 5 | | | N | N | N | N |
| 27 | 1 | RV +++ | P | P | P | N | N |
| | 2 | | | P | P | N | N |
| | 3 | | | P | P | P | N |
| | 4 | | | P | P | P | N |
| | 5 | | | P | P | N | N |
| 35 | 1 | RV ++ | P | N | P | N | N |
| | 2 | | | N | P | N | N |
| | 3 | | | N | P | N | N |
| | 4 | | | N | P | N | N |

Table 22: Chain 1 – flock 3 results of EM, RT-PCRs grouped by age of sampling (N=negative; P=positive; NP=not performed)

| Age (d) | Sample | EM | AsTV RT-PCR | AvRV RT-PCR | | | |
|---|---|---|---|---|---|---|---|
| | | | | A | D | F | G |
| 7 | 1 | AsV ++ | P | N | N | N | N |
| 14 | 1 | N | P | N | N | N | N |
| | 2 | | | N | N | N | N |
| | 3 | | | N | N | N | N |
| | 4 | | | N | N | N | N |
| | 5 | | | N | N | N | N |
| | 6 | | | N | N | N | N |
| 21 | 1 | RV++++ | P | N | P | N | N |
| | 2 | | | N | P | N | N |
| | 3 | | | N | P | N | N |
| | 4 | | | P | P | N | N |
| 28 | 1 | N | P | N | N | N | N |
| | 2 | | | N | N | N | N |
| | 3 | | | N | N | N | N |
| 35 | 1 | N | P | N | N | N | N |
| | 2 | | | N | N | N | N |
| | 3 | | | N | N | N | N |
| | 4 | | | N | N | N | N |
| 45 | 1 | N | P | NP | NP | NP | NP |
| | 2 | | | NP | NP | NP | NP |
| | 3 | | | NP | NP | NP | NP |
| | 4 | | | NP | NP | NP | NP |

Table 23: Chain 1 –flock 4 results of EM, RT-PCRs grouped by age of sampling

| Age (d) | Sample | EM | AsTV RT-PCR | AvRV RT-PCR | | | |
|---|---|---|---|---|---|---|---|
| | | | | A | D | F | G |
| 5 | 1 | N | N | P | N | P | N |
| | 2 | | | P | N | N | N |
| | 3 | | | P | N | N | N |
| | 4 | | | P | N | P | N |
| | 5 | RV+++ | N | P | N | N | N |
| | 6 | | | P | N | N | N |
| | 7 | | | P | N | N | N |
| | 8 | | | P | N | P | N |
| 13 | 1 | N | P | N | N | N | N |
| | 2 | | | N | N | N | N |
| | 3 | | | N | N | N | N |
| | 4 | | | N | N | N | N |
| | 5 | | | P | N | N | N |
| | 6 | | | N | N | N | N |
| | 7 | | | N | N | N | N |
| | 8 | | | N | N | P | N |
| 19 | 1 | N | P | N | N | N | N |
| | 2 | | | N | N | N | N |
| | 3 | | | N | N | N | N |
| | 4 | | | N | N | N | N |
| | 5 | | | N | N | N | N |
| | 6 | | | N | N | N | N |
| | 7 | | | N | N | N | N |
| | 8 | | | N | N | N | N |
| 27 | 1 | N | N | N | N | N | N |
| | 2 | | | N | N | N | N |
| | 3 | | | N | N | N | N |
| | 4 | | | N | N | N | N |
| | 5 | | | N | N | N | N |
| | 6 | | | N | N | N | N |
| | 7 | | | N | N | N | N |
| | 8 | | | N | N | N | N |

Table 24: Chain 1 –flock 5 results of EM, RT-PCRs grouped by age of sampling

| Age (d) | Sample | EM | AsTV RT-PCR | AvRV RT-PCR | | | |
|---|---|---|---|---|---|---|---|
| | | | | A | D | F | G |
| 3 | 1 | N | N | P | P | N | N |
| | 2 | | | P | P | N | P |
| | 3 | | | P | P | N | N |
| | 4 | | | P | P | P | N |
| | 5 | N | P | P | P | N | N |
| | 6 | | | N | N | N | N |
| | 7 | | | N | N | N | N |
| 10 | 1 | AsTV+ | P | P | N | N | N |
| | 2 | | | N | P | N | P |
| | 3 | | | N | P | P | N |
| | 4 | | | P | P | N | N |
| | 5 | | | P | N | N | N |
| | 6 | | | P | P | P | P |
| | 7 | | | N | N | N | P |
| | 8 | | | N | P | N | N |
| 17 | 1 | N | P | P | N | N | N |
| | 2 | | | P | P | N | N |
| | 3 | | | P | N | N | N |
| | 4 | | | P | P | N | N |
| | 5 | RV++ | | P | P | N | N |
| | 6 | | | P | P | N | N |
| | 7 | | | P | P | N | N |
| | 8 | | | P | N | N | N |
| 24 | 1 | N | N | P | N | N | N |
| | 2 | | | P | N | N | N |
| | 3 | | | P | N | P | N |
| | 4 | | | P | N | N | N |
| | 5 | | | P | N | N | N |
| | 6 | | | P | N | N | N |
| | 7 | | | P | N | N | N |
| | 8 | | | P | N | N | N |
| 33 | 1 | RV++ | N | P | P | P | N |
| | 2 | | | P | P | N | N |
| | 3 | | | N | P | N | P |
| | 4 | | | N | P | P | N |
| | 5 | | P | N | P | P | N |
| | 6 | | | N | P | P | P |
| | 7 | | | P | P | P | N |
| 38 | 1 | N | N | N | P | N | N |
| | 2 | | | P | P | N | N |
| | 3 | | | N | P | P | N |
| | 4 | | | P | N | P | P |
| | 5 | | | N | N | N | P |

The results of single positive samples sequenced were summarized in Table 25. In particular group/s identified per flock and sampling time were showed. Moreover also the presence of one or more strains belonging to the different groups was reported.

Table 25: Phylogenetic analysis results grouped per flock

| Flock | Days | Identified Group | Infection type | Strain |
|---|---|---|---|---|
| Flock 1 | 13 | A-D-F | Co-infection | A1-D1-F1 |
| | | A | Single | A1 |
| | | A-F | Co-infecton | A1-F2 |
| | 21 | A | Single | A2! |
| | | D | Single | D2 |
| | | A-D | Co-infection | A2-D2 |
| | 28 | D | Single | D2 |
| | | F | Single | F3! |
| | 34 | A-D | Co-infection | A1-A2-D2 |
| | 42 | A-D-G | Co-infection | A3!-D1-D2-G1 |
| Flock 2 | 5 | A | Single | A1-A2 |
| | 14 | D-A | Co-infection | D1-D2-A1 |
| | 21 | D-A | Co-infection | D2-A1 |
| | 27 | D-A | Co-infection | D2-A1 |
| | 35 | D | Single | D2 |
| Flock 3 | 21 | D | Single | D1 |
| Flock 4 | 5 | A-F | Co-infection | A1-F1 |
| | | A | Single | A1 |
| | 13 | A | Single | A1 |
| | | F | Single | F1 |
| Flock 5 | 3 | A-D | Co-infection | A1-D1-D2 |
| | 10 | A-D-F-G | Co-infection | A2-D1-D2-F1-G1 |
| | 17 | A-D | Co-infection | A3!-D1-D2 |
| | 24 | A | Single | A3 |
| | 33 | D | Single | D3 |
| | | D-A | Co-infection | D3-A3 |
| | | D-F | Co-infection | D3-F1-F2-F3 |
| | 42 | A-D-F-G | Co-infection | A3-D3-F4-G2 |

In order to understand the in-herd situations, combined results of RT-PCRs and sequencing for every flock were listed below, and then summarized in Figure 17. Moreover, phylogenetic trees of the VP6A, VP6D, VP6F and VP6G segments isolated in each flocks (only for flocks 1, 2 and 5) were reported (Figures 18 to 20).

*Flock 1*

13 days: RV-A, D and F were detected, alone RV-A or in double (RV-A, RV-D) or triple co-infections (RV-A, RV-D, RV-F), with one strain for group A and D and two different strains for group F.

21 days: RV-A and D were present in single and in co-infections, with strains different from which observed in the previous time point.

28 days: single infection with RV-D and F, for D the strain was the same reported at 21 days, for F there was a new strain.

34 days: co-infection of RV-A and D, with two different strains of group A and one of group D, previously reported.

42 days: co-infection of RV-A, D, G, with the new introduction of a strain of RV-G and a new strain of group A.

*Flock 2*

5 days: only RV-A was present, with two different strains.

14 days: the introduction of RV-D was observed, with two different strains, in co-infection with one of the previous strains of RV-A.

21 days: the same strain of RV-D present at 14 days was identified, and in one sample the co-infection with RV-A was observed.

27 days: co-infection of RV-A and D, with the same strains detected in previous time points.

35 days: only RV-D, with the same strain observed at 27 days.

*Flock 3*

Only RV-D at 21 days was observed and the RV-D strains of this flock clustered together, in a different cluster from RV-D of other flocks. Moreover, these strains were more closely related to the reference strains than others.

*Flock 4*

5 days: RV-A was present in all samples, only in three samples in co-infection with RV-F.

13 days: RV-A and RV-F were identified singularly in two samples.

*Flock 5*

3 days: co-infection with RV-A (only one strain) and D (two different strains).

10 days: RV-A, D, F and G were present in different combinations. Only a single strain of RV-A different from the one of day 3, but phylogenetic similar to the RV-A isolated from pheasant. For RV-D two strains were observed, different from those of 3 days.

17 days: co-infection with RV-A and RV-D, with the introduction of a new RV-A strain phylogenetic different from previous ones and from reference strains. RV-D strains were the same of ones of previous time points.

24 days: only one strain (the same of 17 days) from RV-A was detected.

33 days: reintroduction of a new strain of RV-D was observed and, only in three samples, RV-A was also present, with the same strain of day 24. In 5 samples out of 7, co-infection with RV-F was identified (three different strains).

42 days: all groups (A, D, F and G) were present; RV-A and D with the same strains observed at 33 days, RV-F and G showed strains different from previous time points.

Figure 17: Distribution of RV groups and strains within the five turkey flocks analyzed. Colour represents rotavirus group (red=RV-A; blue=RV-D; purple=RV-F; green=RV-G), pattern represents different RV strains among the same group

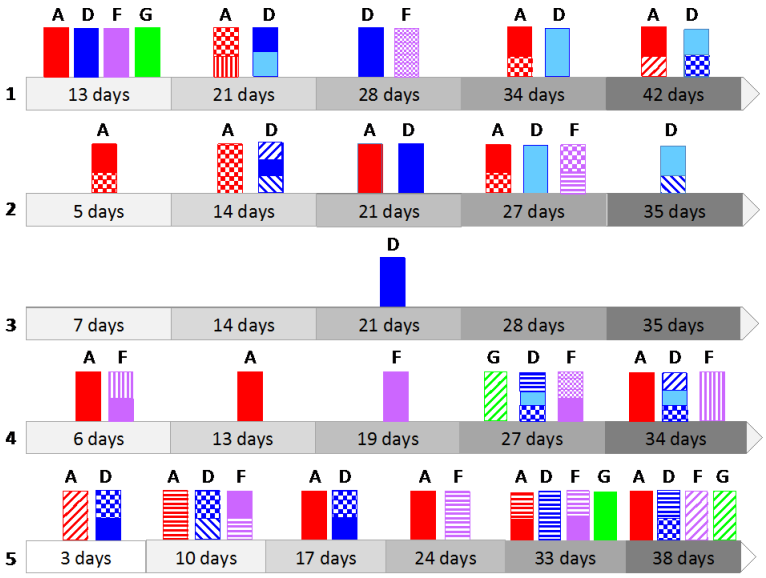


Figure 18: Phylogenetic analysis of the VP6A, VP6D, VP6F and VP6G segments isolated in the flock 1

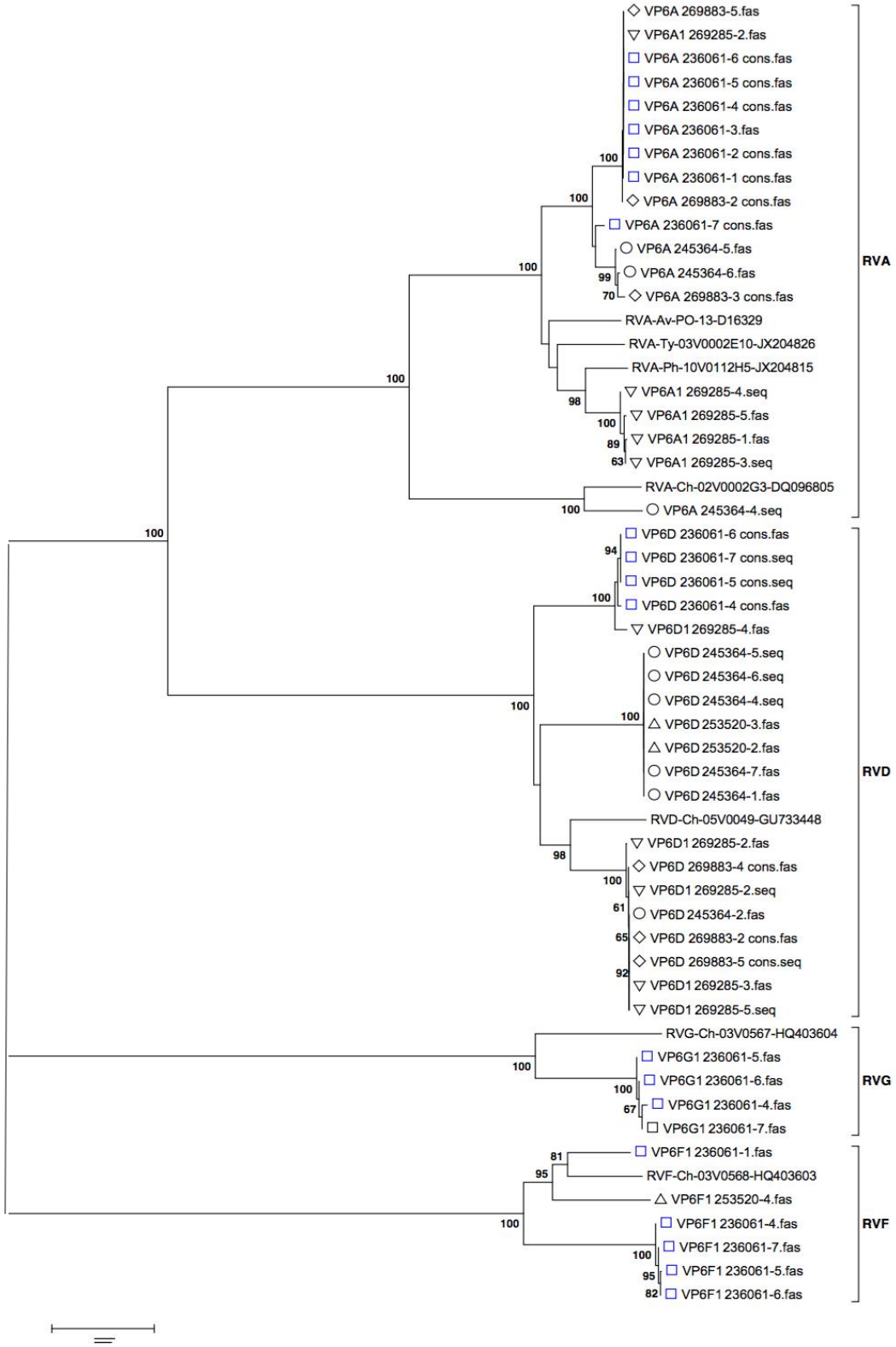


Figure 19: Phylogenetic analysis of the VP6A, VP6D, VP6F and VP6G segments isolated in the flock 2

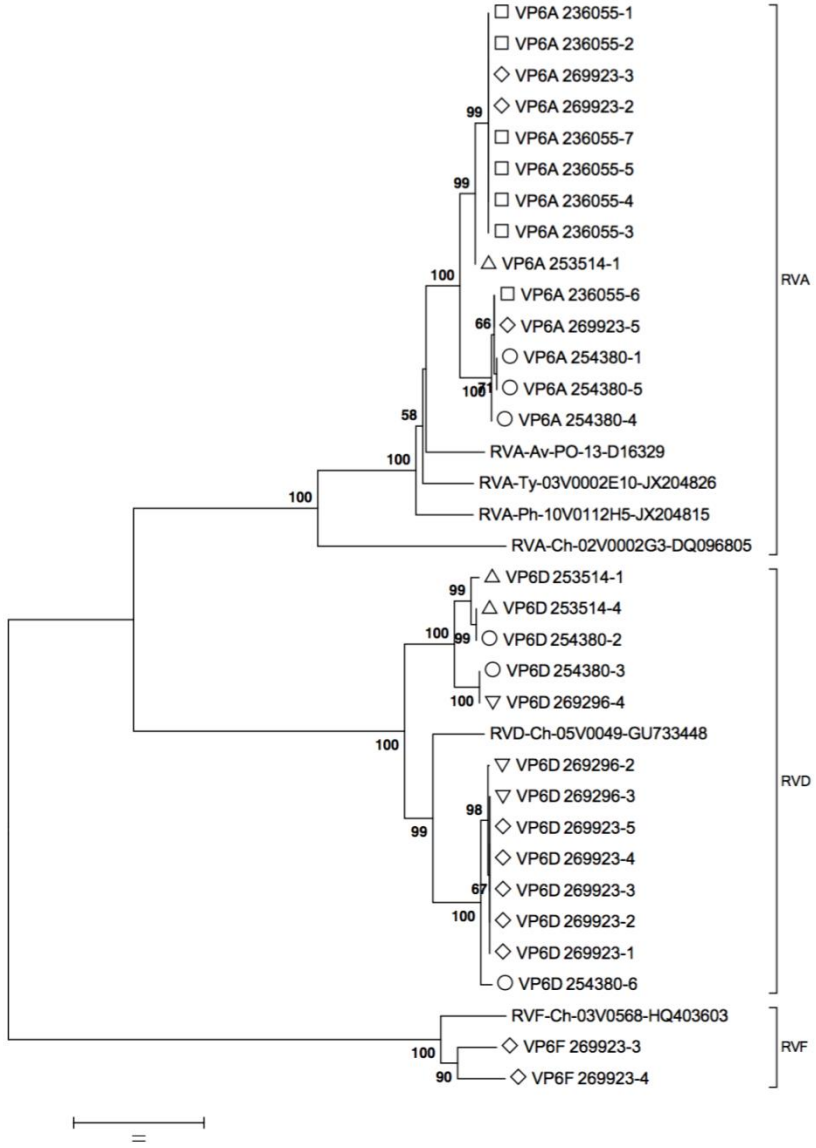
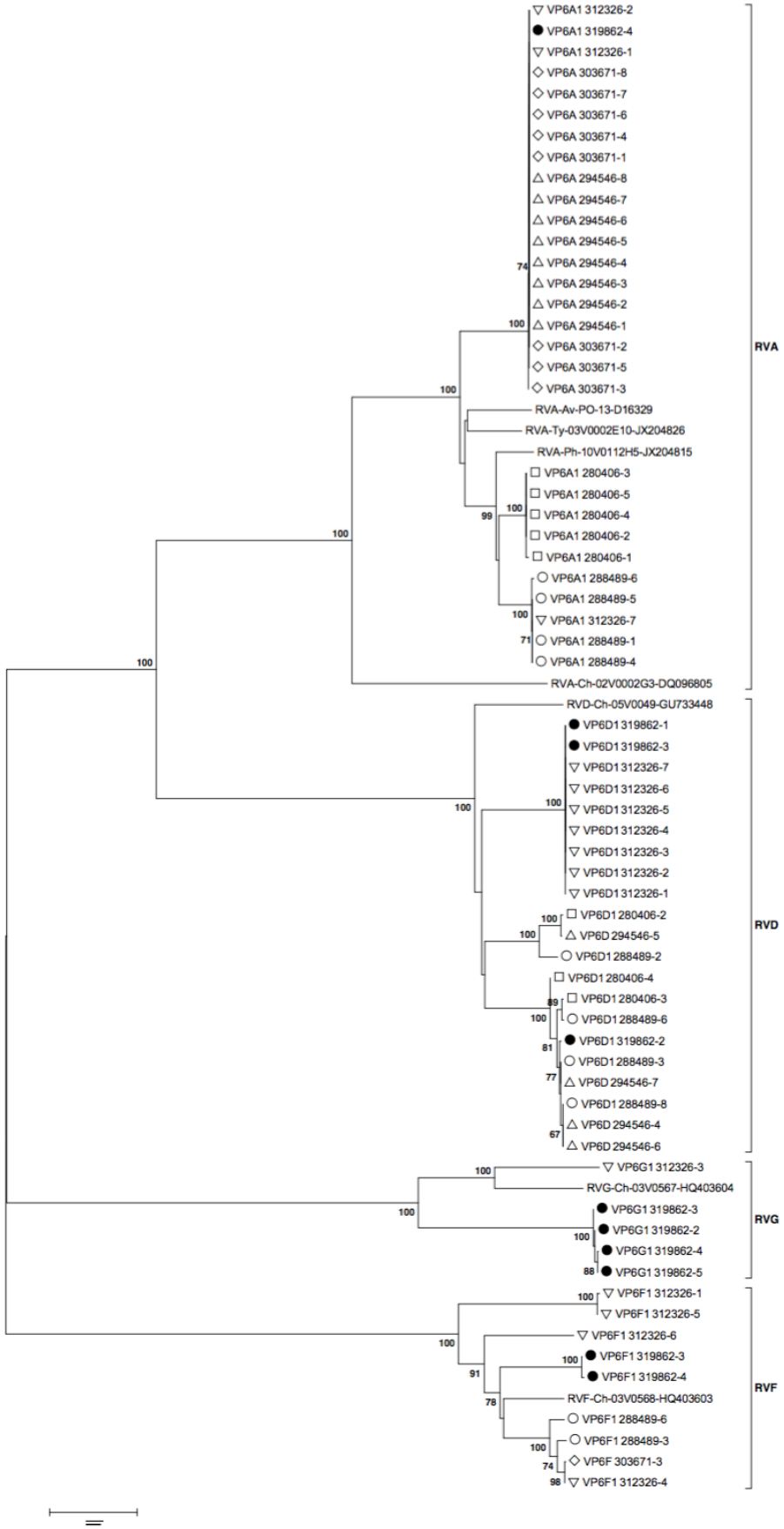


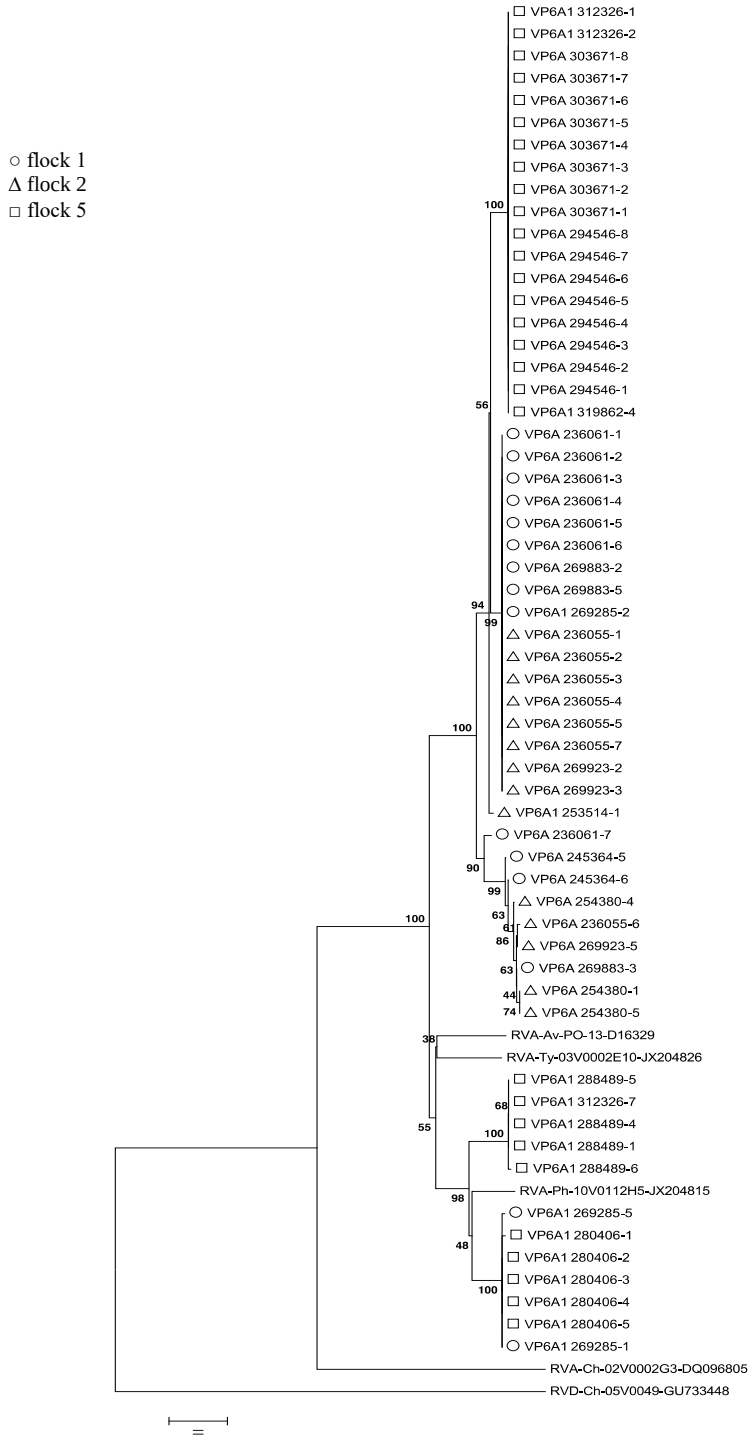
Figure 20: Phylogenetic analysis of the VP6A, VP6D, VP6F and VP6G segments isolated in the flock 5



Phylogenetic analyses of RV-A (Figure 21)

In flocks 1 and 2 different strains of RV-A have been circulating, two were in common for the two flocks; in the flock, 3 different strains were present, and were observed at different times, one of these was also detected in the flock 1. In general, for RV-A the circulation of 5 different strains was observed, one strain present only in the flock 5 and 3 detected in two different flocks.

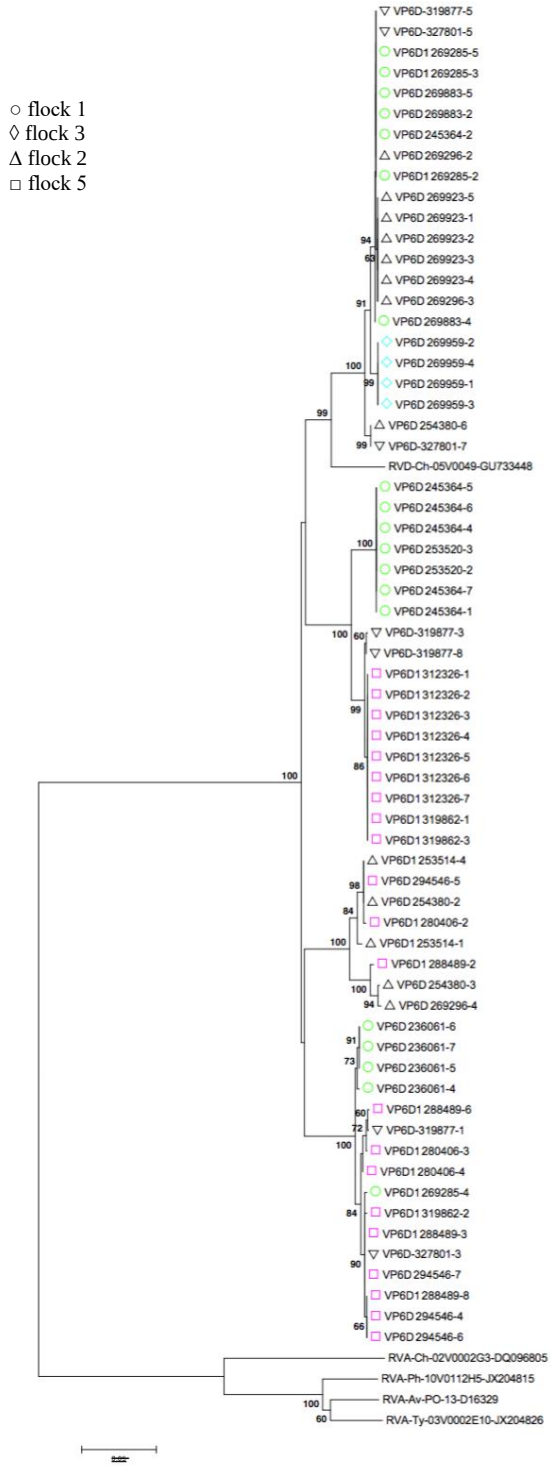
Figure 21: Phylogenetic analysis of RV-A vp6 from all different flocks



Phylogenetic analyses of RV-D (Figure 22)

RV-D showed strains phylogenetic different; in particular only two strains (one exclusively present in the flock 3 and one present at different time in the flocks 1 and 2) were similar to the reference one. All the others were not correlated with the reference strains: three of these were in the same flock at different times; the others were detected in different flocks.

Figure 22: Phylogenetic analysis of RV-D VP6 from all different flocks



### *Chain 2: two chicken flocks*

Results obtained for this chain are summarized in the following table.

Table 26: Results of the longitudinal study in chicken flocks, (N= negative; P= positive; NP = not performed)

| **CASE** | | | | |
|---|---|---|---|---|
| **Samples** | **Age (d)** | **EM** | **AsTV RT-PCR** | **AvRV RT-PCR** |
| 1-4 | 8 | enterolike (astro?) ++ | P | NP |
| 5-8 | 12 | enterolike (astro?) ++ | P | NP |
| 9-12 | 16 | enterolike (astro?) + | P | NP |
| 13-16 | 20 | enterolike (astro?) + | P | NP |
| 17-20 | 24 | rotavirus ++ | N | P |
| 21-24 | 28 | rotavirus +++ | N | P |
| **CONTROL** | | | | |
| **Samples** | **Age (d)** | **EM** | **AsTV RT-PCR** | **AvRV RT-PCR** |
| 1-4 | 8 | Neg | NP | NP |
| 5-8 | 12 | Neg | NP | NP |
| 9-12 | 16 | enterovirus-like + | P | N |
| 13-16 | 20 | Neg | NP | NP |
| 17-20 | 24 | rotavirus + | N | P |
| 21-24 | 28 | Neg | NP | NP |
| 25-28 | 32 | Neg | NP | NP |
| 29-32 | 36 | Neg | NP | NP |

## 6. DISCUSSION AND CONCLUSIONS

Rotaviruses have been associated with intestinal disease in commercial poultry, although their exact role in the pathogenesis of disease has not yet been completely defined (Reynolds *et al.*,1987; Mc Nulty, 2003; Day *et al.*, 2007; Pantin-Jackwood *et al.*, 2007; Reynolds & Schultz- Cherry, 2008). Despite the wide distribution among several avian species and the economic impact due to rotavirus associated enteritis in poultry flocks, AvRVs have not been investigated as thoroughly as mammalian rotaviruses (Guy, 1998; Pantin-Jackwood *et al.*, 2007). Epidemiological information and genetic characterization of circulating AvRVs may instead be helpful in managing enteric disease outbreaks and in the implementation of control measures in affected flocks.

This PhD study was aimed at monitoring rotavirus distribution in different avian species, not only commercial, but also game/hunting ones. The results are important because uncovering diversity among strains is a crucial point, in order to better understand RV ecology in the field and to obtain the best management solutions on practice.

Overall, the data collected during nine years, from 2006 to 2014, indicate that the infection has an average prevalence of 20% in commercial flocks and 15% in game-hunting species. The infection is widespread in both fields and in the Italian production in general (data are representative of the Central-Northern part of Italy).

Regarding the analysis of symptoms and lesions, all the 117 samples analysed in the present study originated from poultry flocks that presented one or more clinical signs and lesions associated with enteric diseases. Clinical manifestations consisted mostly of diarrhoea, dehydration, anorexia, weight loss and increased mortality. Pathological lesions similar to those observed in the analyzed sample set have been previously reported in the course of viral intestinal diseases of young birds (Barnes *et al.*, 2000; Mc Nulty, 2003). The high frequency of single RV infection detected by nsEM analysis of faeces and intestinal contents of birds in this study seems to confirm the important role of RVs in the aetiology of enteric diseases in commercial avian species in Italy. At the same time, the concomitant detection of other enteric viruses and entero-pathogens in analyzed sample set supports the hypothesis of the multifactorial aetiology of enteric disease, as already highlighted in several studies (Villareal *et al.*, 2006; Roussan *et al.*, 2012; Mettifogo *et al.*, 2014; Moura-Alvarez *et al.*, 2014).

By statistical analysis it was not observed any significant correlation between the general clinical signs/lesions or gastroenteric lesions observed in the field and at *post mortem* examination with the presence of different AvRV groups or different combinations of groups, even with regard to the different species involved ($p>0.05$).

Going more into details of the strains characterization, from the 117 samples tested by group-specific RT-PCRs, AvRV-D was detected in 107 (91.5%), AvRV-A in 70 (59.9%), AvRV-F in 61 (52.1%) and AvRV-G in 31 (26.5%). Only 20 samples (17.1%) showed the presence of a single rotavirus group (AvRV-A or AvRV-D), but dual or multiple presence of rotaviruses of different AvRV groups was observed in the majority of samples.

Obtained results of a higher prevalence of group D than group A avian rotavirus in northern Italy confirms previous data on group D avian rotavirus in birds obtained mostly by PAGE typing in different countries (Otto *et al.*, 2006; Karim *et al.*, 2007; Islam *et al.*, 2009; Otto *et al.*, 2012). Group F and G avian rotaviruses have previously been detected in sick chickens and turkeys, although their incidence in birds is generally lower than that for avian group A and D rotaviruses (Otto *et al.,* 2006; Johne *et al.,* 2011; Otto *et al.,*2012). In this study, the identification of several groups F and G avian rotaviruses in partridge, pheasant and guinea fowl, in addition to chicken and turkey species, is also noteworthy, and suggests that the lack of specific detection methods could have underestimated the real distribution of these two additional virus groups in the past.

A fundamental point of this study was to try and give answers to the need for screening diagnostic protocols; in order to rapidly and correctly detect all circulating strains. New methods for a correct, punctual and more rapid grouping were also addressed. In the present study all the 117 RV-positive clinical samples within the current avian rotavirus groups (A, D, F and G) were successfully characterized by using new, updated group-specific RT-PCRs, developed on circulating strains. Although it does not distinguish between different rotavirus groups, screening of avian samples by direct EM with negative staining has prompted to investigate further by molecular approaches, the faecal samples where RVs had been detected. No typing by genomic RNA migration profile in polyacrylamide gel electrophoresis was possible for all the analyzed samples. The low sensitivity obtained by PAGE in the analyzed sample set could be explained by the fact that the electropherotyping technique needs micrograms of undamaged viral RNA, as already described (Bezerra *et al.*, 2012, Otto *et al.*, 2012). Moreover, the presence of mixed RNA migration patterns by PAGE may complicate the correct interpretation of the results (Todd & McNulty, 1986; Desselberger, 1996), which in fact occurred in two of the samples tested in this survey.

The use of new group-specific RT-PCRs developed and performed on a set of clinical specimens tested positive for RVs by EM, allowed the rapid genetic screening of AvRVs circulating within the avian rotavirus main groups.

In this study, 175 complete sequences were obtained (17 of NSP4A, 20 of VP6A, 5 of VP4A, 35 of NSP4D, 34 of VP6D, 9 of VP4D, 17 of VP7D, 21 of VP6F and 13 of VP6G). No correlation between year of isolation or avian species and the different RV-groups was observed. On the other hand, a high degree of heterogenicity for the four segments analyzed was reported. Interestingly, four segments of the same sample clustered in different clades, phylogenetically distant, making suppose of a re-assortment phenomena.

Regarding results obtained from longitudinal studies in turkeys, the infection was already present in the first weeks of life; after 2/3 weeks a second infection outbreak happened in the same herds. The detection of a high number of samples from both individual birds and from pooled samples, with multiple AvRVs of different groups might explain the high variability of clinical signs and lesions recorded in the turkey flocks investigated. However, since most samples consisted of pools originating from two or more birds, this finding indicates the simultaneous infection of AvRVs belonging to different groups in the same poultry flock, but does not necessary imply possible co-infections occurring in a same animal. Therefore, different pathogenicity of different AvRVs groups cannot be inferred from this report. Nevertheless, it should be considered that co-circulation of different AvRV strains within a flock may favour multiple infection of the same bird, which may eventually result in the generation of novel RV strains by reassortment of genome segments (Desselberger, 1996). This possibility was suggested to occur between group A, D and F AvRVs, due to similar terminal sequences in the genome segments of these rotavirus groups (Trojnar *et al.,* 2010; Johne *et al.,* 2011). The hypothesis that hatchery and/or the breeding flock could serve as possible risk factors, needs more investigations.

In chicken longitudinal study, the same viruses were found in both flocks (9-12 days for AstV and 17-20 days for RVs), but with a lower frequency in the “control” one. In the “case” flock both RVs and clinical manifestations were detected, with a higher frequency and for a longer time. These results reinforce the hypothesis of a primary pathogenetic role of astrovirus-rotavirus infections in enteric sindrome during first weeks of life.

The results of this study provide the basis for further genomic studies. In the future, complete genome sequencing of AvRVs isolates might allow the identification of strains belonging to groups that are less common in the bird population and may contribute to finding possible correlations with rotaviruses isolated in mammals. Epidemiological information and genetic characterization of circulating AvRVs may instead be helpful in managing enteric disease outbreaks and in the implementation of control measures in affected flocks.

## 7. REFERENCES


1. Agresti A. 2002. Categorical Data Analysis. Hoboken, New Jersey: John Wiley &Sons.
2. Ahmed M.S., Ahmed M.U. 2006. Detection of avian rotavirus-like virus in broiler chickens in Bangladesh. Bang J Vet Med. 4:73-77.
3. Andral B., Toquin D., L'haridon R., Jestin A., Metz M.H., Rose R. 1985. Les diarrhees du dindonneau: Unbilan des recherches virales effectuees (rotavirus, reovirus, adenovirus, pseudopicornavirus). Avian Pathol. 14:147-162.
4. Attoui H., Mertens P.P.C., Becnel J., Belaganahalli S., Bergoin M., Brussaard C.P., Chappell J.D., Ciarlet M., del Vas M., Dermody T.S., *et al.* 2012. Reoviridae. In: King A, Adams M, Carstens E, Lefkwitz E, editors. Virus taxonomy, Ninth report of the international committee on taxonomy of viruses. Waltham (MA): Elsevier Academic Press; p. 497-650.
5. Ball J.M., Tian P., Zeng C.Q., Morris A.P., Estes M.K.1996. Age-dependent diarrhea induced by a rotaviral nonstructural glycoprotein. Science. 272:101-104.
6. Barnes H.J. 1997. Virology of enteric infections. Diseases of poultry. 10th Edn., Ames: Iowa state University press; pp. 685-686.
7. Barnes H.J., Guy J.S. & Vaillancourt J.P. 2000. Poult enteritis complex. Rev Sci Tec. 19: 565-588.
8. Barrios H., Viora S., de Franceschi M., Fliess E. 1991. Incidence of rotavirus in intensive-production poultry farms. Rev Argent Microbiol 23: 15-21.
9. Bergeland M.E., McAdaragh J.P., Stotz I. 1977. Rotaviral enteritis in turkey poults. In: Proceedings of the 26th Western Poultry Diseases Conference. Davis (CA): University of California. pp. 129-120.
10. Beserra L.A.R., Barbosa B.R.P., Bernardes N.T.C.G., Brandao P.E., Gregori F. 2014. Occurrence and characterization of rotavirus a in broilers, layers, and broiler breeders from Brazilian Poultry Farms. Avian Dis. 58:153-157.
11. Beserra L.A. & Gregori F. 2014. Description of rotavirus F in broilers from Brazilian poultry farms. Avian Dis. 58: 458-461.
12. Bezerra D.A., da Silva R.R., Kaiano J.H., Silvestre R.V., de Souza Oliveira D., Linhares A.C., Gabbay Y.B., Mascarenhas J.D. 2012. Detection of avian group D rotavirus using the polymerase chain reaction for the VP6 gene. J Virol Methods.185:189-192.

13. Boone S.A., Gerba C.P. 2007. Significance of fomites in the spread of respiratory and enteric viral disease. App Env Microbiol.73:1687-1696.
14. Borgan M.A., Mori Y., Ito N., Sugiyama M., Minamoto N. 2003. Antigenic analysis of nonstructural protein (NSP) 4 of group A avian rotavirus strain PO-13. Microbiol Immunol 47: 661-668.
15. Brussow H., Nakagomi O., Gerna G., Eichhorn W. 1992a. Isolation of an avian like group A rotavirus from a calf with diarrhea. J Clin Microbiol. 30: 67-73.
16. Brussow H., Nakagomi O., Minamoto N., Eichhorn W. 1992b. Rotavirus 993/83, isolated from calf feces, closely resembles an avian rotavirus. J Gen Virol. 73: 1873-1875.
17. Cao D., Barro M., Hoshino Y. 2008. Porcine rotavirus bearing an aberrant gene stemming from an intergenic recombination of the NSP2 and NSP5 genes is defective and interfering. J Virol. 82: 6073-6077.
18. Chandra R., Rao V.D.P., Gomez Villamandos J.C., Garg S.K. 2001. Astrovirus infections. In: Diseases of poultry and their control. Lucknow: International Book Distributing Co. p. 283.
19. Chauhan R.S., Dhama K., Mahendran M. 2008. Pathobiology of rotaviral diarrhea in calves and its diagnosis and control: A review. J Immunol Immunopathology 10: 1-13.
20. Cowley D., Donato C.M., Roczo-Farkas S., Kirkwood C.D., 2013. Novel G10P[14]rotavirus strain, Northern Territory, Australia. Emerg Infect Dis. 19: 1324-1327.
21. Day J.M., Spackman E., Pantin-Jackwood M. 2007. A multiplex RT-PCR test for the differential identification of turkey astrovirus type 1, turkey astrovirus type 2, chicken astrovirus, avian nephritis virus, and avian rotavirus. Avian Dis. 51:681-684.
22. De Grazia S., Bonura F., Colomba C., Cascio A., Di Bernardo F., Collura A., Terranova D.M., Martella V., Giammanco G.M., 2014. Data mining from a 27-years rotavirus surveillance in Palermo, Italy. Infect Genet Evol. pii:S1567-1348(14)00083-5.
23. Desselberger U. 2014. Rotaviruses. Virus Res. 190, 75-96.
24. Desselberger U., 1996. Genome rearrangements of rotaviruses. Adv Virus Res., 46: 69-95.
25. Dey B.K. 2003. Molecular epidemiologic studies on rotaviruses in calves, goats and poultry birds [dissertation]. Bangladesh: Department of Medicine, Mymensingh: Agricultural University.

26. Dhama K., Chauhan R.S., Mahendran M., Malik S.V.S. 2009. Rotavirus diarrhea in bovines and other domestic animals. Vet Res Commun. 33: 1-23.
27. Dhama K.,Saminathan M., Kumaragurubaran K., Tiwari R., Shabbir M.Z., Kumar N., Malik Y. S. and Singh R. K. 2015. Avian rotavirus enteritis - an updated review, Vet Quart. 35(3): 142-158.
28. Dodet B., Heseltine E., Mary C., Saliou P. 1997. Rotaviruses in human and veterinary medicine. Sante 7: 195-199.
29. Donker N.C., Boniface K., Kirkwood C.D., 2011. Phylogenetic analysis of rotavirus ANSP2 gene sequences and evidence of intragenic recombination. Infect Genet Evol. 11: 1602-1607.
30. Estes M.K. & Cohen J. 1989. Rotavirus gene structure and function. Microbiol Rev. 53: 410-449.
31. Estes M.K. & Kapikian A.Z. 2007. Rotaviruses. In Knipe, D.M., Howley, P.M., Griffin, M.A., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E. (eds.). Fields Virology. 5th ed. Lippincott, Williams and Wilkins, Philadelphia, PA. pp. 1917-1974.
32. Estes M.K., Greenberg H.B., 2013. Rotaviruses. In: Knipe, D.M., Howley, P.M., *et al.*(eds.). Fields Virology. 6th ed. Wolters Kluwer Health/Lippincott Williams and Wilkins, Philadelphia, PA. pp. 1347-1401.
33. Estes M.K., Palmer E.L., Obijeski J.F. 1983. Rotaviruses: a review. Current Topics in Microbiol Immunol. 105: 123-184.
34. Flewett T.H., Bryden A.S., Davies H., Woode G.N., Bridger J.C., Derrick J.M. 1974. Relation between viruses from acute gastroenteritis of children and new born calves. Lancet. 7872: 61-63.
35. Gough R.E., Collins M.S., Alexander D.J., Cox W.J. 1990. Viruses and virus-like particles detected in samples from diseased game birds in Great Britain during 1988. Avian Pathol. 19: 331-343.
36. Greenberg H.B., Estes M.K., 2009. Rotaviruses: from pathogenesis to vaccination. Gastroenterol. 136: 1939-1951.
37. Guy J.S. 1998. Virus infections of the gastrointestinal tract of poultry. Poultry Sci. 77: 1166-1175.
38. Hamilton J.R., Gall D.G. 1982. Pathophysiological and clinical features of viral enteritis. In: Tyrrell DAJ, Kapikian AZ, eds. Virus infections of the gastrointestinal tract. New York (NY): Marcel Dekker; pp. 227-238.

39. Haynes J.S., Reynolds D.L., Fagerland J.A., Fix A.S.1994. Morphogenesis of enteric lesions induced by group D rotavirus in ring neck pheasant chicks (Phasianus colchicus). Vet Pathol. 31:74-81.
40. Hemida M.G. 2013. Detection and molecular characterization of group D avian rota virus in Kafrelsheikh and Gharbia Governorates. Alexandria J Vet Sci. 39(1):145-154.
41. Hemming M., Vesikari T., 2013a. Genetic diversity of G1P[8] rotavirus VP7 and VP8*antigens in Finland over a 20-year period: No evidence for selection pressure by universal mass vaccination with RotaTeq®vaccine. Infect Genet Evol. 19: 51-58.
42. Holland R.E. 1990. Some infectious causes of diarrhea in young farm animals. Clinical Microbiol Rev 3: 345-375.
43. Horrox N.E. 1980. Some observations and comments on rotaviruses in turkey poults. 29th West Poult Dis Conf. pp. 162-164.
44. Hoshino Y., Kapikian A.Z. 1996. Classification of rotavirus VP4 and VP7 serotypes. Arch Virol. 12: 99-111.
45. Ianiro G., Heylen E., Delogu R., Zeller M., Matthijnssens J., Ruggeri F.M., Van Ranst M., Fiore L., 2013. Genetic diversity of G9P[8] rotavirus strains circulating in Italy in 2007 and 2010 as determined by whole genome sequencing. Infect Gen Evol. 16: 426-432.
46. Islam M.S., Alam M.M., Ahmed M.U., Saifuzzaman A.B.M., Kobayashi N., Kayesh M.E.H. and Uddin M.S. 2009. Molecular epidemiological study on rotavirus infection in human and birds in association with gastroenteritis. Bangladesh J Vet Med. 7(1): 233-237.
47. Iturriza-Gomara M., Desselberger U., Gray J. 2003. Molecular epidemiology of rotaviruses: genetic mechanisms associated with diversity. In: Desselberger, U., Gray, J. (Eds.), Viral Gastroenteritis. Elsevier Science, Amsterdam, pp. 317-344.
48. Iturriza-Gómara M., Green J., Brown D.W., Desselberger U., Gray J.J., 2000. Diversity within the VP4 gene of rotavirus P[8] strains: implications for reverse transcription-PCR genotyping. J Clin Microbiol 38: 898-901.
49. Iturriza-Gómara M., Isherwood B., Desselberger U., Gray J., 2001. Reassortment in vivo: driving force for diversity of human rotavirus strains isolated in the United Kingdom between 1995 and 1999. J Virol. 75: 3696-3705.
50. Jackwood M.J., Spackman E., Day J.M., Rives D. 2007. Periodic monitoring of commercial turkeys for enteric viruses indicates continuous presence of astrovirus and rotavirus on the farms. Avian Dis. 51: 674-680.

51. Jayaram H., Estes M.K., Prasad B.V., 2004. Emerging themes in rotavirus cell entry, genome organization, transcription and replication. Virus Res. 101: 67-81.
52. Jere K.C., Mlera L., Page N.A., van Dijk A.A., O'Neill H.G., 2011. Whole genome analysis of multiple rotavirus strains from a single stool specimen using sequence-independent amplification and 454®pyrosequencing reveals evidence of inter genotype genome segment recombination. Infect Genet Evol. 11: 2072-2082.
53. Jindal N., Chander Y., Patnayak D.P., Mor S.K., Ziegler A.F., Goyal S.M.. 2012. A multiplex RT-PCR for the detection of astrovirus, rotavirus, and reovirus in turkeys. Avian Dis 56: 592-596.
54. Jindal N., Patnayak D.P., Chander Y., Ziegler A.F., Goyal S.M. 2009. Experimental reproduction of poult enteritis syndrome: Clinical findings, growth response, and microbiology. Poult Sci. 88: 949-958.
55. Jindal N., Patnayak D.P., Chander Y., Ziegler A.F., Goyal S.M. 2010. Detection and molecular characterization of enteric viruses from poult enteritis syndrome in turkeys. Poult Sci. 89: 217-226.
56. Johne R., Otto P., Roth B., Lohren U., Belnap D., Reetz J., Trojnar E. 2011. Sequence analysis of the VP6-encoding genome segment of avian group F and G rotaviruses. Virol. 412: 384-391.
57. Jones R.C., Hughes C.S., Hentry R.R. 1979. Rotavirus infection in commercial laying hens. Vet Rec. 104: 22.
58. Kapikian A.Z., Hoshino Y., Chanock R.M. 2001. Rotaviruses. In Knipe, D.M., Howley, P.M., Griffin, M.A., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E. (Eds.). Fields Virology. 5th ed. Lippincott, Williams and Wilkins, Philadelphia, PA. pp.1787-1833.
59. Karim M.R., Rume F.I., Alam M.M., Ahmed M.U. 2007. Molecular epidemiologic study on avian rota virus prevailing in Bangladesh. Bang J Vet Med. 5: 43-48.
60. Kattoor J.J., Malik Y.S., Sharma K., Kumar N., Batra M., Jindal N., Singh A. 2013b. Molecular evidence of group D rota virus in commercial broiler chicks in India. Avian Biol Res 6:313-316.
61. Kattoor J.J., Sharma K., Kumar N., Batra M., Jindal N., Yadav A., Sand Malik Y.S. 2013a. Distribution frequency of avian rotaviruses in India, 2011-2013. Adv Anim Vet Sci.1 (5): 148-150.

62. Kindler E., Trojnar E., Heckel G., Otto P.H. & Johne R. 2013. Analysis of rotavirus species diversity and evolution including the newly determined full-length genome sequences of rotavirus F and G. Inf Gen Evol. 14: 58-67.
63. Kusumakar A.L., Savita, Malik Y.P.S., Minakshi Prasad G. 2010. Genomic diversity among group A rotaviruses from diarrheic children, piglets, buffalo and cow calves of Madhya Pradesh. Indian J Microbiol. 50: 83-88.
64. Lavazza A. 1989. Tecniche di colorazione negativa in microscopia elettronica per la diagnosi di Rotavirus: raffronto con il metodo ELISA. Selezione Veterinaria, 30: 755-765.
65. Lavazza A., Cerioli M., Catelli E., Cecchinato M., Murgia M.V., Battilani M., Tosi G., Moreno Martin A. & Vinco L. 2005. Electron Microscopy Identification of Virus Detected in Cases of Enteritis of Birds in Italy and Preliminary Characterization of Rotavirus Identified in Turkey. Proceedings of the 14th Congress of the World Veterinary Poultry Association Istanbul (Turkey). p.228.
66. Lavazza A., Pascucci S. & Gelmetti D. 1990. Rod-shaped virus-like particles in intestinal contents of three avian species. Vet Rec. 126: 581.
67. Legrottaglie R., Rizzi V. & Agrimi P. 1997. Isolation and identification of avian rotavirus from pheasant chicks with signs of clinical enteritis. Comp Immunol, Microbiol and Infectious Dis. 20: 205-210.
68. Lublin A., Mechani S., Bumbarov V. 2004. Involvement of rotavirus in intestinal infections of poultry and pet birds. Abstract presented at the 28th annual Israel veterinary symposium in memory of Dr. Ora Egozi, 2004. Isr J Vet Med. 59: 3.
69. Lundgren O., Peregrin A.T., Persson K., Kordasti S., Uhnoo I., Svensson L., 2000. Role of the enteric nervous system in the fluid and electrolyte secretion of rotavirus diarrhea. Science 287: 491-495.
70. Maes P., Matthijnssens J., Rahman M., Van Ranst M., 2009. Rota C: a web-based tool for the complete genome classification of group A rotaviruses. BMC Microbiol. 9: 238.
71. Malik Y.P.S., Kumar N., Sharma K., Sharma R., Kumar H.B., Kusumakar A., Kumari S., Shukla S., Chandrashekar, K.M. 2013. Epidemiology and genetic diversity of rotavirus strains associated with acute gastroenteritis in bovine, porcine, poultry and human population of Madhya Pradesh, Central India, 2004-2008. Adv Anim Vet Sci 1: 111-115.

72. Malik Y.P.S., Sharma K., Vaid N., Chakravarti S., Chandrashekar K.M., Basera S.S., Singh R., Minakshi Prasad S., Gulati B.R., *et al.* 2012. Frequency of group A rotavirus with mixed G and P genotypes in bovines: predominance of G3 genotype and its emergence in combination with G8/G10 types. J Vet Sci 13: 271-278.
73. Marlier D. and Vindevogel H. 2006. Viral infections in pigeons. Vet J. 172: 40-51.
74. Martella V., Bányai K., Matthijnssens J., Buonavoglia C., Ciarlet M., 2010. Zoonotic aspects of rotaviruses. Vet Microbiol. 140: 246-255.
75. Martínez-Laso J., Román A., Rodriguez M., Cervera I., Head J., Rodríguez-Avial I., Picazo J.J., 2009. Diversity of the G3 genes of human rotaviruses in isolates from Spain from 2004 to 2006: cross-species transmission and inter-genotype recombination generated alleles. J Gen Virol. 90: 935-943.
76. Matthijnssens J., Ciarlet M., Heiman E., Arijs I., Delbeke T., McDonald S.M., Palombo E.A., Iturriza-Gómara M., Maes P., Patton J.T., Rahman M., Van Ranst M., 2008a. Full genome-based classification of rotaviruses reveals a common origin between human Wa-Like and porcine rotavirus strains and human DS-1-likeand bovine rotavirus strains. J Virol. 82: 3204-3219.
77. Matthijnssens J., Ciarlet M., McDonald S.M., Attoui H., Bányai K., Brister J.R., Buesa J., Esona M.D., Estes M.K., *et al.* 2011a. Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG). Arch Virol. 156: 1397-1413.
78. Matthijnssens J., Ciarlet M., Rahman M., Attoui H., Bányai K., Estes M.K., Gentsch J.R., Iturriza-Gómara M., Kirkwood C.D., Martella V., Mertens P.P., Nakagomi O., Patton J.T., Ruggeri F.M., Saif L.J., Santos N., Steyer A., Taniguchi K., Desselberger U., Van Ranst M., 2008b. Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. Arch Virol. 153: 1621-1629.
79. Matthijnssens J., De Grazia S., Piessens J., Heylen E., Zeller M., Giammanco G.M., Bányai K., Buonavoglia C., Ciarlet M., Martella V., Van Ranst M., 2011b. Multiple reassortment and interspecies transmission events contribute to the diversity of feline, canine and feline/canine-like human group A rotavirus strains. Infect Genet Evol. 11: 1396-1406.
80. Matthijnssens J., Otto P.H., Ciarlet M., Desselberger U., Van Ranst M. & Johne R. 2011c. VP6-sequence-based cutoff values as a criterion for rotavirus species demarcation. Arch of Virol. 157: 1177-1182.

81. Matthijnssens J., Van Ranst M., 2012. Genotype constellation and evolution of group A rotaviruses infecting humans. Curr Opin Virol. 2: 426-433.
82. McNulty M.S. 1997. Rotavirus infections. In: Saif Y.M., Barnes H.J., Glisson J.R., Fadly A.M., McDougald L.R., Swayne D.E. editors. Diseases of poultry. 10th Edn. Ames: Iowa State University Press. pp. 692-701.
83. McNulty M.S. 2003. Rotavirus infections. In Saif, Y.M., Barnes, H.J., Glisson, J.R., Fadly, A.M., McDougald, L.R., Swayne, D.E. editors. Diseases of poultry 11th Edn. Ames: Iowa State University Press. pp. 308-317.
84. McNulty M.S., Allan G.M., Stuart J.C. 1978. Rotavirus infection in avian species. Vet Rec. 103: 319-320.
85. McNulty M.S., Allan G.M., Todd D., McFerran J.B. 1979. Isolation and cell culture propagation of rotavirus from turkeys and chickens. Arch Virol. 61: 13-21.
86. McNulty M.S., Allan G.M., Todd D., McFerran J.B., McCracken R.M. 1981. Isolation from chickens of a rotavirus lacking the rotavirus group antigen. J Gen Virol. 55: 405-413.
87. McNulty M.S., Todd D., Allan G.M., McFerran J.B., Greene J.A. 1984. Epidemiology of rotavirus infection in broiler chickens: recognition of four serogroups. Arch Virol. 81: 113-21.
88. Mettifogo E., Nuñez L.F., Chacón J.L., Santander Parra S.H., Astolfi-Ferreira C.S., Jerez J.A., Jones R.C., Piantino Ferreira A.J. 2014. Emergence of enteric viruses in production chickens is a concern for avian health. Scientific World J. 2014: 450423.
89. Minamoto N., Yuki M. 1988. Inactivation of several animal viruses by glutaraldehyde. J Jap Vet Med Assoc. 41: 497-501.
90. Moon H.W. 1978. Mechanisms in the pathogenesis of diarrhea: a review. J Am Vet Med Assoc. 172: 443-446.
91. Mori Y., Borgan M.A., Ito N., Sugiyama M., Minamoto N. 2002. Diarrhea-inducing activity of avian rotavirus NSP4 glycoproteins, which differ greatly from mammalian rotavirus NSP4 glycoproteins in deduced amino acid sequence, in suckling mice. J Virol. 76: 5829-5834.
92. Mori Y., Sugiyama M., Takayama M., Atoji Y., Masegi T., Minamoto N. 2001. Avian-to-mammal transmission of an avian rotavirus: analysis of its pathogenicity in a heterologous mouse model. Virol. 288: 63-70.

93. Moura-Alvarez J., Nuñez L.F., Astolfi-Ferreira C.S., Knöbl T., Chacón J.L., Moreno A.M., Jones R.C., Ferreira A.J. 2014. Detection of enteric pathogens in Turkey flocks affected with severe enteritis, in Brazil. Tropical Animal Health Prod. 46: 1051-1058.
94. Mullick S., Mukherjee A., Ghosh S., Pazhani G.P., Sur D., Manna B., Nataro J.P., Levine M.M., Ramamurthy T., Chawla-Sarkar M., 2013. Genomic analysis of human rotavirus strains G6P[14] and G11P[25] isolated from Kolkata in 2009 reveals interspecies transmission and complex reassortment events. Infect Genet Evol. 14: 15-21.
95. Niture G.S., Karpe A.G., Prasad M., Bhonsle A.V., Patil S.V. 2010. Detection of group D avian rotaviruses among layer poultry from Western India. Int J Poultry Sci. 9: 72-76.
96. Ntafis V., Fragkiadaki Eir., Xylouri E., Omirou A., Lavazza A., Martella V. 2010. Rotavirus-associated diarrhoea in foals in Greece. Vet Microbiol. 144: 461- 465.
97. Otto P., Liebler-Tenorio E.M., Elschner M., Reetz J., Löhren U. & Diller R. 2006. Detection of rotaviruses and intestinal lesions in broiler chicks from flocks with runting and stunting syndrome (RSS). Avian Dis. 50: 411-418.
98. Otto P.H., Ahmed M.U., Hotzel H., Machnowska P., Reetz J., Roth B., Trojnar E. & Johne R. 2012. Detection of avian rotaviruses of groups A, D, F and G in diseased chickens and turkeys from Europe and Bangladesh. Vet Microbiol. 156: 8- 15.
99. Pantin-Jackwood M.J., Spackman E., Day J.M. & Rives D., 2007. Periodic monitoring of commercial turkeys for enteric viruses indicates continuous presence of astrovirus and rotavirus on the farms. Avian Dis. 51: 674-680.
100. Papp H., Borzák R., Farkas S., Kisfali P., Lengyel G., Molnár P., Melegh B., Matthijnssens J., Jakab F., Martella V., Bányai K., 2013. Zoonotic transmission of reassortant porcine G4P[6] rotaviruses in Hungarian pediatric patients identified sporadically over a 15 year period. Infect Genet Evol. 19: 71-80.
101. Parra G.I., Bok K., Martínez M., Gomez J.A., 2004. Evidence of rotavirus intragenic recombination between two sub-lineages of the same genotype. J Gen Virol. 85: 1713-1716.
102. Pascucci S. & Lavazza A. 1994. A survey of enteric viruses in commercial avian species: experimental studies of transmissible enteritis of guinea fowl. In: M.S. McNulty, J.B. McFerran, editors. New and Evolving Virus Diseases of Poultry. Brussels: Commission of the European Communities. pp. 225-241.

103. Perry R.W., Rowland G.N., Glisson J.R., Steffens W.L. and Quinn J.A. 1991. Skeletal lesions associated with a naturally occurring poult enteritis. Avian Dis. 35: 158-164.

104. Phan T.G., Okitsu S., Maneekarn N., Ushijima H., 2007. Evidence of intra-genic recombination in G1 rotavirus VP7 genes. J Virol. 81: 10188-10194.

105. Reynolds D.L., Saif Y.M., Theil K.W. 1987. A survey of enteric viruses of turkey poults. Avian Dis. 31: 89-98.

106. Reynolds D.L., Schultz-Cherry S.L. 2008. Astrovirus infections. In: Diseases of Poultry. 12th Ed, Blackwell Publishing, Ames, Iowa. pp. 351-355.

107. Reynolds D.L., Theil K.W. & Saif Y.M. 1987. Demonstration of rotavirus and rotavirus-like virus in the intestinal contents of diarrheic pheasant chicks. Avian Dis 31: 376-379.

108. Rodriguez C.A.R., Brandao P.E., Ferreira F., Buzinaro M.G., Jerez J.A. 2004. Improved animal rotavirus isolation in MA-104 cells using different trypsin concentrations. Arquivos Do Instituto Biologico. 71: 437-441.

109. Rohwedder A., Hotop H., Minamoto N., Ito H., Nakagomi O., Brussow H. 1997. Bovine rotavirus 993/83 shows a third subtype of avian VP7 protein. Virus Genes. 14: 147-151.

110. Rohwedder A., Schutz K.I., Minamoto N., Brussow H. 1995. Sequence analysis of pigeon, turkey, and chicken rotavirus VP8 _identifies rotavirus 993/83, isolated from calf feces, as a pigeon rotavirus. Virol. 210: 231-235.

111. Rotavirus Classification Working Group, 6th meeting, Valencia, October 2013.

112. Roussan D.A., Shaheen I.A., Khawaldeh G.Y., Totanji W.S., Al-Rifai R.H. 2012. Simultaneous detection of astrovirus, rotavirus, reovirus and adenovirus type I in broiler chicken flocks. Pol J Vet Sci. 15: 337-344.

113. Saif L.J., Fernandez F.M.. 1996. Group A rotavirus veterinary vaccines. J Infect Dis. 174: 98-106.

114. Saif L.J., Jiang B. 1994. Non-group A rotaviruses of humans and animals. Curr Top Microbiol Immunol. 185: 339-371.

115. Saif L.J., Saif Y.M., Theil K.W. 1985. Enteric viruses in diarrhoeic turkey poults. Avian Dis. 29: 798-811.

116. Santos N., Hoshino Y., 2005. Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine. Rev Med Virol. 15: 29-56.

117. Savita, Kusumakar A.L., Malik Y.P.S., Prasad M. 2008a. Detection and characterization of group A and D avian rotaviruses in India. Indian J Biotechnol. 7: 554-556.

118. Schumann T., Hotzel H., Otto P., Johne R. 2009. Evidence of interspecies transmission and reassortment among avian group A rotaviruses. Virol. 386: 334-343.

119. Shawky S.A., Saif Y.M., McCormick J. 1994. Transfer of maternal anti-rotavirus IgG to the mucosal surfaces and bile of turkey poults. Avian Dis. 38: 409-417.

120. Shawky S.A., Saif Y.M., Swayne D.E. 1993. Role of circulating maternal anti-rotavirus IgG in protection of intestinal mucosal surface in turkey poults. Avian Dis. 37: 1041-1050.

121. Simmonds M.K., Armah G., Asmah R., Banerjee I., Damanka S., Esona M.,Gentsch J.R., Gray J.J., Kirkwood C., Page N., Iturriza-Gómara M., 2008. New oligonucleotide primers for P-typing of rotavirus strains: Strategies for typing previously un typeable strains. J Clin Virol. 42: 368-373.

122. Soma J., Tsunemitsu H., Miyamoto T., Suzuki G., Sasaki T., Suzuki T. 2013. Whole-genome analysis of two bovine rotavirus C strains: Shintoku and Toyama. J Gen Virol. 94: 128-135.

123. Spackman E., Day J.M., Pantin Jackwood M.J. 2010. Astrovirus, reovirus and rotavirus concomitant infection causes decreased weight gain in broad-breasted white poults. Avian Dis. 54: 16-21.

124. Steyer A., Poljsak-Prijatelj M., Barlic-Maganja D., Marin J., 2008. Human, porcine and bovine rotaviruses in Slovenia: evidence of interspecies transmission and genome reassortment. J Gen Virol. 89: 1690-1698.

125. Steyer A., Sagadin M., Kolenc M., Polj˘sak-Prijatelj M., 2013. Whole genome sequence analysis of bovine G6P[11] rotavirus strain found in a child with gastroenteritis. Infect Genet Evol. 13: 89-95.

126. Svensson L., Uhnoo I., Garndien M., Wadell G. 1986. Molecular epidemiology of rotavirus infection in Upsala, Sweden, 1981: Disappearance of a predominated electropherotype. J Clin Microbiol. 18: 101-111.

127. Takase K., Nonaka F., Sakaguchi M. & Yamada S. 1986. Cytopathic avian rotavirus isolated from duck faeces in chicken kidney cell cultures. Avian Path. 15: 719-730.

128. Takehara K., Kiuchi H., Kuwahara M., Yanagisawa F., Mizukami M., Matsuda H., Yoshimura M. 1991. Identification and characterization of a plaque forming avian rotavirus isolated from a wild bird in Japan. J Vet Med Sci. 53: 479-486.

129. Tamehiro C.Y., Alfhyieri A.F., Medici K.C., Alfieri A.A. 2003. Segmented double-stranded genomic RNA viruses in fecal samples from broiler chicken. Braz J Microbiol. 34: 344-348.

130. Tang Y., Ismail M.M., Saif Y.M. 2005 Development of antigen-capture enzyme-linked immunosorbent assay and RT-PCR for detection of turkey astroviruses. Avian Dis. 49: 182-188.

131. Theil K.W., Reynolds D.L., Saif Y.M. 1986. Isolation and serial propagation of turkey rotaviruses in a fetal rhesus monkey kidney (MA104) cell line. Avian Dis. 30: 93-104.

132. Theil K.W., Saif Y.M. 1987. Age-related infections with rotavirus, rotaviruslike virus, and atypical rotavirus in Turkey flocks. J Clin Microbiol. 25: 333-337.

133. Todd D., McNulty M.S. 1986. Electrophoretic variation of avian rotavirus RNA in polyacrylamide gels. Avian Pathol. 15: 149-159.

134. Todd S., Page N.A., Duncan Steele A., Peenze I., Cunliffe N.A., 2010. Rotavirus strain types circulating in Africa: review of studies published during 1997–2006. J. Infect. Dis. 202 (Suppl.), S34-S42.

135. Trojnar E., Otto P., Johne R. 2009. The first complete genome sequence of a chicken group A rotavirus indicates independent evolution of mammalian and avian strains. Virol. 386: 325-333.

136. Trojnar E., Otto P., Roth B., Reetz J., Johne R. 2010. The genome segments of a group D rota virus possess Group-A like conserved termini but encode group-specific proteins. J Virol. 84: 10254-10265.

137. Urasawa S., Hasegawa A., Taniguchi T., Wakasugi F., Suzuki H., Inouve S., Pongprot B., Supawadee J., Suprasert S., Rangsiaynond P., Tonusin S., Yamazi Y. 1992. Antigenic and genetic analyses of human rotaviruses in Chiang Mai, Thailand: evidence for a close relationship between human and animal rotaviruses. J Infect Dis. 166: 227-234.

138. Ursu K., Papp H., Kisfali P., Rigo D., Melegh B., Martella V., Banyai K. 2011. Monitoring of group A rotaviruses in wild living birds in Hungary. Avian Dis. 55: 123-127.

139. Villarreal L.Y.B., Uliana G., Valenzuela C., Chacon J.L.V., Saidenberg A.B.S., Sanches A.A., Brandao P.E., Jerez J.A., Ferreira A.J.P. 2006. Rotavirus detection and isolation from chickens with or without symptoms. Braz J Poultry Sci. 8: 187-191.

140. Wani S.A., Bhat M.A., Ishaq S.M., Ashrafi M.A., Buchh A.S., Haq M. 2003. Detection of a mammalian-like group A rotavirus in diarrhoeic chicken. Vet Microbiol. 94:13-18.

141. Woolcocka P.R. and Shivaprasad H.L. 2008. Electron microscopic identification of viruses associated with poult enteritis in turkeys grown in California 1993-2003. Avian Dis. 52: 209-213.

142. Yamamoto D., Ghosh S., Kuzuya M., Wang Y.H., Zhou X., Chawla-Sarkar M., Paul S.K., Ishino M. and Kobayashi N. 2011. Whole-genome characterization of human group C rotaviruses: identification of two lineages in the VP3 gene. J gen virol 92: 361-369.

143. Yang H., Makeyev E.V., Kang Z., Ji S., Bamford D.H., van Dijk A.A. 2004. Cloning and sequence analysis of dsRNA segments 5, 6 and 7 of a novel non-group A, B, C adult rotavirus that caused an outbreak of gastroenteritis in China. Virus Res, 106: 15-26.

144. Yason C.V., Schat K.A. 1985. Isolation and characterization of avian rotaviruses. Avian Dis. 29: 499-508.

145. Yason C.V., Schat K.A. 1986a. Pathogenesis of rotavirus infection in turkey poults. Avian Pathol. 15: 421-435.

146. Yason C.V., Schat K.A. 1986b. Experimental infection of specific pathogen- free chickens with avian rotaviruses. Avian Dis. 30: 551-556.

147. Yason C.V., Schat K.A. 1987. Pathogenesis of rotavirus infection in various age groups of chickens and turkeys: clinical signs and virology. Am J Vet Res. 48: 977-983.

148. Yegani M., Korve D.R. 2008. Factors affecting intestinal health in poultry. Poultry Sci. 87: 2052-2063.

149. Yu M., Ismail M.M., Qureshi M.A., Dearth R.N., Barnes H.J., Saif Y.M. 2000. Viral agents associated with poult enteritis and mortality syndrome: the role of a small round virus and a turkey coronavirus. Avian Dis. 44: 297-304.

## 8. ACKNOWLEDGMENTS

The first thank is always to my family that supports me as a real fan, and is always on my side in every decision and path that I undertake, helping me without affecting my freedom, I love you all.

Thanks to all my collaborators in this project; they helped me in filling my gaps and limits.

A special thank to my tutor Prof. Turin, who was always available and present in helping me and who was extremely patient with all my delays. Thanks a lot also to Dr. Lavazza, who went along with me through the Ph.D, and who was responsible of introducing me to the avian pathology world and who helped me to go over the exciting challenges of electron microscopy. Thanks also to Dr. Chiara Busi, who worked a lot for sequencing and phylogenetically analyzing samples. Thanks to Cristina and Gianni from the EM lab; they helped me in searching for very “ancient” samples not only on papers, but also in the depths of freezers.

Finally thanks to Dr. Paolo Cordioli, who decided with me for this Ph.D program and who allowed me to start this path; my work is dedicated to him.