Szent István University

Doctoral School of Veterinary Science

Epidemiological study of viral pathogens incriminated in enteric disease complexes in Hungarian broiler flocks, with special emphasis on the newly identified parvovirus

Doctoral thesis

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Content

Abbreviations

aa	amino acid
AAdV1	avian adenovirus group 1
AAAV	avian adeno-associated virus
AN	avian nephritis
ANV	avian nephritis virus
ARV	avian reovirus
AvRV	avian rotavirus
BLAST	basic local alignment search tool
bp	base pair
CAstV	chicken astrovirus
ChPV	chicken parvovirus
$CsCl_2$	cesium chloride
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
ds RNA	double stranded ribonucleic acid
E. coli	*Escherichia coli*
ED	enteric disease
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
GI	gastrointestinal
h	hour
H&E	hematoxylin and eosin
HE	hemorrhagic enteritis
HEV	hemorrhagic enteritis virus
IB	infectious bronchitis
IBV	infectious bronchitis virus
IBDV	infectious bursal disease virus
IF	immunofluorescence
IHC	immunohistochemistry
ISH	*in situ* hybridization

kb	kilobase
$MgCl_2$	magnesium chloride
M&H	Mayer's hematoxylin
min	minute
mM	micromolar
mPCR	multiplex polymerase chain reaction
mRT-PCR	multiplex reverse transcriptase polymerase chain reaction
mRNA	messenger RNA
N	nucleocapsid (gene/protein)
nm	nanometer
no	number
ORF	open reading frame
PCR	polymerase chain reaction
PEC	poult enteritis complex
PEMS	poult enteritis and mortality syndrome
pi	post infection
RDP	recombination detection program
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RSS	runting-stunting syndrome
RT-PCR	reverse transcription polymerase chain reaction
sec	second
SN	serum neutralization
TAstV	turkey astrovirus
TEM	transmission electron microscopy
TuPV	turkey parvovirus
USA	United States of America
UV	ultraviolet
VN	virus neutralization
VP	viral protein
µl	microlitre
µm	micrometer
°C	degrees Celsius

Summary

Samples collected between 2007 and 2010 from chicken and turkey carcasses, originating from Hungarian commercial flocks with clinical signs of enteric disease (ED), increased mortality and poor production parameters, were tested by histopathology, indirect immunohistochemistry (IHC), electron microscopy and methods applying polymerase chain reaction (PCR) techniques, with the goal of determining the epidemiology and pathogenesis of the ED syndrome. PCR and reverse transcription PCR (RT-PCR) were employed to directly demonstrate viral pathogens currently associated with ED syndromes in chickens and turkeys: astroviruses, reoviruses, rotaviruses, coronaviruses, adenoviruses and parvovirus. Among the objectives of this study was to directly demonstrate the presence of the scarcely known chicken parvovirus (ChPV) and turkey parvovirus (TuPV) in Hungarian chicken and turkey flocks experiencing clinical signs of ED. ChPV and TuPV infection was demonstrated in 15 chicken and 25 turkey flocks. The histopathological investigation revealed enteritis in the duodenum and jejunum, and atrophy of the lymphoid organs.

The work also presents an indirect IHC method for the diagnosis of ChPV and TuPV. The results obtained by indirect IHC suggested the intestinal epithelium of chickens and turkeys as a potential replication site for the viruses, similarly to other parvoviruses, while in case of the turkey samples, IHC positivity was also observed in the bursa of Fabricii, liver and pancreas.

Phylogenetic analysis on a 524 base pairs (bp) long region from the NS1 gene of the ChPV strains, and 527 bp long region from TuPV strains respectively, revealed two main clusters, a ChPV and a TuPV group, but also the presence of a divergent branch of tentatively named “TuPV-like ChPV strains”. The 23 Hungarian TuPV strains were positioned separately from the American origin sequences in the TuPV cluster, in two groups, while the 15 Hungarian ChPV strains did not present any specific grouping.

A restriction fragment length polymorphism (RFLP) assay was developed for the fast differentiation of TuPV, ChPV and divergent, TuPV-like ChPV strains. The differences between the *AvaII* digestion pattern of parvovirus strains belonging to the TuPV, ChPV and TuPV-like groups, seem to provide a quick and reliable differentiation of all these strains

without the need for nucleic acid sequencing. In the future, the newly described protocol could be used to rapidly obtain valuable epidemiological data, and will turn out to be even more practical in case potential differences in the pathogenicity of these strains should be revealed by future pathogenesis studies.

A high number of Hungarian chicken broiler flocks were determined to be coinfected with avian nephritis virus (ANV), infectious bronchitis virus (IBV) and infectious bursal disease virus (IBDV). Due to the high prevalence of this coinfection, a diagnostic method based on a multiplex RT-PCR (mRT-PCR) assay was also developed in this study for the direct demonstration in field samples of ANV, IBV and IBDV.

A total of 28 chicken flocks and 51 turkey flocks from distinct geographical regions of Hungary, were investigated by direct methods for the presence of viral pathogens incriminated in ED, with the goal of determining the epidemiology and pathogenesis of the syndrome. By including a broad range of viral agents in the determination, we are confident that the present study contributes in clarifying the epidemiology of a current economic threat for the poultry industry, the ED syndrome, with the potential of being useful for to the elaboration of comprehensive preventive measures, to avoid in the future the serious losses that the poultry industry is suffering due to this syndrome.

Összefoglalás (Summary in Hungarian)

Kutatásaink során enterális kórkép (enteric disease: ED) tüneteit mutató, fokozott elhullással és nem megfelelő takarmányhasznosítási paraméterekkel küszködő állományok csirke és pulyka tetemeiből származó, 2007 és 2010 között gyűjtött mintákat kórszövettani, immunhisztokémiai, (IHC) elektronmikroszkópos és polimeráz láncreakció (PCR) alapú vizsgálatnak vetettük alá, a korábban említett ED kórkép járványtanának és kórfejlődésének vizsgálata és jobb megismerése céljából. A PCR-alapú eljárások segítségével az ED kórkép csirke- és pulykaállományokban történő kialakulásával szoros összefüggésbe hozott vírusos kórokozók, pontosabban astrovírusok, reovírusok, rotavírusok, coronavírusok, adenovírusok és parvovírusok kimutatását kíséreltük meg. Vizsgálataink további célja a kevésbé ismert csirke és pulyka parvovírus (chicken parvovirus: ChPV, illetve turkey parvovirus: TuPV) kimutatása volt ED tüneteit mutató magyarországi csirke- és pulykaállományokban. ChPV és TuPV fertőzést 15 csirke- és 25 pulykaállományban sikerült igazolni. A pozitív állományokból származó mintákban a kórszövettani vizsgálat során vékonybélgyulladást és a lymphoid szervek sorvadása volt megfigyelhető.

Vizsgálataink során a ChPV és TuPV kimutatására alkalmas IHC eljárás kidolgozására is sor került. Az újonnan kidolgozott IHC eljárás alapján a többi parvovírushoz hasonlóan, a ChPV és TuPV multiplikációja nagy valószínűséggel a csirke és pulyka bélhámsejtjeiben zajlik. A pulyka eredetű minták esetében IHC pozitivitás a Fabricius-tömlőben, a májban és a hasnyálmirigyben is megfigyelhető volt.

A ChPV törzsek esetében az NS1 gén 524 bázispár (bp), míg a TuPV törzsek 527 bp hosszú szakaszán végzett filogenetikai vizsgálat alapján a vírustörzsek két, jól elkülönülő, ChPV illetve TuPV csoportba rendeződtek, ugyanakkor a filogenetikai fán egy külön ágon helyeződő, „TuPV-szerű ChPV" törzsek csoportja is felismerhető volt. A 23 magyarországi minta a TuPV csoport két alcsoportjába rendeződött, az amerikai eredetű mintáktól elkülönülve, míg a 15 magyarországi ChPV törzs esetében semmilyen jellegzetes csoportosulás nem volt megfigyelhető.

A restrikciós fragmentumhossz polimorfizmus (restriction fragment length polymorphism: RFLP) alapú eljárást a TuPV, ChPV, illetve a TuPV-szerű ChPV törzsek gyors elkülönítése céljából dolgoztuk ki. A TuPV, ChPV, illetve TuPV-szerű ChPV törzsek

estében kapott PCR termékek különböző *Ava*II hasítási tulajdonságai alapján az ezekbe a csoportokba tartozó törzsek nukleinsav-szekvencia meghatározása nélkül is gyorsan, megbízható módon elkülöníthetők. Az újonnan kidolgozott RFLP alapú eljárás a jövőben nagy segítséget nyújthat a járványtani felmérésekben, főleg abban az esetben, főleg ha a későbbiekben a különböző törzsek pathogenitásában különbségek megállapítására sor kerül.

A magyarországi brojlerállományok vizsgálata során számos esetben igazoltuk az állományok a csirkék fertőző vesegyulladásának vírusával (avian nephritis virus: ANV), a fertőző bronchitis virusával (infectious bronchitis virus: IBV), illetve a fertőző bursitis vírusával (infectious bursal disease virus: IBDV) történő egyidejű fertőződését. Az egyidejű fertőzések viszonylag nagy gyakorisága következtében az említett kórokozók klinikai, vagy szervmintákban történő gyors és megbízható kimutatásának céljából egy multiplex RT-PCR (mRT-PCR) alapú diagnosztikai eljárást dolgoztunk ki.

Összesítve, vizsgálataink során Magyarország különböző földrajzi régióiban található 28 csirke- és 51 pulykaállományból származó mintákat vizsgáltunk az ED kórképben szerepet játszó vírusos kórokozók kimutatása céljából. Meggyőződésünk, hogy a számos kórokozóra kiterjedő vizsgálataink eredményei hozzájárulnak a baromfiállományokban fokozott gazdasági veszteségeket okozó kórkép járványtanának és kórfejlődésének jobb megismeréséhez, és ezáltal hozzájárul a kórkép által okozott veszteségek megelőzéséhez szükséges intézkedések feltárásában.

1. Introduction

Expansion of modern farming techniques and facilities has lead to more than 50 billion chickens raised annually as a source of food. The increasing need for poultry meat at affordable price for the general population created farms with impressive capacities, which lead to an increasing infectious pressure on individuals.

Due to intense research, vaccines were developed to control and prevent common diseases in poultry flocks, hence to avoid economic losses in the form of mortality, poor production or poor feed conversion efficiency. However in the last decade new, complex syndromes have appeared, due to the still increasing pressure on individuals, who are now living in farms with up to 30 000 individuals.

As the general European trend for the poultry meat consumption is increasing, one can easily conclude that poultry meat is, and will continue to be part of the population's menu for years to come, making poultry production and meat biosecurity a constant challenge for veterinarians and researchers alike.

The newly emerged enteric disease (ED) syndromes, causing serious economic losses throughout the world, have become a challenge for researchers in the field of poultry diseases. Determining their exact etiology, and elucidating their pathogenesis, has the potential of finding treatment and prevention strategies, hence avoiding economic losses and improving the biosecurity of the poultry meat. The purpose of this study is to contribute to the clarifying of the etiology and pathogenesis of the ED syndromes of chicken and turkey raised under intensive conditions, by providing research data regarding these syndromes, from Hungarian commercial flocks.

2. Literature survey

2.1. Poultry production trends

According to the Food and Agriculture Organization of the United Nations (FAO) statistics, the poultry meat consumption in Hungary has decreased in the last years and in the same time the population number has been constant or slightly decreased, however the meat production quantity has increased (Figure 1). On European level, even if the population has been fairly constant in the last 10 years, the poultry meat consumption per person has doubled (http://www.fao.org/corp/statistics/en/, accessed on 4th of February, 2011).

	Human population (millions)			Poultry consumption (kg/person/year)	
	2000	2010	2015	2000	2007
Albania	3.1	3.2	3.3	5.8	9.9
Andorra	0.1	0.1	0.1	-	-
Austria	8.0	8.4	8.5	17.1	17.5
Belarus	10.1	9.6	9.4	9.1	17.8
Belgium	10.2	10.7	10.9	19.7	25.1
Bosnia/Herzegovina	3.7	3.8	3.7	4.2	7.6
Bulgaria	8.0	7.5	7.3	16.9	20.0
Croatia	4.5	4.4	4.4	6.9	12.8
Czech Rep	10.2	10.4	10.5	22.3	24.7
Denmark	5.3	5.5	5.5	17.4	18.3
Estonia	1.4	1.3	1.3	17.7	17.3
Faroe Isl	0.1	0.1	0.1	-	-
Finland	5.2	5.4	5.4	12.6	17.2
France	59.1	62.6	63.9	26.5	21.1
Germany	82.1	82.1	81.4	13.1	15.6
Gibralter	#	#	#	-	-
Greece	10.9	11.2	11.3	13.4	13.8
Holy See	#	#	#	-	-
Hungary	10.2	10.0	9.9	34.1	27.6
Iceland	0.3	0.3	0.4	11.3	25.8
Ireland	3.8	4.6	4.9	30.9	25.5
Italy	57.1	60.1	60.6	18.9	15.9
Latvia	2.4	2.2	2.2	10.3	20.7
Liechtenstein	#	#	#	-	-
Lithuania	3.5	3.3	3.1	9.8	25.0
Luxembourg	0.4	0.5	0.5	46.7	39.9
Macedonia Rep	2.0	2.0	2.1	12.3	19.5
Malta	0.4	0.4	0.4	15.8	24.8
Moldova	4.1	3.6	3.5	5.1	12.1
Monaco	#	#	#	-	-
Montenegro	-	0.6	0.6	-	5.4
Netherlands	15.9	16.7	16.9	12.6	14.9
Norway	4.5	4.9	5.0	9.6	14.9
Poland	38.4	38.0	37.8	14.5	20.3
Portugal	10.2	10.7	10.8	26.8	25.3
Romania	22.1	21.2	20.8	13.0	19.5
Russian Federation	146.7	140.4	138.0	9.8	22.3
SanMarino	#	#	#	-	-
Serbia	-	9.9	9.8	-	7.0
Serbia/Montenegro	10.8	-	-	8.7	-
Slovakia	5.4	5.4	5.4	12.4	18.1
Slovenia	2.0	2.0	2.0	29.0	19.9
Spain	40.3	45.3	47.2	25.3	27.6
Sweden	8.9	9.3	9.5	10.1	14.8
Switzerland	7.2	7.6	7.7	12.8	15.0
Ukraine	48.9	45.4	44.2	4.4	17.4
United Kingdom	59.1	62.1	63.8	28.5	29.1
European Union	**48.1**	**49.8**	**50.3**	**19.9**	**20.8**
Europe	**72.7**	**73.3**	**73.4**	**11.2**	**20.3**
World	**611.5**	**690.9**	**730.2**	**10.9**	**12.6**

Figure 1: Human population of Europe and poultry meat consumption (based on FAO)

2.2. Enteric disease syndrome in chickens

The viral ED complex is a serious economic problem in the poultry industry. In broiler chickens the major ED complex is known as malabsorbtion or runting-stunting syndrome (RSS) (Page *et al*., 1982; Goodwin *et al*., 1993; Barnes *et al*., 2000; Barnes and Guy, 2003).

The RSS is characterized by diarrhea, depression, ingestion of litter, increased vocalization and huddling. Morbidity and mortality are variable, and the economic impact is primarily due to poor production, failure of affected birds to grow, as well as increase in costs of therapy, and poor feed conversion efficiency, but in the severe forms, immune dysfunction and increased mortality have been reported (Day and Zsak, 2010).

Viruses from numerous families have been identified in the intestinal tracts of poultry with ED: *Astroviridae*, *Coronaviridae*, *Reoviridae*, *Rotaviridae* (Pass *et al*., 1982; Reynolds *et al*., 1987a, b; Goodwin *et al*., 1993; Guy, 1998; Koci *et al*., 2000; Yu *et al*., 2000; Spackman *et al*., 2005a, b; Pantin-Jackwood *et al*., 2007a, b; Day *et al*., 2007a, b; Jones, 2008; Reynolds and Shultz-Cherry, 2008; Pantin-Jackwood *et al*., 2008a), and more recently *Parvoviridae* (Kisary *et al.*, 1984; Woolcock and Shivaprasad, 2008; Zsak *et al*., 2008; Zsak *et al*., 2009; Day and Zsak 2010). The role of these viruses in the ED is not fully understood (Zsak *et al*., 2008), but is supported by the syndrome reproducibility with preparations from the intestinal contents of affected birds, that do not contain bacteria or protozoa (Barnes and Guy, 2003).

Most enteric viral infections in chicken broilers occur in the first three weeks of life, but it was reported that under certain conditions some could occur later (Saif, 2008). Since the clinical signs and lesions induced by the different viral agents are similar, it is difficult to attribute a specific viral disease to a given virus. In addition to this difficulty, different combination of the incriminated viruses will result in different presentation of the same disease, making the diagnosis extremely difficult. In general it is considered that a combination of high morbidity and low mortality happens when only one virus is detected, and high mortality combined with various economic losses, takes place when several viruses are detected (Saif, 2008). Different viruses replicate in different parts of the small intestine, and different sites on the intestinal villi, but due to the secondary infections, most of them of bacterial origin, the histopathological findings will be masked, hence these information are difficult to interpret in naturally occurring ED. Epidemiological studies have indicated that these viruses do not persist for long in birds, and no evidence of egg

transmission of enteric viruses exists (Saif, 2008). Active immunity seems to play a role in limiting these infections; still the benefits of passive immunity are limited to the first days of life.

However, because the incriminated viruses have been directly demonstrated in both healthy and diseased flocks, suggesting that a certain combination of pathogens and/or factors can lead to the ED (Pantin-Jakwood *et al*., 2008b; Zsak *et al*., 2009), the diagnosis of this syndrome remains difficult. No comprehensive treatment or preventive measures have been determined for RSS because of the lack of information regarding the exact etiology of the disease and its pathogenesis, and no commercial vaccines are available for most of these infections (Saif, 2008).

Most information regarding ED were derived from studies on turkeys, because of the higher economic importance of these syndromes caused by enteric viruses in commercial turkey poults, but lately there has been an increasing interest in studying ED in young chicken broilers because the emergence of ED as an economically significant problem in chicken broiler production (Saif, 2008).

2.3. Enteric disease syndrome in turkeys

Despite the intense research regarding the ED, a major disease complex seriously threatening the turkey industry especially by the produced economic losses, the causative agents of the syndrome are not completely identified, and the pathogenesis still remains unclear.

In turkey poults up to 6 weeks of age, the syndrome is referred to as poult enteritis complex (PEC), characterized by diarrhea, depression, ingestion of litter, immunosuppression and increased mortality. In case of high mortality the disease is referred to as poult enteritis and mortality syndrome (PEMS). In the most severe forms of PEMS, up to 100% morbidity and mortality was reported (Page *et al.*, 1982; Guy, 1998; Goodwin *et al.*, 1993; Barnes *et al.*, 2000; Barnes and Guy, 2003).

The etiology of the disease is not completely understood, but is considered multifactorial. Numerous viral and bacterial agents were identified in the intestine of poults with PEC and PEMS. From the bacterial group, enteropathogenic strains of *Escherichia*

coli (*E. coli*) are considered to contribute in the manifestation of the disease (Guy *et al.*, 2000).

Turkey coronaviruses, astroviruses and reoviruses have been isolated from flocks suffering from PEMS (Guy *et al.*, 2000; Heggen-Peay *et al.*, 2002; Koci *et al.*, 2002), and the potential involvement of turkey parvovirus (TuPV) has also been suspected (Zsak *et al.*, 2009). As a food animal, the integrity of the gastrointestinal (GI) tract in turkeys is of extreme importance. The efficient utilization of nutrients is primarily dependent on a healthy GI tract, and is considered especially true in case of young animals; hence any damage to the GI tract in the early stages of life will result in irreversible economic damage to the flock (Saif, 2008).

Turkey coronavirus (TCV), member of the group 3 coronaviruses (Cavanagh, 2001a, b; Cavanagh, 2005), was identified as the etiological agent of a highly contagious enteric disease described for the first time in 1951 named “bluecomb disease” (Ritchie *et al.*, 1973). In recent years TCV has been increasingly identified as an important cause of ED in turkeys and has been associated with PEMS (Barnes and Guy, 2003; Pantin-Jackwood *et al.*, 2007b), however studies revealed that TCV was not required for installation of PEMS (Barnes *et al.*, 1997).

Astroviruses are non-enveloped positive sense RNA, small round particles of 25-35 nm in diameter (Koci *et al.*, 2002; Koci *et al.*, 2000), and have been associated with acute gastroenteritis in mammals and turkeys as well as with hepatitis in ducks (Reynolds *et al.*, 1987a, b; Reynolds *et al.*, 2008). Astroviruses have been detected in birds with PEMS, although their exact role remains unclear (Imada *et al.*, 2000; Koci *et al.*, 2000; Yu *et al.*, 2000). Turkey astroviruses (TAstV) have been isolated from 1 to 3 weeks of age turkey poults experiencing viral enteritis (Reynolds *et al.*, 1986).

Rotaviruses, 70 to 75 nm particles with ds RNA genome, are a major cause of enteritis in a wide range of mammalian and bird species (Kapikian and Hoshino, 2001). Avian reoviruses (ARV) have been isolated from turkeys with PEMS (Heggen-Peay *et al.*, 2002; Reynolds *et al.*, 1987a, b; Jones, 2008) and also from chickens with RSS (Pass *et al.*, 1982).

Hemorrhagic enteritis virus (HEV) is a group 2 siadenovirus, which causes an acute disease in turkeys of 4 weeks of age and older, characterized by depression, bloody droppings and death (Hess *et al.*, 1999).

TCV and HEV are the only viral pathogens incriminated in ED which are known to cause disease with well identifiable clinical signs in absence of any other pathogens. No treatment or preventive measures are known for PEC or PEMS, and besides HEV, no commercial vaccines are currently available for most of these infections.

2.4. Viral pathogens incriminated in the enteric disease syndromes

2.4.1. Small round viruses

An increasing number of so-called "small round viruses" (SRVs) are implicated in decreased production and increased mortality in poultry, hence it is crucial that they be characterized to completely understand distribution and design effective control mechanisms for the poultry flocks (Reynolds *et al.*, 1987a, b; Qureshi *et al.*, 1997, 2000; Imada *et al.*, 2000; Koci *et al.*, 2000; Schultze-Cherry *et al.*, 2000; Todd *et al.*, 2000; Yu *et al.*, 2000; Cavanagh, 2001a, b; Saif *et al.*, 1990).

SRVs are typically represented by the following viral families: *Astroviridae, Parvoviridae, Circoviridae, Picornaviridae, Caliciviridae,* each with characteristic size, shape and morphologies visible by electron microscopy (EM) (Caul and Appleton, 1982). Ever since the expansion of molecular techniques, one by one the so-called SRVs are starting to be clearly determined and their involvement in ED is intensely studied.

2.4.2. Astroviruses

Astroviruses have been associated with acute gastroenteritis in mammals and turkeys as well as with hepatitis in ducks (Pomeroy *et al.*, 1978; Reynolds and Schultz-Cherry, 2008). Avian astroviruses were first identified in 1980, in intestinal content from 11-days old turkey poults with diarrhea and increased mortality (McNulty *et al.*, 1980).

Astroviruses are small icosahedral viruses, typically 25 to 35 nm in diameter. They have received their name from the 5 or 6 pointed star-like surface projections observed by EM. However only 10% of astroviruses in a population may present this morphology and visualization depends on sample preparation. Astroviruses are non-enveloped, positive sense RNA viruses, with a viral genome of 6.5-7.5 kb long and consisting of three open

reading frames (ORF). Human astrovirus, different strains of TAstV, swine astrovirus and different strains of ANV have their genomes fully sequenced (Reynolds and Schultz-Cherry, 2008).

Avian astroviruses are molecularly distinct, with little sequence similarity on different gene segments. Still TAstVs cluster into a distinct group from ANV. Astrovirus infections typically occur in the first 4 weeks of life. Astroviruses have been frequently detected in birds with ED, although their exact role remains unclear (Imada *et al.*, 2000; Koci *et al.*, 2000; Tang *et al.*, 2005)

2.4.2.1. ANV

ANV is the causative agent of a disease in young chickens evolving with distinct kidney lesions and enteritis (Mándoki *et al.*, 2006), and was recently identified for the first time in commercial turkey flocks, although its importance in this species is not yet fully determined (Pantin-Jackwood *et al.*, 2008b).

Based on its genetic structure, ANV is classified as a new genus member of the *Astroviridae* virus family. Young chickens are the only animals known to develop clinical disease and distinct kidney lesions due to ANV. Different serotypes and even strains from the same serotype can produce different clinical signs. Recent studies reported that infectious bursal disease virus (IBDV) infections can enhance the pathogenicity of ANV infection in chickens (Reynolds and Schultz-Cherry, 2008).

Transmission of ANV occurs by direct or indirect contact with infected feces. Clinical signs have been described in 1 day old chicks and consist of transient diarrhea (Narita *et al.*, 1990; Imada and Kawamura, 1997). The clinical signs under field conditions may vary from none, in case of subclinical infections, to the so-called RSS and “baby chick nephropathy”. No data exist regarding clinical signs in turkeys (Pantin-Jackwood *et al.*, 2008a).

Gross lesions

Macroscopic changes include: mild to severe discoloration of the kidneys and sometimes visceral urate deposits. Mortality is considered to be influenced by the ANV strain, bird hybrid, other concurrent infections, and environmental conditions (Reynolds and Schultz-Cherry, 2008).

Histopathological lesions

In case of ANV infections, histopathological lesions have been studied mainly experimentally. In the initial stage of infection, necrosis and degeneration of the epithelial cells of the proximal convoluted tubules with granulocytic infiltration have been observed. Interstitial lymphocyte infiltration and moderate fibrosis was also described. Distinct microscopic lesions were not observed in the intestine, but studies employing immunofluorescence (IF) have found specific viral antigens in the small intestine (Reynolds and Schultz-Cherry, 2008).

Diagnosis, treatment, prevention

For confirmation and direct demonstration of an ANV infection RT-PCR-based methods are most recommended (Mándoki *et al.*, 2006; Reynolds and Schultz-Cherry, 2008).

There is no specific treatment, prevention or control methods for ANV infections, and lately, big emphasis is put on the possible economic implications for the poultry industry, especially in cases of coinfections with two or more enteric pathogens, or with immunosuppressive agents such as chicken anemia virus (CAV) or IBDV (Reynolds and Schultz-Cherry, 2008).

2.4.2.2. TAstV

TAstVs have been isolated from turkey poults 1 to 3 weeks of age experiencing viral enteritis (Reynolds and Schultz-Cherry, 2008). Clinical signs of disease usually develop between 1 and 3 weeks of age and last 10 to 14 days. They typically include diarrhea, litter eating, and nervousness. Severity of the disease ranges from mild to moderate and generally there is only a slight mortality.

The main concern in case of infections with TAstVs is the morbidity, occurring as decreased growth, known also as stunting. A TAstV from turkey flocks with severe signs of PEMS was isolated and referred to as TAstV-2, and proven to be molecularly distinct from the original TAstVs. TAstV infection results in an overall growth depression of the affected birds (Reynolds and Schultz-Cherry, 2008). TAstV is considered widely distributed (Saif *et al.*, 1985; Reynolds and Saif, 1986; Reynolds *et al.*, 1987b).

The PEMS-associated TAstV-2 was originally isolated from the thymus of infected poults (Schultz-Cherry *et al.*, 2000). In experimentally infected poults thymus and bursal atrophy was observed, and previous studies have shown that the virus can be isolated from

other tissues, although replication is only routinely detected in the intestines (Behling-Kelly *et al.*, 2001).

Gross lesions

In experimental infections, turkeys infected with TAstV-2 develop a profuse watery diarrhea by 2 days post infection (pi), which can continue through 12 days pi. The intestine of infected poults was found 3 to 5 times larger then their control, and were dilated, distended and filled with fluid. Characteristic pathological changes in case of TAstV infection are dilated ceca with yellowish frothy content and gaseous fluid (Reynolds and Schultz-Cherry, 2008).

Histopathological lesions

Mild crypt hyperplasia resulting in increased crypt depth and area is the main histopathological lesion in case of TAstV infection. The lesions were observed in the proximal jejunum as early as day 1 pi and by 5 days pi in all of the small intestine. *In situ* hybridization (ISH) studies revealed that replication of TAstV-2 is restricted to the intestine. Still TAstV-2 was isolated from bursa, spleen, thymus, kidney, skeletal muscle, pancreas and plasma.

In case of TAstV-2 infections the histopathological lesions include: degenerating enterocytes along the basal edge of the villi, mild shortening of the villi and occasional clusters of necrotic enterocytes along the villous base. The lack of inflammatory response may result from the increase in transforming growth factor-beta, a suppressor of inflammation in infected intestine (Reynolds and Schultz-Cherry, 2008).

Diagnosis, treatment, prevention

Immune EM was the main method of diagnosis in case of astrovirus infections, but ever since the expansion of molecular methods, RT-PCR is considered a far more reliable, faster and more sensitive technique. No vaccine, treatment or other measures are reported to be effective for control and/or prevention of TAstVs infections (Reynolds and Schultz-Cherry, 2008).

2.4.3. Parvoviruses

2.4.3.1. ChPV and TuPV

In the 1980's the potential involvement of parvoviruses in the ED was suspected (Trampel *et al.*, 1983; Kisary *et al.*, 1984). Kisary and his co-workers (1984) demonstrated by EM the presence of parvovirus-like particles in intestinal homogenates of 10 days old chickens with ED (Kisary *et al.*, 1984), and by inoculating 1 day old chickens with the purified viral particles, obtained the characteristic clinical signs of RSS (Kisary, 1985a). The following molecular biological studies revealed that the virus belongs to the *Parvoviridae* family (Kisary *et al.*, 1985).

Parvoviruses have a linear single-stranded DNA, between 4 and 6 kb; they are non-enveloped, icosahedral virions of approximately 20 nm in diameter (Tattersall, 2006). They encode two major genes (Figure 2): a nonstructural gene (NS1), which appears to be conserved within parvoviruses, and it is used as a target for PCR-based diagnosis, and a structural viral protein (VP1) gene (Cotmore and Tattersall, 2006). An ELISA based test was also developed to demonstrate the presence of maternally derived parvovirus-specific antibodies in chicken serum samples and virus-specific antibodies in chicken sera following infection (Strother and Zsak, 2009).

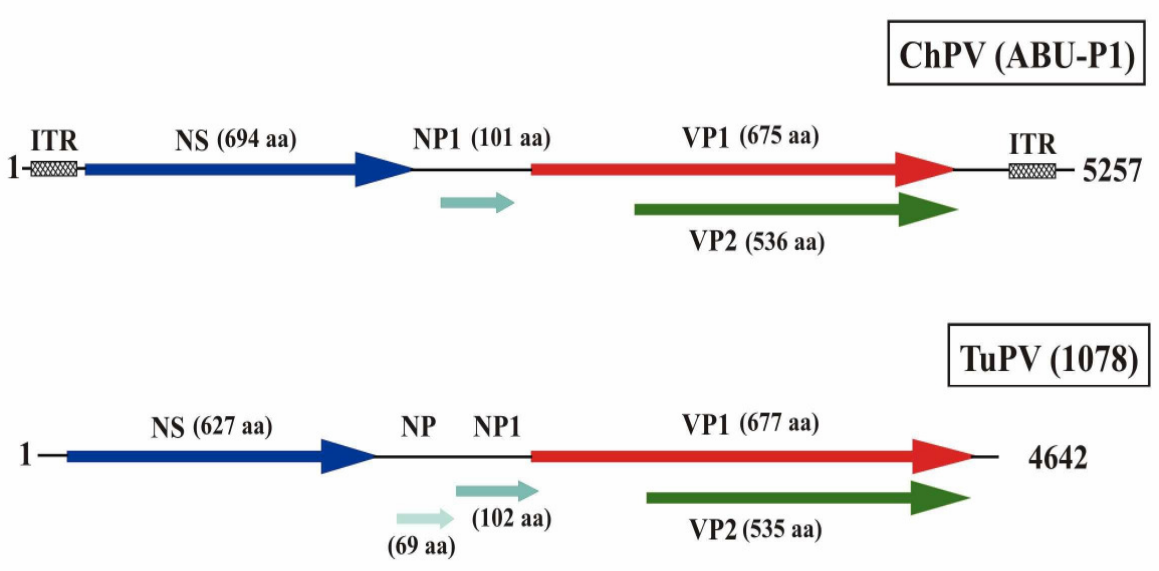


Figure 2: Graphical presentation of the genome organization of ChPV (ABU P1) and TuPV (1078). NP: nonstructural protein. ITR: inverted terminal repeat (based on Day and Zsak, 2010)

The full length genome of the newly involved parvoviruses (Figure 2) was recently determined and analyzed (Day and Zsak, 2010). Day and Zsak (2010) compared the ChPV and the closely related TuPVs across their entire coding region with representative members of the *Parvovirinae* subfamily: *Dependovirus*, *Bocavirus*, *Erythrovirus*, *Amdovirus* and *Parvovirus* genera. From the multiple sequence alignments and phylogenetic analysis they concluded that ChPV, along with the closely related TuPVs, represents a distinct member of the *Parvovirinae* subfamily and suggest it should be recognized as the prototypical member of a novel genus within the *Parvovirinae* (Figure 3).

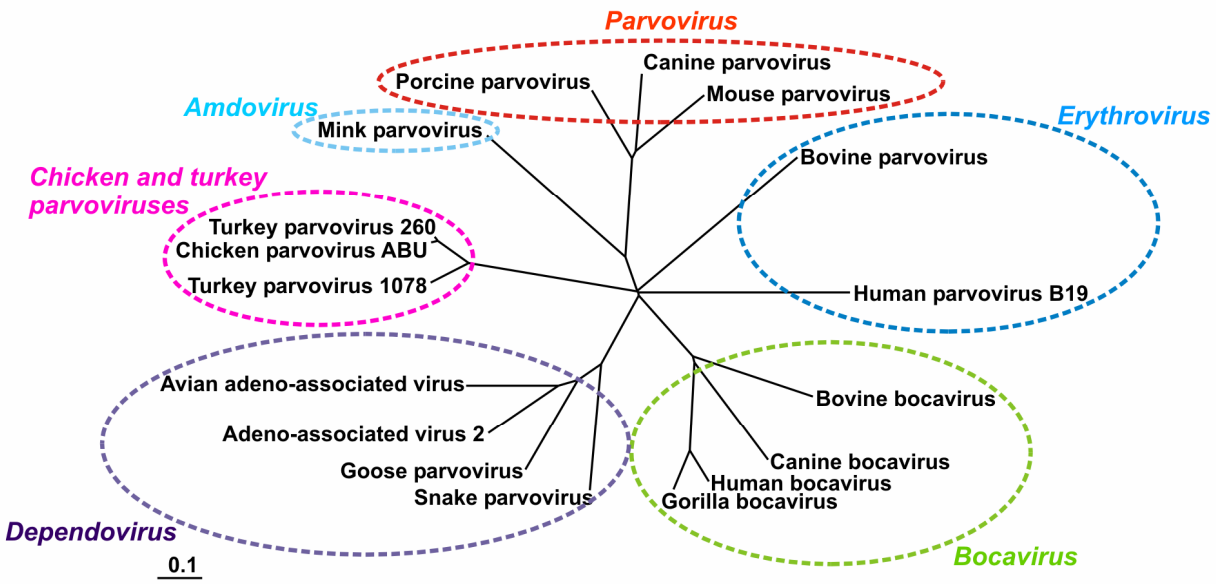


Figure 3: Phylogenetic tree constructed using the full coding sequence of the indicated parvoviruses. Representative genera within the *Parvovirinae* are indicated. GenBank Accession numbers for the used sequences: canine parvovirus: NC001539; porcine parvovirus: NC001718; mink parvovirus: NC001662; turkey parvovirus 260: GU214706; chicken parvovirus ABU: GU214704; turkey parvovirus 1078: GU214705; AAAV: NC004828; adeno-associated virus 2: NC001401; goose parvovirus: NC001701; snake parvovirus 1: NC006148; gorilla bocavirus: NC014358; human bocavirus: NC007455; canine bocavirus: NC004442; bovine bocavirus: NC001540; human parvovirus: B19 NC000883; bovine parvovirus: NC006259; mouse parvovirus 1: NC001630. (based on Day and Zsak, 2010)

The high amino acid identities noted by the authors among the turkey and chicken isolates were considered suggestive for the divergence at sometime in the recent past from a common ancestor.

As a comparison with a similar situation, another member of the *Parvovirinae*, canine parvovirus (CPV), despite its single stranded DNA genome, has a mutation rate that approaches that of RNA viruses, which allows rapid evolution and host adaptation (Hoelzer *et al.*, 2008; Shackelton *et al.*, 2005). In the same study, following phylogenetic analysis it was expected that ChPV and TuPV will group with the parvoviruses that infect geese (GoPV) and Muscovy ducks (MdPV), but that hypothesis proved to be false. The authors found that the ChPV NS protein shares only 19.1% amino acid identity with the MdPV NS protein. Based on the strong genome sequence homology, it was concluded that chicken and turkey parvoviruses could have similar, or potentially identical, pathogenic properties in their respective hosts (Day and Zsak, 2010).

However the authors found no definite correlation between virus presence and disease (Zsak *et al.*, 2009). The finding was not surprising, as in case of other parvovirus infections, including GoPV and human boca parvoviruses, the DNA can be frequently detected from both healthy and diseased individuals, especially at an early stage of their life (Gough, 2003, Kahn *et al.*, 2008). A comparison was considered with the GoPV infection, where maternally acquired virus-specific antibodies play a significant role in the epidemiology of clinical disease, and the level of passive immunity determines the susceptibility of the progeny following virus infection (Gough, 2003).

According to a previous study the ChPV NS1 deduced amino acid sequence contains highly conserved motifs important for the initiation of parvovirus replication (Zsak *et al.*, 2008), including a well-conserved phosphate-binding loop motif specifically involved in the binding of nucleoside triphosphates by this protein (Saraste *et al.*, 1990). Previous studies have determined that this motif is required for pathogenesis in case of the human parvovirus B19 and is found in many parvovirus isolates (Momoeda *et al.*, 1994). The 5′ major ORF of ChPV appears to encode the viral capsid proteins VP1, VP2, and VP3. These three proteins together comprise the parvovirus virion (Muzyczka and Berns, 2001), and are responsible for the production of neutralizing antibodies during an infection (Saikawa *et al.*, 1993). Positive nuclear staining was obtained by indirect immunoperoxidase staining in the epithelial cells of the small intestine of chickens experimentally infected with the designated prototype ABU strain (Kisary *et al.* 1984;

Kisary, 2001). Immunofluorescens staining was suggested as a diagnostic tool for parvoviral infection in chicken broilers (Kisary, 1985; Kisary *et al.*, 1985).

A recent survey revealed the presence of parvovirus infection in chicken and turkey samples from 8 different states of the USA. PCR-positive samples were identified in both chicken and turkey enteric samples, between 5 days and 8 weeks of age, however the tested flocks did not presented any clinical signs of ED (Zsak *et al*., 2009). The phylogenetic analysis comparing NS1 gene segments revealed a strong similarity between the chicken and turkey parvoviruses from the USA, and that the ChPV and TuPV isolates formed distinct phylogenetic groups (Zsak *et al.*, 2009). According to the previously mentioned study (Zsak *et al.*, 2009), all of the major poultry producing states have poultry flocks where parvovirus infection was detected.

Like in the case of other parvovirus infections, chicken and turkey parvoviruses were present in very young birds, as early as 4 days of age, confirming the possibility of a potential vertical transmission of the virus. The rapid growth characteristics of parvoviruses and the horizontal transmission of the virus from infected birds to healthy susceptible individuals may also play an important role in the spread of virus infections within flocks.

The high prevalence of parvovirus infection in young birds was considered to have a potential role of these viruses in the etiology of enteric disease of poultry (Zsak *et al.*, 2009).

2.4.4. Coronaviruses

The *Coronaviridae* family is in the order *Nidovirales* and is made up of RNA viruses that infect a wide variety of avian and mammalian species. It contains viruses with linear, nonsegmented, positive-sense, single-stranded RNA genomes. Coronaviruses are spherical, pleomorphic, enveloped particles with a diameter of 60-200 nm. They possess a characteristic surface structure composed of long, widely spaced, club-shaped peplomers (Ritchie *et al.*, 1973; Siddell, 1995; Cavanagh *et al.*, 2005).

The coronavirus genome consists of an RNA molecule of approximately 30 kb, with four major structural proteins: the surface glycoprotein, an integral membrane (M) protein, a small envelope protein, and a nucleocapsid (N) protein. Based on antigenic differences identified by serological analyses coronaviruses have been divided into 3 major antigenic subgroups. Group 1 coronaviruses, which contains: human coronavirus, porcine

transmissible gastroenteritis virus, canine coronavirus, and feline infectious peritonitis virus. Group 2 coronaviruses: a second human coronavirus, murine hepatitis virus, porcine hemagglutinating encephalomyelitis virus, and bovine coronavirus. Infectious bronchitis virus (IBV) and TCV comprise group 3 coronaviruses (Cavanagh, 2005).

If TCV is a known enteric pathogen in turkeys, IBV has no involvement in the ED of poultry, however due to its nephropathogenic strains which are causing confusing clinical signs, the frequent intestinal signs found in case of infection with specific strains of IBV, and the numerous secondary infections in broiler flocks, the need of a definite differential diagnosis has emerged in case of chicken flocks with signs of ED.

2.4.4.1. TCV

TCV was identified in 1951 as the etiological agent of a highly contagious ED named "bluecomb disease". The enteritis caused by TCV is often referred to as mud fever, transmissible enteritis and coronavirus enteritis. TCV may infect turkeys of all ages, but clinical disease is observed in young turkeys, during the first few weeks of life (Guy, 2008).

Infected birds will exhibit depression, anorexia, decreased water consumption, diarrhea, dehydration and weight loss (Guy, 2008). In recent years TCV has been increasingly identified as an important cause of ED in turkeys and has been associated with PEMS, however studies revealed that TCV is not required for installation of PEMS (Carver *et al.*, 2001).

Flocks infected with TCV present increased mortality, growth depression and poor feed conversion efficiency. Mortality and morbidity are variable and depend on age, secondary infections, management practice and environmental conditions. Viral antigen in intestinal enterocytes was found predominately in enterocytes lining the upper half to two thirds of intestinal villi (Adams *et al.*, 1972; Breslin *et al.*, 2000; Guy, 2008). In the bursa of Fabricii, viral antigens are found in both the follicular and the interfollicular epithelium. In inoculated embryos, virus replication occurs exclusively in intestinal epithelial cells and epithelium of the bursa of Fabricii (Nemes *et al.*, 2008a, b). TCV is shed in feces of infected birds and spreads horizontally through ingestion of feces and feces contaminated materials. Experimental attempts to infect turkeys with homogenates of liver, heart, spleen, kidney, and pancreas of infected turkeys were unsuccessful. Turkeys were infected readily using filtered or non-filtered intestinal materials or filtered homogenates of the bursa of

Fabricii of infected turkeys. Darkling beetle larvae have been demonstrated to be potential mechanical vectors of TCV (Calibeo-Hayes *et al.*, 2003; Watson *et al.*, 2000).

Gross lesions

Gross lesions are seen primarily in intestines and bursa of Fabricii. Duodenum and jejunum generally are pale and flaccid; ceca are distended and filled with watery contents. Atrophy of the bursa of Fabricii may be observed. Emaciation and dehydration also may be observed in infected turkeys (Guy, 2008).

Histopathological lesions

Microscopic lesions are observed in intestines and bursa of Fabricii of TCV infected turkeys. In intestines, microscopic lesions in experimentally infected turkeys consist of a decrease in villous length, increase in crypt depth, and decreased intestinal diameter (Adams *et al.*, 1972; Gonder *et al.*, 1976). In poults infected with TCV the columnar epithelium of intestinal villi changes to a cuboidal epithelium, and the cells exhibit a loss of microvilli. There is a decrease in number of goblet cells, separation of enterocytes from lamina propria, and infiltration of lamina propria with heterophils and lymphocytes (Guy, 2008).

Moderate lymphoid atrophy of bursal follicles is observed; however, as TCV antigens are not detected within lymphoid follicles, it is unlikely that TCV directly damages bursal lymphoid tissue (Guy, 2008).

Diagnosis, treatment, prevention

As for most viral pathogens, molecular methods are preferred for the direct demonstration of TCV presence in a flock, however the presence of the virus does not necessarily implies clinical signs. Antibiotic treatment has been shown to reduce mortality, most likely by controlling secondary bacterial infections. At present, there is no specific treatment for TCV enteritis, and there is no approved vaccine against TCV (Guy, 2008).

2.4.4.2. IBV

IBV is a highly infectious and contagious pathogen of chicken worldwide causing a severe respiratory infection, infectious bronchitis (IB). The genome of IBV contains a single-stranded, positive sense RNA of 27.6 kb coding for three major virus-encoded structural

proteins, which include the spike (S) glycoprotein, the membrane (M) protein, and the nucleocapsid (N) protein (Lai and Cavanagh, 1997).

IBV replicates in the respiratory, GI and urogenital tract, causing various disease manifestations, and affecting both production and egg quality (Boltz *et al.*, 2004). The virus has also been shown to replicate in the Harderian gland after eyedrop inoculation. Virulence for the reproductive tract may differ among IBV strains. Presence of maternal antibody could prevent damage to the oviduct during an early-age infection. All ages are susceptible to IB, but the disease is most severe in baby chicks, causing even mortality. As age increases, chickens become more resistant to the nephropathic effects, oviduct lesions, and mortality due to infection (Cavanagh and Gelb Jr., 2008).

Gross lesions

Broiler chickens infected with one of the nephropathic viruses may appear to recover from the typical respiratory phase and then show signs of depression, ruffled feathers, wet droppings, and increased water intake (Cavanagh and Gelb Jr., 2008). Infected chickens may have serous, catarrhal or caseous exudate in the trachea, nasal passages and sinuses. Nephropathogenic infections produce changes in the urinary sytem: the tubules and ureters of affected birds will become swollen and distended by urates (Cavanagh and Naqui, 2003).

Histopathological lesions

The kidney lesions of IB are principally those of an interstitial nephritis. The virus causes granular degeneration, vacuolization and desquamation of the tubular epithelium, and massive infiltration of heterophils in the interstitium in acute stages of the disease. The lesions in tubules are most prominent in the medulla. Focal areas of necrosis may be seen as well as indications of attempted regeneration of the tubular epithelium (Dhinaker and Jones, 1997; Cavanagh and Gelb Jr., 2008).

Diagnosis, treatment, prevention

The molecular identification of IBV is based mainly on the analysis of the S1 protein gene (Lai and Cavanagh, 1997; Handberg *et al.*, 1999; Kingsham *et al.*, 2000; deWit, 2000; Cavanagh, 2001a, b, 2006; Li and Yang, 2001).

All applied treatments are generally targeting the secondary infections, most of them bacterial.

Since the first description by Schalk and Hawn (1931) in the US, many strains, which vary widely in virulence, have been identified (Fabricant, 1998). The control of IB is based on the use of live attenuated vaccines. Live vaccines are used in broilers and for the initial vaccination of breeders and layers. The live vaccines prepared from Massachusetts serotype strains provide good immunity against the homologous serotype. However, the disadvantage of the live vaccine strains is that they are spreading in the field (Meulemans *et al.*, 2001).

2.4.5. Adenoviruses

Adenoviruses are 70 to 90 nm in diameter, and are considered common infectious agents in poultry. Most of the viruses replicate in healthy birds with little or no apparent signs of infection, even if they can quickly take on the role of opportunistic pathogens when additional factors, particularly concurrent infections, affect the health of the avian host (Adair and Fitzgerald, 2008).

The adenoviruses incriminated to participate in the ED in poultry are the avian adenovirus group I (AAdVI), with the potential of causing ED in chickens, hence participating in RSS, and the hemorrhagic enteritis virus (HEV) from the group II avian adenoviruses, which causes a well determined and intensely studied disease in turkey, hemorrhagic enteritis (HE), hence having a definite role in PEC/PEMS (Table 1).

Table 1: Classification of adenoviruses

Family *Adenoviridae*		
Genus *Mastadenovirus*	Mammalian adenoviruses	human, simian, bovine, equine, murine, porcine, ovine, caprine, etc.
Genus *Aviadenovirus*	**Group I Avian adenoviruses**	conventional adenoviruses of: **chicken**, turkey, duck and goose
Genus *Siadenovirus*	**Group II Avian adenoviruses**	**hemorrhagic enteritis virus (turkeys)** marble spleen disease (pheasant) AASV (Chickens)
Genus *Atadenovirus*	Group III Avian adenoviruses	egg drop syndrome virus and related viruses

2.4.5.1. AAdV I

The subgroup I avian adenoviruses comprise the genus *Aviadenovirus* within the *Adenoviridae* virus family. The role of subgroup I adenoviruses as primary pathogens is not clearly established as different serotypes, and also strains of the same serotype, can vary in their ability to produce illness and death (Adair and Fitzgerald, 2008).

Many viral isolates have failed to cause disease when inoculated by natural routes. This finding may suggest that many adenoviruses are potential pathogens but require the presence of some other agents to allow them to cause disease. This hypothesis is of major importance in case of RSS, as the viruses incriminated in the disease have been isolated from both healthy and diseased flocks, with no apparent genetic difference in the involved strains. Previous reports have shown that coinfection with IBDV can enhance the pathogenicity of some aviadenoviruses (Adair and Fitzgerald, 2008). Aviadenovirus infection in chickens is represented by the following syndromes: food conversion and growth deficiency, respiratory disease and tenosynovitis.

Food conversion and growth deficiency

There have been reports of adenovirus infection resulting in decreased food consumption (Cook *et al.*, 1974; Cowen *et al.*, 1978). Although birds inoculated with adenovirus may have depressed body weights and even high mortality, there is little evidence to suggest that naturally occurring infection causes either reduced food utilization or growth. However, growth retardation did occur in naturally infected birds kept under experimental conditions (Adair and Fitzgerald, 2008).

Respiratory disease

Subgroup I aviadenoviruses have been frequently isolated from both the upper and lower respiratory tract of birds with respiratory disease. However a survey of records of virus isolations, clinical, and necropsy findings over a 20-year period, involving hundreds of adenovirus isolates, indicated no primary role for adenoviruses in fowl respiratory disease (Cook *et al.*, 1974; Cowen *et al.*, 1978).

Tenosynovitis

Adenoviruses have been isolated from chickens with tenosynovitis, but experimental work has not confirmed their involvement (Jones and Georgiou, 1984).

2.4.5.2. HEV

HE is an acute viral disease of turkeys 4 weeks of age and older characterized by depression, bloody droppings, and death. Due to the immunosuppressive nature of HE, secondary bacterial infections may extend the course of illness and mortality for an additional 2-3 weeks (Pierson and Fitzgerald, 2008).

HE is characterized by a rapid progression of clinical signs over a 24 hours period. These include depression, bloody droppings, and death. Feces containing frank blood are frequently present on the skin and feathers surrounding the vents of moribund and dead birds. Bloody feces may also be forced from the vent if moderate pressure is applied to the abdominal area.

In field outbreaks, all or nearly all birds are infected, as indicated by seroconversion and resistance to experimental challenge. Depressed, clinically affected poults usually die within 24 hours or recover completely. Field mortality ranges from less than 1 to slightly more than 60% with the average being approximately 10-15%. Mortality of 80% is often seen in laboratory experiments where 100% infection is achieved (Pierson and Fitzgerald, 2008).

Gross lesions

Dead poults routinely appear pale due to blood loss but are often in good flesh and have feed in their crops. The small intestine is commonly distended, grossly discolored, and filled with bloody contents.

The intestinal mucosa is congested and in some individuals covered with a yellow, fibrinonecrotic membrane. Lesions are usually more pronounced in the proximal small intestine (duodenal loop) but often extend distally in severe cases. Spleen of infected birds are characteristically enlarged, friable, and marbled or mottled in appearance; however, those of dead poults tend to be smaller presumably due to blood loss and subsequent splenic contraction. Lungs may be congested, but other organs are generally pale (Pierson and Fitzgerald, 2008).

Histopathological lesions

Pathologic changes that characterize HE are most evident in lymphoreticular and GI systems. Splenic lesions present at death include hyperplasia. Typical lesions in the GI tract include severe congestion of intestinal mucosa, degeneration and sloughing of villus

epithelium, and hemorrhage in the villus tips. Hemorrhage is believed to result from endothelial disruption rather than destruction, because blood vessels in the lamina propria appear intact, and red blood cells have been observed exiting these vessels by diapedesis (Pierson and Fitzgerald, 2008).

Diagnosis, treatment, prevention

Corroboration of clinical signs, histopathological findings and also molecular methods are used for diagnosis. While there is no treatment once the disease has installed, it can be controlled by vaccination.

Maternal antibody can provide protection from clinical HE for up to 6 weeks post-hatching and has been reported to interfere with vaccination for up to 5 weeks. However, in a commercial setting, it has been observed that maternal antibody declines sufficiently by 4 weeks to permit vaccination with turkey propagated strains. Passive immunity can also be conferred by injection. Avirulent isolates of HEV have been successfully used as live, water-administered vaccines (Pierson and Fitzgerald, 2008).

2.4.6. Reoviruses

ARVs are members of the *Orthoreovirus* genus in the *Reoviridae* virus family. They have a double stranded RNA genome, with 10 segments arranged into a non-enveloped icoshaedral double-capsid shell. Orthoreoviruses are classified based on biological proprieties and host range. Certain members have the ability to induce cell-fusion. ARVs belong to subgroup II of the family. They are ubiquitous in commercial poultry, and can be differentiated by antigenic configuration, pathotype, relative pathogenicity, growth in cell culture, sensitivity to trypsin, and host specificity (Jones, 2008).

Reoviruses have been isolated from a variety of tissues in chickens affected by various disease conditions, including viral arthritis/tenosynovitis, stunting syndrome, respiratory disease, ED, immunosuppression, and malabsorption syndrome (Jones, 2008). They have also been found in chickens that were clinically normal (Pantin-Jackwood *et al.*, 2008a)

The nature of the disease that occurs following reovirus infection is considered to be very much dependent upon host age, immune status, virus pathotype, and route of exposure (Spackman *et al.*, 2005b). Interactions with other infectious agents have been documented

and may result in differences in both the nature and severity of reovirus induced disease expression. In young meat-type chickens, economic losses related with reovirus infections are frequently associated with increased mortality, viral arthritis/tenosynovitis, and a general lack of performance including diminished weight gains, poor feed conversions, uneven growth rates, and reduced production of affected birds (Jones, 2008).

Commercial reovirus vaccines, which have claimed to protect against RSS have been produced, however they are considered unlikely to protect against the primary causes (Jones, 2008).

Enteric disease in chickens

Several descriptions exist of reovirus-associated ED. An agent characterized as a reovirus was isolated from young chicks suffering from ulcerative enteritis but it was not confirmed that this virus was the cause of the disease. Additionally, early reports described ED, cloacal pasting and mortality in young chicks. Reoviruses have been isolated from the intestines of normal chickens and turkeys with enteric disorders. In the late 1970's a disease syndrome has been linked with several viral agents, including reoviruses. The diseased was called RSS, brittle bone, malabsorbtion-maldigestion syndrome (MMS), and pale bird syndrome (Goodwin *et al.*, 1993; Palya *et al.*, 2003; Pass *et al.*, 1982).

Various attempts were made to demonstrate the link between reovirus and the clinical signs of ED. Despite proving that the reovirus is replicating in the intestinal epithelium, causing small intestine lesions including denudation of the intestinal villi, the data regarding the weight gain depression and economic losses were insufficient (Jones, 2008).

Enteric disease in turkeys

Reoviruses have been isolated from the intestinal content of healthy and diseased turkeys. Some strains have been found to be pathogenic and other not or with low pathogenicity (Goodwin *et al.*, 1993).

A condition called PEMS, which caused severe losses in the turkey industry, was described in 1990 in USA. Originally the disease was referred to as PEC, and its main features include stunting, poor feed utilization and enteritis (Barnes *et al.*, 2000). Among the agents incriminated in this multifactorial syndrome, reoviruses were also included (Heggen-Peay *et al.*, 2002). The gross and microscopic lesions are present in various forms

and are considered non-specific. Reports of several viruses proven to cause PEC and PEMS have lead to development of multiplex RT-PCR tests for diagnostic purposes (Spakman *et al.*, 2005a).

2.4.7. Rotaviruses

Rotaviruses are now established as a major cause of enteritis and diarrhea in a wide range of mammalian species, including humans (Kapikian *et al.*, 2001). Rotavirus infection in avian species was first reported in 1977 (Bergeland *et al.,* 1977), when particles morphologically indistinguishable from rotaviruses were found in the intestinal contents of poults with watery droppings and increased mortality.

Rotaviruses are classified as a genus in the family *Reoviridae*, and have 11 segments of double-stranded RNA. Rotaviruses can only infect vertebrates and are only transmitted by the fecal-oral route. Group A rotaviruses have been isolated from mammals and birds, groups B, C, and E have been found only in mammals, and groups D, F, and G have been detected only in birds. Rotaviruses are causing enteritis and diarrheal disease in avian and mammalian species. Even if rotavirus infections in avian species are associated with outbreaks of ED, subclinical infections are also common (McNulty and Reynolds, 2008). Commercially available vaccines for the protection against avian rotaviruses have not yet been developed.

Virus morphology

Intact rotavirus virions are approximately 70-75 nm in diameter when visualized by negative contrast EM. They have frequently been described as reovirus-like particles, but can be distinguished from reoviruses by their more clearly defined smooth outer edge. Some negatively stained rotavirus particles may resemble a wheel with short spokes, hence the derivation of the name rotavirus. The outer capsid shell may be lost, producing noninfectious or poorly infectious particles (Bridger, 1987).

The advances in understanding of the structure of the rotavirus virion, have revealed that the so-called single-shelled particles are double-layered, and similarly, intact virions, previously referred to as double-shelled particles, are triple layered (McNulty and Reynolds, 2008).

Gross lesions

The most common finding at necropsy is the presence of abnormal amounts of fluid and gas in the intestinal tract and ceca. Pallor of the intestinal tract and loss of tonicity may also be found. Secondary findings are frequent, and they include: dehydration, stunting of growth, pasted, and inflamed vents, anemia due to vent pecking, litter in the gizzard, and inflammation and encrustation with droppings of the plantar surfaces of the feet (McNulty *et al.*, 1983; Reynolds *et al.*, 1987b; Yason and Schat, 1986).

Histopathological lesions

Immunofluorescence (IF) studies using chickens and turkeys experimentally infected with rotavirus have demonstrated that the principal site of virus replication is the cytoplasm of mature villus absorptive epithelial cells of the small intestine. Microscopic lesions in the small intestines of turkey experimentally infected with group A rotaviruses are basal vacuolization of enterocytes, separation of enterocytes from the lamina propria and subsequent desquamation, villus atrophy and widening of the lamina propria, fusion of the villi, and leukocyte infiltration in the lamina propria (Reynolds *et al.*, 1987b). The average villus lengths were decreased and crypt depths were increased following experimental infection, these changes resulted in decreased villus to crypt ratios (Reynolds *et al.*, 1987a).

2.5. Frequent coinfection with viral pathogens causing similar clinical signs

Nephroso-nephritis is an expression used to describe concurrent degenerative and inflammatory renal changes in poultry. Viral infections are frequently related with primary or secondary inflammatory kidney lesions in birds (Siller *et al.*, 1981).

IBV and ANV can lead to nephropathy, subsequent gout (Frazier *et al.*, 1990), and enteritis. IB is an acute, highly contagious disease that affects the respiratory, renal, intestinal and reproductive systems, causing severe economic loss in the broiler and in the egg layer industry. Although IBV causes respiratory disease, the virus also replicates in many non-respiratory epithelial surfaces, such as: kidneys, gonads, oviduct, and intestinal tract, where it may cause pathological changes (Cosgrove *et al.*, 1962; Cavanagh and Naqui, 2003; Cavanagh, 2006).

The possibility for IB and AN to evolve simultaneously in a flock should not be overlooked (Imada and Kawamura, 2003). IBDV is one of the most important immunosuppressive agents in modern poultry production (Allan *et al.*, 1972; Faraghner *et al.*, 1974; Lukert and Saif, 2003). The disease was first recognized as a specific entity in 1962 and was named “avian nephrosis” due to the kidney damage found in the birds that succumbed (Cosgrove, 1962), but the renal malfunction was never reproducible in experimental infections (Beckman, 2003). It was later demonstrated that the kidney lesions were produced by the dehydration, hence having no direct connection with IBDV infection. Still due to the immunosuppression caused by the IBDV, the flocks become infected with secondary agents that are frequently manifesting with clinical signs of enteritis.

The rather high incidence of cases with similar clinical signs and pathological renal and/or enteric lesions in different flocks, and age groups, raises the possibility of simultaneous infection by IBV, IBDV and ANV. As there is a practical need to detect the three pathogens in tissue samples from chickens presenting the corresponding lesions by employing quick, reliable and cost efficient protocols, one of our distinct goals was the development of a rapid and reliable multiplex RT-PCR (mRT-PCR) assay.

3. Materials and methods

3.1. Samples

3.1.1. Chicken farms

The cases considered in this study were collected between January 2007 and March 2010, from flocks experiencing signs of ED.

Carcasses of 6 days to 3 weeks old birds from 15 Hungarian chicken broiler flocks, from several counties of Hungary (Figure 4), experiencing increased mortality, and/or poor production parameters were sent to the Department of Pathology and Forensic Veterinary Medicine (Szent Isván University, Faculty of Veterinary Science, Budapest, Hungary) for diagnostic purposes. In order to obtain a better insight into the epidemiology of ED, carcasses from 13 flocks free of ED clinical signs, received for routine assessment of the flock's status were included in this study. These samples were only tested by PCR for the direct demonstration of enteric viruses (Table 2). Details on the chicken flocks included in this thesis are summarized in Table 2, and the geographical origin is presented in Figure 4.

Figure 4: Geographical origin of the chicken flocks examined in this study. Samples numbered according to Table 2

Table 2: Information regarding the chicken flocks included in the thesis

no	Sample	Age (days)	Region	Mortality (%)	Anamnesis
1	Ch 1514/07$_{\beta}$	6	Letenye	10	growth depression, diarrhea, huddling
2	Ch 1515/07 $_{\beta}$	8	Letenye	15	growth depression, diarrhea, huddling
3	Ch 347/08$_{\S}$	7	Hernád	10	uneven growth, diarrhea, huddling,
4	Ch 348/08$_{\S}$	7	Hernád	8	uneven growth, diarrhea, huddling,
5	Ch A1/09$_{\delta}$	14	Újkígyós	6	growth depression, huddling, dehydration
6	Ch B1/09 $_{\delta}$	14	Újkígyós	6	growth depression, huddling, dehydration
7	Ch 189/09	7	Újkígyós	du	uneven growth, diarrhea, poor production
8	Ch 528/09	21	Budapest	du	growth depression, diarrhea, huddling
9	Ch 538/09	21	Budapest	du	growth depression, huddling,
10	Ch 841/3/09	8	Budapest	10	diarrhea, growth depression
11	Ch 852/09	10	Budapest	10	huddling, dehydration
12	Ch 5596/7/10$_{\lambda}$	21	Pálháza	3	growth depression, diarrhea, huddling
13	Ch 5596/10/10 $_{\lambda}$	21	Pálháza	8	growth depression, increased mortality, diarrhea, huddling
14	Ch 5596/13/10 $_{\lambda}$	21	Pálháza	7	growth depression, increased mortality, diarrhea, huddling
15	Ch 5598/18/10 $_{\lambda}$	21	Pálháza	7	growth depression, increased mortality, diarrhea, huddling
16	*Ch 847/07*	*8*	*Szolnok*	*wtl*	*routine assessment*
17	*Ch 1253/07*	*14*	*Szolnok*	*wtl*	*routine assessment*
18	*Ch 59/08*	*7*	*Hernád*	*wtl*	*routine assessment*
19	*Ch 72/08*	*21*	*Hernád*	*wtl*	*routine assessment*
20	*Ch 642/08*	*14*	*Kecskemét*	*wtl*	*routine assessment*
21	*Ch 694/08*	*2*	*Lajosmizse*	*wtl*	*routine assessment*
22	*Ch 715/08*	*21*	*Tárkány*	*wtl*	*routine assessment*
23	*Ch 375/09*	*10*	*Pálháza*	*wtl*	*routine assessment*
24	Ch 482/09	7	*Budapest*	wtl	*routine assessment*
25	Ch 661/09	21	*Budapest*	wtl	*routine assessment*
26	Ch 669/09	2	*Budapest*	wtl	*routine assessment*
27	Ch 813/09	21	*Hernád*	wtl	*routine assessment*
28	Ch 888/09	21	*Esztergom*	wtl	*routine assessment*

du: data unknown, due to confidentiality; wtl: within technological limits
β, §, δ, λ: samples originating from the same flock, but different houses
italic: routine assessment, free of ED clinical signs
up to 10%: increased mortality; >10%: high mortality

3.1.2. Turkey farms

The cases were collected between January 2008 and December 2010 from commercial Hungarian turkey flocks experiencing signs of PEC or PEMS combined with high mortality.

Pooled intestinal tissue (duodenum and ileum) was collected from 49 Hungarian turkey flocks in the Central Agricultural Office, Veterinary Diagnostic Directorate (MgSzH ÁDI) Kaposvár, kindly provided by Dr. Csaba Nemes. One sample corresponds to one flock or house and contains pooled tissue from 5 birds with clinical signs of ED. The age of the birds varied from 6 to 43 days.

Two samples (50, 51) were collected in 2010 from the carcasses sent to the Department of Pathology and Forensic Veterinary Medicine (Szent Isván University, Faculty of Veterinary Science, Budapest, Hungary) for diagnostic purposes. Details on the turkey flocks included in this thesis (age, anamnesis) are summarized in Table 3, and the geographical origin is presented in Figure 5.

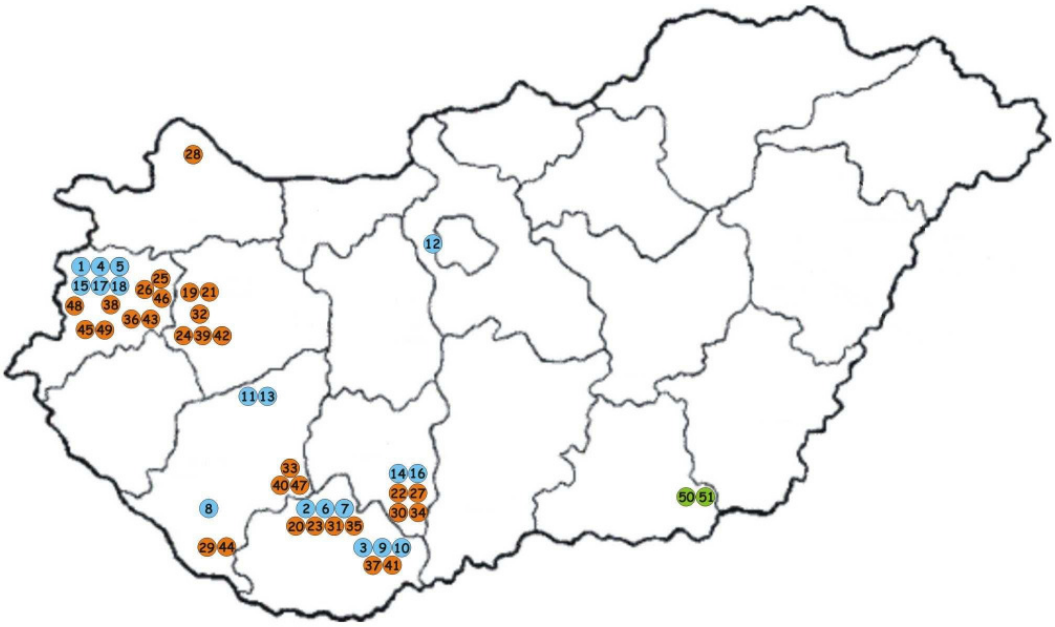


Figure 5: Geographical origin of the turkey flocks examined in this study.
Blue: samples collected in 2008, orange: samples collected in 2009, green: samples collected in 2010. Samples numbered according to Table 3

Table 3: Information regarding the turkey flocks included in this thesis

Year	Farm/House	no.	Age	Region	Anamnesis
2008	B-369/4	1	13	Ikervár	uneven development
	B-284	2	<42	Mágocs	increased mortality
	B-486/2	3	6	Geresdlak	high mortality, growth depression
	B-369/9	4	29	Ikervár	uneven development
	B-23/1	5	35	Ikervár	growth depression
	B-281	6	30	Mágocs	increased mortality
	B-122	7	<42	Mágocs	growth depression
	B-496	8	11	Mike	increased mortality, poor production
	B-589/4	9	23	Geresdlak	increased mortality
	B-589/1	10	23	Geresdlak	increased mortality
	B-547	11	21	Lengyeltóti	increased mortality
	B-470/2	12	15	Nádorliget	increased mortality
	B-288	13	21	Lengyeltóti	increased mortality, diarrhea
	B-584	14	16	Bogyiszló	increased mortality, huddling
	B-369/1	15	43	Ikervár	uneven development, diarrhea
	B-501	16	26	Bogyiszló	increased mortality
	B-23/8	17	21	Ikervár	growth depression
	B-23/3	18	28	Ikervár	growth depression
2009	B-291/11	19	16	Kerta	increased mortality, diarrhea
	B-87	20	9	Mágocs	high mortality, growth depression
	B-280	21	25	Kerta	diarrhea
	B-232	22	21	Bogyiszló	high mortality, growth depression, diarrhea
	B-160	23	38	Mágocs	high mortality, growth depression, diarrhea
	B-164/3	24	43	Szentimrefalva	diarrhea
	B-307/2	25	24	Jánosháza	increased mortality, exsiccosis
	B-307/8	26	20	Jánosháza	increased mortality, exsiccosis
	B-649	27	15	Bogyiszló	high mortality
	B-557	28	13	Kimle	uneven growth, poor production
	B-297	29	16	Lad	increased mortality, diarrhea
	B-893	30	24	Bogyiszló	increased mortality
	B-125	31	24	Mágocs	high mortality, growth depression
	B-169/7	32	30	Karakószörcsök	diarrhea, huddling
	B-184	33	14	Nagyberki	growth depression, poor production
	B-27	34	9	Bogyiszló	increased mortality, growth depression
	B-70	35	15	Mágocs	increased mortality, uneven growth, diarrhea
	B-31/2	36	42	Bögöte	diarrhea, huddling, poor production
	B-315	37	7	Geresdlak	high mortality, diarrhea
	B-465/9	38	<42	Ikervár	immunosuppression
	B-164/1	39	28	Szentimrefalva	high mortality, growth depression
	B-346	40	20	Nagyberki	increased mortality
	B-320/4	41	10	Geresdlak	high mortality, poor production
	B-164/4	42	42	Szentimrefalva	high mortality, growth depression
	B-31/5	43	35	Bögöte	diarrhea, huddling
	B-121	44	11	Lad	restlessness, high mortality
	B-648/1	45	<42	Nemesbőd	uneven growth, poor production
	B-307/6	46	20	Jánosháza	increased mortality, exsiccosis
	B-546	47	19	Nagyberki	increased mortality, uneven development
	B-671	48	<42	Gencsapáti	immunosuppression
	B-648/2	49	15	Nemesbőd	uneven growth, diarrhea
2010	33/10	50	22	Nagyér	high mortality, diarrhea, uneven growth
	762/10	51	14	Nagyér	increased mortality, diarrhea, uneven growth

3.2. Macroscopic examination and histopathology

Following routine dissection, tissue samples from various organs collected from chicken carcasses submitted for diagnosis (intestine, pancreas, bursa Fabricii, liver, spleen, thymus) were stored in 8 % neutral buffered formaldehyde solution for histological examination, processed, sectioned and stained with hematoxylin and eosin (H&E) (Stevens, 2007).

3.3. Bacteriology

Aseptically collected fresh liver samples were inoculated into blood agar and Drigalski plates. Cultures were incubated at 37ºC under aerobic conditions, and examined after 24 h for microbial colonies growth.

3.4. Immunohistochemistry for ChPV and TuPV

Paraffin embedded sections were initially dewaxed in xylene and graded ethanol. After treatment with appropriate antigen retrieval (Target Retrieval Solution, pH 6.0; DAKO, Glostrup, Denmark; 30 min in 880 W microwave oven), the sections were treated with 3% peroxide for 10 min and incubated with unlabelled primary antibody against ChPV (chicken polyclonal antibody 1:500 dilution) for 12 h at 4ºC.

The primary antibody was obtained on SPF chickens, Spafas, line 22 (Charles River Ltd., UK), following oral infection at 1 day of age and subcutaneously at 21 days, with the ABU strain. Intestinal samples were collected at 1 day of age, before the oral infection, and at sacrification (28 days), tested by PCR for viral pathogens known to be involved in ED: ANV, chicken astrovirus (CAstV), ARV, AvRV, and AAdV1, according to previously described protocols (Table 4) and were found negative at both times. The designated ABU strain was isolated from chickens in Hungary in 1984 (Kisary *et al*., 1984), and was purified by cesium chloride ($CsCl_2$) density gradient centrifugation prior to inoculation of the chickens (Kisary, 1985b). Antigen bound primary antibody was detected using goat anti-chicken IgG (Fc) HRP conjugate (Alpha Diagnostic Intel. Inc.). DAB (3,3´-diaminobenzidine tetrahydrochloride) was added to the slides for 30 min at room

temperature. Sections were counterstained with Mayer's hematoxylin (M&H) for 10 sec. The specific occurrence of the reaction was ensured by the use of positive and negative control intestinal tissue samples in each reaction. The IHC method was applied for all chicken samples and for the two turkey samples collected in 2010.

3.5. Electron microscopy

Intestinal homogenates of 100 ml in 1/10 dilution with sterile phosphate buffered saline (PBS) were centrifuged at 3000×g for 5 minutes to sediment cell debris, bacteria and fungi followed by sterile filtration by 0.22 µm filter.

Pelletizing ultracentrifugation of the collected supernatants was carried out at 25000 rpm for 20 hours through sucrose cushion of 20% at 4°C. Preformed density gradient was prepared containing cesium chloride ($CsCl_2$) in a continuous concentration gradient from % w/w 20 to 60 following pellet collections in 2 ml Tris-EDTA (TE) buffer pH 8. Gradient density ultracentrifugation with $CsCl_2$ was performed at 36 000 rpm for 30 hours at 4°C.

Fractionated collection of the bands with hypodermic needle and syringe was accomplished by puncturing the bottom of the ultracentrifuge tube, and dialyzed through dialysis sack against TE buffer for 48 hours. The buffer was changed three times during the process. Fractions were stored at -80°C until use. The samples were prepared according to the single-droplet negative staining technique (Harris, 2007) and examined at a transmission electron microscope (TEM). The TEM method employing negative staining was applied for all chicken flocks and for the two turkey samples collected in 2010.

3.6. Genetic investigations

3.6.1. Nucleic acid purification

Tissue samples were homogenized in 10 ml PBS and centrifuged at 1500 x g for 10 min. The viral nucleic acid was isolated from the supernatants using the QIAamp viral RNA Mini Kit (Qiagen, Germany) and High Pure Viral Nucleic Acid Kit (Roche, Switzerland), according to the manufacturers' instructions.

3.6.2. Primers

All the primers and their details regarding design and thermal profile, target gene, sequence, expected amplicon size, annealing temperature, used for the direct demonstration of the viral pathogens incriminated in ED in chicken and turkey are presented in Table 4. Details on the primers used for the multiplex RT-PCR are presented in Table 5.

Table 4: Details on the primers used for the direct demonstration of viral pathogens incriminated in ED

Targeted virus	Targeted gene	Primer name and sequence (5'-3')	Amplicon size (bp)	Annealing (°C)
Chicken/Turkey parvovirus[f] **(ChPV/TuPV)**	NS1	PVF1: TTCTAATAACGATATCACTCAAGTTTC PVR1: TTTGCGCTTGCGGTGAAGTCTGGCTCG	561	55
Avian nephritis virus[a] **(ANV)**	GP1	ANV-ORF1-f: AGATACGCTTGCTCGTCTTG ANV-ORF1-r: CCTCTAACCGGCGATATTCT	608	53
Chicken astrovirus[b] **(CAstV)**	ORF 1b	ANVpol1F: GYT GGG CGC YTC YTT YGA YAC ANVpol1R: CRT TTG CCC KRT ART CTT TRT	473	53
Turkey astrovirus[c] **(TAstVs)**	ORF-1b	TAPG-L1: TGG TGG TGY TTY CTC AAR A TAPG-R1: GYC KGT CAT CMC CRT ARC A	601	55
Turkey astrovirus 2[i] **(TAstV-2)**	capsid ORF 2	MKCAP8F: TCA TCA TCC TCT CAC ACT GG MKCAP19R: AGC AGC AGT AGG TGG CAG TG	802	53
Turkey coronavirus[j] **(TCV)**	nucleocapsid	TCV-NF: GGT AGC GGT GTT CCT GA TCV-NR: CCC TCC TTA CCT TTA GT	598	53
Hemorrhagic enteritis virus[h] **(HEV)**	CP1	HEV-CP1-F: GGTTCGTGAACATTGGAGAC HEV-CP1-R: CAGGCACAACAGGTGTAACT	155	48
Avian reovirus[d] **(ARV)**	S4	S4-F13: GTG CGT GTT GGA GTT TCC CG S4-R1133: TAC GCC ATC CTA GCT GGA	1120	53
Avian rotavirus[b] **(AvRV)**	NSP4	NSP4-F30: GTG CGG AAA GAT GGA GAA C NSP4-R660: GTT GGG GTA CCA GGG ATT AA	630	55
Avian adenovirus group 1[g] **(AAdV1)**	Hexon	H1: TGG ACA TGG GGG CGA CCT A H2: AAG GGA TTG ACG TTG TCC A	1219	53

a: Mándoki *et al.*, 2006; b: Day *et al.*, 2007a, b: Tang *et al.*, 2005; d: Pantin-Jackwood *et al.*, 2008b; e: Day *et al.*, 2007a; f: Zsak *et al.*, 2009; g: Hess *et al.*, 1999; h: designed for this study; i: Koci *et al.*, 2000; j: Sellers *et al.*, 2004

Table 5: Details on the primers used for the multiplex RT-PCR

Targeted virus	Targeted gene	Primer name and sequence (5' to 3')	Amplicon size (bps)	Annealing (°C)
Infectious bronchitis virus[a] **(IBV)**	N	IBV-OutN-f: GTGATGACAAGATGAATGAGGA IBV-OutN-r: CAGATGAGGTCAATGCTTTATC	402	60
Avian nephritis virus[b] **(ANV)**	GP1	ANV-ORF1-f: AGATACGCTTGCTCGTCTTG ANV-ORF1-r: CCTCTAACCGGCGATATTCT	608	55
Infectious bursal disease virus[c] **(IBDV)**	VP2	IBVD-VP2-f: GCCCAGAGTCTACACCAT IBVD-VP2-r: CCCGGATTATGTCTTTGA	743	53

a: Farsang *et al.*, 2002
b: Mandoki *et al.*, 2006
c: Jakwood and Sommer-Wagner, 2007

3.6.3. Amplifications

3.6.3.1. Classical PCR assays

The 50 µl reaction mixtures contained sterile deionized water, 10 x PCR buffer without $MgCl_2$, 2 mM dNTP mix with a final concentration of 0.2 mM of each dATP, dCTP, dGTP, dTTP, 25 mM $MgCl_2$ with a final concentration of 1-4 mM, 0.8 µM of the appropriate forward and reverse primers (Table 4), 1 µl of 1.25 u/50 µ1 Taq DNA polymerase (Fermentas, Lithuania) and 2.5 µl of template DNA.

Following an initial denaturation at 95°C for 10 min, the reaction mixture was subjected to 35 cycles of heat denaturation at 94°C for 45 sec, primer annealing at the corresponding temperatures (Table 4) for 45 sec, and DNA extension at 72°C for 1 or 2 min, followed by a final extension at 72°C for 10 min. Positive and negative (distilled water) controls were added to each run. For the direct demonstration of ChPV and TuPV, the designated ABU strain kindly provided by Dr. János Kisary was used as a positive control.

3.6.3.2. Diagnosis of HEV by a newly developed protocol

Following an initial denaturation at 95°C for 10 min, the reaction mixture (Chapter 3.6.3.1.) was subjected to 40 cycles of heat denaturation at 94°C for 45 sec, primer annealing at 48ºC for 50 sec, and DNA extension at 68°C for 2 min, followed by a final extension at 68°C for 10 min. Primer design was developed with the help of SE Central (version 4.10 Scientific & Educational Software). Positive (commercial live vaccine) and negative (distilled water) controls were added to each run.

3.6.3.3. RT-PCR assays

Reverse transcription and amplifications were performed in a continuous RT-PCR method by using the Qiagen OneStep RT-PCR Kit (Qiagen, Germany). The 25 µl reaction mixtures contained 5 µl of 5 x buffer (final $MgCl_2$ concentration 1.5 mM), 0.4 mM of each deoxynucleozide triphosphate (dNTP), 10 U rRNasin™ RNase Inhibitor (Promega, USA), 0.8 µM of the appropriate forward and reverse primers, 1 µl of enzyme mix and 2.5 µl of template RNA.

Reverse transcription was carried out at 50 °C for 30 min. Following an initial denaturation at 95°C for 15 min, the reaction mixture was subjected to 35 cycles of heat denaturation at 94°C for 45 sec, primer annealing for 45 sec at the corresponding temperature (Table 4) and DNA extension at 72°C for 1 or 2 min, followed by a final extension of 10 min at 72°C. The reactions were performed in a PCR Sprint Thermal Cycler SPRT001 (Hybaid, UK). Positive and negative (distilled water) controls were added to each run.

3.6.3.4. Multiplex RT-PCR

The 25 µl reaction mixtures contained 5 µl of 5 x buffer (final $MgCl_2$ concentration 1.5 mM), 0.4 mM of each deoxynucleotide triphosphate (dNTP), 10 U RNase Inhibitor, 0.8 µM of each appropriate forward and reverse primers (Table 5), 1 µl of enzyme mix and 2.5 µl of template RNA.

The thermal profile was modified according to the performance of the assay obtained in the different trials of the test. Reverse transcription was carried out at 50 °C for 30 min. Following an initial denaturation at 95°C for 15 min, the reaction mixture was subjected to 40 cycles of heat denaturation at 94°C for 45 sec, primer annealing at 53°C for

55 sec and DNA extension at 72°C for 2 min and 30 sec, followed by a final extension of 10 min at 72°C.

The three primer pairs were selected considering their close annealing temperature, the fact that they did not produce any primer dimers and the amplicons (Table 5) produced were gel separable. To test for possible repetitive sequences, the primers used were aligned with the sequence databases at the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST/), as previously recommended (Henegariu *et al.*, 1997).

The specificity of the primers was first tested against a positive ANV isolate kindly provided by Vilmos Palya, against a commercial IB vaccine (strain H120) and a commercial IBD vaccine (strain D78), by individual RT-PCR reactions, under the thermal profile previously recommended (Table 5), and the amplicones were visualized by gel electrophoresis.

To determine the sensitivity of the mRT-PCR for different levels of starting templates, 10-fold serial dilution was made from a positive control containing Vaccine 1 (strain H120), from 8.87 x 10^{5} µg to 8.87 x 10^{-6} µg for IBV, from Vaccine 2 (strain D78), from 8.45 x 10^{5} µg to 8.45 x 10^{-6} for IBDV and from ANV isolate, from 8.92 x 10^{5} µg to 8.92 x 10^{-6} µg for ANV. The concentration of the nucleic acid for the positive control was determined by spectrophotometry.

3.6.4. Electrophoresis

Following PCRs, 7.5 µl of the amplicons were electrophoresed in a 1.2% agarose gel (SeaKem® LE Agarose, Cambrex, USA) at 80 V for 80 min. The gel was stained with GR safe nucleic acid stain (InnoVita, USA) and the bands were visualized at 312 nm using a TFX 35M UV transilluminator (Life Technologies, UK), and using the Kodak Digital Science 1D software program (Kodak, Japan). Product sizes were determined with reference to 50 bp, 100 bp and 1 kb molecular weight markers (Fermentas, Lithuania).

3.6.5. Nucleic acid sequencing and phylogenetic analysis

Following electrophoresis with 50 µl, the amplicons were cut out from the gel and DNA was extracted with the QiaQuick Gel Extraction Kit (Qiagen, Germany). Fluorescence-

based direct sequencings were performed in both directions on the amplicons at Biogon Kft. (Budapest, Hungary) employing an ABI 3100 genetic analyzer (Applied Biosystems, USA).

All obtained nucleotide sequences were identified by BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/), against GenBank databases. Nucleotide sequences were compiled and aligned using the Align Plus 4 software (Scientific & Educational Software, USA).

Phylogenetic analysis was performed with the help of the ClustalX program. ChPV and TuPV phylogenetic tree of the nucleotide sequences and deduced aa sequences were created using sequence data of the 15 chicken and 25 turkey Hungarian parvovirus strains, 7 strains retrieved from the GenBank, and 20 strains kindly provided by L. Zsak (Zsak *et al*., 2009). Blocks of sequence data leading to 524 nucleotides for ChPV and 527 nucleotides for TuPV were used for the analysis, and 174 aa for ChPV and 175 aa for TuPV respectively. Alignments of nucleotides and aa were performed using the Align Plus 4 program (Align Plus 4, 1995; Scientific & Educational software).

The phylogenetic tree was constructed by neighbourjoining with a two-parameter distance matrix using the Phylip program. Goose parvovirus strain HG5 was used as outgroup. Eventual recombination events were investigated by using the Recombination Detection Program (RDP) (http://darwin.uvigo.es/rdp/rdp.html; Martin and Rybicki, 2000).

3.6.6. RFLP-based technique

Discrimination of TuPV, ChPV and ChPV strains with unique sequences grouped in the TuPV cluster (TuPV-like ChPV strains) was performed using the amplicons produced by the primer pair used for the diagnosis of ChPV and TuPV (Table 4) as follows: 5 µl of the PCR amplicons was mixed with 3.5 µl ddH_2O, 1 µl of *AvaII* enzyme (New England BioLabs, USA) and 1 µl of buffer solution 1XNEB4, provided by the manufacturer.

The mixture was then heated to 37ºC for 60 min and mixed at 100 x g, at intervals of 30 sec in a Thermomixer Comfort device (Eppendorf, Germany). The amplicons were then detected by electrophoresis in a 2% Tris acetate-EDTA-agarose gel at 80 V for 80 min and fragment sizes were determined using the previously described protocol (Chapter 3.6.4.).

4. Results

4.1. Enteric disease syndrome in chickens

4.1.1. Macroscopic examination

According to the history submitted by the owners and/or treating veterinarians along with the carcasses, the broilers presented slightly higher than normal daily mortality, stunted growth, and diarrhea (Table 2).

At necropsy obvious signs of uneven growth between the members of the same flock with overall growth retardation in the entire farm was present, and signs of diarrhea were observed (Figures 6 A and B).

The segments of the small intestine were partially filled with fluid-mucoid content, and large amount of gas (Figure 6 C). Dilatation of the intestinal and mesenteric blood vessels (Figure 6D, Figure 7) and the dark-reddish discoloration of the mucous membrane were present in the duodenum and jejunum, and atrophy of the immune organs (bursa Fabricii, thymus, spleen) was identified.

Macroscopic signs representative for multifocal pancreatitis (Figure 7) were also observed.

4.1.2. Bacteriology

Routine aerobic bacteriological investigations, performed from fresh liver tissue, on bloody agar and Drigalski media, were negative in case of all the chicken samples included in the thesis.

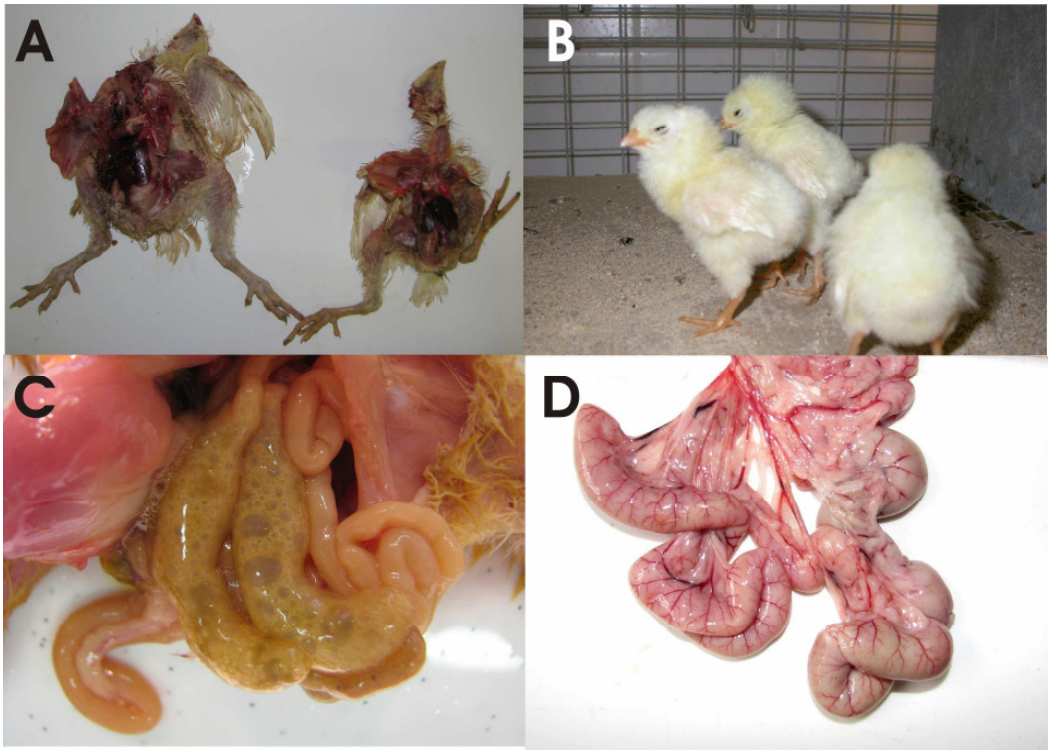


Figure 6: A: signs of evident uneven growth and development between members of the same flock, 14 days old broiler chickens. **B:** broilers with apathy, presence of watery feces on the flooring; **C:** signs of dysbacteriosis, due to the maldigestion and enteritis; **D:** dilated intestinal and mesenteric blood vessels

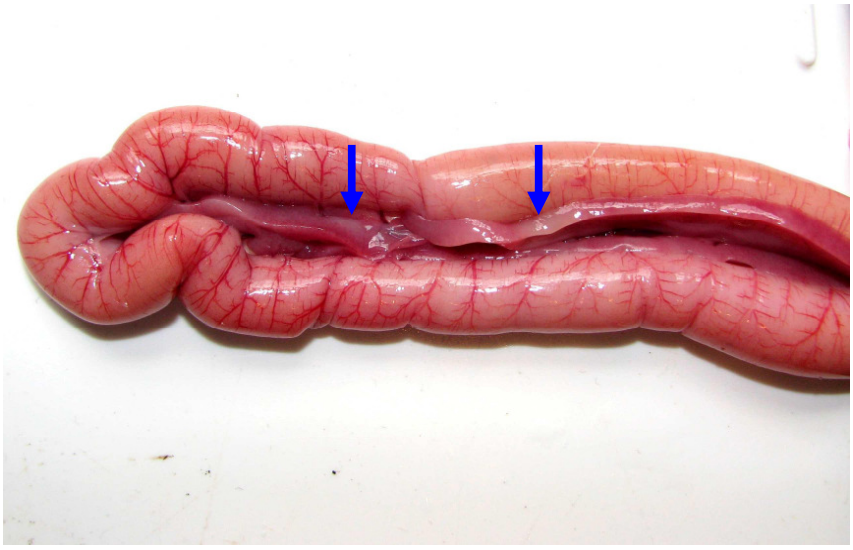


Figure 7: Dilated blood vessels of the duodenum and multifocal pancreatitis (blue arrows)

4.1.3. Histopathology

The histological examination revealed moderate to severe distention of the intestinal crypts which were lined with flattened epithelium and were containing desquamated cells (Figure 8). Acute catarrhal enteritis with a mixed inflammatory cell population (with evident lymphohistiocytic dominance, and lower number of heterophil granulocytes) was observed in the jejunum and duodenum (Figures 9 and 10), with a low incidence of enterocyte desquamation.

Shortening, moderate denudation and fusion of the intestinal villi was observed (Figures 11 and 12). In a few cases active regeneration in the small intestine was present (Figure 13). Lymphohistiocytic nodular pancreatitis was also observed (Figure 14).

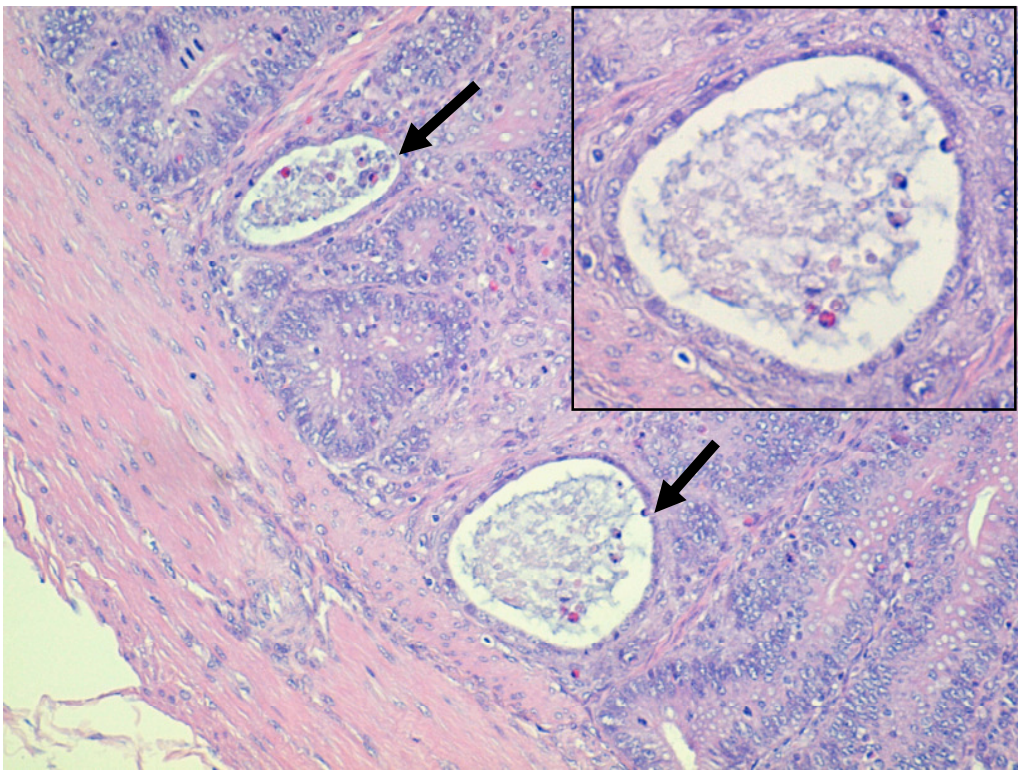
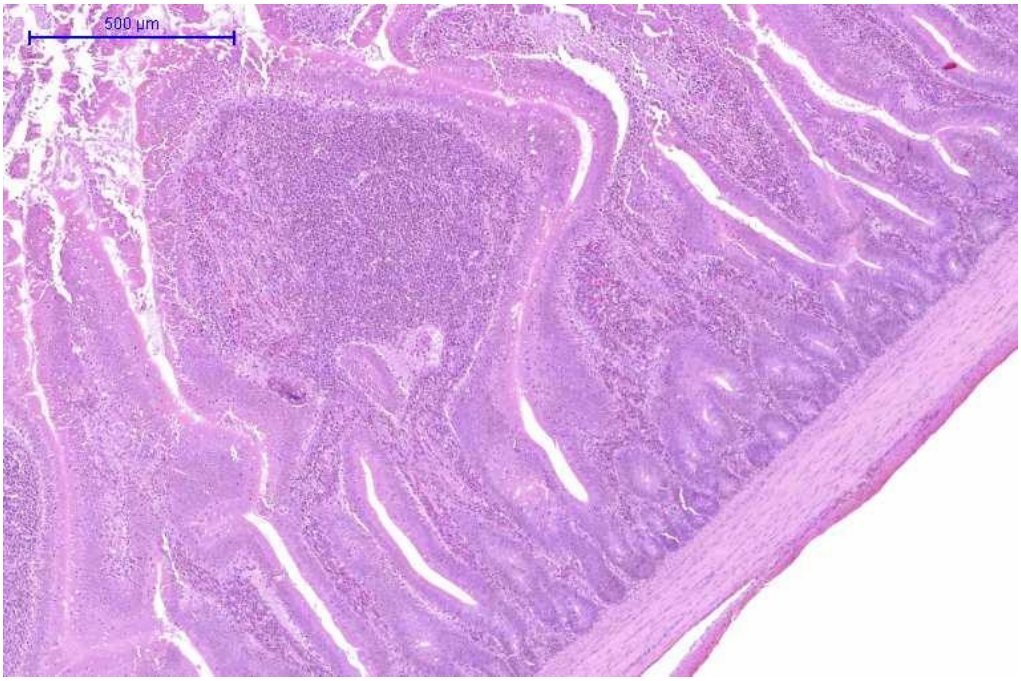


Figure 8: Distention of the intestinal crypts (arrows), lined with flattened epithelium and containing desquamated cells. Insert: higher magnification presenting the flattened epithelium and desquamated cells in the distended crypt (jejunum, 100 x, H&E staining)



Figures 9: Enteritis with evident, mixed inflammatory cell infiltrate, (jejunum, H&E staining)

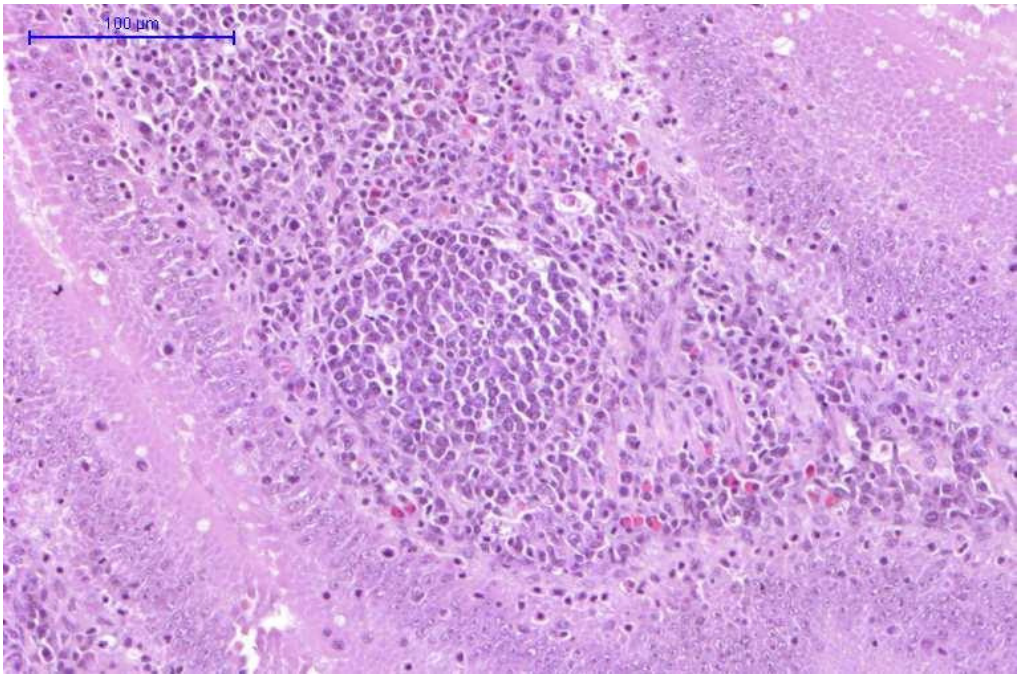
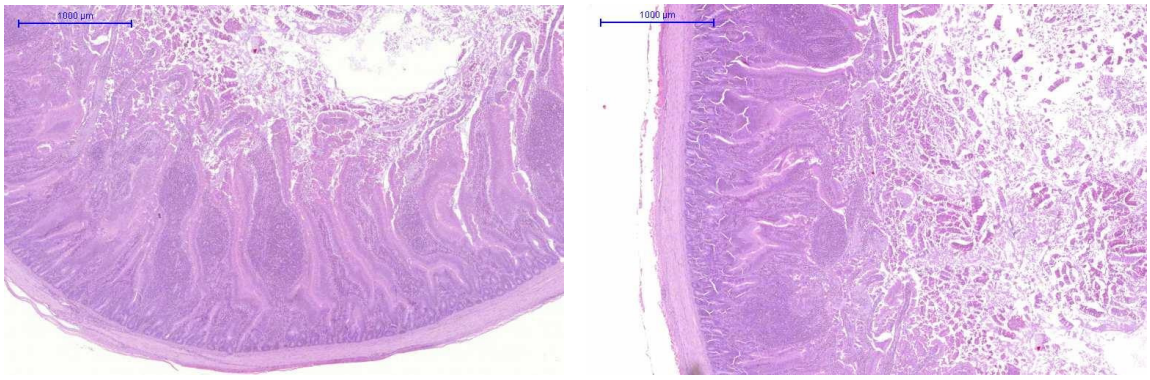


Figure 10: Inflammatory cell infiltrate made up of lymphocytes, histiocytes and a few heterophily granulocytes (jejunum, H&E staining)



Figures 11 and 12: Shortening, denudation and fusion of the intestinal villi (jejunum, H&E staining)

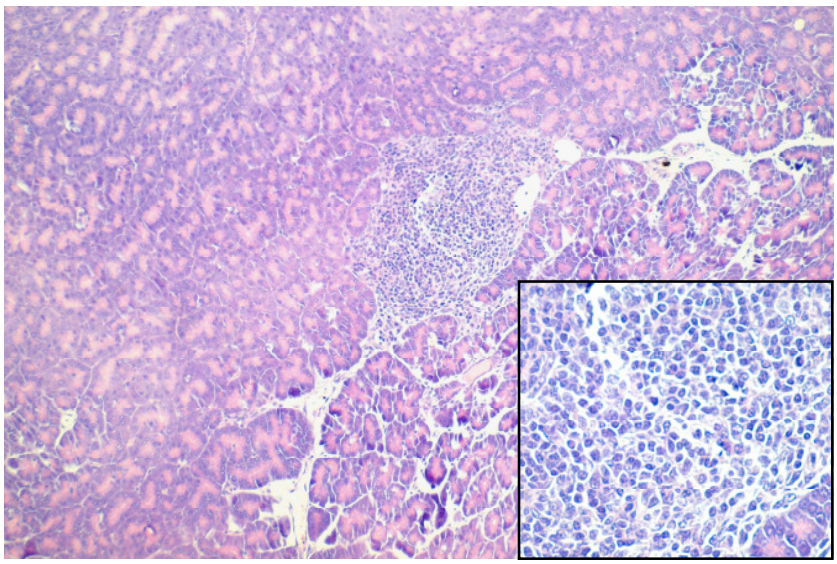


Figure 13: Nodular multifocal pancreatitis. Insert: higher magnification presenting the inflammatory cells, lymphocytes and histiocytes (pancreas, 40 x, H&E staining)

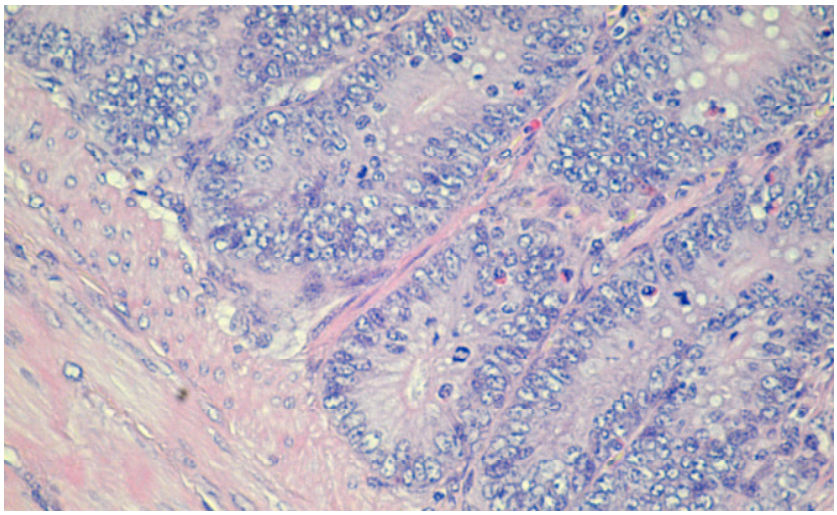


Figure 14: Signs of active regeneration in the small intestine (jejunum, 100 x, H&E staining)

4.1.4. Immunohistochemistry for ChPV

Positive nuclear staining was detected at indirect IHC in the epithelial cells and inflammatory cells from the lamina propria of the duodenum (Figure 15) and jejunum (Figure 16) in case of all chicken samples directly demonstrated to be positive for ChPV, by PCR. The summarized results are representative for all the chicken flocks demonstrated positive for ChPV by PCR.

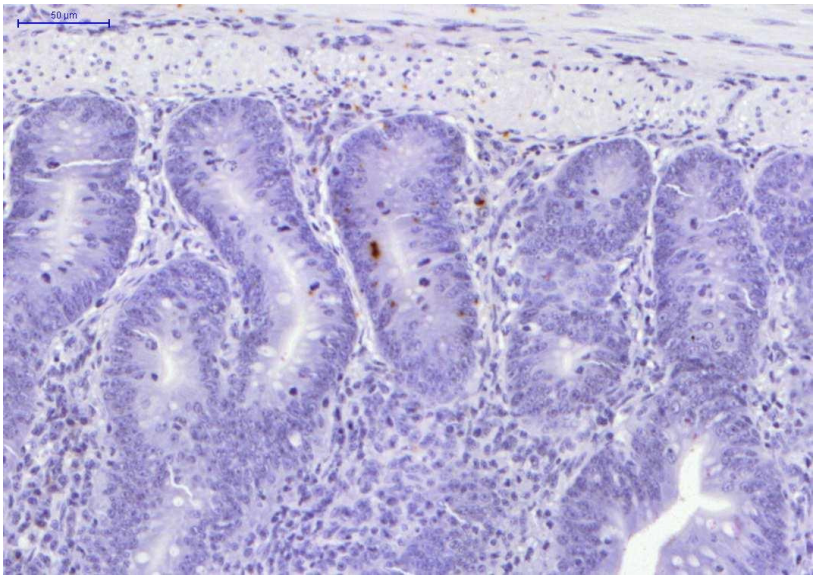


Figure 15: Positive staining at indirect IHC, counterstained with M&H, in the epithelial cells from the lamina propria of the duodenum

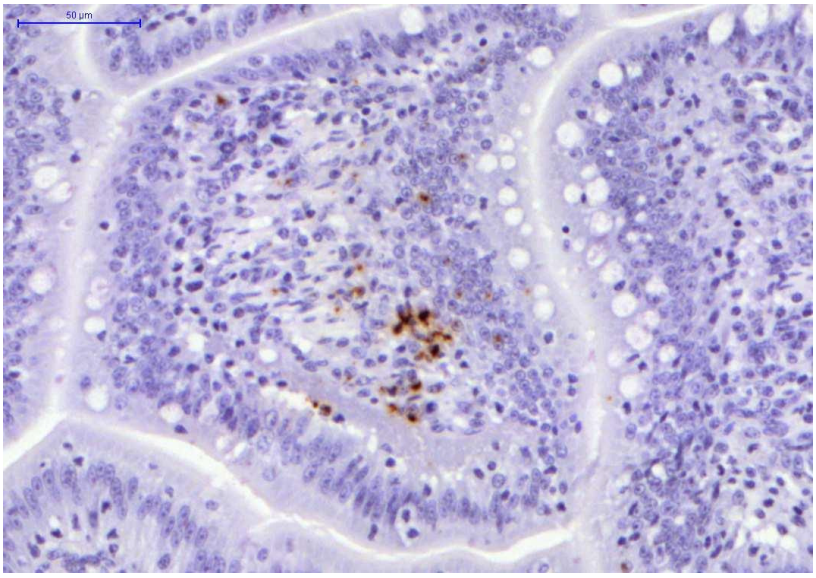


Figure 16: Positive staining at indirect IHC, counterstained with M&H, in the epithelial cells of the jejunum

4.1.5. Electron microscopy

The TEM examination of the fractions obtained from the intestinal homogenate revealed the presence of numerous icosahedral, non-enveloped viral particles, measuring about 25-35 nm in diameter (Figure 17), 22-25 nm (Figure 18), 60-65 nm (Figure 19) and 70-75 nm (Figure 20).

Based on their size and ultrastructural morphology, the viral particles were identified as members of the *Astroviridae*, *Parvoviridae,* and *Reoviridae* virus families. No fraction was found containing particles with size shape and morphology typical for *Coronaviridae* or *Adenoviridae* virus families.

As they present similar size and shape, rotaviruses and reoviruses were distinguished on the basis of outer capsid layer morphology; in the case of rotaviruses, the arrangement of the capsomers of the inner capsid and the well defined outer layer provides the characteristic spoke-like appearance, whereas the outer layer of reovirus particles is featureless.

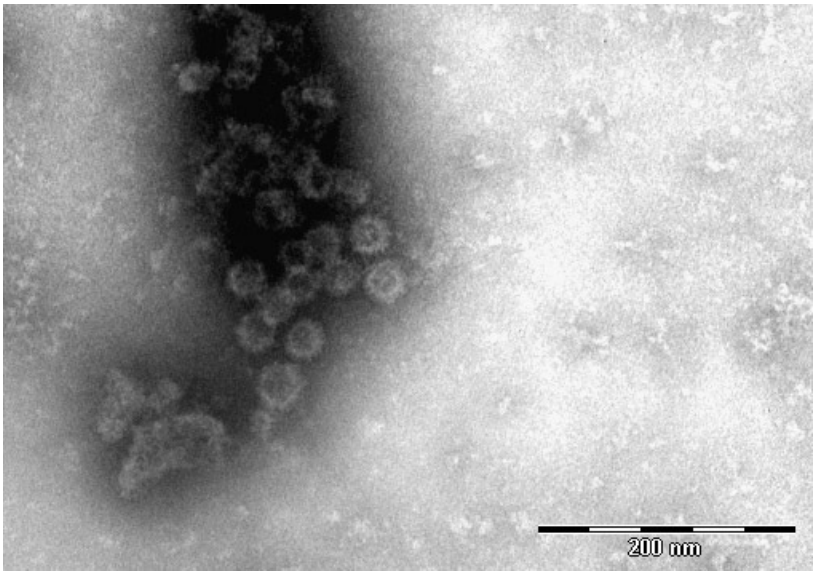


Figure 17: Electron micrograph showing viral particles with size, shape and morphology typical for astroviruses. Magnification: 75 000 x

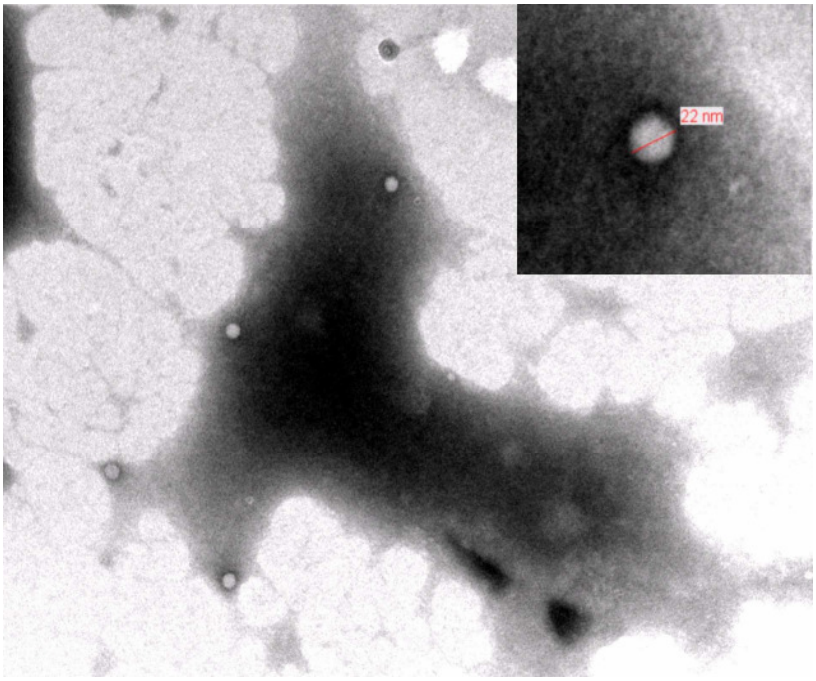


Figure 18: Electron micrograph presenting viral particles with size, shape and morphology typical for parvoviruses. Insert: higher magnification, the diameter of one particle (22 nm). Magnification: 100 000 x

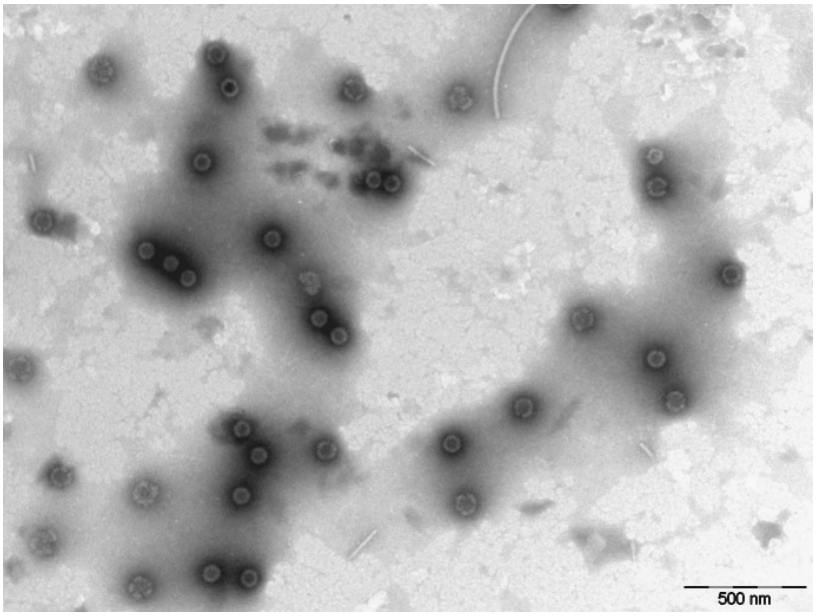


Figure 19: Electron micrograph presenting viral particles with size, shape and morphology typical for the *Reoviridae* family. Magnification: 40 000 x

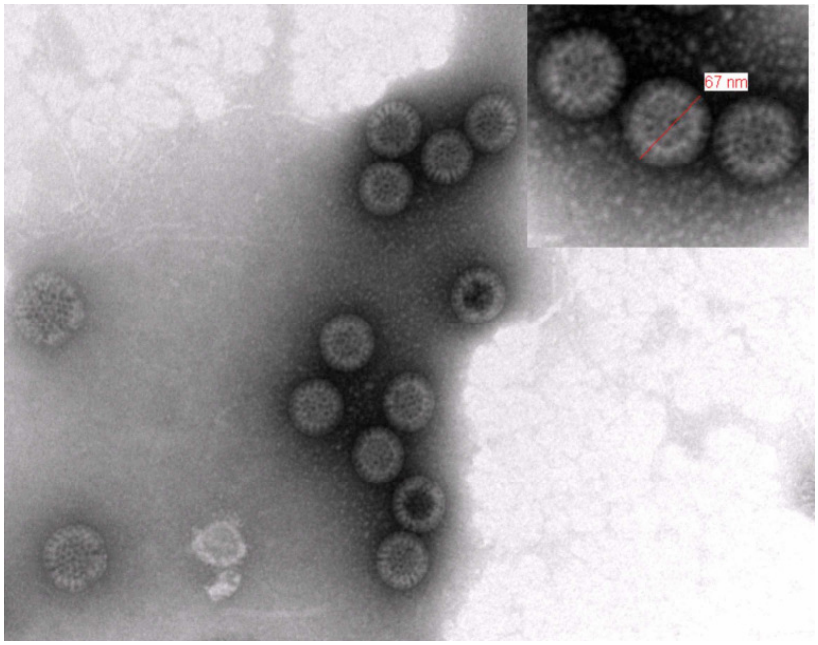


Figure 20: Electron micrograph presenting viral particles with size, shape and morphology typical for rotaviruses. Insert: higher magnification, the diameter of one particle (67 nm). Magnification: 120 000 x

4.1.6. PCR and RT-PCR results for the chicken flocks

The results of the PCR and RT-PCR applied for the chicken samples are summarized in Table 6. No sample was found positive for AAdV1 and AvRV.

A high incidence of ChPV was determined: 17 out of 28 samples were positive. Eight chicken flocks, were free from any other enteric viral infection besides ChPV, as determined by PCR. Astroviruses were found to have a high prevalence also: 12 out of 28 samples, with ANV being more frequent. In the case of the samples collected from clinically healthy flocks only two chicken flocks were found positive for ChPV, while 6 were positive for ANV and 4 for ARV.

Table 6: Results of the PCR and RT-PCR applied for the chicken samples. Samples without clinical signs of ED presented in italic

no	Sample	ANV	CAstV	ARV	ChPV	AvRV	AAdV1
1	Ch 1514/07$_{\beta}$	+	-	-	+	-	-
2	Ch 1515/07 $_{\beta}$	-	-	+	+	-	-
3	Ch 347/08$_{\S}$	+	-	+	+	-	-
4	Ch 348/08$_{\S}$	+	-	-	+	-	-
5	Ch A1/09$_{\delta}$	-	-	-	+	-	-
6	Ch B1/09 $_{\delta}$	-	-	-	+	-	-
7	Ch 189/09	-	-	-	+	-	-
8	Ch 528/09	-	-	-	+	-	-
9	Ch 538/09	-	-	-	+	-	-
10	Ch 841/3/09	-	+	+	+	-	-
11	Ch 852/09	-	+	-	+	-	-
12	Ch 5596/7/10$_{\lambda}$	-	-	-	+	-	-
13	Ch 5596/10/10 $_{\lambda}$	-	+	-	+	-	-
14	Ch 5596/13/10 $_{\lambda}$	-	-	-	+	-	-
15	Ch 5598/18/10 $_{\lambda}$	-	-	-	+	-	-
16	*Ch 847/07*	+	-	-	-	-	-
17	*Ch 1253/07*	-	-	+	-	-	-
18	*Ch 59/08*	+	-	+	-	-	-
19	*Ch 72/08*	-	-	-	-	-	-
20	*Ch 642/08*	-	-	-	-	-	-
21	*Ch 694/08*	+	-		+	-	-
22	*Ch 715/08*	-	-	-	-	-	-
23	*Ch 375/09*	+	-	+	-	-	-
24	*Ch 482/09*	-	-	-	-	-	-
25	*Ch 661/09*	+	-	-	+	-	-
26	*Ch 669/09*	-	-	-	-	-	-
27	*Ch 813/09*	-	-	+	-	-	-
28	*Ch 888/09*	+	-	-	-	-	-

β, §, δ, λ: samples originating from the same flock, but different houses
italic: routine assessments, free of clinical signs of ED

4.2. Enteric disease syndrome in turkeys

4.2.1. Macroscopic examination

The poults presented higher than normal daily mortality, stunted growth (Figure 21 A), diarrhea (figure 21 B), dehydration and high variation in weight amongst the individuals of one flock.

At necropsy the small intestine was partially filled with fluid-mucoid content (Figure 21 C and D), and large amount of gas. Dilatation of the intestinal blood vessels and catarrhal enteritis were identified in the jejunum and ileum and atrophy of the bursa Fabricii was observed.

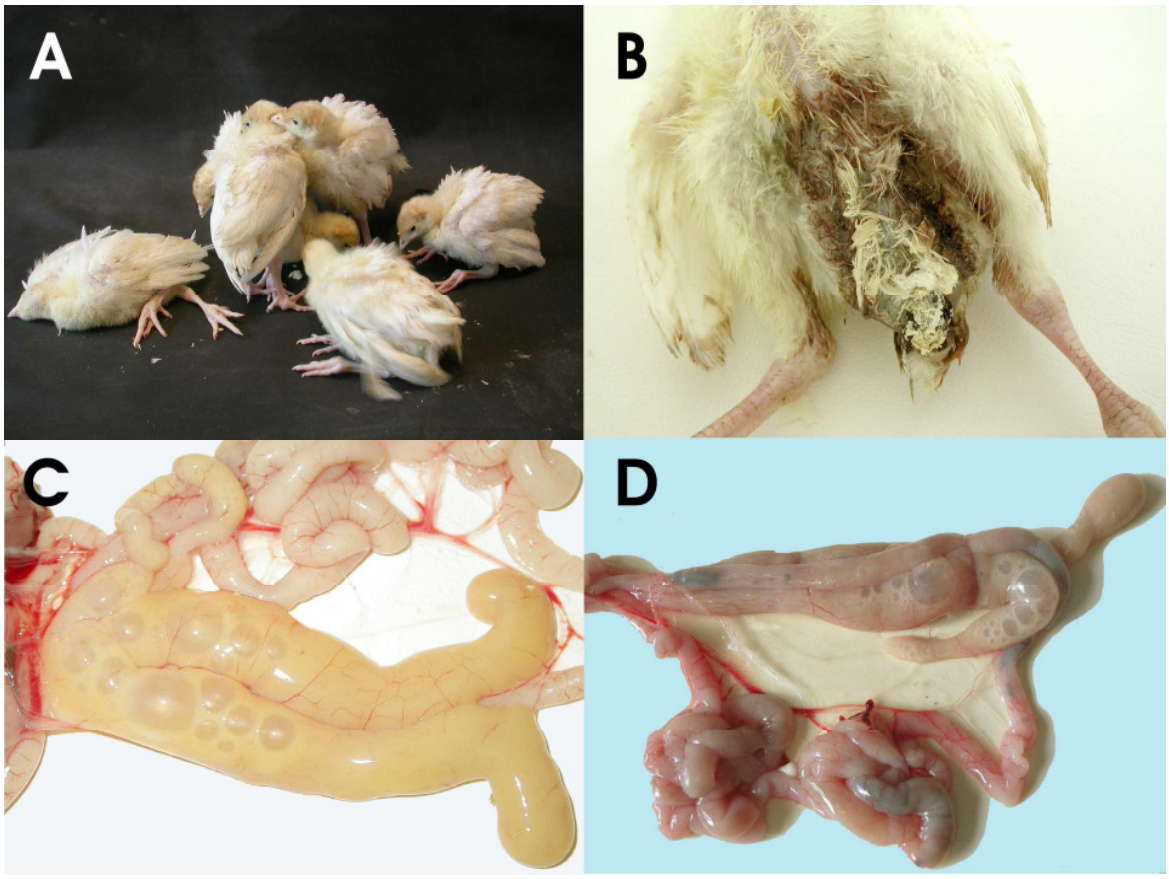


Figure 21: A: signs of stunted growth and uneven development between members of the same flock; **B:** poult with hemorrhagic diarrhea **C:** anemic intestines with signs of disbacteriosis, due to the maldigestion and enteritis; **D:** dilated intestinal and mesenteric blood vessels, enteritis

4.2.2. Bacteriology

Routine aerobic bacteriological investigations, performed from fresh liver tissue, on bloody agar and Drigalski media, were positive for *E. coli* in case of 2 turkey samples.

4.2.3. Histopathology

Shortening, partial denudation and fusion of the intestinal villi was observed in the jejunum (Figure 22), with evident inflammation. The inflammatory cell infiltrate was made up of a mixed population of lymphocytes, histiocytes and a few heterophil granulocytes (Figure 23).

In case of the two samples collected in 2010, mild lymphocyte depletion was observed in the follicles of the bursa Fabricii, without signs of inflammation or epithelial destruction (Figure 24).

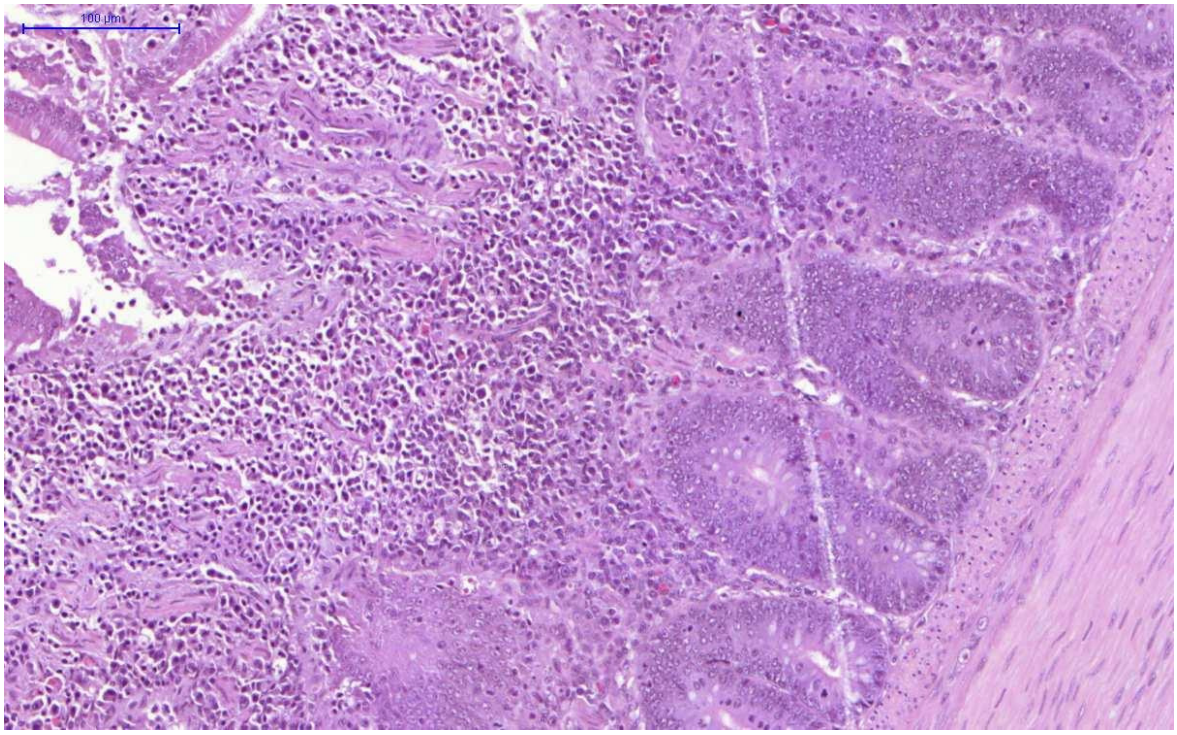


Figure 22: Fusion of the intestinal villi and inflammation (jejunum, H&E staining)

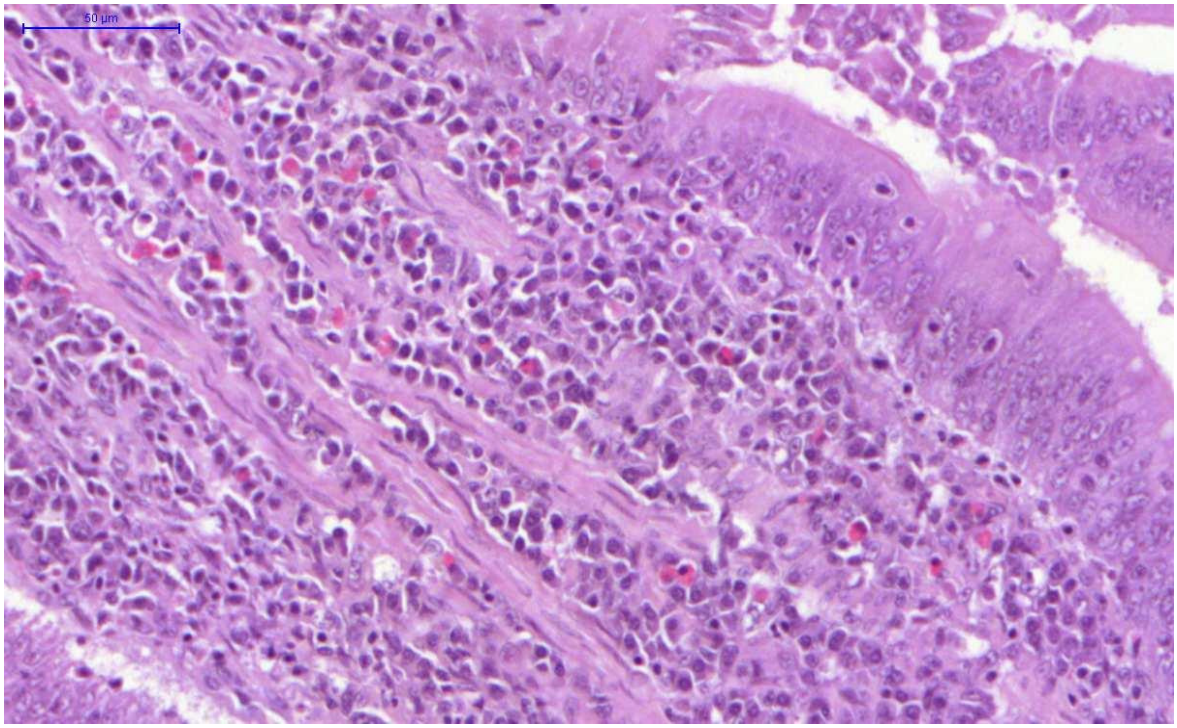


Figure 23: Inflammatory cell infiltrate made up of lymhocytes, histiocytes and a few heterophil granulocytes (jejunum, H&E staining)

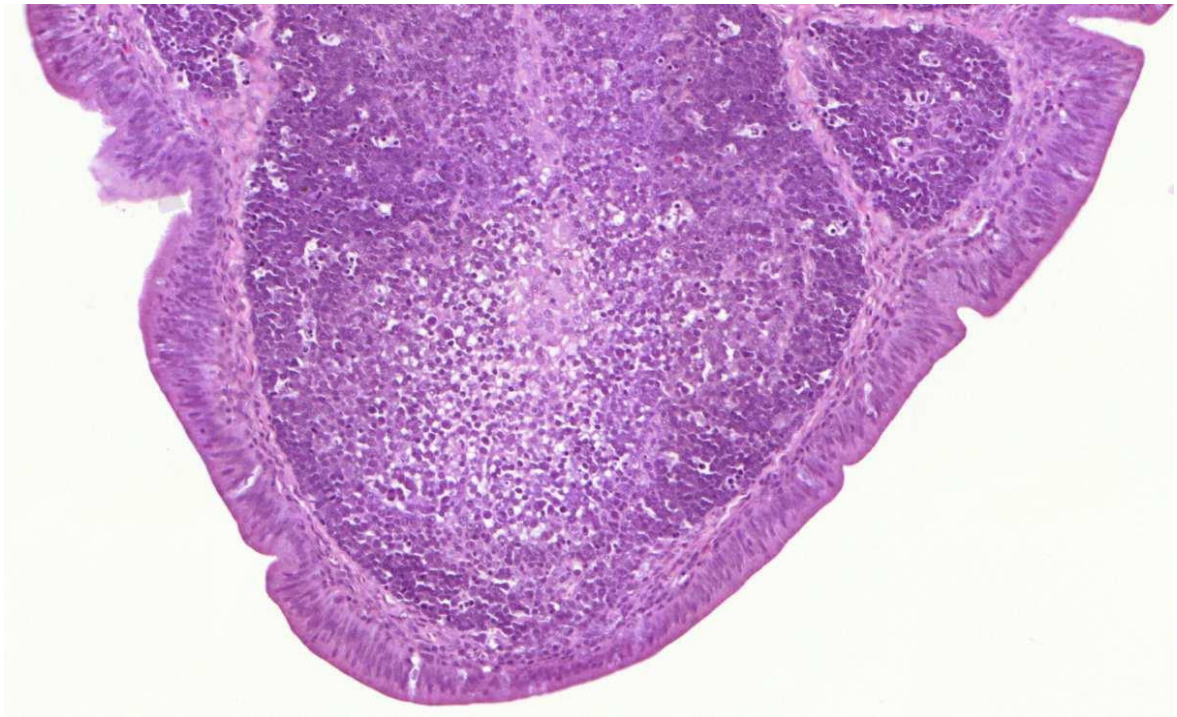


Figure 24: Mild lymphocyte depletion in a follicle (bursa Fabricii, H&E staining)

4.2.4. Immunohistochemistry for TuPV

Positive nuclear staining was detected by indirect IHC in the epithelial cells and inflammatory cells from the lamina propria of the duodenum and jejunum (Figure 25) in case of the two turkey samples collected in 2010, and directly demonstrated to be positive for TuPV by PCR.

Positive reaction was also observed in the follicles of the bursa Fabricii, liver and exocrine pancreas (Figures 26, 27, 28). The summarized results are representative for the changes observed in the samples collected from the turkey flocks from 2010.

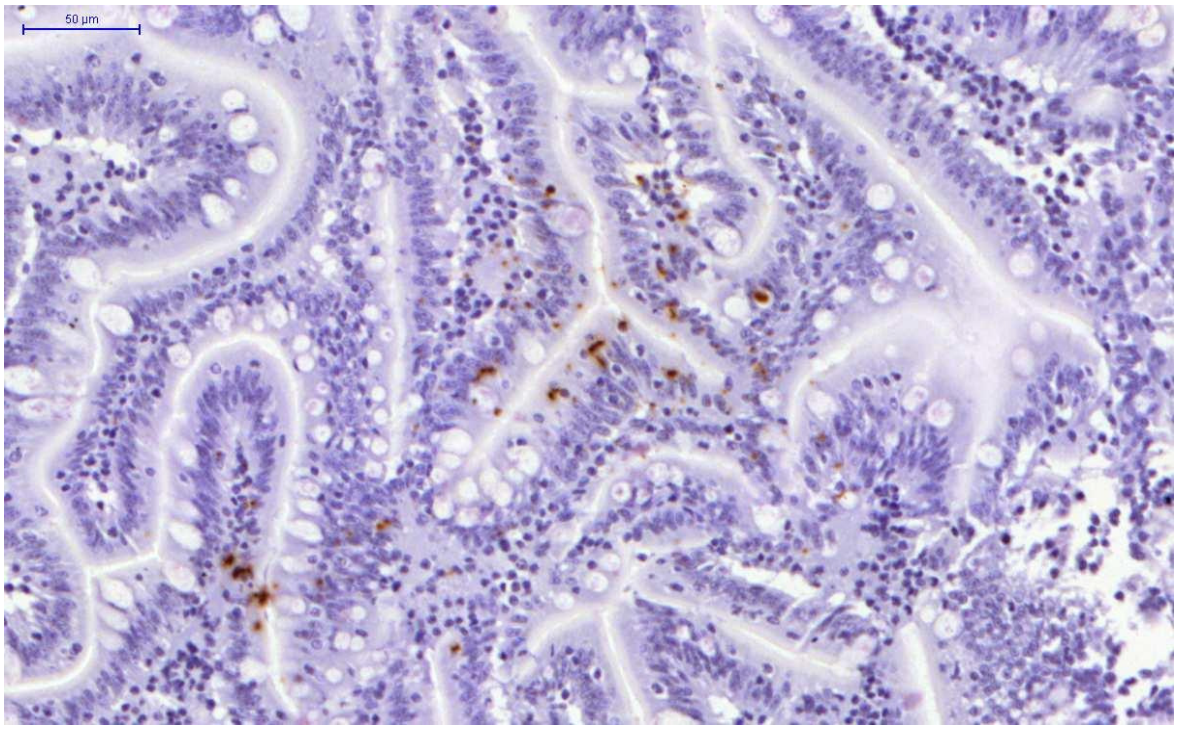


Figure 25: Positive staining by indirect IHC, counterstained with M&H, in the epithelial cells of the jejunum

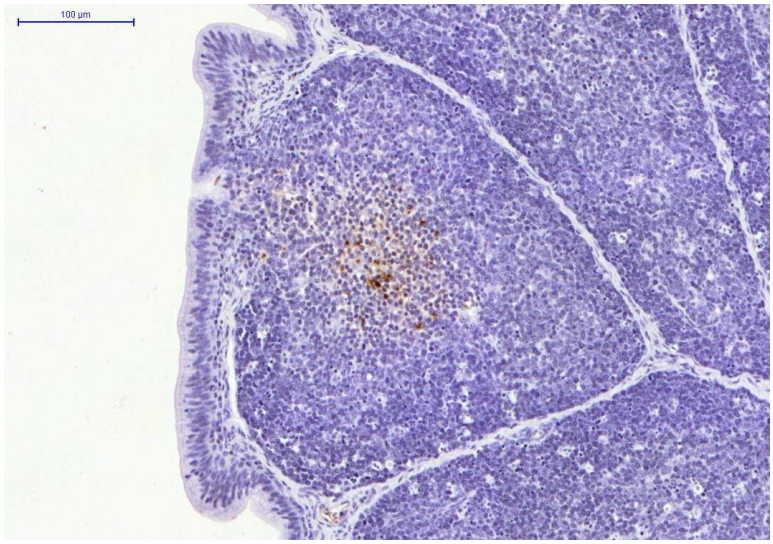


Figure 26: Positive staining by indirect IHC, counterstained with M&H, in the follicles of the bursa Fabricii

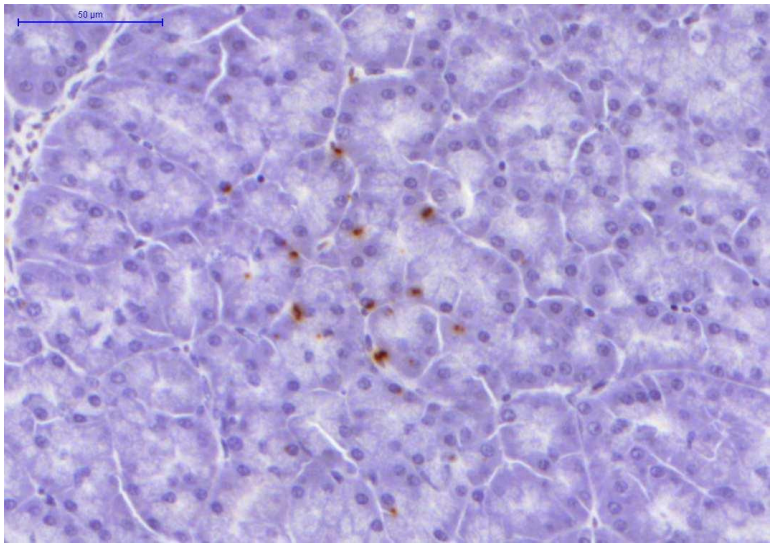


Figure 27: Positive staining by indirect IHC, counterstained with M&H, in the cells of the pancreas

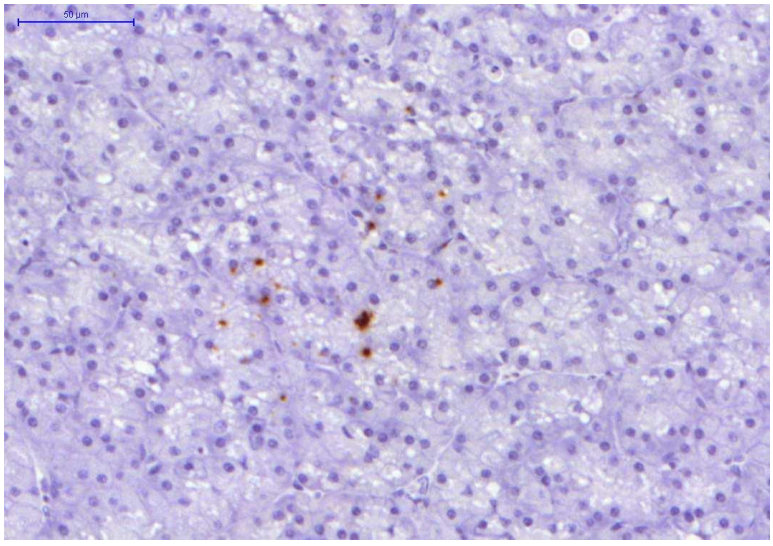


Figure 28: Positive staining by indirect IHC, counterstained with M&H, in the liver

4.2.5. PCR and RT-PCR for the enteric disease in turkeys

Results of the PCR and RT-PCR amplifications are presented in Table 7. HEV was not identified in any of the samples, and only 14.28% positivity was found for TCV and ARV respectively. Turkey astroviruses were found in 83.67% of the cases, and TAstV-2 was found in 26.53% of the cases. Two samples were positive for ANV. Due to the unusual positivity for ANV, as it is considered a virus able to infect and produce clinical signs only in chickens, the positive results were confirmed by direct sequencing.

From a total of 51 samples 25 were found positive for TuPV (49.01%). Singular infections were found in case of 12 flocks (23.52%), from those 6 cases were TAstV positive (11.76%), 5 TuPV positive (9.80%) and only one TCV positive (1.9%).

4.2.6. Statistical analysis

In order to establish whether there is any significant connection and correlation in the incidence of the investigated pathogens, statistical analysis was performed using the correlation testing, and P-values were determined. As the incidence of the pathogens was compared in pairs and type I error rate can increase, therefore P-values were corrected using the Holm correction.

The statistical analysis has revealed that with only a few exceptions there are no significant statistical correlations between the incidences of the investigated pathogens (Tables 8 and 9). The only statistically significant negative values were observed between the incidence of TAstV-2 and TuPV (corrected p value: 0.02) and TCV and TAstVs (corrected p value: 0), while the only positive, “almost” significant correlation was between the incidence of TAstV-2 and ARV (corrected p value: 0.057).

Table 7: PCR and RT-PCR results of the turkey samples included in the thesis

Year	Farm/House	TuPV	ARV	AvRV	TastV-2	HEV	TCV	ANV	TAstVs
2008	B-369/4	+	-	-	-	-	+	-	-
	B-284	+	-	-	-	-	-	-	-
	B-486/2	-	-	+	-	-	-	-	+
	B-369/9	+	-	-	-	-	-	-	+
	B-23/1	+	-	-	-	-	+	-	-
	B-281	+	-	-	-	-	-	-	-
	B-122	-	-	-	-	-	-	-	+
	B-496	-	-	+	-	-	-	-	+
	B-589/4	+	-	+	-	-	-	-	+
	B-589/1	+	-	+	-	-	-	-	+
	B-547	+	-	-	-	-	+	-	+
	B-470/2	-	-	-	-	-	-	-	+
	B-288	+	-	+	-	-	-	-	+
	B-584	+	-	-	-	-	-	-	+
	B-369/1	-	-	+	-	-	+	-	-
	B-501	+	+	-	-	-	-	+	+
	B-23/8	-	-	-	+	-	-	-	+
	B-23/3	+	-	-	-	-	-	-	+
2009	B-291/11	-	-	-	-	-	-	-	+
	B-87	-	-	+	-	-	-	-	+
	B-280	+	-	-	-	-	-	-	+
	B-232	+	-	-	-	-	-	-	+
	B-160	-	-	-	-	-	-	+	+
	B-164/3	-	-	-	-	-	+	-	-
	B-307/2	+	-	-	-	-	-	-	-
	B-307/8	-	-	-	-	-	-	-	+
	B-649	-	+	+	+	-	-	-	+
	B-557	-	-	-	+	-	-	-	+
	B-297	-	-	-	+	-	-	-	+
	B-893	-	-	-	+	-	-	-	+
	B-125	+	-	+	-	-	-	-	+
	B-169/7	+	-	+	+	-	+	-	+
	B-184	-	+	-	+	-	-	-	+
	B-27	-	-	-	+	-	-	-	+
	B-70	+	-	-	-	-	-	-	+
	B-31/2	+	-	+	-	-	-	-	+
	B-315	-	+	-	+	-	-	-	+
	B-465/9	+	-	-	-	-	-	-	+
	B-164/1	+	-	-	-	-	-	-	+
	B-346	+	-	+	-	-	-	-	+
	B-320/4	-	-	-	-	-	-	-	+
	B-164/4	-	+	-	-	-	+	-	-
	B-31/5	+	-	-	-	-	-	-	+
	B121	-	+	-	+	-	-	-	+
	B-648/1	-	-	-	-	-	-	-	+
	B-307/6	-	-	+	-	-	-	-	+
	B-546	-	+	-	+	-	-	-	+
	B-671	-	-	+	+	-	-	-	+
	B-648/2	-	-	-	+	-	-	-	+
2010	33/10	+	-	-	-	-	-	-	-
	762/10	+	-	-	-	-	-	-	-

Table 8: Statistical correlation between the viral pathogens directly demonstrated in turkey samples. NA: not determined. Relevant values highlighted in bold and colors

Statistical correlations							
	TuPV	**ARV**	**AvRV**	**TAstV-2**	**TCV**	**ANV**	**TAstVs**
TuPV	NA	-0.267	0.039	**-0.473**	0.083	-0.27	-0.138
ARV	NA	NA	-0.129	**0.415**	0	0.108	0.023
AvRV	NA	NA	NA	-0.073	0	-0.126	0.157
TAstV-2	NA	NA	NA	NA	-0.113	0.102	0.265
TCV	NA	NA	NA	NA	NA	-0.271	**-0.609**
ANV	NA	NA	NA	NA	NA	NA	0.054
TAstVs	NA	NA	NA	NA	NA	NA	NA

Table 9: Corrected P-value for the viral pathogens directly demonstrated in turkey samples. NA: not determined. Relevant values highlighted in bold and colors

Corrected P-value							
	TuPV	**ARV**	**AvRV**	**TAstV-2**	**TCV**	**ANV**	**TAstVs**
TuPV	NA	1	1	**0.02**	1	1	1
ARV	NA	NA	1	**0.057**	1	1	1
AvRV	NA	NA	NA	1	1	1	1
TAstV-2	NA	NA	NA	NA	1	1	1
TCV	NA	NA	NA	NA	NA	1	**0**
ANV	NA	NA	NA	NA	NA	NA	1
TAstVs	NA	NA	NA	NA	NA	NA	NA

4.3. Sequence analysis and phylogeny of ChPV and TuPV strains

4.3.1. Nucleotide based sequence analysis and phylogeny

A total of 15 ChPV and 25 TuPV positive samples were directly sequenced and analyzed. The obtained sequences were 524 bp long for ChPV strains and 527 bp long for TuPV strains respectively. The phylogenetic tree constructed based on the nucleotide sequence of the analyzed NS1 gene segment revealed an evident clustering of the virus strains of different species origin, ChPV group and TuPV group (Figure 29).

Two ChPV strains (1515/07 from Hungary and Ch841AR05 from USA) proved to be more closely related to TuPV strains than to ChPV strains. Samples 1514/07 and 1515/07 were collected from the same flock, but different houses, still they did not cluster together as expected. The same situation was found in case of several samples collected at the same time from the same flock but different houses (Table 2). With the exception of two samples (33/10 and 762/10) the Hungarian turkey strains clustered separately from the American strains, in two groups. No time connected clustering was observed.

At nucleotide level the identity between the relevant strains considered for comparison, varied from 88.8% to 99.8%, being the lowest when comparing sample 1515/07 with the considered reference strain (EU 304808), and the highest between samples B307/2/09 and B31/6/09 (Table 10). The RDP based investigation of the analyzed gene segment did not reveal any recombination events between the examined strains.

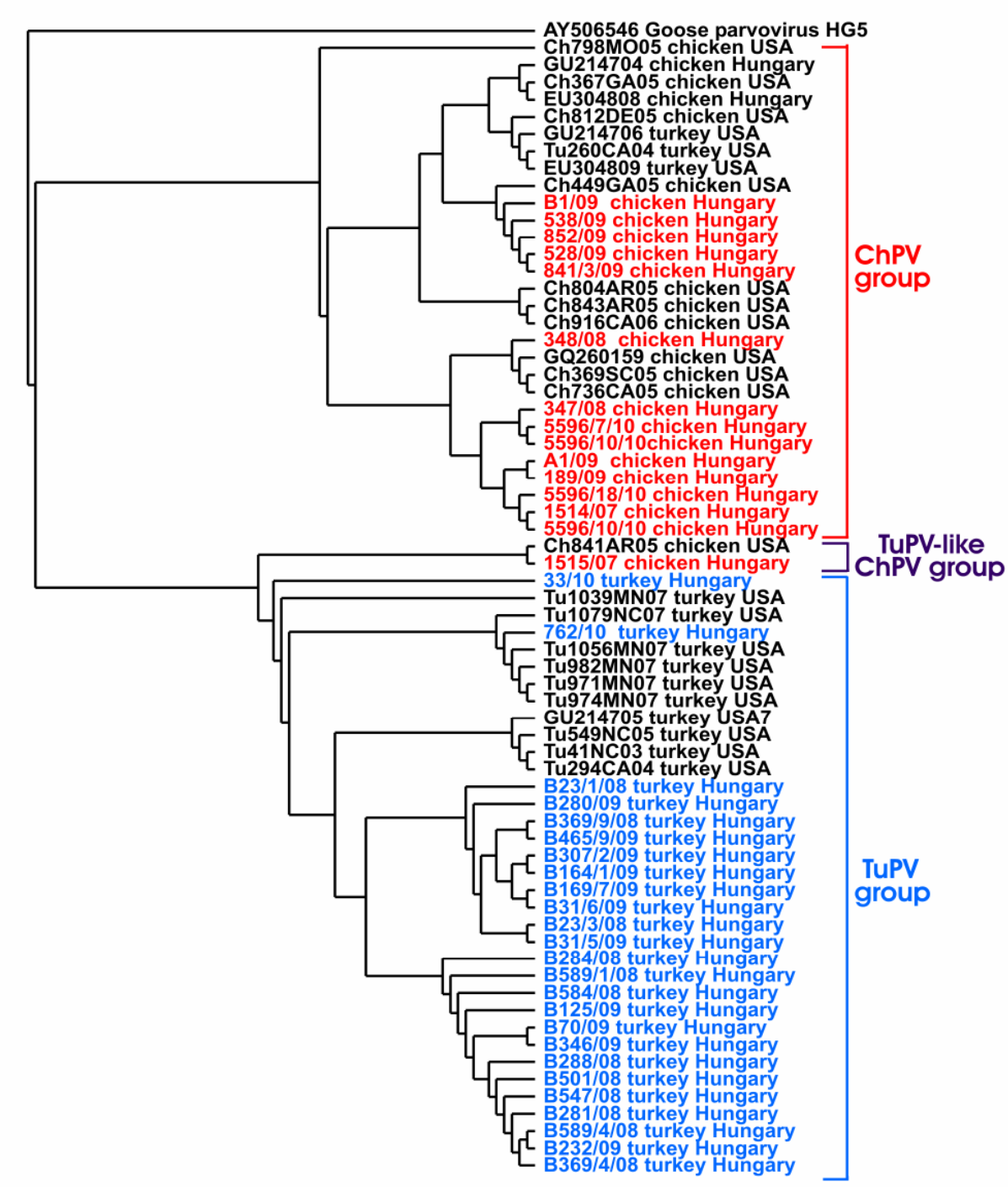


Figure 29: Phylogenetic relationship of the investigated strains based on the nucleotide sequence of the examined region of the Hungarian ChPV (red) and TuPV (blue) strains from this study, the sequences retrieved from the GenBank (accession numbers are indicated for each strain) and the American-origin sequences (no accession numbers available). TuPV-like ChPV group highlighted in purple. Goose parvovirus strain HG5 was used as outgroup

4.3.2. Amino acid sequence analysis and phylogeny

The analysis of the deduced amino acid sequence of the ChPV strains resulted in 174 aa long sequences, and 175 aa in case of the TuPV strains (Figure 30).

An amino acid substitution appeared in almost all chicken origin sequences at position 524, compared with the reference strain (EU304808), where aspartic acid (D) is replaced by glutamic acid (E). Various amino acid substitutions were found throughout the chicken origin amino acid sequences, but with less constant appearance (Figure 31). The same analysis revealed that the two separately clustered ChPV strains presented unique sequence at four aa sites. In case of the turkey origin amino acid sequences a similar situation as for the chicken sequences was found in position 489, where serine (S) is replaced by asparagine (N). In case of the turkey samples similar constant substitutions were found at different positions. All those substitutions were also present in case of the 2 chicken origin samples (1515/07 and 841AR05) that grouped in the TuPV cluster. In addition to all the previously mentioned substitutions the two chicken origin sequences presented two extra unique extra substitutions at positions 524 were instead of aspartic acid (D), asparagine (N) was present, and 651 were glutamic acid (E) was replaced by glycine (G). The Hungarian origin chicken sequence 1515/07 had in addition to all these substitutions two more at position 536 where glutamic acid (E) was replaced by aspartic acid, and 540 where threonine (T) was replaced by serine (S). Positions are according the full length of the NS1 gene of the reference strain (EU304808).

At aa level the identity between the relevant strains considered for comparison varied from 82.3% to 99.6%, being the lowest when comparing sample B70/09 with sample 189/09, and the highest between samples B33/10 and B31/6/09 (Table 10). Compared with the reference strain the lowest level of identity was found for sample B70/09 and the highest for 5596/7/10. Overall the levels of identity in case of aa comparison were lower than for the nucleotide sequence comparisons.

The phylogenetic tree constructed based on the deduced aa sequence of the analyzed NS1 gene segment revealed similar arrangement as described in case of the nucleotide based phylogeny, and the evident clustering of the virus strains of different species origin, ChPV group and TuPV group, was maintained (Figure 30). The two ChPV strains, 1515/07 from Hungary and Ch841AR05 from USA, remained more closely related to TuPV strains than to ChPV strains (Figure 30).

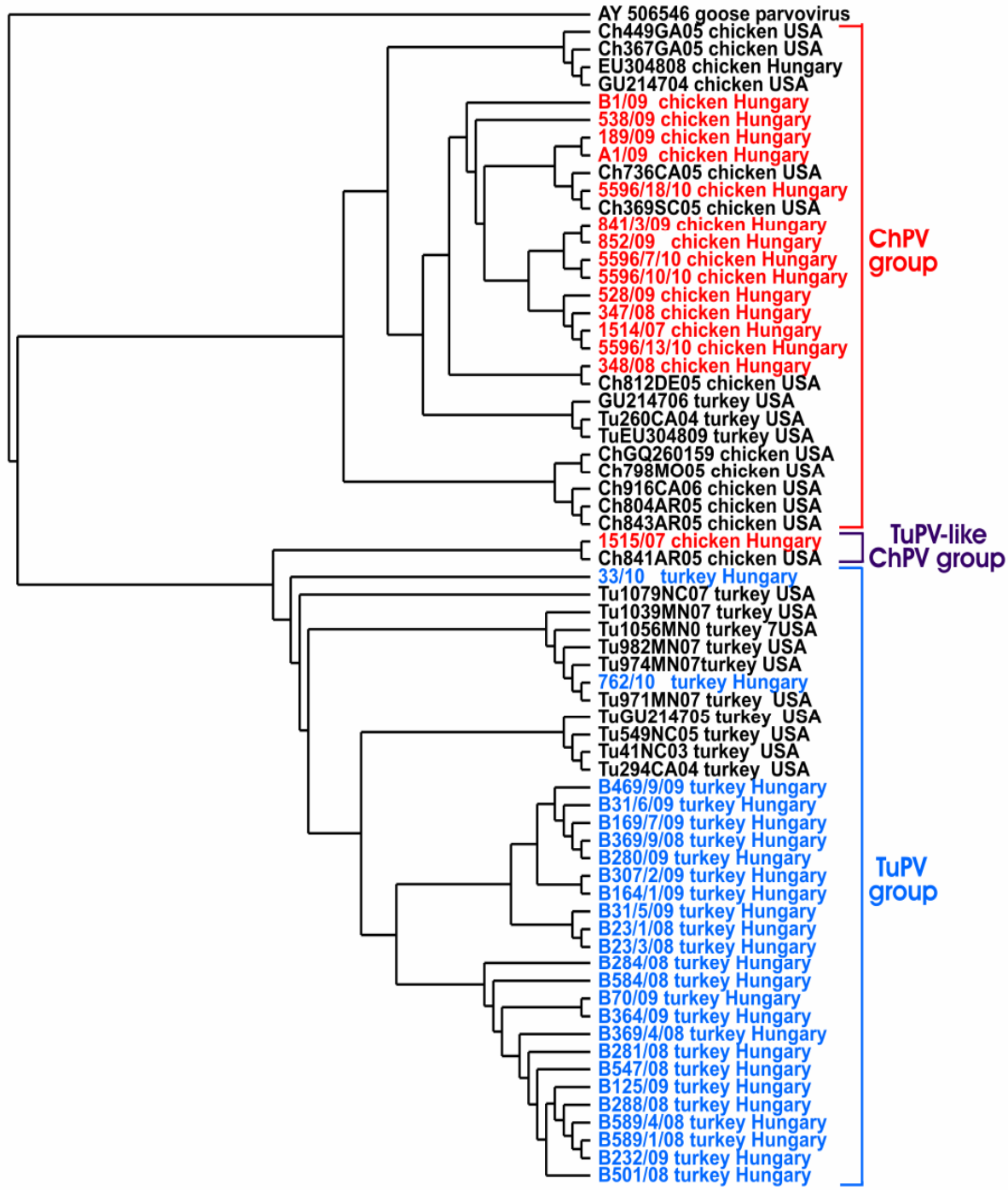


Figure 30: Phylogenetic relationship of the investigated strains based on the amino acid sequence of the examined region of the Hungarian ChPV (red) and TuPV (blue) strains from this study, the sequences retrieved from the GenBank (accession numbers are indicated for each strain) and the American-origin sequences (no accession numbers available). TuPV-like ChPV group highlighted in purple. Goose parvovirus strain HG5 was used as outgroup

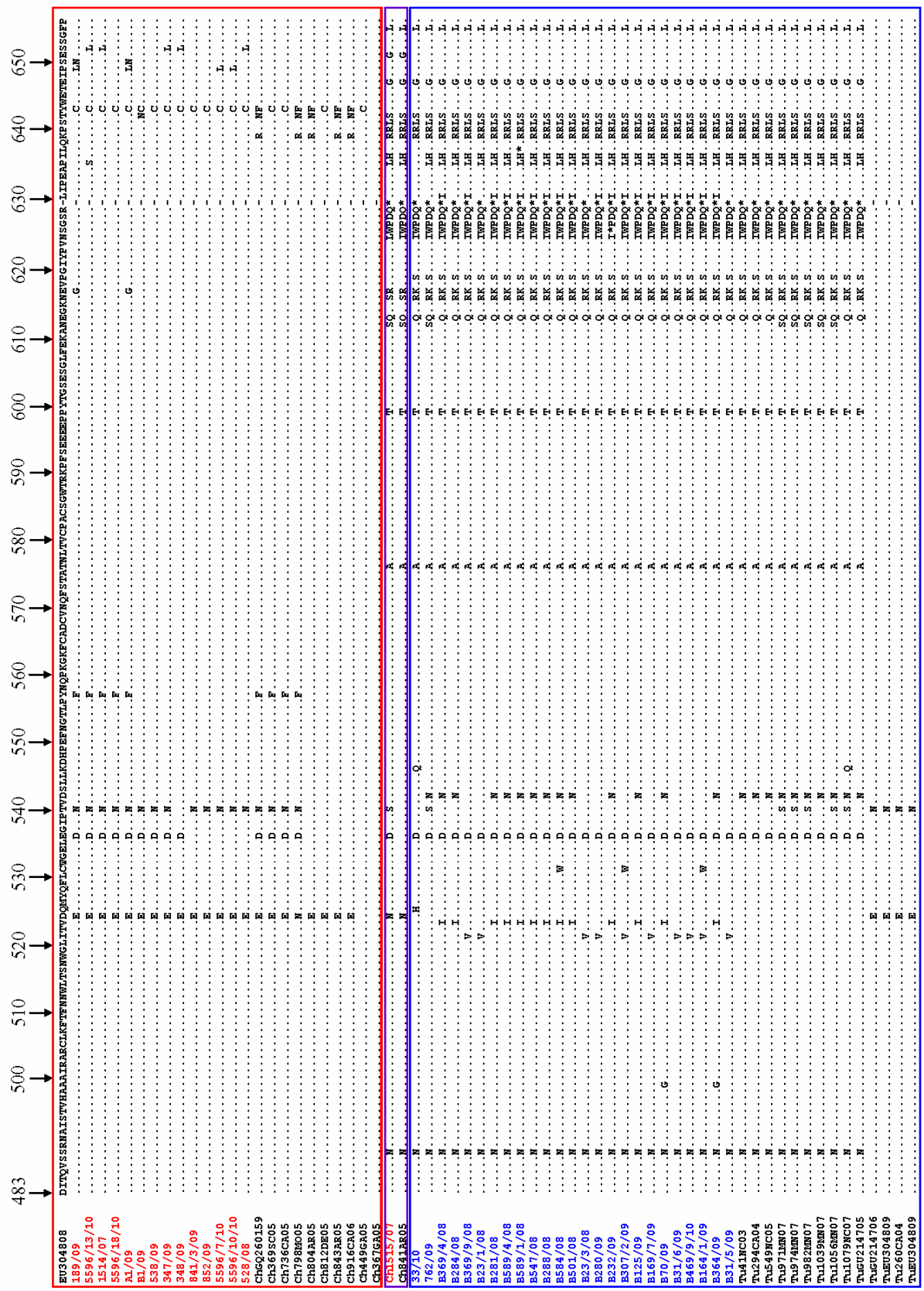


Figure 31: Alignement of the deduced aa sequences included in this study, ChPV strains red square, TuPV-strains blue squeare, and TuPV-ChPV strains purpule square. Hungarian ChPV stains in red and TuPV strains in blue.

Table 10: Level of identity between the nucleotide and deduced aa sequences of representative Hungarian ChPV (red), TuPV (blue), and strains retrieved from the data base. Values in italic represent the level of identity at aa level. Lowest levels of identity are marked in bold

	EU304808	189/09	347/09	A1/09	5596/7/10	841AR05	1515/07	GU214705	1039MN07	B288/08	B70/09	B23/1/08	B307/2/09	B31/6/09	33/10
EU304808	-	*95.4*	*97.1*	*95.4*	*97.7*	*86.8*	*85.7*	*86.8*	*86.3*	*85.7*	*85.1*	*86.8*	*85.7*	*86.3*	*87.4*
189/09	95.0	-	*97.1*	*100*	*97.7*	***83.4***	*84.0*	*84.0*	*83.4*	***82.8***	***82.3***	*84.0*	*82.8*	*83.4*	*84.6*
347/09	96.4	97.7	-	*97.1*	*98.3*	*84.6*	*85.1*	*85.1*	*84.6*	*84.0*	*83.4*	*85.1*	*85.1*	*84.6*	*85.7*
A1/09	95.0	99.2	97.3	-	*97.7*	*83.4*	*84.0*	*84.0*	*83.4*	***82.8***	***82.3***	*84.0*	*82.8*	*83.4*	*84.6*
5596/7/10	96.9	97.1	98.3	97.1	-	*85.1*	*84.6*	*84.6*	*84.0*	*83.4*	*82.8*	*84.6*	*83.4*	*84.0*	*85.1*
841AR05	89.7	89.7	90.1	89.7	89.7	-	*98.3*	*95.4*	*96.0*	*94.3*	*93.7*	*95.4*	*94.3*	*94.8*	*93.7*
1515/07	**88.8**	89.2	89.6	89.2	**89.0**	99.0	-	*94.8*	*95.4*	*93.7*	*93.1*	*94.8*	*93.7*	*94.3*	*93.1*
GU214705	89.9	89.4	90.1	89.4	89.6	96.8	96.6	-	*99.4*	*98.8*	*98.3*	*98.8*	*97.7*	*98.3*	*97.1*
1039MN07	89.9	89.4	90.1	89.4	89.6	96.8	96.6	99.6	-	*98.3*	*97.7*	*98.3*	*97.1*	*97.7*	*96.6*
B288/08	89.6	89.0	89.7	89.0	89.2	96.2	96.0	99.4	99.0	-	*99.4*	*97.7*	*97.7*	*98.3*	*96.0*
B70/09	89.4	**88.8**	89.6	**88.8**	89.0	96.0	95.8	99.2	98.9	99.8	-	*97.1*	*97.1*	*97.7*	*95.4*
B23/1/08	89.7	89.2	89.9	89.2	89.4	96.6	96.4	99.4	99.0	98.9	98.7	-	*98.8*	*99.4*	*97.1*
B307/2/09	89.4	89.2	89.6	89.2	89.4	96.0	95.8	98.9	98.5	98.7	98.5	99.4	-	*99.4*	*96.0*
B31/6/09	89.6	89.4	89.7	89.4	89.6	96.2	96.0	99.0	98.7	98.9	98.7	99.6	99.8	-	*96.6*
33/10	90.1	90.1	90.3	89.9	90.1	95.4	95.2	97.9	97.9	97.3	97.1	97.7	97.5	97.7	-

4.4. Differentiation of ChPV, TuPV and TuPV-like ChPV strains

According to the nucleotide sequences alignment of all existing strains deposited in the GenBank, the sequences of American origin and the Hungarian sequences included in this study, an enzyme site recognition is present on the 561 bp fragment, which has the potential of differentiation of ChPV strains, TuPV strains and TuPV-like ChPV strains that formed a unique subgroup in the TuPV cluster (TuPV-like ChPV), by employing a RFLP-based techinque.

The alignment of the all sequences ChPV, TuPV and Tu-PV-like ChPV, on the fragments in question, with the cleavage site of the *AvaII* enzyme is shown in Figure 32.

Following the newly developed and previously described protocol (Chapter 3.6.6), in case of the ChPV strains two clearly differentiable bands at the predicted sizes of 415 bp and 146 bp were obtained, in case of the TuPV strains two clearly differentiable bands at 323 bp and 238 bp, while in case of the amplicons of the two chicken origin samples that clustered in the TuPV-like ChPV group, three differentiable bands were obtained at 323 bp, 146 bp and 92 bp (Figure 33).

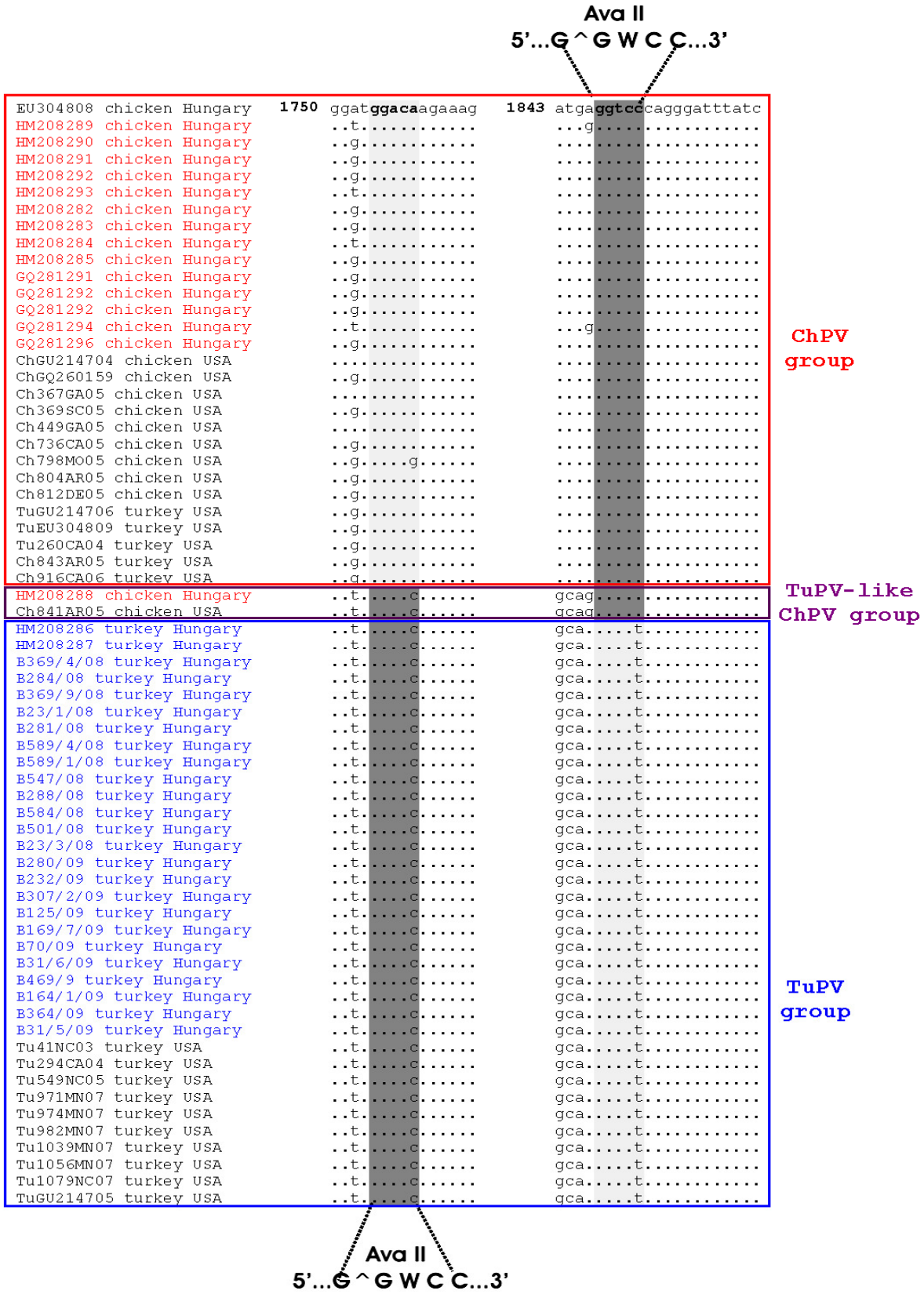


Figure 32: The *AvaII* enzyme cleavage site (dark grey) that allows the differentiation of ChPV, TuPV and TuPv-like ChPV strains

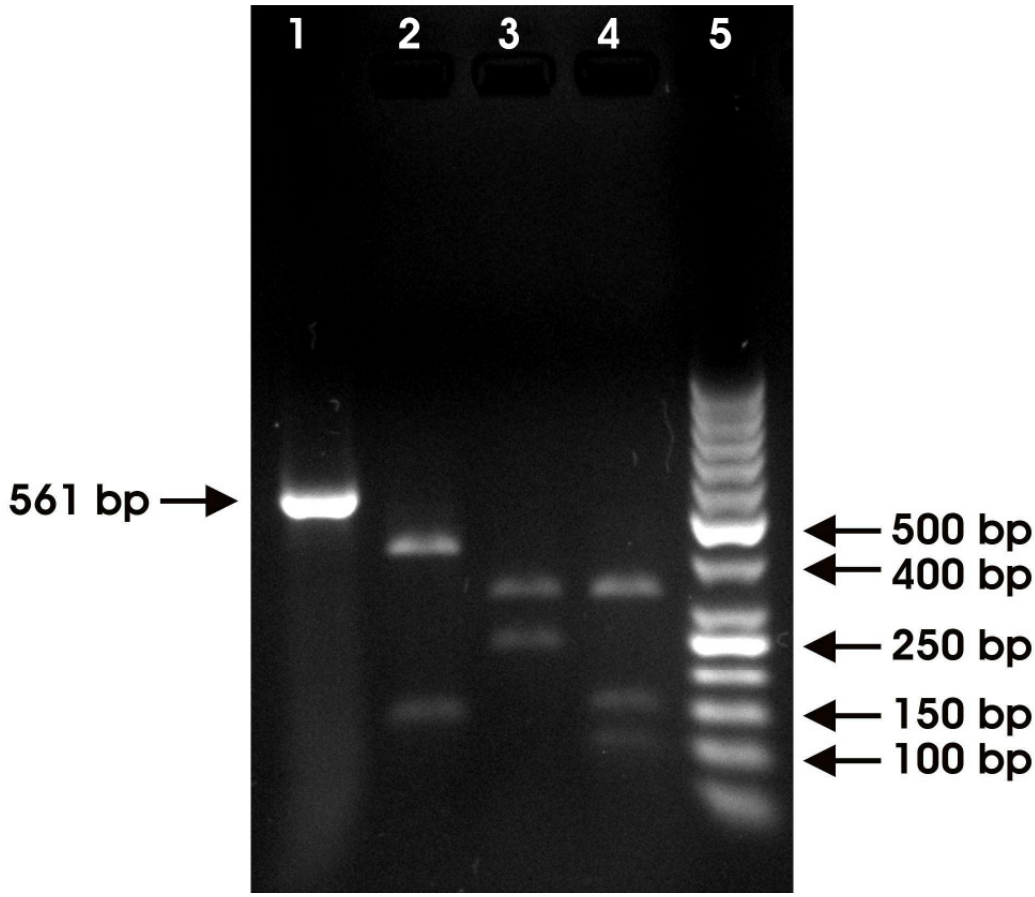


Figure 33: Result of the RFLP analysis based on *AvaII* restriction enzyme recognition sites of the ChPV and TuPV amplicons produced by the diagnosis primers (Table 4). Lane 1: PCR product of the diagnostic primers; Lane 2: ChPV strain digested in two fragments 415 bp and 146 bp; Lane 3: TuPV strain digested in two fragments 323bp and 238 bp; Lane 4: TuPV-like strain 1515/07 (accession number: HM208288) digested in three fragments 323 bp, 146 bp and 92 bp; Lane 5: molecular marker (50 bp.)

4.5. Accession numbers

The Hungarian ChPV/TuPV sequences from this study were uploaded to the GenBank and the following accession numbers were assigned.

Sequences originating from Hungarian chicken broiler flocks

528/09: GQ281293; A1/09: GQ281294; B1/09: GQ281296; 347/08: GQ281291; 348/08: GQ281292; 5596/7/10: HM208282; 5596/10/10:HM208283; 5596/13/10: HM208284;

5596/18/10: HM208285; 1515/07: HM208288; 189/09: HM208289; 538/09: HM208290; 841/3/09: HM208291; 852/09: HM208292; 1514/07: HM208293

Sequences originating from Hungarian turkey flocks

33/10: HM208286; 762/09: HM208287;

A total of 23 TuPV sequences are in the course of receiving accession numbers.

Other sequences used in this study:

Codes of the American origin sequences

Ch367GA05; Ch798MO05; Ch843AR05; Ch369AR05; Ch804AR05; Ch916CA06; Ch449GA05; Ch812DE05; Ch736CA05; Ch741AR05; Tu294CA04; Tu982MN07; Tu549NC05; Tu1030MN07; Tu41NC03; Tu971MN07; Tu1056MN07; Tu260CA04; Tu974MN07; Tu1079NC07 (no accession numbers available)

Sequences retrieved from the GenBank

AY506546; EU304808; EU304809; GU214704; GU214705; GU214706; GQ260159

4.6. Simultaneous detection of ANV, IBV and IBDV by mRT-PCR

The diluted sample templates were subjected to mRT-PCR under the previously described optimized thermal profile (Chapter 3.6.3.3.), and the amplified products were analyzed by agarose gel electrophoresis. The specificity of the primer pairs was proven by the successful amplification of the expected products (Table 5), without the presence of non-specific bands. The initial results were verified by individual RT-PCRs applied according to the described protocols (Table 5).

The mRT-PCR was able to detect the RNA template at levels as low as 8.45×10^{-2} µg for IBDV, 8.92×10^{-3} µg for ANV, and 8.87×10^{-3} µg for IBV (Figure 34), without producing any nonspecific amplicons that may interfere with the diagnostic purpose of the test (Figure 35).

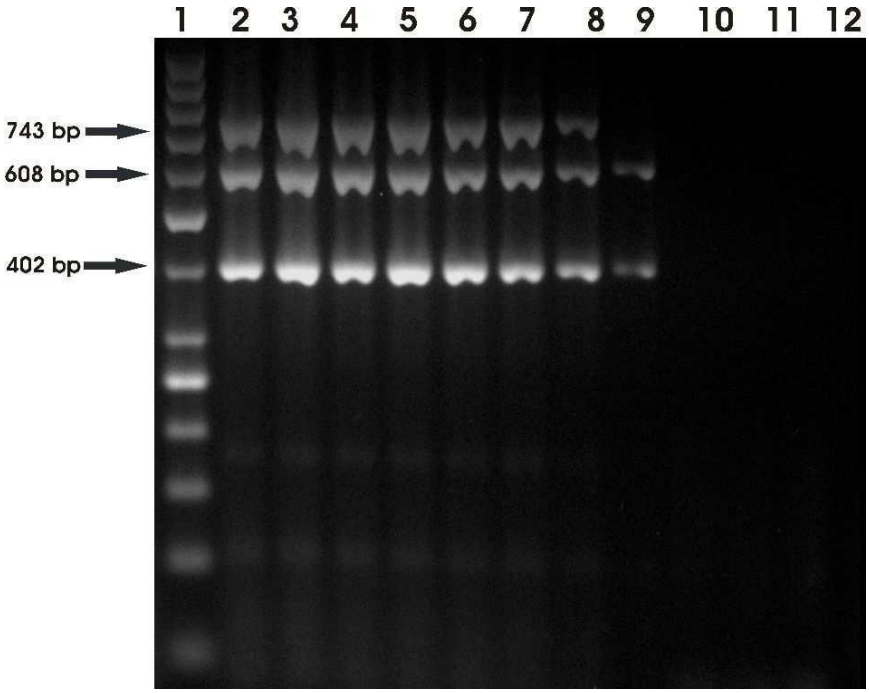


Figure 34: Sensitivity of the newly developed mRT-PCR.
Lane 1: molecular marker; lane 2: starting template, IBVD RNA at 8.45 x 10^5 µg, ANV RNA at 8.92 x 10^5 µg, IBV RNA at 8.87 x 10^5 µg; lanes 3-12: serial 10-fold dilution from the starting template

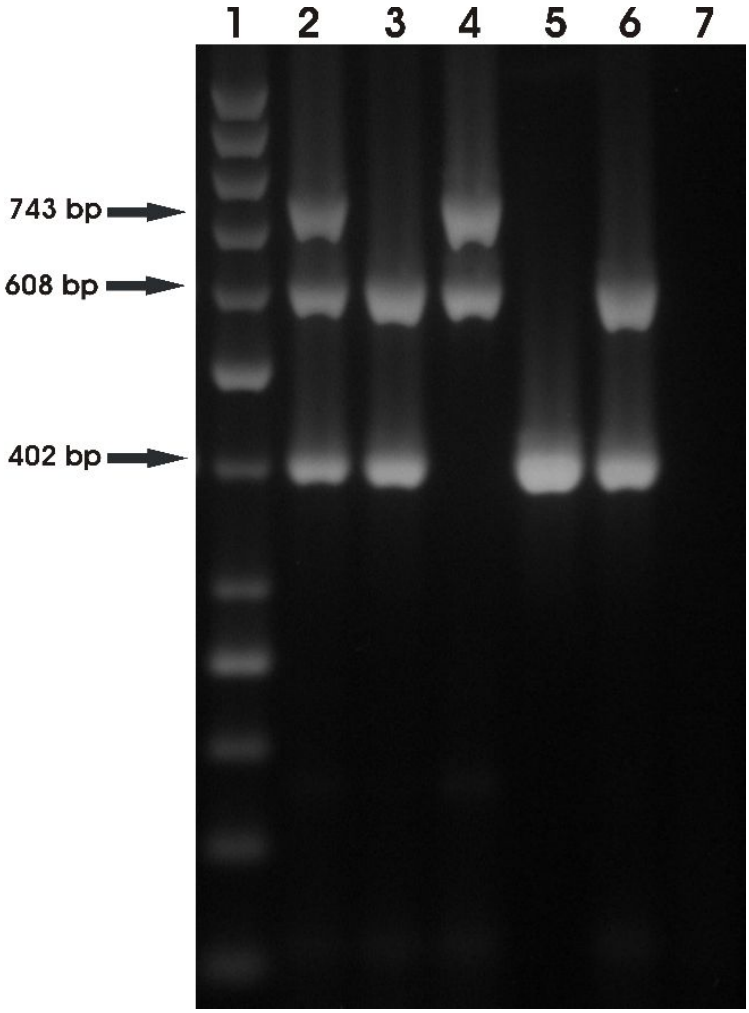


Figure 35: Results of the diagnostic mRT-PCR applied for four flocks.
Lane 1: molecular marker, lane 2: positive control, lanes 3 to 6: field samples, lane 7: negative control, no template

5. Discussions

Despite the intense research regarding the ED, a major disease complex still seriously threatening the poultry industry, the causative viral pathogens are not accurately identified, and its pathogenesis is not fully understood. Numerous viruses have been incriminated as causing or participating in the etiology of the ED: astroviruses, coronaviruses, reoviruses, and rotaviruses (Pass *et al.*, 1982; Reynolds *et al.*, 1987a, b; Goodwin *et al.*, 1993; Guy, 1998; Koci *et al.*, 2000; Yu *et al.*, 2000; Spackman *et al.*, 2005a, b; Pantin-Jackwood *et al.*, 2007a, b; Day *et al.*, 2007a, b; Jones, 2008; Reynolds and Shultz-Cherry, 2008; Pantin-Jackwood *et al.*, 2008b;).

The most recently accepted causative viral pathogen is a new member of the *Parvoviridae* family (Woolcock and Shivaprasad, 2008; Zsak *et al.*, 2008; Zsak *et al.*, 2009; Day and Zsak, 2010) that was suspected decades ago as causative agent in RSS (Kisary *et al.*, 1984; Kisary *et al.*, 1985a).

Epidemiological survey of viral pathogens incriminated in the ED of chickens

A total of 28 chicken broiler flocks were included in the thesis, 15 with clinical signs of RSS, and 13 healthy flocks, included with the purpose of comparison, and selected from the cases received for routine health flock evaluation.

In case of the 15 chicken flocks presenting RSS, the histological examination revealed the moderate to severe distention of the intestinal crypts and acute catarrhal enteritis with a mixed inflammatory cell population in the jejunum and duodenum, with a low incidence of enterocyte desquamation. Shortening, moderate denudation and fusion of the intestinal villi were observed. All these findings are typical for ED, and similar changes were described by previous studies (Page *et al.*, 1982; McNulty *et al.*, 1983). In a few cases active regeneration in the small intestine was present, suggestive of the fact that the birds from which the samples were collected probably have passed through the disease and recovered, but the viral pathogens were still present in their tissues as determined by PCR testing. The regeneration is not an uncommon finding as the main problem in case of RSS

is not the high mortality, but the general weakening of the flock, hence most chickens will survive and the affected gastrointestinal tract will undergo regeneration.

Lympho-histiocytic nodular pancreatitis was also observed in the chicken samples; unfortunately due to the lack of data regarding the pathogenesis of this syndrome the causative agent of this lesion cannot be reliably determined. Reoviruses are known to cause lesions in the pancreas; however, only 3 chicken flocks from the ones presenting clinical signs of RSS were infected with ARV, hence this pathogen cannot be considered the only cause for the pancreatitis. Routine aerobic bacteriological investigation was negative for all the chicken flocks included in this study, demonstrating that all macroscopic and histopathological changes found, were due to the presence of viral pathogens.

EM was successfully used for a long time in the diagnosis of ED (McNulty *et al.*, 1980; Kisary *et al.*, 1984; Reynolds *et al.*, 1987a). The only viral particles observed by EM in the intestinal content samples collected from 6 Hungarian broiler flocks, which were also found positive by PCR only for ChPV, were parvoviruses, according to their size, shape, and morphology (Figure 18).

The epidemiologic study attempted in this thesis included a wide range of viral pathogens previously incriminated in ED, such as: astroviruses, coronaviruses, reoviruses, rotaviruses, adenoviruses, but also newly suggested agents as: ChPV. The viruses directly identified in the chicken flocks are similar with previous reports. No sample was found positive for AAdV1, an interesting result, considering that most commercial flocks are known to be infected with adenoviruses. A low incidence was found in case of ARV. Only 3 cases of positivity were found in the chicken flocks with clinical signs of RSS, and 4 in case of the flocks without clinical signs. This finding was similar with other studies that have reported similar incidence of ARV in healthy and diseased chicken flocks (Pantin-Jackwood *et al.*, 2008a). CAstV was found only in case of the flocks with signs of RSS, 4 out of 15 (26.66%), an interesting finding considering that previous studies determined a high incidence of astroviruses in healty chicken flocks as well (Pantin-Jackwood *et al.*, 2008a, b). A high incidence of ChPV was determined, 17 out of 28 samples were positive if all chicken flocks are considered, and all flocks with clinical signs of RSS were infected with ChPV (100%). Unfortunately there are only few studies regarding the incidence of ChPV in healthy chicken flocks (Zsak *et al.*, 2009), and to our knowledge no data concerning the prevalence of ChPV in flocks with RSS clinical signs.

Beside ChPV, astroviruses were found to have the highest prevalence in the chicken flocks (12 positive samples out of 28), with ANV being the most frequent. This is an expected finding as according to previous studies involving both healthy and diseased chicken flocks, from the enteric viruses the highest prevalence was observed in case of astroviruses. Interestingly ANV was found in 6 out of the 13 chicken flocks included only as routine assessment, with no clinical signs, and only in 3 out of the 15 chicken flocks with clinical signs of RSS. ANV is known to cause clinical disease especially in day old chickens (Mándoki *et al.*, 2005 and 2006), but no age correlation could be detected for the chicken flocks. No difference was observed regarding epidemiology between the two groups of chicken samples.

All chicken flocks with clinical signs of RSS proved to be positive for ChPV, however this pathogen was also found positive in case of two samples with no clinical signs, hence no correlation can be made. The completely viral etiology of the RSS is supported by the syndrome reproducibility with preparations from the intestinal contents of affected birds that do not contain bacteria or protozoa (Barnes and Guy, 2003).

Epidemiological survey of viral pathogens incriminated in the ED of turkeys

The viruses identified in the 51 turkey flocks included in this study are also similar with previous reports; however a broader range of pathogens was incorporated, including the scarcely known TuPV, for a better understanding of the epidemiology of ED in turkeys.

EM was successfully used for a long time in the diagnosis of ED and the identification of the viral pathogens present in the intestinal tract of chickens and turkeys, but ever since the expansion of the molecular techniques, PCR and RT-PCR are considered far more reliable and sensitive for this purpose, permitting also viral characterization by sequencing techniques.

By employing EM no fraction was found containing particles with size, shape and morphology typical for coronaviruses or adenoviruses. All flocks have been negative for HEV by PCR; however 7 out of 49 flocks were positive for TCV. The failure to identify coronavirus particles by EM can only be explained by the reduced sensitivity of this method compared with PCR, regarding the identification of viral pathogens. Molecular methods have become routinely used in most diagnostic laboratories, and have been

developed for many avian enteric viruses, as they offer a sensitive and specific alternative for virus demonstration, compared with the EM methods.

The most recently incriminated causative viral pathogen in ED in turkeys is a new member of the *Parvoviridae* family. Our investigations directly demonstrated the presence of the scarcely known TuPV in 25 Hungarian turkey flocks (49.01%) experiencing clinical signs of ED, making this pathogen the second most identified after astroviruses. The wide distribution of TuPV in American commercial flocks was recently reported (Zsak *et al.*, 2009). Recent studies have shown the presence of TuPV in Hungarian turkey flocks presenting clinical signs of ED. To our knowledge there is no data on the role of TuPV in the ED of turkeys, still the results of the present survey accentuate their potential involvement, as suggested earlier.

Astroviruses were the most identified viral pathogens in the investigated turkey flocks, nevertheless previous reports have shown a high prevalence of astroviruses in healthy turkey flocks (Pantin-Jackwood *et al.*, 2008b), making this finding difficult to interpret. Avian nephritis virus, a pathogen known to infect and cause disease in young chickens and pheasants, was found in 15 flocks but no data is available concerning any possible involvement of this pathogen in PEMS, as it was only recently directly identified for the first time in healthy commercial turkey flocks (Pantin-Jackwood *et al.*, 2008b). Due to the novelty of this infection, and because ANV is known to infect and cause clinical diseases only in chickens (Mándoki *et al.*, 2005 and 2006), positivity for ANV was confirmed by direct nucleotide sequencing and BLAST search against GenBank data base (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Singular infections were found in case of 12 flocks (23.52%), from those 6 cases were TAstV positive (11.76%), 5 TuPV positive (9.80%) and only one TCV positive (1.9%). It is not considered surprising the fact that flocks with singular infections will develop clinical signs of PEC or PEMS, as TAstVs infections are known to cause even severe losses in a flock especially when the TAstV-2 subtype is present. In the early research on PEC and PEMS it was determined that TCV is a pathogen without which the clinical signs cannot appear, being the first viral agent associated with ED (Lin *et al.*, 1996), however following studies and experimental infections proved that its presence was not necessary for the disease (Barnes *et al.*, 1997).

Not detecting HEV in any of the flocks is not unexpected as the disease is well controlled and prevented by the extensive use of vaccination.

The role of reoviruses in PEC and PEMS was intensely debated. The first investigations about pathogenicity of the reovirus in the stunting syndrome were made by Yu *et al.* (2000) who concluded that it is not an important pathogen for clinical symptoms of the disease. However, Heggen-Peay *et al.* (2002) demonstrated that strain ARV CU98 isolated from PEMS poults does cause clinical signs in PEMS.

The statistical analysis results are suggesting a negative correlation between ARV and TuPV, and TCV and TAstVs respectively, hence if one of the viruses is present it is less likely for the second one to appear. The only "almost" positive correlation was found between TAstV-2 and ARV, as they were found most frequently together. Comprehensive experimental infections are still needed to determine if there is indeed a positive correlation between those pathogens.

Identification of the newly incriminated avian parvovirus in the ED complexes

Our investigations revealed the presence of the scarcely known ChPV and TuPV in 15 Hungarian chicken flocks and 25 Hungarian turkey flocks experiencing clinical signs of ED. The wide distribution of ChPV and TuPV in American commercial flocks was recently reported (Zsak *et al.*, 2009), however in the mentioned study there was no correlation between virus presence and disease or unsatisfactory feed conversion efficiency in the included farms. The mortality in the affected Hungarian chicken flocks was assessed to be just above the allowed level, while morbidity was 50-60%, observations sustained by previous reports in case of ED (Barnes *et al.*, 2000; Barnes and Guy, 2003).

Infection with ChPV was directly demonstrated in chickens as young as 6 days, presenting clinical signs of ED: this finding emphasizes the potential vertical transmission of the virus, as previously suggested (Kisary *et al.*, 1985b; Zsak *et al.*, 2009). No correlation was observed between age, intensity of the clinical signs and pathological findings. The common pathological finding was the enteritis in the duodenum and jejunum with dilatation of the crypts without villus atrophy, as found in reovirus and rotavirus intestinal viral infection, considered to be widespread in chicken flocks, especially in the first two weeks of life (Pantin-Jackwood *et al.*, 2008a; Day *et al.*, 2007b).

IHC positive nuclear staining for ChPV and TuPV was obtained constantly in the duodenum and jejunum, correlated with the inflammation: this finding is suggestive for a

possible multiplication site for the parvovirus. To our knowledge there is no study in the literature regarding the multiplication site for ChPV and TuPV. Still the small intestine can be easily suspected as a multiplication site, as it is for most parvoviruses (Hoelzer *et al.*, 2008). Positive nuclear staining was obtained at IHC in the intestinal epithelium of broiler chickens, after experimental infection with the reference ABU strain (Kisary, 2001), but no data is available regarding the natural infection.

Multiplex nodular pancreatitis was observed in all chicken cases with clinical signs of RSS, however positive nuclear staining at IHC for pancreas along with bursa Fabricii and liver was observed only in turkeys. Currently there is no literature data to explain these findings, in case of TuPV; however analogies can be made with immunosuppressive parvoviruses, where lymphoid tissue is a known site for virus multiplication (Hueffer and Parrish, 2003; Parrish and Hueffer, 2003; Bloom and Kerr, 2006).

The concomitant enteritis and IHC nuclear staining in the intestinal epithelial cells confirms the intestinal epithelium as a possible multiplication site for ChPV and TuPV, as described for most parvoviruses (Hueffer and Parrish, 2003). Furthermore in case of two turkey flocks and in six chicken flocks no other viral pathogens with potential effect on the small intestine were found as determined by PCR (Tables 6 and 7), hence one can only conclude that the macroscopic and histological lesions in case of those samples are due to the presence of ChPV or TuPV.

Genetic characterization of Hugarian avian parvovirus strains

A total of 15 ChPV positive samples and 25 TuPV positive samples were directly sequenced and analyzed in this study. The phylogenetic tree constructed based on the nucleotide sequence of the analyzed NS1 gene segment revealed an evident clustering of the virus strains of different species origin, ChPV group and TuPV group (Figure 29). This finding is consistent with the characteristics of parvoviruses of other species (e. g. canine/feline parvoviruses).

Two ChPV strains (1515/07 from Hungary and 841AR05 from USA) were more closely related to the TuPV group; furthermore they presented unique sequences at several aa sites. This finding could be relevant, as parvoviruses are typically small viruses of approximately 5000 nucleotides long, and minor mutations resulting in only a few key aa

changes can lead to drastic changes in their infective behavior (Truyen *et al.*, 1995; Parrish and Hueffer, 2003), therefore the possibility that ChPV and TuPV could have evolved from a common ancestor cannot be ruled out. Even if according to the RDP based investigation of the analyzed gene segment no recombination events were found between the examined strains (data not shown), the probability of a recombinant virus should not be excluded, as these two strains seem to form a clearly distinctive cluster, tentatively named here TuPV-like ChPV group, but they could have also evolved separately from other ChPVs.

The chicken and turkey samples analyzed in the present study were collected from geographically isolated regions. However, samples 5596/7/10 HUN, 5596/10/10 HUN, 5596/13/10 HUN, 5596/18/10 HUN were collected at the same time and originated from a single breeder with different age group flocks, still only two of them were 100% identical on the examined region. There are two possible explanations for this finding: either this particular flock got infected with three different stains at the same time, or in the short period of time, dramatic changes occurred with the viral strains, which is an unlikely situation for parvoviruses. The presented case was not singular, as samples 347/08 HUN and 348/08 HUN also originated from one breeder, and were collected at the same time.

With the exception of two turkey samples (33/10 and 762/10) the Hungarian turkey stains clustered separately from the American strains, in two groups. No time connected clustering was observed throughout the phylogenetic tree; however the Hungarian origin turkey sequences collected in 2008 and 2009 clustered evidently in two groups in both phylogenetic trees (nucleotide and amino acid based). On the other hand, in spite of the observed genetic diversity of the analyzed strains, as due to several reasons (including confidentiality issues) no reliable data were available regarding the production performance (e.g. feed conversion, production index etc.), no objective, scientifically substantiated conclusions can be drawn regarding the variation in pathogenicity of the Hungarian ChPV and TuPV strains.

At aa level the identity between the relevant strains considered for comparison varied from 82.3% to 99.6%, being the lowest when comparing sample B70/09 with sample 189/09, and the highest between samples 33/10 and B31/6/09 (Table 10). Compared with the reference strain the lowest level of identity was found for samples B70/09 and the highest for 5596/7/10. Overall the levels of identity in case of aa comparison were lower then for the nucleotide sequence comparisons, data that implies a low number of “silent” mutation on the examined region from the NS1 gene.

The phylogenetic tree constructed based on the deduced aa sequence of the analyzed NS1 gene segment revealed similar arrangement as emphasized in case of the nucleotide based phylogeny, and the same separate clustering of the two ChPV strains, 1515/07 from Hungary and Ch841AR05 from USA, now named TuPV-like ChPV strains (Figure 30). These findings are emphasizing the same fact regarding the number of "silent" mutations.

There is little data in the literature regarding the definite correlation between virus presence and disease. One study was made to determine the prevalence of the virus in healthy American broiler flocks (Zsak *et al.*, 2008), however there are no reports currently available directly connecting the natural infection with clinical symptoms and/or specific enteric pathological changes.

The role of the ChPV and TuPV in the ED is far from understood, but the results of the present study indicate the fact that these viruses are more frequently present in chicken flocks experiencing ED than in flocks free from ED (Table 6), suggesting a potential role in the pathogenesis of ED. They have also revealed that the situation is similar in case of turkey flocks experiencing PEMS, and that the virus strains circulating in Hungary are genetically similar to ChPV and TuPV strains demonstrated in the USA. However, as most of the currently used diagnostic techniques have their specific limitations, and a considerable genetic diversity among the circulating virus strains can be observed in case of all currently known pathogens, all conclusions regarding the potential implication and role of these viruses in the pathogenesis of ED should be carefully formulated and sustained by extensive investigations of naturally occurring RSS and PEC/PEMS and meticulously planned experimental infections.

Most enteric viruses incriminated in ED have been directly demonstrated in healthy turkey flocks as well; therefore the epidemiology of this disease complex remains unclear, suggesting that a certain combination of pathogens, viral strains and/or environmental and individual flock factors can lead to the development of clinical signs.

Differentiation of ChPV, TuPV, and TuPV-like ChPV strains by RFLP

The analysis of all nucleic acid sequences available from the GenBank and of those determined in the present study revealed that strains clustered in the TuPV, ChPV and TuPV-like ChPV groups have different *AvaII* cleavage patterns on the segment amplified

by the diagnostic primer pair (Figure 32). Enzymatic digestion using *AvaII* enzyme of products amplified by these primers was performed according to the described protocol. Following the *AvaII*-based RFLP, products of various sized were obtained in case of strains belonging to the previously mentioned groups. In case of TuPV strains the enzymatic digestion resulted in clearly differentiable bands at the predicted sizes of 323 and 238 bps. In case of ChPV strains the sizes were 415 and 146 bps, while in case of the TuPV-like ChPV strains the enzymatic digestion resulted in three bands at different levels: 92, 146 and 323 bps (Figure 33). The differences between the *AvaII* digestion pattern of parvovirus strains belonging to the TuPV, ChPV and TuPV-like ChPV groups seem to provide a quick and reliable differentiation of all these strains without the need for nucleic acid sequencing.

At the moment there are only two chicken origin sequences in the so called TuPV-like ChPV group, with clearly different geographic origin, one American and one Hungarian. Due to their unique nucleotide sequence on the analyzed region, one can only assume that as diagnosis and research will progress, more such sequences will be identified. In the future, the newly described protocol could be used to rapidly obtain valuable epidemiological data, and will turn out to be even more practical in case potential differences in the pathogenicity of these strains should be revealed by future studies.

Simultaneous detection of ANV, IBV, and IBDV by mRT-PCR

Due to its sensitivity the PCR technique is widely used for diagnostic purposes. A single copy of a given template can be amplified exponentially up to 10 (Frazier *et al.*, 1990) applying this technique, by repeating cycles of heat denaturation, annealing and primer extension. The mPCR is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction (Henegariu *et al.*, 1997). Since its first description (Chamberlain *et al.*, 1988) this method has been applied successfully in both research and clinical laboratories (Henegariu *et al.*, 1997). By employing mPCR-based diagnostic techniques, the time and effort invested in obtaining an extremely specific and reliable diagnostic result can be significantly reduced.

The high incidence of cases with similar clinical signs and pathological renal and/or enteric lesions in different Hungarian flocks, and age groups, raises the possibility of simultaneous infection by IBV, IBDV and ANV, hence requiring the development of a

rapid and reliable multiplex RT-PCR (mRT-PCR) assay for the diagnosis of the three viral pathogens by one diagnostic test. However one should remain cautious when considering viral pathogens which are controlled by vaccination, as IBV and IBDV. The present test did not have as an objective the strain origin differentiation of the mentioned pathogens.

During the trials and optimization of the mRT-PCR the reaction conditions recommended by the commercial kit's manufacturer were modified according to the suggestion in the literature (Elnifro *et al.*, 2000; Henegariu *et al.*, 1997). The critical parameters for the mRT-PCR assay are the primer selection, extension time and annealing temperature. As previously mentioned (Elnifro *et al.*, 2000), the primers play a crucial role in designing a successful mPCR. The primers used in the present study had lengths of 18 to 22 bp and an ideal GC content of 35% to 60%, as previously recommended (Cha and Thilly, 1993).

According to previous studies (Henegariu *et al.*, 1997), as more loci are simultaneously amplified more time is necessary for the polymerase molecule to complete synthesis of all the products. A longer extension time, 2 min and 30 sec, was used with the purpose of obtaining higher yields of PCR products. Although for the three individual loci the annealing temperature was 53°C for ANV and 55 and 60°C for IBV and IBDV respectively as previously described (Table 5), the trials demonstrated that by lowering it to 53°C the same loci in multiplex mixtures could be co-amplified successfully. Following these changes the amplicons of all targeted pathogens became visible, without the presence of any bands at non specific levels (Figures 34 and 35). These results demonstrate that the newly designed mRT-PCR-based test can be used under the optimized conditions to identify and differentiate the infections caused by the previously mentioned viruses. The mRT-PCR-based test method can be used for differential diagnostic purposes in cases when clinical and pathological changes suggestive of infections with any of these pathogens are present.

The uricosis and the proceeding enteritis due to viral multiplication, is a multi-factorial, often fatal disease of chickens. It occurs in any age, causing severe economic losses. The "baby chick nephropathy complex" has been observed in Hungarian broiler flocks for many years now. Decades ago in Hungary the causative agent in the suspected viral nephritis cases was identified as IBV, which also may cause nephritis in young chickens (Tanyi and Sári, 1970), but there was no reliable method to detect ANV at that time. The newly developed test represents a useful, fast and reliable diagnostic method for

the simultaneous detection of ANV, IBDV and IBV from tissue samples, and has great applicability in samples originating from Hungarian chicken flocks, as the similar coinfections have proven to exist in a high prevalence in this geographic region.

Final conclusions

In this thesis a total of 28 chicken flocks and 51 turkey flocks from distinct geographical regions of Hungary, were investigated by direct methods for the presence of viral pathogens incriminated in the enteric disease syndromes, with the goal of determining the epidemiology and pathogenesis of the syndrome. In the same time various tissue samples collected from the birds succumbed due to the enteric disease syndromes were assessed to identify the pathological changes appeared as a consequence of the viral infections.

By including a broad range of viral agents in the determination, we are confident that the present study contributes in clarifying the epidemiology of a current economic threat for the poultry industry, the enteric disease syndromes, with the potential of being useful to the elaboration of comprehensive preventive measures, to avoid in the future the serious losses that the poultry industry is suffering due to these syndromes.

6. New scientific results

Most important scientific results of the present study:

1. Epidemiological evaluation of the enteric disease syndromes in Hungarian chicken and turkey commercial flocks.

2. Genetic analysis of the newly involved pathogens in enteric disease, chicken parvovirus (ChPV) and turkey parvovirus (TuPV).

3. Determining the existence of a new subgroup, the TuPV-like ChPV, besides the two existing ones, ChPV and TuPV groups.

4. Fast and reliable differentiation of chicken parvovirus, turkey parvovirus and turkey parvovirus-like chicken parvovirus strains, by a newly designed *AvaII* based restriction fragment length polymorphism assay.

5. Detection of chicken parvovirus and turkey parvovirus in infected tissues employing a new indirect immunohistochemical protocol.

6. Simultaneous detection of infectious bronchitis virus (IBV), infectious bursal disease virus (IBDV) and avian nephritis virus (ANV) in coinfected Hungarian field samples, employing a newly developed multiplex RT-PCR (mRT-PCR) protocol.

7. References

1. Adair B.M., and Fitzgerald S.D. 2008. Group I Adenovirus Infections. In: Diseases of Poultry, ed. Saif Y.M., 12th ed., pp. 352-366. Blackwell Publishing, Ames, IA.

2. Adams N.R., Ball R.S., Annis C.L., and Hofstad M.S. 1972. Ultrastructural changes in the intestines of turkey poults and embryos affected with transmissible enteritis. *Journal of Comparative Pathology*, 82: 187-192.

3. Allan W.H., Faraghner J.T., and Cullen G.A. 1972. Immunosuppression by the infectious bursal agent in chickens immunized against Newcastle disease. *Veterinary Record*, 90:511-512.

4. Barnes H.J., Guy J.S., Weaver J.T., and Jennings S.R. 1997. Turkey flocks with high spiking mortality that are negative for turkey coronavirus. In: Proceedings of the 134th Annual Convention of the American Veterinary Association, Reno, Nevada, USA, pp. 169.

5. Barnes H.J., and Guy J.S. 2003. Poult-enteritis mortality syndrome. In: Diseases of Poultry, ed. Saif Y.M., 11th ed., pp. 1171-1180. Iowa State University Press, Ames, IA.

6. Barnes H.J., Guy J.S., and Vaillancourt J.P. 2000. Poult enteritis complex. *Science and technology review*, 19: 565-588.

7. Beckman B. 2003. Avian Urolithiasis (Gout). Hy-line International: *Technical bulletin* HLST 18 2 03.

8. Behling-Kelly E., Schultz-Cherry S., Koci M.D., Kelley L.A., Larsen D.L. and Brown C. 2001. Astrovirus replication in experimentally infected turkeys, as determined by in situ hybridization. *Journal of Veterinary Pathology*, 39: 595-598.

9. Bergeland M.E., McAdargh J.P., and Stotz I. 1977. Rotaviral enteritis in turkey poults. Proceedings 26th West Poultry Disease Conference, 129-130.

10. Bloom M.E., and Kerr J.R. 2006. Pathogenesis of parvovirus infection. In: Kerr J.R., Cotmore S.F., Bloom M.E., Linden R.M., and Parish C.R. Parvoviruses eds. pp. 323-341. London U.K: Hodder Arnold.

11. Boltz D.A., Nakai M., and Bahra J.M. 2004. Avian infectious bronchitis virus: a possible cause of reduced fertility in the rooster. *Avian Diseases*, 48: 909-915.

12. Breslin J.J., Smith L.G., Fuller F.J., and Guy J.S. 2000. Comparation of virus isolation, immunohistochemistry, and reverse transcription-polymerase chain reaction procedures for detection of turkey coronavirus. *Avian Diseases*, 44: 624-631.

13. Bridger J.C. 1987. Novel rotaviruses in animals and men. *Ciba Found Symposium*, 5-23.

14. Calibeo-Hayes D., Denning S.S., Stringham S.M., Guy J.S., Smith L.G., and Watson D.W. 2003. Mechanical transmission of turkey coronavirus by domestic house flies (*Musca domestica Linneatus)*. *Avian Diseases,* 47: 149-153.

15. Carver, D.K., J.P Vaillancourt, M. Stringham, J.S. Guy, and H.J. Barnes. Mortality patterns associated with poult enteritis mortality syndrome (PEMS) and coronaviral enteritis in turkey flocks raised in PEMS-affected regions. *Avian Dis* 45: 985-91. 2001.

16. Caul E.O., and Appleton H. 1982. The electron microscopical and physical characteristics of small round human fecal viruses: an interim scheme for classification. *Journal of Medical Virology*, 9: 257-265.

17. Cavanagh D. 2001a. A nomenclature for avian coronavirus isolates and the question of species status. *Avian Pathology*, 30: 109-115.

18. Cavanagh, D. 2001b. Technical Review: innovation and discovery: the application of nucleic acid-based technology to avian virus detection and characterisation. *Avian Pathology*, 30: 581-598.

19. Cavanagh D. 2005. Coronaviruses in poultry and other birds. *Avian Pathology*, 34: 439-448.

20. Cavanagh D. 2006. Coronavirus avian infectious bronchitis virus. *Veterinary Research*, 38: 281-297.

21. Cavanagh D., and Gelb Jr. J. 2008. Infectious bronchitis. In: Diseases of Poultry, ed. Saif Y.M., 12th ed., pp. 117-137. Blackwell Publishing, Ames, IA.

22. Cavanagh D., and Naqi S.A. 2003. Infectious bronchitis In: Saif Y.M., Barnes H.J., Fadly A.M., Glisson J.R., McDougald L.R., Swayne D.E. (Eds): Diseases of Poultry, 11th edition, pp. 101-119. Iowa State University Press, Ames, IA.

23. Cha R.S., and Thilly W.G. 1993. Specificity, efficiency, and fidelity of PCR. *PCR Methods and Applications*, 3: S18-S29.

24. Chamberlain J.S., Gibbs R.A., Ranier J.E., Nguyen P.N., and Caskey C.T. 1988. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic Acids Research,* 16: 11141-11156.

25. Cook J.K.A. 1974. Pathogenicity of avian adenoviruses for day-old chicks. *Journal of Comparative Pathology*, 84: 505-515.

26. Cosgrove A.S. 1962. An apparently new disease of chickens – avian nephrosis. *Avian Diseases*, 6: 385-389.

27. Cotmore S.F., and Tattersall P. 2006. Structure and organization of the viral genome. In: Parvoviruses, ed. Kerr J.R., Cotmore S.F., Bloom M.E., Linden R.M., and Parish C.R., 1st ed., pp. 73-94. Hodder Arnold Publication, London, UK.

28. Cowen B., Calnek B.W., Menedez N.A., and Ball R.F. 1978. Avian adenoviruses-effect on egg production, shell quality and feed consumption. *Avian Diseases*, 22: 459-470.

29. Day J.M., Pantin-Jackwood M.J., and Spackman E. 2007a. Sequence and phylogenetic analysis of the S1 genome segment of turkey-origin reoviruses. *Virus Genes, 35*: 235-242.

30. Day J.M., Spackman E., and Pantin-Jackwood M.J. 2007b. 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 Diseases*, 51: 681-684.

31. Day J.M., and Zsak L. 2010. Determination and analysis of the full-length chicken parvovirus genome. *Virology,* 399: 59-64.

32. deWit J.J. 2000. Technical Review. Detection of infectious bronchitis virus. *Avian Pathology,* 29: 71-93.

33. Dhinaker R.G., and Jones R.C. 1997. Infectious bronchitis virus: Immunopathogenesis of infection in the chicken. *Avian Pathology*, 26: 677-706.

34. Elnifro E.M., Ashshi A.M., Cooper R.J., and Klappner P.E. 2000. Multiplex PCR: Optimization and application in diagnostic virology. *Clinical Microbiology Reviews*, 4: 559-570.

35. Fabricant J. 1998. The early history of infectious bronchitis. *Avian Diseases*, 42: 648-650.

36. Faraghner J.T., Allan W.H., and Wyeth C.J. 1974. Immunosuppressive effect of infectious bursal agent on vaccination against Newcastle disease. *Veterinary Record*, 95: 385-388.

37. Farsang A., Ros C., Renström L.H.M., Baule C., Soós T., and Belák S. 2002. Molecular epizootiology of infectious bronchitis virus in Sweden indicating the involvement of a vaccine strain. *Avian Pathology*, 31: 229-236.

38. Frazier J.A., Howes K., Reece R.L., Kidd A.W., and Cavanagh D. 1990. Isolation of non-cytopathic viruses implicated in the aetiology of nephritis and baby chick nephropathy and serologically related to avian nephritis virus. *Avian Pathology*, 19: 139-160.

39. Gonder E., Patel B.L., and Pomeroy B.S. 1976. Scanning electron, light and immunoflorescent microscopy of coronaviral enteritis of turkeys (bluecomb). *American Journal of Veterinary Research*, 37: 1435-1439.

40. Goodwin M.A., Davis J.F., McNulty M.S., Brown J., and Player E.C. 1993. Enteritis (so called runting stunting syndrome) in Georgia broiler chicks. *Avian Diseases*, 37:451-458.

41. Gough R.E. 2003. Goose parvovirus infection. In: Diseases of poultry, ed. Saif Y.M., 11th ed., pp. 367–374. Blackwell Publishing, Ames, IA.

42. Guy J.S. 1998. Virus infections of the gastrointestinal tract of poultry. *Poultry Scienc*e, 77: 1166-1175.

43. Guy J.S. 2008. Turkey coronavirus enteritis. In: Diseases of Poultry, ed. Saif Y.M., 12th ed., pp. 330-338. Blackwell Publishing, Ames, IA.

44. Guy J.S., Smith L.G., Breslin J.J., Vaillancourt J.P., and Barnes H.J. 2000. High mortality and growth depression experimentally produced in young turkeys by dual infection with enteropathogenic *Escherichia coli* and turkey coronavirus. *Avian Diseases*, 44: 105-113.

45. Handberg K.J., Nielsen O.L., Pedersen M.W., and Jorgensen P.H. 1999. Detection and strain differentation of infectious bronchitis virus in tracheal tissues from experimentally infected chickens by reverse transcription-polymerase chain reaction. Comparison with an immunohistochemical technique. *Avian Pathology*, 28: 327-335.

46. Harris J.R. 2007. Negative Staining of Thinly Spread Biological Samples. In: Electron Microscopy Methods and Protocols, ed. Kuo J., 2nd ed., pp. 116-119. Humana Press Inc., Totowa, HJ.

47. Heggen-Peay M.A., Qureshi F.W., Edens B., Sherry B., Wakenell P.S., O'Connell P.H., and Schat K.A. 2002. Isolation of a reovirus from poult enteritis and mortality syndrome and its pathogenicity in turkey poults. *Avian Diseases*, 46: 360-369.

48. Henegariu O., Heerema N.A., Dlouhy S.R., Vance G.H., and Vogt P.H. 1997 Multiplex PCR: critical parameters and step-by-step protocol. *Biotechniques*, 23: 504-511.

49. Hess M., Raue R., and Hafez H.M. 1999. PCR for specific detection of haemorrhagic enteritis of turkeys, an avian adenovirus. *Journal of Virological Methods*, 81: 199-203.

50. Hoelzer K., Shackelton L.A., Parrish C.R., and Holmes E.C. 2008. Phylogenetic analysis reveals the emergence, evolution and dispersal of carnivore parvoviruses. *Journal of General Virology*, 89: 2280-2289.

51. Hueffer K., and Parrish C.R. 2003. Parvovirus host range, cell tropism and evolution. *Current Opinion in Microbiology*, 6: 392-398.

52. Imada T., and Kawamura H. 1997. Avian nephritis. In: Diseases of Poultry, ed. Calnek B.W., Barnes H.J., Beard C.W., McDougald L.R., Saif Y.M., 10th ed., pp. 379-383. Iowa State University Press, Ames, IA.

53. Imada T., and Kawamura H. 2003. Avian Nephritis. In: Diseases of Poultry, ed. Saif Y.M., Barnes H.J., Fadly A.M., Glisson J.R., McDougald L.R., Swayne D.E., 11th ed., pp. 101-119. Ames, Iowa State University Press, USA.

54. Imada T., Yamaguchi S., Mase M., Tsukamoto K., Kubo M., and Morookaet A. 2000. Avian nephritis virus (ANV) as a new member of the family Astroviridae and construction of infectious ANV cDNA. *Journal of Virology*, 18: 8487-8493.

55. Jackwood D.J., and Sommer-Wagner S. 2007, Genetic characteristics of infectious bursal disease viruses from four continents. *Virology,* 365: 369-375.

56. Jones R.C. 2008. Other reovirus infections. In: Diseases of Poultry, ed. Saif YM, 12th ed., pp. 322-328. Blackwell Publishing, Ames, IA.

57. Jones R.C., and Georgiou K. 1984. Experimental infection of chickens with adenoviruses isolated from tenosynovitis. *Avian Pathology*, 13: 13-23.

58. Kahn J.S., Kesebir D., Cotmore S.F., D'Abramo Jr. A., Cosby C., Weibel C., and Tattersall P. 2008. Seroepidemiology of human bocavirus defined using recombinant virus-like particles. J*ournal of Infectious Diseases,* 198: 41-50.

59. Kapikian A.Z., and Hoshino Y. 2001. Rotaviruses. In: Fields Virology, ed. Knipe D.M., 4th ed., pp. 1787-1833. Lippincott Williams and Wilkins, Philadelphia, PA.

60. Kingsham B.E., Keeler Jr. C.L., Nix W.A., Landman B.S., and Gelb Jr. J. 2000. Identification of avian infectious bronchitis virus by direct automated cycle sequencing of the S-1 gene. *Avian Diseases,* 44: 325-335.

61. Kisary J. 1985. Experimental infection of chicken embryos and dayold chickens with parvovirus of chicken origin. *Avian Pathology*, 14: 1-7.

62. Kisary J., Avalosse B., Miller-Faures A., and Rommelaere J. 1985. The genome structure of a new chicken virus identifies it as a parvovirus. *Journal of General Virology,* 66: 2259-2263.

63. Kisary J., Nagy B., and Bitay Z. 1984. Presence of parvoviruses in the intestine of chickens showing stunting syndrome. *Avian Pathology*, 13: 339-343.

64. Kisary J. 2001. Csirkeparvovirus-antigén kimutatása immunperoxidáz eljárással (Detection of chicken parvovirus antigen by immunoperoxidase staining). *Magyar Állatorvosok Lapja*, 123: 518-521. (article in Hungarian with English abstract)

65. Koci M.D., and Schultz-Cherry S. 2002. Avian astroviruses. *Avian Pathology,* 31: 213-227.

66. Koci M.D., Seal B.S., and Schultz-Cherry S. 2000. Molecular characterization of an avian astrovirus. *Journal of Virology,* 7: 6173-6177.

67. Lai M.M.C., and Cavanagh D. 1997. The molecular biology of coronaviruses. *Advances in Virus Research*, 48: 1-100.

68. Li H., and Yang H. 2001. Sequence analysis of nephropathogenic infectious bronchitis virus strains of the Massachusetts genotype in Beijing. *Avian Pathology*, 30: 535-541.

69. Lin T.L., Wu C.C., Porter R.E., Thacker H.L., Bryan T.A., Kanitz C.L., Hermes D., Schrader D.L., Woodruff M.M., and Hooper T.A. 1996. Turkey poult enteritis caused by turkey coronavirus. *Journal of the American Veterinary Medical Association,* 209: 372.

70. Lukert P.D., and Saif Y.M. 2003. Infectious bursal disease. In: Diseases of Poultry, ed. Saif Y.M., Barnes H.J., Fadly A.M., Glisson J.R., McDougald L.R., and Swayne D.E. 11th ed., pp. 161-181. Ames, Iowa State University Press, USA.

71. Mándoki M.., Dobos-Kovács M., Ivanics É., Nemes Cs., Bakonyi T., and Rusvai M., 2005. Az avian nephritis vírus okozta kórkép elıfordulásának elsı hazai leírása és elterjedtségének vizsgálata (First description and distribution of the avian nephritis infection in Hungary). *Magyar Állatorvosok Lapja*, 127: 720-726. (article in Hungarian with English abstract)

72. Mandoki M., Bakonyi T., Ivanics É., Nemes Cs., Dobos-Kovács M., and Rusvai M. 2006. Phylogenetic diversity of avian nephritis virus in Hungarian chicken flocks. *Avian Pathology*, 35: 224-229.

73. Martin D., and Rybicki E. 2000. RDP: detection of recombination amongst aligned sequences. *Bioinformatics, 16:* 562-563.

74. McNulty M.S., and Reynolds D.L. 2008. Rotavirus infections. In: Diseases of Poultry, ed. Saif Y.M., 12th ed., pp. 338-351. Blackwell Publishing, Ames, IA.

75. McNulty M.S., Curran W.L., and McFerran J.B. 1980. Detection of astroviruses in turkey faeces by direct electron microscopy. *Veterinary Record*, 106: 561.

76. McNulty M.S., Allan G.M., and McCracken R.M. 1983. Experimental infection of chickens with rotaviruses: clinical and virological findings. *Avian Pathology*, 12: 45-54.

77. Meulemans G., Boschmans M., Decaesstecker M., van den Berg T.P., Denis P., and Cavanagh D. 2001. Epidemiology of infectious bronchitis virus in Belgian broilers: a retrospective study, 1986 to 1995. *Avian Pathology*, 30: 411-421.

78. Momoeda M., Wong S., Kawase M., Young N.S., and Kajigaya S. 1994. A putative nucleoside triphosphate-binding domain in the nonstructural protein of B19 parvovirus is required for cytotoxicity. *Journal of Virology*, 68: 8443-8446.

79. Muzyczka N., and Berns K.I. 2001. Parvoviridae: the viruses and their replication, In: Fields Virology, ed. Knipe D.M., and Howley P.M. 4th ed., pp. 2327–2359. Lippincott, Williams and Wilkins.

80. Narita M., Kawamura H., Furuta K., Shirai J., and Nakamura K. 1990. Effects of cyclophosphamide in newly hatched chickens after inoculation with avian nephritis virus. *American Journal of Veterinary Research*, 51: 1623-1628.

81. Nemes Cs., Szalay D., Ursu K., Palya V., and Glávits R. 2008 a. Pulykák coranavírus okozta bélgyulladásának vizsgálata Magyarországon (Turkey coranavirus enteritis in Hungary). *Magyar Állatorvosok Lapja*, 130: 148-156. (article in Hungarian with English abstract)

82. Nemes Cs., Ivanics É., Szalay D., Ursu K., Simonyai E., Glávits R.2008 b. Kispulykák astrovírus és rotavírus okozta bélgyulladásának hazai vizsgálata. Irodalmi áttekintés és saját megfigyelések. (Turkey astrovirus and rotavirus enteritis in Hungary) *Magyar Állatorvosok Lapja*, 130: 148-156 464. (article in Hungarian with English abstract)

83. Page R.K., Fletcher O.J., Rowland G.N., Gaudry D., and Villegas P. 1982 Malabsorption syndrome in broiler chickens. *Avian Diseases*, 26: 618-624.

84. Palya V., Glávits R., Dobos-Kovács M., Ivanics E., Nagy E., Banyai K., Reuter G., Szűcs G., Dán Á. and Benkő M. 2003. Reovirus identified as cause of disease in young geese. *Avian Pathology*, 32: 129-138.

85. Pantin-Jackwood M.J., Day J.M., Jackwood M.W., and Spackman E. 2008a. Enteric viruses detected by molecular methods in commercial chicken and turkey flocks in the United States between 2005 and 2006. *Avian Diseases,* 52: 235-244.

86. Pantin-Jackwood M.J., Spackman E., and Day J.M. 2008b. Pathogenesis of type 2 turkey astroviruses with variant capsid genes in 2-day-old specific pathogen free poults. *Avian Pathology*, 37: 193-201.

87. Pantin-Jackwood M.J., Spackman E., Day J.M., and Rives D. 2007a. Periodic monitoring of commercial turkeys for enteric viruses indicates continuous presence of astrovirus and rotavirus on the farms. *Avian Diseases*, 51: 674-680.

88. Pantin-Jackwood M.J., Spackman E., Day J.M. 2007b. Pathology and virus tissue distribution of turkey origin reoviruses in experimentally infected turkey poults. *Veterinary Pathology*, 44: 185-195.

89. Parrish C.R., and Hueffer K. 2003. Parvovirus host range, cell tropism and evolution – studies of canine and feline parvoviruses, minute virus of mice, porcine parvovirus, and Aleutian mink disease virus. In: Parvoviruses, ed. Kerr J.R., Cotmore S.F., Bloom M.E., Linden R.M., and Parrish C.R. pp. 343-351. London, U.K.: Edward Arnold Limited.

90. Pass D.A., Robertson M.D., and Wilcox G.E. 1982. Runting syndrome in broiler chickens in Australia. *Veterinary Record,* 110: 386-387.

91. Pierson F.W., and Fitzgerald S.D. 2008. Hemorrhagic enteritis and related infections. In: Diseases of Poultry, ed. Saif Y.M., 12th ed., pp. 276-287. Blackwell Publishing, Ames, IA.

92. Pomeroy K.A., Patel B.L., Larsen C.T., and Pomeroy K.A. 1978. Combined immunoflorescence and transmission electron microscopic studies of sequential intestinal samples from turkey embryos and poults infected with turkey coronavirus. *American Journal of Veterinary Research*, 39: 1348-1354.

93. Qureshi M.A., Edens F.W., and Havenstein G.B. 1997. Immune system dysfunction during exposure to poult enteritis and mortality syndrome agents. *Poultry Science,* 76: 564-569.

94. Qureshi M.A., Yu M., and Saif Y.M. 2000. A novel 'small round virus' inducing poult enteritis and mortality syndrome and associated immune alterations. *Avian Diseases*, 44: 275–283.

95. Reynolds D.L., and Schultz-Cherry S.L. 2008. Astrovirus Infections. In: Diseases of Poultry, ed. Saif Y.M., 12th ed., pp. 351-356. Blackwell Publishing, Ames, IA.

96. Reynolds D.L., and Saif Y.M. 1986. Astrovirus: a cause of an enteric disease in turkey poults. *Avian Diseases,* 30: 728–735.

97. Reynolds D.L., and Schultz-Cherry S.L. 2008. Astrovirus Infections. In: Diseases of Poultry, ed. Saif Y.M., 12th ed., pp. 351-356. Blackwell Publishing, Ames, IA.

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

99. Reynolds D.L., Saif Y.M., and Theil K.W. 1987b. Enteric viral infections of turkey poults: incidence of infection. *Avian Diseases*, 31: 272-276.

100. Ritchie A.E., Deshmukh D.R., Larsen C.T., and Pomeroy B.S. 1973. Electron microscopy of coronavirus-like particles characteristic of turkey bluecomb disease. *Avian Diseases*, 17: 546-558.

101. Saraste M., Sibbald P.R., and Wittinghofer A. 1990. The P-loop-a common motif in ATP and GTP-binding proteins. *Trends in Biochemical Sciences*, 15: 430-434.

102. Saif L.J., Saif Y.M., and Theil K.W. 1985. Enteric viruses in diarrheic turkey poults. *Avian Diseases*, 29: 798–811.

103. Saif Y.M. 2008. Viral enteric infections, Introduction. In: Diseases of Poultry, ed. Saif Y.M., 12th ed., pp. 329-330. Blackwell Publishing, Ames, IA.

104. Saif Y.M., Saif L.J., Hofacre C.L., Hayhow C., Swayne D.E., and Dearth R.N. 1990. A small round virus associated with enteritis in turkey poults. *Avian Diseases*, 34: 762–764.

105. Saikawa T., Anderson M., Momoeda M., Kajigaya S., and Young N.S. 1993. Neutralizing linear epitopes of B19 parvovirus cluster in the VP1 unique and VP1–VP2 junction regions. *Journal of Virology*, 67: 3004–3009.

106. Schalk A.F., and Hawn M.C. 1931. An apparently new respiratory disease of chicks. *Journal of American Veterinary Medical Association*, 78: 413-422.

107. Sellers H.S., Koci M.D., Linnemann E., Kelley L.A., and Schultz-Cherry, S. 2004. Development of a multiplex reverse transcription-polymerase chain reaction diagnostic test specific for turkey astrovirus and coronavirus. *Avian Diseases*, 48: 531-539.

108. Schultz-Cherry S., Kapczynski D.R., Simmons V.M., Koci M.D., Brown C., and Barnes H.J. 2000. Identifying agent(s) associated with poult enteritis mortality syndrome: importance of the thymus. *Avian Diseases*, 44: 256-265.

109. Shackelton L.A., Parrish C.R., Truyen U., and Holmes E.C. 2005. High rate of viral evolution associated with the emergence of carnivore parvovirus. *Proceedings of the National Academy of Science U.S.A.* 102: 379-384.

110. Siddell S.G. 1995. The Coronaviridae: an introduction. In: Coronaviridae, ed. Siddell S.G., pp. 1-9. Plenum Press, Inc., New York.

111. Siller W.G.: 1981. Renal pathology of the fowl-a review. *Avian Pathology*, 10: 187-262.

112. Spackman E., Kapczynski D., and Sellers H. 2005a. Multiplex real-time reverse transcription-polymerase chain reaction for the detection of three viruses associated with poult enteritis complex: turkey astrovirus, turkey coronavirus, and turkey reovirus. *Avian Diseases*, 49: 86-91.

113. Spackman E., Pantin-Jackwood M., Day J.M., and Sellers H. 2005b. The pathogenesis of turkey origin reoviruses in turkeys and chickens. *Avian Pathology,* 34: 291-296.

114. Stevens A. 2007. The haematoxylins. In: Theory and Practice of Histological Techniques, ed. Bancroft J.D, and Stevens A. 6th ed., pp.107-118. Edinburgh: Churchill Livingstone.

115. Strother K.O. and Zsak L. 2009. Development of an Enzyme-Linked Immunosorbent Assay to Detect Chicken Parvovirus-Specific Antibodies. *Avian Diseases,* 53: 585-591.

116. Tang Y., Ismail M.M., Saif Y.M. 2005. Development of antigencapture enzyme-linked immunosorbent assay and RT-PCR for detection of turkey astroviruses. *Avian Diseases*, 49: 182-188.

117. Tanyi J., and Sári I. 1970. A csirkék vírusos vesegyulladásának hazai előfordulása (Occurrence in Hungary of virus induced nephritis of chicken). *Magyar Állatorvosok Lapja* 25: 545-547. (in Hungarian with English summary)

118. Tattersall P. 2006. The evolution of parvovirus taxonomy. In: Parvoviruses, ed. Kerr J.R., Cotmore S.F., Bloom M.E., Linden R.M., and Parish C.R., 1st ed., pp. 73-94. Hodder Arnold Publication, London, UK.

119. Todd D., McNulty M.S., Lukert A., Randles J.W., and Dale J.L. 2000. Family Circovirus. In: Virus Taxonomy: Classification and Nomenclature of Viruses: Seventh Report of the International Committee on Taxonomy of Viruses, ed. van Regenmortel M.H.V., Fauquet C.M., and Bishop D.H.L. pp. 299–303. New York: Academic Press.

120. Trampel D.W., Kinden D.A., Solorzano R.F., and Stogsdill P.L. 1983. Parvovirus like enteropathy in Missouri turkeys. *Avian Diseases*, 27: 49-54.

121. Truyen U., Gruenberg A., Chang S.F., Obermaier B., Veialainen P, and Parrish R. 1995. Evolution of the feline sub-group parvoviruses and the control of canine host range in vivo. *Journal of Virology, 69:* 4702-4710.

122. Watson D.W., Guy J.S., and Stringham S.M. 2000. Limited transmission of turkey coronavirus in young turkeys by adult Alphitobius diapernius (Coleoptera: Tenebrionidae). *Journal of Medical Entomology,* 37: 480-483.

123. Woolcock P.A., and Shivaprasad H.L. 2008. Electron microscopic identification of viruses associated with poult enteritis in turkeys grown in California 1993–2003. *Avian Diseases*, 52: 209-213.

124. Yason C.V., and Schat K.A. 1986. Pathogenesis of rotavirus infection in turkey poults. *Avian Pathology*, 15: 421-435.

125. Yu M., Ismail M.M., Qureshi M.A., Dearth R.N., Barnes H.J., and 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 Diseases*, 44: 297-304.

126. Zsak L., Strother K.O., and Day J.M. 2009. Development of a Polymerase Chain Reaction Procedure for Detection of Chicken and Turkey Parvoviruses. *Avian Diseases*, 53: 83-88.

127. Zsak L., Strother K.O., and Kisary J. 2008. Partial genome sequence analysis of parvoviruses associated with enteric disease in poultry. *Avian Pathology*, 37: 435-441.

8. Scientific publications of the thesis

Palade E.A., Demeter Z., Dobos-Kovács M., Rusvai M., Mándoki M.: A fertőző bronchitis vírus, a csirke nephritis vírus és a fertőző bursitis vírus kimutatása multiplex RT-PCR alapú diagnosztikai eljárással (Demonstration of infectious bursal disease virus, chicken nephritis virus and infectious bronchitis virus by multiplex RT-PCR diagnostic technique). *Magyar Állatorvosok Lapja*, 2008, 130: 559-564. (in Hungarian, with English abstract)

Palade E.A., Kisary J., Benyeda Zs., Mándoki M., Balka Gy., Jakab Cs., Végh B., Demeter Z., Rusvai M. Naturally occurring parvoviral infection in Hungarian broiler flocks. *Avian Patholog*y, 2011, 40 (2): 191-197.

Palade E.A., Demeter Z., Hornyák Á., Nemes Cs., Kisary J., Rusvai M. High prevalence of turkey parvovirus in turkey flocks from Hungary experiencing enteric disease syndrome. *Avian Diseases,* in press.

9. Other publications in peer reviewed journals

Palade E.A, Gál J., Mándoki M.: Avian encephalomyelitis vírus okozta megbetegedés magyarországi importált brojlerállományban (Avian encephalomyelitis in imported Hungarian broiler flocks). *Magyar állatorvosok lapja*, 2011, 133, 220-223. (in Hungarian, with English abstract)

Mándoki M., **Palade E.A.**, Kléh Zs., Dobos-Kovács M., Gál J.: Derzsy betegség okozta tömeges elhullás libaállományban (Multitudineuss loss due to Derzsy's disease in goose flocks). *Magyar állatorvosok lapja*, 2011, 133, 13-18. (in Hungarian, with English abstract)

Demeter Z., **Palade E.A.**, Soós T., Farsang A., Jakab Cs., Rusvai M.: Misleading results of the *Mbo*II-based identification of type 2a canine parvovirus strains from Hungary reacting as type 2c strains. *Virus Genes,* 2010, 41, 37-42.

Demeter Z., **Palade E.A.**, Hornyák Á., Rusvai M.: Controversial results of the genetic analysis of a canine distemper vaccine strain. *Veterinary Microbiology,* 2010, 142, 420-426.

Demeter Z., **Palade E.A.**, Jakab Cs., Hornyák Á., Rusvai M., Mándoki M.: Macskaparvovírus által okozott, végzetes kimenetelű járvány egy kis oroszláncsoportban *(Panthera leo)* Magyarországon. Esetismertetés (Fatal outbreak in a small lion *(Panthera leo)* group caused by feline parvovirus in Hungary. Case report.) *Magyar Állatorvosok Lapja,* 2010, 132, 32-38. (in Hungarian, with English abstract)

Palade E.A., Bajnok L., Dobos-Kovács M., Demeter Z., Rusvai M.: A csirkék fertőző anaemiáját okozó, Magyarországon előforduló vírustörzsek genetikai jellemzése (Genetic characterization of Hungarian chicken anaemia virus strains). *Magyar Állatorvosok Lapja,* 2009, 131, 154-161. (in Hungarian, with English abstract)

Gál J., Demeter Z., **Palade E.A.,** Rusvai M., Géczy Cs.: Harderian gland adenocarcinoma in a Florida red-bellied turtle *(Pseudemys nelsoni). Acta Veterinaria Hungarica,* 2009, 57, 275-282.

Gál J., Landauer K., **Palade E.A.**, Ivaskevics K., Rusvai M., Demeter Z.: Squamous cell carcinoma and consequent otitis in a Long-eared Hedgehog *(Hemiechinus auritus). Acta Veterinaria Hungarica,* 2009, 57, 69-74.

Demeter Z., Gál J., **Palade E.A.**, Rusvai M.: Feline parvovirus infection in an Asian palm civet *(Paradoxurus hermaphroditus). Veterinary Record,* 2009, 164, 213-215.

Palade E.A., Biró N., Dobos-Kovács M., Demeter Z., Mándoki M., Rusvai M.: Poxvirus infection in Hungarian great tits *(Parus major). Acta Veterinaria Hungarica,* 2008, 56, 539-546.

Gál J., Pásztor I., Demeter Z., **Palade E.A**., Ursu K., Bálint Á., Pap T., Farkas Sz.: Amursikló (*Elaphe schrenki*) vírus okozta savós-fibrines tracheitise és következményes fulladása (Viral serofibrinous tracheitis and resulting suffocation in an Amur ratsnake – *Elaphe schrenki*). *Magyar Állatorvosok Lapja,* 2008, 130, 421-424. (in Hungarian, with English abstract)

Gál J., **Palade E.A.**, Majoros G., Landauer K., Pásztor I.: Cryptosporidiosis első hazai megállapítása leopárd gekkóban (*Eublepharis macularius*) (First detection of cryptosporidiosis in leopard gecko in Hungary – *Eublepharis macularius*). *Magyar állatorvosok lapja*, 2008, 130, 535-542. (in Hungarian, with English abstract)

Demeter Z., Lakatos B., **Palade E.A**., Kozma T., Forgách P., Rusvai M.: Genetic diversity of Hungarian canine distemper virus strains. *Veterinary Microbiology,* 2007, 122, 258-269.

Demeter Z., **Palade E.A.**, Lakatos B., Kozma T., Rusvai M.: Az endémiás szopornyicafertőzés leküzdésével kapcsolatos tapasztalatok a Fővárosi Ebrendészeti Telepen (Experiences on the control of endemic distemper at the flaying-house of Budapest). *Magyar Állatorvosok Lapja*, 2006; 128, 665-673. (in Hungarian, with English abstract)

10. Congress abstracts

Palade E.A., Demeter Z., Benyeda Zs., Mándoki M, Rusvai M.: Parvoviral Infection in Hungarian Broiler Flocks and the genetic diversity of the causative agent. Congress of the Hungarian Academy of Sciences and the Doctoral (PhD) School of Veterinary Medicine, January 26, 2011, Budapest, Hungary (in Hungarian)

Balázs M., Törökné Kozma A., Pándics T., **Palade E.A.**: Nanoszennyezők vizsgálata krónikus daphniateszttel. Vízmikrobiológusok IX. országos konferenciája, November 9, 2010, Budapest, Hungary (in Hungarian)

Palade E.A., Demeter Z., Dobos-Kovács M., Rusvai M., Kisary J., Mándoki M.: Etiological investigations of naturally occurring runting stunting syndrome in hungarian flocks. Congress of the Hungarian Academy of Sciences and the Doctoral (PhD) School of Veterinary Medicine, January 26, 2010, Budapest, Hungary

Hornyák Á., Gál J., **Palade E.A.**, Demeter Z., Forgách P., Bakonyi T., Rusvai M.: Demonstration of a novel adenovirus infection in a pygmy marmoset *(Cebuella pygmaea)*. Congress of the Hungarian Academy of Sciences and the Doctoral (PhD) School of Veterinary Medicine, January 26, 2010, Budapest, Hungary (in Hungarian)

Demeter Z., **Palade E.A.**, Rusvai M.: Canine distemper: still a major concern in Central Europe. *Lucrări Ştiinţifice Medicină Veterinară* Vol. XLII (1), 2009, Timişoara, Romania, p. 136-150.

Palade E.A., Hornyák Á., Demeter Z., Dobos-Kovács M., Benyeda Zs., Rusvai M., Kisary J.: Etiological investigations of naturally occurring runting stunting syndrome in hungarian flocks. *Acta Microbiologica et Immunologica Hungarica*, 2009, 56: 114-263.

Palade E.A., Hornyák Á., Demeter Z., Dobos-Kovács M., Benyeda Zs., Rusvai M., Kisary J.: Genetic characterization of Chicken parvovirus strains from naturally infected Hungarian flocks. 8th International Congress of Veterinary Virology, Budapest, Hungary, 23-26 August, 2009

Palade E.A., Mándoki M., Dobos-Kovács M., Demeter Z., Rusvai M.: Diagnosis of infectious bronchitis, avian nephritis and infectious bursal disease by multiplex RT-PCR, and the phylogenetical analysis of infectious bursal disease virus strains circulating in Hungary. Congress of the Hungarian Academy of Sciences and the Doctoral (PhD) School of Veterinary Medicine, January 22, 2008

Demeter Z., **Palade E.A.**, Gál J., Rusvai M.: Demonstration of distemper and parvovirus infection in different species. Congress of the Hungarian Academy of Sciences and the Doctoral (PhD) School of Veterinary Medicine, January 22, 2008, Budapest, Hungary (in Hungarian)

Demeter Z., **Palade E.A.**, Rusvai M.: Development of a multiplex RT-PCR test for the simultaneous detection of some of the major respiratory viral pathogens of the dog. In: *Acta Microbiologica et Immunologica Hungarica,* 2007, 54, 23-23.

Demeter Z., **Palade E.A.**, Farsang A., Soós T., Balka Gy., Rusvai M.: Controversial results of the genetic analysis of a canine distemper vaccine strain. Vaccine Congress, December 9-11, 2007, Amsterdam, The Netherlands. Abstract Book. Poster abstract P151.

Palade E.A., Biró N., Dobos-Kovács M., Demeter Z., Benyeda Zs., Mándoki M., Rusvai M.: Poxvirus infection in great tit *(Parus major)* in Hungary. In: *Acta Microbiologica et Immunologica Hungarica,* 2007, 54, 97-98.

Demeter Z., **Palade E.A.**, Rusvai M.: Differentiation of vaccine and wild-type strains of CDV by restriction fragment length polymorphism. Congress of the Hungarian Academy of Sciences and the Doctoral (PhD) School of Veterinary Medicine, January 23, 2007, Budapest, Hungary (in Hungarian)

Palade E.A., Mándoki M., Demeter Z., Dobos-Kovács M., Benyeda Zs., Rusvai M.: Development of a multiplex PCR assay for the simultaneous detection of infectious bronchitis virus and avian nephritis virus. Congress of the Hungarian Academy of Sciences and the Doctoral (PhD) School of Veterinary Medicine, January 23, 2007, Budapest, Hungary (in Hungarian)

Demeter Z., **Palade E.A.**, Lakatos B., Kozma T., Rusvai M.: Endemic canine distemper infection at a dog shelter: epizootology and viral variability. In: *Acta Microbiologica et Immunologica Hungarica,* 2006, 53, 28-29.

Palade E.A., Dobos-Kovács M., Demeter Z.: Different diagnostic methods for turkey rhinotracheitis virus infections. In: *Acta Microbiologica et Immunologica Hungarica,* 2006, 53, 326.

Forgách, P., **Palade, E.A**, Tapaszti, Zs., Bakonyi, T., Rusvai, M.: Demonstartion of chronic bee paralysis virus of honey bees using RT-PCR and an electon-microscopy survey of the causative agent. In: *Acta Microbiologica et Immunologica Hungarica*, 2005, 52, 41-42.

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