

EVALUATION OF A NEW CHICKEN ASTROVIRUS IN THE ETIOLOGY OF RUNTING  
STUNTING SYNDROME (RSS) IN BROILER CHICKENS

by

KYUNG-IL KANG

(Under the Direction of Holly S. Sellers and Egbert Mundt)

ABSTRACT

Runting stunting syndrome (RSS) is an enteric disease of young broiler chickens characterized by weight suppression and poor flock uniformity. Cystic lesions in the crypt of Lieberkühn in the small intestines are typical for RSS. Despite indications of viral etiology, the etiologic agent(s) were unknown. Based on recently described vaccination experiments, a novel chicken astrovirus (CkAstV) was suggested as an RSS pathogen. The aim of these studies was to investigate the etiological role of the novel CkAstV in RSS. Three different astroviruses (ANV-1, ANV-2, new CkAstV) were detected in the intestines of RSS-affected chickens by *in situ* hybridization. In subsequent studies, only the CkAstV-specific signals were detected in the cystic lesions, implying a close association with the formation of the RSS hallmark lesion. Furthermore, the full length nucleotide sequence of the viral genome of a CkAstV present in gut homogenates obtained from RSS-affected chickens was determined. The sequence showed significant differences compared to published sequences of other chicken astroviruses. Additionally, a different replication mechanism for this virus was postulated based on differences for the initiation of expression of the viral RNA-dependent RNA polymerase. In experiments performed in parallel, a new CkAstV was isolated in cell culture. This new CkAstV

(cCkAstV) replicated in chickens in the intestinal crypt epithelial cells during the first few days of life. However, during the first passage in chickens, the cCkAstV did not induce typical clinical signs of RSS. A serial chicken-to-chicken passage of the cCkAstV resulted in an increased virulence during the 4<sup>th</sup> passage and showed all signs of RSS. Comparison of the complete genomic sequence of the cCkAstV with the chicken passaged cCkAstV revealed only minor genetic modification as indicated by a >99% homology which resulted in a total of four amino acid exchanges in two viral proteins. The consequences of the observed mutations need to be further evaluated utilizing a reverse genetics approach. In conclusion, the results indicated that the cCkAstV has the potential to cause clinical signs of RSS in chickens. As an etiological agent of the disease, the involvement of the cCkAstV in RSS was strongly supported by this study.

INDEX WORDS:     runting stunting syndrome, chicken astrovirus, chicken parvovirus, weight retardation, cystic enteropathy, cystic crypt, *in situ* hybridization, etiology, pathogenicity, pathogenesis

EVALUATION OF A NEW CHICKEN ASTROVIRUS IN THE ETIOLOGY OF RUNTING  
STUNTING SYNDROME (RSS) IN BROILER CHICKENS

by

KYUNG-IL KANG

DVM, Kangwon National University, Korea, 1996

M.S., University of Georgia, 2007

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial

Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2012

© 2012

Kyung-il Kang

All Rights Reserved

EVALUATION OF A NEW CHICKEN ASTROVIRUS IN THE ETIOLOGY OF RUNTING  
STUNTING SYNDROME (RSS) IN BROILER CHICKENS

by

KYUNG-IL KANG

Major Professor: Holly Sellers  
Committee: Egbert Mundt  
Jeff Hogan  
Eric R. Lafontaine  
Biao He

Electronic Version Approved:

Maureen Grasso  
Dean of the Graduate School  
The University of Georgia  
August 2012

## DEDICATION

To my wife, Joeeun, my son, Andrew, and my parents  
whose love and support enabled me to complete this work.

## ACKNOWLEDGEMENTS

I am deeply thankful to Dr. Egbert Mundt for giving me the opportunity to study under his guidance. It was a real honor and I will never forget the time I worked with him. I wish I could find a better way to express this appreciation, but I cannot. Again, thank you so much for all of his dedication, enthusiasm, and support, which will remain in my heart forever.

I also express great appreciation to Dr. Holly Sellers, who served as my major professor in lieu of Dr. Mundt. Thank you for your direction and contributions for my graduate work.

Thanks are also extended to Drs. Jeff Hogan, Eric R. Lafontaine, and Biao He who served as my committee members and provided me wise suggestions and valuable guidance for my professional development.

My sincere thanks also go to my colleagues VJ, Callie, Alan, Taejoong, Lauren, JT, and Erich for their helpful cooperation. Finally, I would like to thank the animal caretakers and all other staff at the PDRC for their unselfish technical assistance.

## TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS .....	v
LIST OF TABLES .....	vii
LIST OF FIGURES .....	viii
CHAPTER	
1 INTRODUCTION AND LITERATURE REVIEW .....	1
2 MANUSCRIPT I .....	37
INVESTIGATION INTO THE ETIOLOGY OF RUNTING AND STUNTING SYNDROME IN CHICKENS	
3 MANUSCRIPT II .....	70
DETERMINATION OF THE FULL LENGTH SEQUENCE OF A CHICKEN ASTROVIRUS SUGGESTS A DIFFERENT REPLICATION MECHANISM	
4 MANUSCRIPT III .....	86
ISOLATION AND CHARACTERIZATION OF A CHICKEN ASTROVIRUS - AN ETIOLOGICAL AGENT OF RUNTING STUNTING SYNDROME IN BROILER	
5 DISCUSSION AND CONCLUSION .....	130

LIST OF TABLES

	Page
Table 2.1: Oligonucleotides used amplification of the sequences for the RNA probes .....	62
Table 2.2: Average scores of microscopic changes in bursa of Fabricius, thymus, and Harderian gland.....	63
Table 3.1: Similarities of amino acid sequences of different astrovirus to sequences of the new chicken astrovirus .....	81

## LIST OF FIGURES

	Page
Figure 1.1: Schematic depiction of astrovirus genomic organization.....	36
Figure 2.1: Weight gain and presence of cystic lesions in RSS-affected chicken.....	64
Figure 2.2: Astrovirus RNA was detected in epithelial cells of RSS-exposed chicken .....	66
Figure 2.3: Presence of viral RNA specific for astroviruses during exposure to RSS- contaminated litter .....	68
Figure 3.1: Conserved nucleotides in the noncoding regions of bird astroviruses .....	82
Figure 3.2: Schematic of the genomic organization of the new chicken astrovirus .....	83
Figure 3.3: The sequence of the new chicken astrovirus forms a new branch .....	85
Figure 4.1: Characterization of a chicken astrovirus in cell culture .....	118
Figure 4.2: Infection of broiler chickens with a chicken astrovirus resulted in viral replication in the crypt region of the duodenum .....	120
Figure 4.3: Dynamics of viral RNA in the duodenum of chickens infected with a chicken astrovirus.....	122
Figure 4.4: Serial passage of a chicken astrovirus in broiler chickens resulted in an increased weight retardation .....	124
Figure 4.5: Presence of bacteria during serial passage of a chicken astrovirus in broiler chickens did not influence weight retardation .....	126
Figure 4.6: Comparison of amino acid sequences of chicken astrovirus.....	128

## CHAPTER 1

### INTRODUCTION & LITERATURE REVIEW

#### INTRODUCTION

Astroviruses are small, non-enveloped RNA viruses measuring approximately 28-30 nm in diameter. Virus particles have been described, based on morphology observed by electron microscopy, as spherical containing short spikes on their surface (Madeley & Cosgrove, 1975; Risco *et al.*, 1995; Mendez & Arias, 2007). The viral capsid contains a single copy of a positive sense, single-stranded RNA genome. The approximately 7.0 kb long genome is polyadenylated and contains three open reading frames (ORFs): ORF1a, ORF1b, and ORF2. ORF1a and ORF1b have been reported to encode for a fusion protein via a ribosomal shift mechanism, while ORF2 encodes for the capsid protein (Jiang *et al.*, 1993).

Astroviruses mainly induce gastroenteritis in animals. Human astroviruses are considered the second leading cause of viral gastroenteritis in young children (Glass *et al.*, 1996; Mendez & Arias, 2007). Similarly, a number of avian astroviruses have been detected and isolated from birds affected with enteric disease. Astroviruses are also associated with hepatitis in ducks and nephritis in chickens (Gough *et al.*, 1984; Imada *et al.*, 2000; Koci & Schultz-Cherry, 2002). Two different groups of astrovirus species have been reported in chickens, avian nephritis virus (ANV) and chicken astrovirus (CkAstV). ANV-like viruses have been linked to a cause of nephritis (Imada *et al.*, 2000), while CkAstV has been isolated from broiler chickens with

runting stunting syndrome (RSS) and considered a causal agent of this disease (Baxendale & Mebatsion, 2004; Sellers *et al.*, 2010).

RSS is an enteric disease in broiler chickens of uncertain etiology (Olsen, 1977; Kouwenhoven *et al.*, 1978). RSS affects broiler chickens early in life and is characterized by severe weight suppression resulting from diarrhea, and results in significant economic losses in the broiler industry (Goodwin *et al.*, 1985; Rebel *et al.*, 2006). Chickens affected with RSS have intestinal disorders. The small intestines of the affected birds macroscopically appear pale and distended. Microscopically, the small intestine is characterized by cystic dilatation of the crypt of Lieberkühn, accompanied by atrophy, clubbing, and fusion of the villi as well as hyperplasia of the crypt (Smart *et al.*, 1988; Goodwin *et al.*, 1993; Otto *et al.*, 2006).

Clinical and pathological signs of RSS can be experimentally reproduced by oral inoculation of young chickens with gut homogenate from previously affected chickens, thus implying a transmissible disease. Moreover, bacteria-free filtrate of the gut homogenate can cause the disease, implying a viral etiology (Montgomery *et al.*, 1997; Songserm *et al.*, 2002b; Sellers *et al.*, 2010). Small round viruses, such as picornaviruses, circoviruses, parvoviruses, caliciviruses, and astroviruses, have been suggested as present in the intestines of affected chickens. In a subtractive metagenomic analysis, conducted in our lab, astroviruses were suspected to be the cause of the disease (Kim *et al.*, 2011; Kim & Mundt, 2011). In addition, a novel chicken astrovirus (CkAstV) was identified as a possible RSS pathogen (Sellers *et al.*, 2010).

Currently, there are no effective vaccines or intervention strategies to control RSS, primarily due to the absence of established etiologic agents. Determination of causal agents in affected birds is essential for the development of effective vaccines. However, the importance of

astroviruses in RSS-affected chickens is still poorly understood, and possible mechanisms must be further elucidated. Since the CkAstV was isolated, it is essential to elucidate the molecular characterization of the virus and its virulence related to the primary pathological changes. The objective of this study was to characterize a novel CkAstV and to evaluate its role in the pathogenesis of RSS. The findings of this study may become the basis for developing effective prevention methods for RSS.

### **Hypothesis**

If astroviruses are associated with RSS in commercial broilers, then vaccination of broiler breeders or the progeny with astroviruses may mitigate the outcome of the disease.

**Specific Aim 1:** Etiologic evaluation of three chicken astroviruses and a chicken parvovirus in runting stunting syndrome in chickens using *in situ* hybridization.

**Specific Aim 2:** Determination and comparison of the full length viral sequences for a chicken astrovirus from intestinal homogenates of RSS-affected chickens and a novel chicken astrovirus isolated in cell culture.

**Specific Aim 3:** In vitro characterization of a novel chicken astrovirus isolate and evaluation of its in vivo role in the pathogenesis of RSS in chickens.

## **LITERATURE REVIEW**

### **RUNTING STUNTING SYNDROME**

Runting stunting syndrome (RSS) is an enteric disease in young broiler chickens with unknown etiology (Olsen, 1977; Kouwenhoven *et al.*, 1978). It has also been referred to as malabsorption syndrome (MAS), infectious stunting syndrome, pale bird syndrome, and

helicopter syndrome. Affected chickens suffer from diarrhea during the first few weeks of age, resulting in severe weight suppression, poor flock uniformity, and considerable economic losses in the broiler industry (Eidson *et al.*, 1985; Goodwin *et al.*, 1985; Rebel *et al.*, 2006).

### **Pathogenesis & Pathophysiology**

Chickens affected with RSS show intestinal disorders. The small intestines of the affected birds macroscopically appear pale and distended. Microscopically, the small intestine is characterized by cystic formation of the crypt of Lieberkühn, as well as, atrophy, clubbing, and fusion of the villi and hyperplasia of the intestinal crypts (Smart *et al.*, 1988; Goodwin *et al.*, 1993). At the initial stage of disease, cystic crypt formation is characteristic for the disease. Therefore, the crypt lesion is a hallmark lesion of RSS, and this phenotype has been referred to as cystic enteropathy. However, the severity of clinical signs of RSS may depend on the extent of villous atrophy resulting from epithelial loss and decreased turnover rate of the cells (Otto *et al.*, 2006).

The actual cause and pathogenesis of the intestinal lesions is still not clear; however, increased apoptosis along the lining of villi and crypt epithelial cells was reported (Zekarias *et al.*, 2005). In the intestines of affected chickens, the crypt epithelial cells become degenerated and detached into the lumen, forming cystic crypts lined by flattened epithelial cells. The resulting crypts have an expanded lumen where cellular debris was observed. About two to three weeks after experimental infection, recovery of the intestinal lesions has been observed (Zekarias *et al.*, 2005; Otto *et al.*, 2006). The cystic crypts were also found in other animal diseases such as canine protein-losing enteropathy (Feron & Mullink, 1971; Willard *et al.*, 2000). Nonetheless, the cause of the cystic crypts is unknown. Interestingly, the cystic crypts are not observed in

poult malabsorption syndrome, a similar enteric disease in young turkeys in which decreased villous length-to-crypt depth ratio is characteristic resulting from villous atrophy and crypt hyperplasia (Perry *et al.*, 1991).

To date, there is little knowledge of pathophysiology in RSS-affected birds. Blood parameters have not shown important changes, and most of them have been related to general disease conditions, such as dehydration and malnutrition. However, increased plasma amylase and alkaline phosphatase (ALP) was detected; interestingly, those changes were attributed to pancreatic degeneration and an ALP isoenzyme originating from the damaged intestine of RSS-affected chickens (Vertommen *et al.*, 1980; Nili *et al.*, 2007). Immunologically, local CD8<sup>+</sup> T cells were more common in the intestinal villi of susceptible broilers compared to those of resistant counterparts (Songserm *et al.*, 2002a; Zekarias *et al.*, 2002). This implies the presence of an immune reaction to RSS agents, but details have not been elucidated.

### **Etiology of RSS**

RSS is a transmissible disease, but the etiology remains unknown. Nevertheless, RSS is reliably reproduced by inoculating young chickens with filtered intestinal homogenates from previously affected chickens (Montgomery *et al.*, 1997; Songserm *et al.*, 2000; Songserm *et al.*, 2002b; Sellers *et al.*, 2010). Even though bacteria may play a role as a secondary agent in this syndrome, these filterable agents imply a viral etiology as a primary causal agent.

A variety of viruses, including reoviruses (Songserm *et al.*, 2000), parvoviruses (Kisary *et al.*, 1984), enterovirus-like viruses (Decaesstecker *et al.*, 1986), rotaviruses (Otto *et al.*, 2006), and astroviruses (Baxendale & Mebatsion, 2004), have been suggested as the etiological candidates. However, a number of the detected viruses have also been detected by PCR/RT-PCR

in healthy flocks (Pantin-Jackwood *et al.*, 2008a; Pantin-Jackwood *et al.*, 2011). Moreover, the relationship between the presence of these viruses and disease occurrence has not been established. For example, reovirus antigen has been detected, but it was only in the epithelial cells of the tip and middle regions of the intestinal villi in RSS-affected chickens (Songserm *et al.*, 2000; Songserm *et al.*, 2002b).

RSS has been described as a multifactorial disease (Goodwin *et al.*, 1993; Rebel *et al.*, 2006). Reproduction of clinical RSS has been partially successful using the enteric small round viruses or in combination with other factors (Kouwenhoven *et al.*, 1988; Smart *et al.*, 1988; Montgomery *et al.*, 1997; Songserm *et al.*, 2002b). In addition, attempts to prevent the disease have been conducted through active immunization or passive transfer of maternal antibody against previously known agents, such as reoviruses and astroviruses. However, RSS symptoms were only partially reduced with neutralization or vaccination against these viruses (Eidson *et al.*, 1985; Sellers *et al.*, 2010). Therefore, currently no effective vaccine or intervention measures to control RSS have been described, primarily due to the absence of the identification of etiologic agents.

Astroviruses have been proposed as a possible RSS pathogen. Subtractive metagenomic analysis of gut contents from healthy and RSS-affected chickens identified astroviruses, implying a possible role in the etiology of RSS (Kim *et al.*, 2011; Kim & Mundt, 2011). An experimental vaccine based on baculovirus, encoding an astrovirus capsid protein, was effective in reducing weight suppression and histological changes (Sellers *et al.*, 2010), supporting the involvement of astrovirus in RSS. Additionally, turkey astroviruses (TAsV) have been detected in the intestinal epithelial cells in TAsV infected turkeys (Koci *et al.*, 2003; Pantin-Jackwood *et al.*

*al.*, 2008b). These findings for astroviruses on other species may imply a similar role of astroviruses in chicken enteric diseases.

## **ASTROVIRUSES**

### **General Background of Astroviruses**

Astroviruses belong to a group of small round viruses with a diameter of approximately 28 to 30 nm (Madeley & Cosgrove, 1975; Snodgrass & Gray, 1977). Astroviruses were first described in fecal samples from children with gastroenteritis (Madeley & Cosgrove, 1975). Since then, eight human astrovirus (HAstV) serotypes have been identified in young and immune compromised patients. In a titration of HAstV-1 by reverse transcription-polymerase chain reaction (RT-PCR), virus genome copy numbers were detected up to  $10^{11}$  in 1 ml of patient feces (Jonassen *et al.*, 1993).

Viral particles observed in fecal samples from patients with diarrhea had a star-like appearance upon electron microscopy (Madeley & Cosgrove, 1975). The presence of capsid spikes was supported by a cryo-electron microscopy and an image reconstruction of astroviruses (Risco *et al.*, 1995; Dong *et al.*, 2011). However, the characteristic surface appearance is not always detected by EM (Baxendale & Mebatsion, 2004), but is induced by alkaline treatment (pH 10 for 10 mins) of cell culture grown HAstV-2 (Risco *et al.*, 1995).

The family *Astroviridae* has two genera: genus *Mammastrovirus* for mammal astroviruses and genus *Avastrovirus* for avian astroviruses. Astroviruses mainly induce gastroenteritis in a wide variety of mammals and birds; however, in birds, extraintestinal diseases have been associated. There is no supporting evidence that animal astroviruses cross-infect humans, and vice versa (Desselberger & Gray, 2003). Avian astrovirus in poultry diseases

include duck astrovirus, TAstV, chicken astrovirus (CkAstV), and avian nephritis virus (ANV). Two genotypes of TAstVs have been reported: TAstV-1 and TAstV-2, which were associated with enteritis and increased mortality in young turkeys (Koci & Schultz-Cherry, 2002; Tang *et al.*, 2005). In chickens, many of these viruses were previously identified as enterovirus-like viruses (ELV), a virus belonging to the genus *Enterovirus* of the family *Picornaviridae* (Todd *et al.*, 2009a). Full length genome sequencing led to the re- classification of ANV as an Astrovirus. Both ANV and chicken astrovirus belong to the genus *Avastrovirus* in the family *Astroviridae*, while avian encephalomyelitis virus and duck hepatitis virus type 1 (DHV-1) remain in the *Picornaviridae* family. The first astrovirus identified in chickens was avian nephritis virus (ANV) (Imada *et al.*, 2000). Later, other astroviruses were reported from chicken flocks with enteric disease (Baxendale & Mebatsion, 2004).

### **Molecular Biology and Genomic Organization of astroviruses**

In the following literature review regarding the general astrovirus molecular characteristics, most of the content is based on the knowledge of HAstVs.

The genome of astroviruses is composed of a non-segmented, positive-sense, single-stranded RNA with a capacity of 6.8 to 7.9 kb (Fig. 1.1). The astrovirus genome contains three open reading frames (ORF): ORF1a, ORF1b, and ORF2 (Jiang *et al.*, 1993; Lewis *et al.*, 1994). The ORFs are flanked by a 5' untranslated region (UTR) and a 3' UTR followed by a poly-A tail. The lengths of UTRs vary by genus. The 5' end UTR is 80 to 85 nt in mammalian astroviruses, while it is 10 to 20 nt in avian astroviruses (Finkbeiner *et al.*, 2008). The sequence at the 5' end UTR in avian astroviruses begins with CCGAA, which are highly conserved among avian astrovirus species (Jonassen *et al.*, 2003). Interestingly, the conserved sequences were also

present at the 5' end of subgenomic mRNAs, implying a presence of common regulatory sequences on both genomic and subgenomic RNAs (Jonassen *et al.*, 2003). The 3'-UTR is longer in avian astroviruses, ranging from 130 to 305 nt. In mammalian species, the 3'-UTR ranged from 58 to 108 nt (Finkbeiner *et al.*, 2008).

ORF1a encodes for nonstructural polyprotein. Interestingly, the RNA dependent RNA polymerase (RdRp) was reported to be expressed via a ribosomal shift as a fusion protein from both ORF1a and ORF1b (Jiang *et al.*, 1993; Lewis *et al.*, 1994). ORF2 at the 3'-part of the genome encodes for the capsid protein and were found in both genomic RNA and subgenomic mRNAs (Monroe *et al.*, 1991; Matsui *et al.*, 1993; Monroe *et al.*, 1993; Koci *et al.*, 2000). Interestingly, proteins encoded by astroviruses seem to lack an RNA helicase domain, which is unusual since proteins with a helicase domain were commonly found in positive strand RNA viruses with genomes larger than 5.8 kb (Kadare & Haenni, 1997). Immunoreactive epitopes in the NS viral proteins were indicated by an expression cloning study. In this study, proteins encoded by genomic regions out of the subgenomic RNA showed immunoreactions against anti-astrovirus rabbit serum (Matsui *et al.*, 1993).

### **Astrovirus Replication**

The viral replication strategy is not yet known in detail, but a proposed replication strategy has been inferred from the replication cycle of alphaviruses, such as Sindbis virus. Astroviruses were regarded as similar to alphaviruses in that they have a similar genome organization and capsid protein production strategy using a subgenomic mRNA. (Matsui *et al.*, 1993; Geigenmüller *et al.*, 2003). Astroviruses attach to an unknown host cell receptor, which has been proposed as a polysaccharide molecule on a target cell membrane (Dong *et al.*, 2011).

Upon binding, astroviruses enter host cells by clathrin-mediated endocytosis (Donelli *et al.*, 1992). When the virus genome is released into the cytoplasm via an unknown mechanism, two nonstructural (NS) viral proteins from ORF1a and 1b are directly translated from the positive stranded viral genomic RNA. A full-length negative strand RNA is transcribed from the genomic RNA by the newly generated viral proteins. The viral RdRp uses this antigenomic RNA as a template for transcription of subgenomic mRNAs and genomic RNAs (Mendez & Arias, 2007). The subgenomic mRNAs are polyadenylated, so that they serve as messenger RNAs for the translation of the capsid protein (Geigenmüller *et al.*, 2003). Positive sense viral RNAs are packaged in the capsid protein. The releasing mechanisms of astroviruses are not known, but caspases, the effectors of apoptosis, are involved in *de novo* capsid maturation and virus release (Banos-Lara Mdel & Mendez, 2010). The replication steps are detailed below.

**Viral Genomic RNA and Subgenomic RNA:** In the replication cycle of astroviruses, three viral RNAs are involved, which have been described as genomic RNAs, antigenomic RNAs, and subgenomic RNAs. The genomic and subgenomic RNAs have been detected by Northern blot (Monroe *et al.*, 1991; Matsui *et al.*, 1993; Monroe *et al.*, 1993; Lewis *et al.*, 1994; Koci *et al.*, 2000), and one subgenomic RNA was identified during astrovirus replication. Monroe *et al.* (1993) detected two viral RNAs with different lengths, 7.2 kb and 2.8 kb, 12 hours after infection of cells with HAstV-2. The small RNA (2.8 kb) is considered a viral subgenomic mRNA containing ORF2 encoding for the capsid protein, while the large RNA molecule (7.2 kb) is the genomic RNA. The reasons are as follows: first, the sequence of the small RNA was part of the large RNA molecule. Second, both of the RNAs contained the same 3' end. Third, the 3' ends of both of the RNAs were polyadenylated. Furthermore, the intensity of the smaller RNA in Northern blot was 10 fold more than that of the large RNA (Monroe *et al.*, 1991), which implies

that the small RNA is a subgenomic mRNA encoding for a large production of capsid proteins necessary for the assembly of progeny viruses. This is corroborated by a study in which genomic, antigenomic, and subgenomic RNAs were titrated from cells infected with different HAstV-1 mutants by end-point RT-PCR (Guix *et al.*, 2005). The amounts of antigenomic RNAs were similar to those of genomic RNAs in all of the mutants. However, in high progeny productive mutants, subgenomic RNAs comprised almost 99% of the cell-associated viral RNAs. On the other hand, in low productive mutants, subgenomic RNAs were even less than their genomic RNAs, supporting the role of the subgenomic RNA in astrovirus replication. Intriguingly, a highly conserved region of astrovirus RNAs is located at the upstream of the ORF1b and ORF2 junction. This highly conserved sequence may imply the presence of a promoter to transcribe the subgenomic RNA (Finkbeiner *et al.*, 2008; Smyth *et al.*, 2009).

Subgenomic RNAs were also found in avian astroviruses (Imada *et al.*, 2000; Koci *et al.*, 2000). Two RNA bands (7.5 & 3 kb) were detected by Northern blot using a 3'-end probe in the ANV-infected cells, while only a large RNA (7.5 kb) was detected using a 5'-end probe (Imada *et al.*, 2000). Additionally, in a TAstV study, one genomic RNA and one subgenomic RNA were detected by Northern blot with an ORF2 specific probe (Koci *et al.*, 2000).

**ORF1a:** ORF1a is preceded by the 5' UTR and spans one third of the genome. ORF1a encodes for a NS polyprotein (nsP1a) of 920 amino acids (aa) that contains a 3C-like serine protease. The protease is functional in nsP1a processing because at least one autocatalytical cleavage site was indicated in a cell-free expression system (Kiang & Matsui, 2002). When nsP1a was expressed in cells, multiple nsP1a derivatives were detected by immunoprecipitation, suggesting there are multiple cleavage sites in nsP1a for the viral protease or cellular enzymes (Geigenmuller *et al.*, 2002a). The protease was also involved in the processing of the ORF1a/1b

fusion protein to RdRp. When a 9 aa substitution was introduced into the protease motif, virus replication was not detected (Geigenmuller *et al.*, 2002a), suggesting the viral protease encoded in ORF1a is required to process RdRp from the ORF1a and 1b fusion protein.

Astroviruses appear to lack a 5' cap structure in the genomic RNAs and mRNAs, because evidence for viral capping enzymes, such as a methyltransferase domain, has not been identified in the astrovirus genome. Most of the other animal viruses modify their RNA 5' ends by attaching a cap structure, while ss (+) RNA viruses, namely picornaviruses and caliciviruses use a protein attachment (Decroly *et al.*, 2011). In astroviruses, a putative VPg-encoding region has been identified in ORF1a (Jiang *et al.*, 1993). It has been suggested that this putative VPg, a viral protein bound to the 5' end of the viral genome, acts as an alternative for a cap structure and a primer for RdRp (Decroly *et al.*, 2011). Nevertheless, in a reverse genetics system, astrovirus RNA with an m<sup>7</sup>G cap at its 5' end showed efficient RNA stability to generate infectious viral particles (Geigenmuller *et al.*, 1997; Imada *et al.*, 2000).

A sequence encoding a nuclear localization signal (NLS) was detected downstream of the viral protease motif. However, its functionality in viral subcellular localization is not fully understood. Nuclear localization was reported by immunofluorescence using anti-ORF1a (NLS region) antiserum (Willcocks *et al.*, 1999), while another immunofluorescence study using different antibodies against the NLS region was clearly confined in the cytoplasm (Geigenmüller *et al.*, 2003).

The C-terminal end of nsP1a proteins seemed to be related to viral replication. The C-terminal part of nsP1a co-localized with viral RNA, as evidenced by the double labeling of viral RNA by fluorescence *in situ* hybridization (FISH) and the viral protein by immunofluorescence. In addition, the C-terminal localized nsP1a proteins co-localized with the endoplasmic reticulum

in the perinuclear region, while viral structural proteins were evenly located in the cytoplasm. These co-localizations with viral RNA and cellular compartments suggested a possible role in the C-terminal nsP1a proteins in viral RNA replication (Guix *et al.*, 2004). The same author generated four recombinant astroviruses differing in their C-terminal nsP1a protein to evaluate the nsP1a's role in viral RNA replication. All were able to replicate in cells, but there were differences in viral RNA replication amounts among the recombinant viruses observed, supporting a role of the C-terminal located nsP1a for virus replication (Guix *et al.*, 2005).

Immunoreactive epitopes located on non-capsid proteins were described in nsP1a/b proteins (Matsui *et al.*, 1993). Immunologically reactive clones from oligo (dT) primed HAstV-1 cDNA libraries were selected by a polyclonal anti-HAstV rabbit serum; then, the immunologically reactive peptides encoding plasmids were further analyzed in order to locate antigenic, protein-coding regions of astroviruses. Two patterns of Northern blot were detected using the screened plasmids: three plasmids hybridized to the genomic RNA (7.2 kb) alone, while the other plasmid hybridized to both the genomic and the subgenomic RNA (2.8 kb). The authors postulated the former plasmids contained sequences which were located at the 5' end of the genomic RNA, while the latter contained sequences which were in the subgenomic RNA at the 3' end of the genomic RNA. These findings provided not only evidence for the presence of epitopes in the capsid protein, but also the presence of immunoreactive regions in the NS viral proteins.

**ORF1b:** ORF1b encodes for a highly conserved RdRp, which subsequently facilitates the transcription of the viral RNAs. However, ORF1b of HAstV-2 overlaps ORF1a by 70 nt at its 5' end. In addition, the first start codon in ORF1b is found 380 nt downstream of the preceding ORF1a stop codon. Furthermore, ORF1b is located at the (-1) reading frame of ORF1a.

Therefore, instead of the classical mechanism that uses a start codon and a stop codon in the given ORF, a programmed -1 ribosomal frameshift mechanism has been postulated for ORF1b expression (Jiang *et al.*, 1993). This process requires two essential *cis* acting sequence elements: a shifty heptamer sequence (AAAAAAC or A<sub>6</sub>C) and a stimulatory RNA secondary structure forming a downstream stem-loop. A downstream pseudoknot region that is required for the process in other viruses is not essential for astroviruses (Lewis & Matsui, 1996).

Translation of ORF1a occurred via the classical mechanisms. When the translation machinery reaches the end of ORF1a, the postulated presence of an RNA secondary structure hinders the further scanning of the ribosomal complex. Via the shifty RNA sequence, the ribosomal complex reinitiates in a (-1) frame (one nucleotide movement to the 5' end) localized in ORF1b, avoiding an in-frame termination codon. After the unwinding of the downstream RNA secondary structure, the ribosome continues the translation of the mRNA in the following frame (Farabaugh, 1996). Via a ribosomal frame shift, the translation of ORF1a and 1b, which encode for two different polyproteins, occurred in a fusion protein (nsP1a/1b) (Jiang *et al.*, 1993; Lewis *et al.*, 1994; Lewis & Matsui, 1996). The resulting fusion proteins (nsP1a/1b) are processed into RdRp and other non-structural proteins by a viral 3C-like serine protease and other cellular proteases (Lewis & Matsui, 1996).

A number of positive stranded RNA viruses are known to use the frameshifting mechanism for the expression of viral replicates. In retroviruses, Gag-Pol polyprotein is translated by ribosome frameshifting by which NS proteins, including reverse transcriptase and integrase, were synthesized (Jacks & Varmus, 1985). Coronaviruses produce their RNA polymerase via a frameshifting between the 1a and 1b coding regions, resulting in a 1a-1b fusion

protein (Brierley *et al.*, 1987). The highly conserved, shifty heptamer and a downstream stem-loop structure were also present in avian astroviruses (Imada *et al.*, 2000; Koci *et al.*, 2000).

**ORF2:** In comparison with other astroviruses, ORF2 is a minimally conserved region with high sequence variability (Jonassen *et al.*, 2003; Guo *et al.*, 2010). ORF2 is found in both genomic and subgenomic RNAs, and located at the 3' end of the genome overlapping ORF1b by 8 nt in the case of HAstV1. However, unlike ORF1a and 1b, both of the frames are in the same reading frame (Geigenmüller *et al.*, 2003).

An 87 to 90 kDa polyprotein (VP90) is encoded as the precursor of the viral capsid protein. An *in vitro* trypsinization revealed the capsid proteins are initially translated as a single 90 kDa polypeptide, which is later separated into 31-34 (VP34), 27-29 (VP27), and 20-26 kDa (VP25) (Monroe *et al.*, 1991; Mendez *et al.*, 2002). The molecular weights were slightly variable among astroviruses. VP90 comprises three domains based on their sequence identity: a highly conserved N-terminal domain, a hypervariable domain, and a highly acidic C-terminal domain (Wang *et al.*, 2001). A conserved basic amino acid region is found in the highly conserved N-terminal domain spanning aa 18 to aa 62 in the case of HAstV-1 ORF2. Deletion of this basic region did prevent viral assembly, thus implying that the N-terminal region attaches to the viral RNA for the genome assembly into the progeny virion (Geigenmuller *et al.*, 2002b; Caballero *et al.*, 2004). The acidic C-terminal domain in VP90 is cleaved by host cellular caspases to yield the mature extracellular virion composed of VP70 (Mendez *et al.*, 2002; Mendez *et al.*, 2004; Banos-Lara Mdel & Mendez, 2010).

After the release of virus particles from infected cells, VP70 is further processed by host extracellular enzymes, such as trypsin. Interestingly, through this trypsin cleavage processing on ORF2 polyproteins, astroviruses acquire enhanced infectivity (Mendez *et al.*, 2002). Finally,

mature infectious virions of astroviruses are composed of three main capsid proteins: VP34, VP27, and VP25 (Mendez *et al.*, 2002). VP34 originates from the highly conserved N-terminal region. On the other hand, VP27 and VP25 are derived from the hypervariable domain beginning at different N termini but ending at the same C terminus (Fig. 1.1). A viral crystal structure revealed that VP27 and VP25 form the dimeric viral surface spikes (Dong *et al.*, 2011). By comparing the domains from eight HAstVs, a large number of conserved amino acid residues were identified on top of the spike, implying its role as a putative receptor binding sites. In support of this, the polar residue composition is also found in other viruses that use a polysaccharide molecule as a cell receptor; thus, cellular sugar molecules were suggested to be part of the host cellular receptor (Dong *et al.*, 2011).

Mutations in ORF2 were demonstrated to cause a defect in virus infectivity. In a study with HAstV-1, two full-length cDNA clones (pAVIC) were able to give rise to their viral progeny, but one of the virus mutants lost infectivity. To find the phenotypic difference between two viral progenies, their sequences were compared, and four point mutations were found in ORF2, among which one is a silent mutation. By amino acid substitution, a highly sensitive amino acid, T227 of HAstV ORF2, was identified, which might have induced aberrant capsid assembly (Matsui *et al.*, 2001).

A stem-loop II like molecule (s2m) is 35 nt of highly conserved region at the 3' end of RNA genome, in which the ORF2 stop codon is included. S2m has been observed in every astrovirus species except TAstV-2 (Koci *et al.*, 2000; Jonassen *et al.*, 2001).

## **Construction of Astrovirus Full Genomic Clones**

Transfection-infection cell systems using astrovirus RNAs have been developed that provided efficient methods for evaluating viral replication requirements and mechanisms via targeted mutations into the viral genome. First of all, astrovirus RNA itself is infectious. Total RNA isolated from HAstV-1 infected cells produced infectious progeny virus particles. In addition, viral RNA transcribed from HAstV-1 genomic cDNA (pAVIC) also generated its viral particles (Geigenmuller *et al.*, 1997). This astrovirus full genomic clone pAVIC allowed systematic exploration of the viral molecular biology via targeted mutations. BHK and CaCo-2 cells have been transfected with RNA from the infectious cDNA clone for HAstV-1, and gave rise to a high titer of infectious virus up to  $1.8 \times 10^8$  IU/ml (Geigenmuller *et al.*, 1997). For avian astroviruses, ANV RNA has been transfected into chicken kidney cells and BHK cells (Imada *et al.*, 2000).

## **ASTROVIRUSES IN CHICKENS**

To date, two species of astroviruses have been described in chickens: ANV and CkAstV. Multiple subtypes of these viruses have been suggested on the basis of a high level of genetic and antigenic variation (Shirai *et al.*, 1991; Smyth *et al.*, 2009). Extensive genetic variation has been described especially in capsid genes among avian astrovirus species, and may be responsible for pathogenicity and antigenicity of the viruses (Jonassen *et al.*, 2001). However, most of these classifications were obtained from a gene analysis of a partial astrovirus genome. Considering the high frequency of RNA virus mutations and recombination between astroviruses (Strain *et al.*, 2008), taxonomic subdivisions should be determined based on multiple genes or the full length genomes (Smyth *et al.*, 2009; Pantin-Jackwood *et al.*, 2011).

## **Avian Nephritis Virus (ANV) and Chicken Astrovirus (CkAstV)**

ANV was the first chicken Astrovirus identified, and initially regarded as a picornavirus, then later classified as an astrovirus by sequence analysis (Yamaguchi *et al.*, 1979; Imada *et al.*, 2000). Since then, a number of ANV strains have been identified by RT-PCR and subsequent sequence analysis (Pantin-Jackwood *et al.*, 2006; de Wit *et al.*, 2011). Currently, two serotypes have been reported (Shirai *et al.*, 1991; Shirai *et al.*, 1992), and another serotype was proposed from new isolates in Europe (de Wit *et al.*, 2011). Interestingly, antibodies to avian nephritis virus (ANV) were found in turkeys (Connor *et al.*, 1987), and ANV specific RT-PCR was positive in turkey flocks (Pantin-Jackwood *et al.*, 2008a; Pantin-Jackwood *et al.*, 2011), implying that turkeys are also susceptible to ANV.

ANV replicates in the intestine as well as in the kidney, inducing a wide range of clinical signs from subclinical infection to severe disease conditions known as RSS and chick nephropathy (Shirai *et al.*, 1990; Shirai *et al.*, 1992; Takase *et al.*, 1994; de Wit *et al.*, 2011). ANV pathogenicity varies among susceptible chickens: one-day-old SPF (line 15I) infected with ANV showed growth retardation at 7 and 14 days post inoculation (PI), while another SPF line (line PDL-1) did not develop growth retardation with the same virus (Shirai *et al.*, 1990). Furthermore, more severe growth retardation was not developed by combination of ANV and reovirus infection in comparison to single infection with ANV or reovirus (Shirai *et al.*, 1990).

CkAstV were described from broiler flocks in the Netherlands which showed weight depression (Baxendale & Mebatsion, 2004). Three new CkAstV isolates that were reported in the study were antigenically distinct from other known avian astroviruses, such as ANV and duck hepatitis virus type 2. In addition, these chicken astroviruses were widely distributed in chickens in the field and also in turkey flocks. However, chickens infected with the isolates showed only

mild diarrhea which was associated with mild macroscopic and microscopic lesions. For this insignificant pathogenicity of the isolated viruses, the authors suggested that the modified virulence of the isolates was caused by passaging in cell culture. Willcocks *et al.* (1994) reported changes in virulence in a cell culture adapted astrovirus. During cell adaptation to HEK, a primary human embryo kidney cells, HAstV lost 45-nucleotide (15 aa) in the C-terminal of ORF1a. The defective virus did not cause cytopathic effects during the passages, suggesting possible modifications of the viral replication. To date, at least two subgroups of CkAstV were postulated to exist based on phylogenetic analyses (Smyth *et al.*, 2009).

### **Intestinal Pathogenicity of Astroviruses**

Chickens were infected with astroviruses in the first few days of life. The detection rate of the virus peaked around day 4 to 5 PI followed by a decrease. A small percentage of birds remained positive after several weeks. Thus, the pathogenicity is age-related, in that the virus infection in older chickens might be not as serious as that in younger chicks (Smyth *et al.*, 2009).

In experimental infections, a mild diarrhea occurred in chickens infected with astroviruses isolated from RSS-affected chickens. CkAstV and ANV replicate in the intestine, which was proven by an immunofluorescent technique to detect viral antigens. However, astroviruses were also detected in other extraintestinal tissues, including the kidney, liver, spleen, and pancreas, suggesting the virus invades other organs beyond the intestine (Smyth *et al.*, 2007). Astroviruses have also been detected in the blood of chickens infected with CkAstV (Baxendale & Mebatsion, 2004) and in the buffy coat of turkeys infected with TAstV (Koci *et al.*, 2003), suggesting a viremic stage during astrovirus infection.

Histologically, only mild lesions have been detected. In the intestines, there were a few individually shrunken degenerate cells in the intestinal epithelial cells and occasional cellular exudates in the dilated crypt lumen with attenuated epithelial cells. The most striking morphologic changes were observed in the ratio of villi and crypts (V:C ratio), which resulted from atrophy of villi and hyperplasia of crypts (Smyth *et al.*, 2007). Interestingly, diarrhea caused by TAstVs neither accompanied inflammation nor morphological changes in the intestine (Koci *et al.*, 2003). In addition, TAstV replication was not related to host cellular apoptosis because the number of TUNEL positive cells in TAstV infected intestines was not different from that in controls (Koci *et al.*, 2003). The lack of inflammation and cell death was also reported after HAstV infection (Sebire *et al.*, 2004).

In another study, the author compared pathogenicity and distribution of two chicken astrovirus isolates with those of ANV and ELV-1 (enterovirus-like virus type 1) in SPF chickens. The virus antigen was mainly detected in the villous epithelial cells. However, virulence and tissue tropism of chicken astroviruses were quite different among isolates (Smyth *et al.*, 2007). ELV-1 was later identified as an ANV isolate (Todd *et al.*, 2009a). Similarly, the majority of TAstV is also present in the villous epithelial cells, but it is detected in a small number of crypt epithelial cells without specific histopathological changes (Pantin-Jackwood *et al.*, 2008b). Likewise, HAstV and other mammal astrovirus species were not observed in crypt cells, thus implying only mature intestinal epithelial cells are susceptible to astrovirus infection (Sebire *et al.*, 2004).

Effects of astroviruses on small intestinal absorptive function have been investigated in chickens and turkeys with malabsorptive diarrhea. D-xylose is a pentose sugar that is absorbed from the small intestine. Once absorbed, D-xylose is poorly metabolized by the body and

excreted via the urine. Intestinal D-xylose absorption was significantly decreased in chickens of uneven weight gain in comparison to healthy control chickens, therefore suggesting a malabsorptive diarrhea (Goodwin *et al.*, 1985). In detail, astroviruses can modify physiological function of intestinal epithelial cells. In a Caco-2 cell culture system, HAstVs increased barrier permeability, suggesting a diarrhea mechanism upon virus infection. Interestingly, the permeability was increased by UV-inactivated virus as well as in the presence of the astrovirus capsid protein alone, indicating that the astrovirus capsid alone can induce the diarrhea mechanism without virus replication (Moser *et al.*, 2007). TAstV-2 caused a reduction in mucosal-to-serosal Na<sup>+</sup> flux, determined by an electrophysiological experiment to measure ion flux. Furthermore, cellular actin rearrangement was observed upon the virus infection by EM, and membrane Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE) protein expression in the mucosa was reduced in TAstV-2 infection in Western blot analysis. These results suggest that malabsorptive diarrhea is associated with the failure of Na and water absorption, caused by actin re-arrangement and reduced NHE function (Nighot *et al.*, 2010).

### **Prevalence and Epidemiology**

CkAstV and ANV are widespread throughout the world, and they have been commonly detected in broiler chickens and even in turkeys (Pantin-Jackwood *et al.*, 2006; Todd *et al.*, 2009b; Pantin-Jackwood *et al.*, 2011). Antibody levels to astroviruses in broilers were high at slaughter (Todd *et al.*, 2009b); however, apparent growth retardation was not detected in the examined flocks, suggesting astroviruses with variable pathogenicity are circulating in the poultry flocks. In addition, more than one astrovirus types have been detected in individual

flocks as well as in individual chickens (Pantin-Jackwood *et al.*, 2006; Pantin-Jackwood *et al.*, 2011).

High seroprevalence against astroviruses was detected in broiler, broiler parent, grandparent, and great-grandparent flocks in the UK (Todd *et al.*, 2009b). The presence of astrovirus antibodies in parent flocks may imply low effects of maternal antibodies or variable serotypes of astroviruses in the field. Smyth *et al.* (2009) reported that astroviruses were not detected at hatching days by RT-PCR, but high prevalence was indicated for the first few days of age. This may indicate a horizontal infection in young chicks in their chicken house or an infection with the virus excreted from a small number of vertically infected chicken.

In longitudinal surveys by RT-PCR, flocks with higher performance had lower chicken astrovirus detection rates than those with low performance, implying that a continuous, high level of astrovirus presence is related to broiler performance (Smyth *et al.*, 2009). Nevertheless, the high correlation between the presence of astroviruses and growth retarded disease in chickens resulting from enteric disorder has not been demonstrated (Todd *et al.*, 2009b).

### **Diagnosis of Astrovirus Infection in Chicken**

There are no convenient, sensitive, and specific diagnostic tests mainly due to the absence of reliable causal relationships between astroviruses and growth retarding disease phenotypes (Baxendale & Mebatsion, 2004; Pantin-Jackwood *et al.*, 2006). Detecting virus particles from fecal samples of affected birds by EM was the primary diagnostic method. However, EM is neither specific nor sensitive. Other small round viruses, such as picornavirus and enterovirus-like virus were also thought to be associated with enteric diseases in poultry

(Parashar & Glass, 2003). Moreover, the characteristic star like surface appearance of astroviruses was not a constant EM finding of astroviruses (Baxendale & Mebatsion, 2004).

**Virus isolation:** A number of astroviruses have been isolated in cell cultures; however, astroviruses may replicate on a very low level, and the cells can be outgrown by other viruses, such as reoviruses and adenoviruses (Smyth *et al.*, 2009). Avian nephritis virus was first isolated in the 1970s as a picornavirus (Yamaguchi *et al.*, 1979). Other astroviruses in chickens were isolated from a flock that showed symptoms of RSS. The virus was mainly isolated from the small intestine. However, the virus isolation was also successful from other extraintestinal tissues, including the blood and the bursa of Fabricius (Baxendale & Mebatsion, 2004).

Isolation of HAstVs required trypsin for continuous replication (Lee & Kurtz, 1981). Trypsin may be required to propagate the virus efficiently from infected cells by releasing the virus from infected cells. Another suggested role for trypsin in astrovirus propagation is the cleavage of capsid polyprotein into small parts for the viral assembles (Monroe *et al.*, 1991). In the absence of trypsin, the majority of viral antigen was detectable only inside of infected cells by 48 h PI (Monroe *et al.*, 1991). However, ANV can be cultured in absence of trypsin, which may imply different trypsin cleavage sites on ANV from other astroviruses, possibly suggesting the reason for the different tissue tropism of ANV (Jonassen *et al.*, 2001).

Astrovirus replicated to 20 to 50 times higher viral titers with presence of serum in the media than without serum (Monroe *et al.*, 1991). The authors proposed that the virus replicated efficiently in metabolically active cells, because there might be growth factors and nutrients for cell growth in serum.

**Serology:** Serological methods have been developed to determine astrovirus prevalence in the chicken industry. Field surveys for astrovirus antibodies were conducted using a viral

neutralization test, which revealed that astrovirus antibodies were widespread throughout the poultry field regardless whether RSS was observed or not (Connor *et al.*, 1987; Baxendale & Mebatsion, 2004). However, the availability of virus neutralization (VN) tests varied between cell culture systems; in a study for CkAstV, a VN test was not available due to poor cell culture replication of astroviruses (Todd *et al.*, 2009b). Indirect immunofluorescence has also been employed as a screening method for astrovirus infection in field surveys (Todd *et al.*, 2009b; de Wit *et al.*, 2011). In this method, chicken embryo cells were grown with an astrovirus on glass coverslips. Each serum was incubated with the coverslips followed by species-specific FITC conjugated antibodies. Likewise, antibodies to astroviruses were commonly detected in chicken flocks regardless whether the chicken flock showed a good performance or not.

**Antigenic detection of astroviruses:** Different astroviruses contain specific epitopes in the capsid proteins, which allowed the classification in different serotypes (van Hemert *et al.*, 2007). The serotype specific epitopes are encoded in the subgenomic RNA which encodes for the capsid protein. In addition, other epitopes which are encoded upstream of the subgenomic region (ORF 1a and ORF1b) were described to be specific for viruses of different serotypes (Matsui *et al.*, 1993; Oh & Schreier, 2001).

Antigenic distribution of astroviruses was studied by an immunofluorescence technique on cryostat tissues. Astroviruses were present in the affected tissues; however, a positive reaction was not detected at 10 days after infection (McNulty *et al.*, 1990; Smyth *et al.*, 2007). In addition, antigenic epitopes of astroviruses in close distance may be shared among different virus isolates (Todd *et al.*, 2009a). However, antibodies against each astrovirus isolate showed low cross reactivity with other astroviruses in an immunofluorescence test, indicating each virus

expresses antigenically different epitopes on the capsid structures (Smyth *et al.*, 2009; Todd *et al.*, 2009a).

**RT-PCR:** The detection of viral nucleic acid and the subsequent sequencing are important tools to detect the presence of target virus without virus isolation. However, the application of RT-PCR has been limited to detect astroviruses because of limited availability of sequence information and the high degree of sequence diversity in astroviruses like other RNA viruses. Nevertheless, RT-PCR with degenerate primers has been used to amplify cDNA from highly conserved RNA sequences. Using this approach the sensitivity of RT-PCR has been improved which resulted in the detection of a broad range of astrovirus isolates. Alternatively, highly conserved regions have been used to develop sensitive RT-PCR primers. Examples of this approach have been used for regions within the RdRp (Pantin-Jackwood *et al.*, 2006), a region upstream of the ORF1b and ORF2 junction (Smyth *et al.*, 2009; Reuter *et al.*, 2011), as well as a stem-loop-II-like region (s2m) located upstream of the ORF2 stop codon (Jonassen *et al.*, 2001). However, s2m has been not found in TAstV-2 (Koci *et al.*, 2000).

**Reporter cell line:** On the basis of the replication strategy, a reporter cell line for astroviruses has been described. A reporter construct was composed of an astrovirus genome cDNA copy with a defect in ORF1b, but with a GFP reporter gene in ORF2; therefore, no subgenomic RNAs were expected in the absence of RdRp. Then, this reporter construct was inserted into the genome of Caco-2 cells under the control of a Rous sarcoma virus promoter for constitutive expression. When the cells were infected with a wild type astrovirus, the NS protein was complemented in *trans*, resulting in transcription which enabled the expression of GFP (Matsui *et al.*, 2001). This reporter cell line would provide large scale detection, and a broad

potential application would be feasible including the purification, isolation, and titration of a viral sample as well as a field sample diagnosis.

### **Prevention and Control of Astrovirus infection**

Astroviruses have been regarded as a candidate causal agent for RSS in chickens. Like HAstV infection, RSS occurrence was prevalent in the winter season (Kurtz *et al.*, 1977; Guix *et al.*, 2002; Otto *et al.*, 2006). Astroviruses are very resistant to artificial inactivation under laboratory conditions, suggesting a high stability under environmental conditions in the field (Abad *et al.*, 1997). Even though it was lower than in other generations of flocks, the presence of astroviruses in broiler great-grand parent flocks, which were maintained at higher levels of biosecurity, indicates difficulties in eradicating astroviruses in the environment (Todd *et al.*, 2009b). TAsTV, an avian astrovirus, remained infective to turkey embryos after treatment at pH 3. Heating the virus at 60 °C for 10 min did not inactivate the virus. In addition, the virus was resistant to commercial disinfectants like hypochlorite, phenolic compounds, quaternary ammonia, aldehydes, and non-ionic detergents. However, it can be completely inactivated with peroxymonosulfate (Virkon S), methanol, hydracrylic acid  $\beta$ -lactone ( $\beta$ -propiolactone) (Schultz-Cherry *et al.*, 2001).

To date, efficient protective vaccines are not available for RSS. One experimental vaccine based on a recombinant capsid protein based on a recombinant baculovirus has been described recently (Sellers *et al.*, 2010). Progeny birds from breeders that received repeated vaccination with the recombinant protein showed protection against RSS challenges in comparison to progenies from nonvaccinated breeders. However, the protection was not complete in comparison to controls, implying the involvement of other factors. Specifically, co-

infection with astroviruses and other enteric agents were very common in a field infection, most frequently with rotaviruses, reoviruses, and coronaviruses (Pantin-Jackwood *et al.*, 2008a).

Progeny with ANV specific maternal antibodies were protected against ANV (Takase *et al.*, 1994). However, maternal antibodies appeared to have a very limited effect on progeny immunization against astroviruses. Chickens with high antibody titers from their parent flocks did not prevent enteric infection in the face of astroviruses. Moreover, astrovirus antibody titers were uneven among individual parent flocks. However, limited spreading of astroviruses in internal organs other than the intestine was proposed in the presence of maternal antibodies (Todd *et al.*, 2009b).

## References

- Abad, F. X., Pinto, R. M., Villena, C., Gajardo, R., & Bosch, A. (1997). Astrovirus survival in drinking water. *Appl Environ Microbiol*, *63*, 3119-3122.
- Banos-Lara Mdel, R., & Mendez, E. (2010). Role of individual caspases induced by astrovirus on the processing of its structural protein and its release from the cell through a non-lytic mechanism. *Virology*, *401*, 322-332.
- Baxendale, W., & Mebatsion, T. (2004). The isolation and characterisation of astroviruses from chickens. *Avian Pathology*, *33*, 364-370.
- Brierley, I., Bournsnel, M. E., Binns, M. M., Bilimoria, B., Blok, V. C., Brown, T. D., & Inglis, S. C. (1987). An efficient ribosomal frame-shifting signal in the polymerase-encoding region of the coronavirus IBV. *EMBO J*, *6*, 3779-3785.
- Caballero, S., Guix, S., Ribes, E., Bosch, A., & Pinto, R. M. (2004). Structural requirements of astrovirus virus-like particles assembled in insect cells. *Journal of Virology*, *78*, 13285-13292.
- Connor, T. J., Mcneilly, F., Mcferran, J. B., & McNulty, M. S. (1987). A Survey of Avian Sera from Northern-Ireland for Antibody to Avian Nephritis Virus. *Avian Pathology*, *16*, 15-20.
- de Wit, J. J., Dam, G. B., de Laar, J. M., Biermann, Y., Verstegen, I., Edens, F., & Schrier, C. C. (2011). Detection and characterization of a new astrovirus in chicken and turkeys with enteric and locomotion disorders. *Avian Pathology*, *40*, 453-461.

- Decaesstecker, M., Charlier, G., & Meulemans, G. (1986). Significance of parvoviruses, enterovirus-like viruses and reoviruses in the aetiology of the chicken malabsorption syndrome. *Avian Pathology*, *15*, 769-782.
- Decroly, E., Ferron, F., Lescar, J., & Canard, B. (2011). Conventional and unconventional mechanisms for capping viral mRNA. *Nat Rev Microbiol*, *10*, 51-65.
- Desselberger, U., & Gray, J. (2003). Astroviruses *Viral Gastroenteritis* (pp. 567-571): Elsevier Science.
- Donelli, G., Superti, F., Tinari, A., & Marziano, M. L. (1992). Mechanism of Astrovirus Entry into Graham-293 Cells. *Journal of Medical Virology*, *38*, 271-277.
- Dong, J., Dong, L., Mendez, E., & Tao, Y. (2011). Crystal structure of the human astrovirus capsid spike. *Proc Natl Acad Sci U S A*, *108*, 12681-12686.
- Eidson, C. S., Kleven, S. H., & Fletcher, O. J. (1985). Performance of broiler progeny of breeder flocks vaccinated with inactivated oil emulsion malabsorption syndrome virus vaccine. *Poultry Science*, *64*, 2081-2086.
- Farabaugh, P. J. (1996). Programmed translational frameshifting. *Microbiol Rev*, *60*, 103-134.
- Feron, V. J., & Mullink, J. W. (1971). Mucosal cysts in the gastrointestinal tract of beagle dogs. *Lab Anim*, *5*, 193-201.
- Finkbeiner, S. R., Kirkwood, C. D., & Wang, D. (2008). Complete genome sequence of a highly divergent astrovirus isolated from a child with acute diarrhea. *Virology*, *375*, 117.
- Geigenmuller, U., Ginzton, N. H., & Matsui, S. M. (1997). Construction of a genome-length cDNA clone for human astrovirus serotype 1 and synthesis of infectious RNA transcripts. *Journal of Virology*, *71*, 1713-1717.
- Geigenmuller, U., Chew, T., Ginzton, N., & Matsui, S. M. (2002a). Processing of nonstructural protein 1a of human astrovirus. *Journal of Virology*, *76*, 2003-2008.
- Geigenmuller, U., Ginzton, N. H., & Matsui, S. M. (2002b). Studies on intracellular processing of the capsid protein of human astrovirus serotype 1 in infected cells. *Journal of General Virology*, *83*, 1691-1695.
- Geigenmüller, U., Mendez, E., & Matsui, S. M. (2003). V, 1. Studies on the molecular biology of human astrovirus. In U. Desselberger & J. Gray (Eds.), *Viral Gastroenteritis* (pp. 573-586): Elsevier Science.
- Glass, R. I., Noel, J., Mitchell, D., Herrmann, J. E., Blacklow, N. R., Pickering, L. K., Dennehy, P., Ruiz-Palacios, G., de Guerrero, M. L., & Monroe, S. S. (1996). The changing epidemiology of astrovirus-associated gastroenteritis: a review. *Arch Virol Suppl*, *12*, 287-300.

- Goodwin, M. A., Dekich, M. A., Latimer, K. S., & Fletcher, O. J. (1985). Quantitation of intestinal D-xylose absorption in normal broilers and in broilers with pale-bird syndrome. *Avian Diseases*, *29*, 630-639.
- Goodwin, M. A., Davis, J. F., McNulty, M. S., Brown, J., & Player, E. C. (1993). Enteritis (so-called runting stunting syndrome) in Georgia broiler chicks. *Avian Diseases*, *37*, 451-458.
- Gough, R. E., Collins, M. S., Borland, E., & Keymer, L. F. (1984). Astrovirus-like particles associated with hepatitis in ducklings. *Vet Rec*, *114*, 279.
- Guix, S., Caballero, S., Villena, C., Bartolome, R., Latorre, C., Rabella, N., Simo, M., Bosch, A., & Pinto, R. M. (2002). Molecular epidemiology of astrovirus infection in Barcelona, Spain. *J Clin Microbiol*, *40*, 133-139.
- Guix, S., Caballero, S., Bosch, A., & Pinto, R. M. (2004). C-terminal nsP1a protein of human astrovirus colocalizes with the endoplasmic reticulum and viral RNA. *Journal of Virology*, *78*, 13627-13636.
- Guix, S., Caballero, S., Bosch, A., & Pinto, R. M. (2005). Human astrovirus C-terminal nsP1a protein is involved in RNA replication. *Virology*, *333*, 124-131.
- Guo, L., Gonzalez, R., Wang, W., Li, Y., Paranhos-Baccala, G., Vernet, G., & Wang, J. (2010). Complete genome sequence of human astrovirus genotype 6. *Virol J*, *7*, 29.
- Imada, T., Yamaguchi, S., Mase, M., Tsukamoto, K., Kubo, M., & Morooka, A. (2000). Avian nephritis virus (ANV) as a new member of the family Astroviridae and construction of infectious ANV cDNA. *Journal of Virology*, *74*, 8487-8493.
- Jacks, T., & Varmus, H. E. (1985). Expression of the Rous sarcoma virus pol gene by ribosomal frameshifting. *Science*, *230*, 1237-1242.
- Jiang, B., Monroe, S. S., Koonin, E. V., Stine, S. E., & Glass, R. I. (1993). RNA sequence of astrovirus: distinctive genomic organization and a putative retrovirus-like ribosomal frameshifting signal that directs the viral replicase synthesis. *Proceedings of the National Academy of Sciences USA*, *90*, 10539-10543.
- Jonassen, C. M., Jonassen, T. O., Saif, Y. M., Snodgrass, D. R., Ushijima, H., Shimizu, M., & Grinde, B. (2001). Comparison of capsid sequences from human and animal astroviruses. *Journal of General Virology*, *82*, 1061-1067.
- Jonassen, C. M., Jonassen, T. T., Sveen, T. M., & Grinde, B. (2003). Complete genomic sequences of astroviruses from sheep and turkey: comparison with related viruses. *Virus Res*, *91*, 195-201.
- Jonassen, T. O., Kjeldsberg, E., & Grinde, B. (1993). Detection of human astrovirus serotype 1 by the polymerase chain reaction. *J Virol Methods*, *44*, 83-88.

- Kadare, G., & Haenni, A. L. (1997). Virus-encoded RNA helicases. *Journal of Virology*, *71*, 2583-2590.
- Kiang, D., & Matsui, S. M. (2002). Proteolytic processing of a human astrovirus nonstructural protein. *Journal of General Virology*, *83*, 25-34.
- Kim, T., Kang, K. I., Linneman, E., Sellers, H. S., & Mundt, E. (2011). Characterization of a novel chicken astrovirus isolated from intestinal homogenates of RSS-affected chickens. *Proceedings of the International Poultry Scientific Forum* (pp. 241). Atlanta, GA.
- Kim, T., & Mundt, E. (2011). Metagenomic analysis of intestinal microbiomes in chickens. *Methods Mol Biol*, *733*, 185-194.
- Kisary, J., Nagy, B., & Bitay, Z. (1984). Presence of parvoviruses in the intestine of chickens showing stunting syndrome. *Avian Pathology*, *13*, 339-343.
- Koci, M. D., Seal, B. S., & Schultz-Cherry, S. (2000). Molecular characterization of an avian astrovirus. *Journal of Virology*, *74*, 6173-6177.
- Koci, M. D., & Schultz-Cherry, S. (2002). Avian astroviruses. *Avian Pathology*, *31*, 213-227.
- Koci, M. D., Moser, L. A., Kelley, L. A., Larsen, D., Brown, C. C., & Schultz-Cherry, S. (2003). Astrovirus induces diarrhea in the absence of inflammation and cell death. *Journal of Virology*, *77*, 11798-11808.
- Kouwenhoven, B., Davelaar, F. G., & Van Walsum, J. (1978). Infectious proventriculitis causing runting in broilers. *Avian Pathology*, *7*, 183-187.
- Kouwenhoven, B., Vertommen, M., & Goren, E. (1988). Investigations into the role of reovirus in the malabsorption syndrome. *Avian Pathology*, *17*, 879-892.
- Krishna, N. K. (2005). Identification of structural domains involved in astrovirus capsid biology. *Viral Immunol*, *18*, 17-26.
- Kurtz, J. B., Lee, T. W., & Pickering, D. (1977). Astrovirus associated gastroenteritis in a children's ward. *J Clin Pathol*, *30*, 948-952.
- Lee, T. W., & Kurtz, J. B. (1981). Serial propagation of astrovirus in tissue culture with the aid of trypsin. *Journal of General Virology*, *57*, 421-424.
- Lewis, T. L., Greenberg, H. B., Herrmann, J. E., Smith, L. S., & Matsui, S. M. (1994). Analysis of astrovirus serotype 1 RNA, identification of the viral RNA-dependent RNA polymerase motif, and expression of a viral structural protein. *Journal of Virology*, *68*, 77-83.
- Lewis, T. L., & Matsui, S. M. (1996). Astrovirus ribosomal frameshifting in an infection-transfection transient expression system. *Journal of Virology*, *70*, 2869-2875.

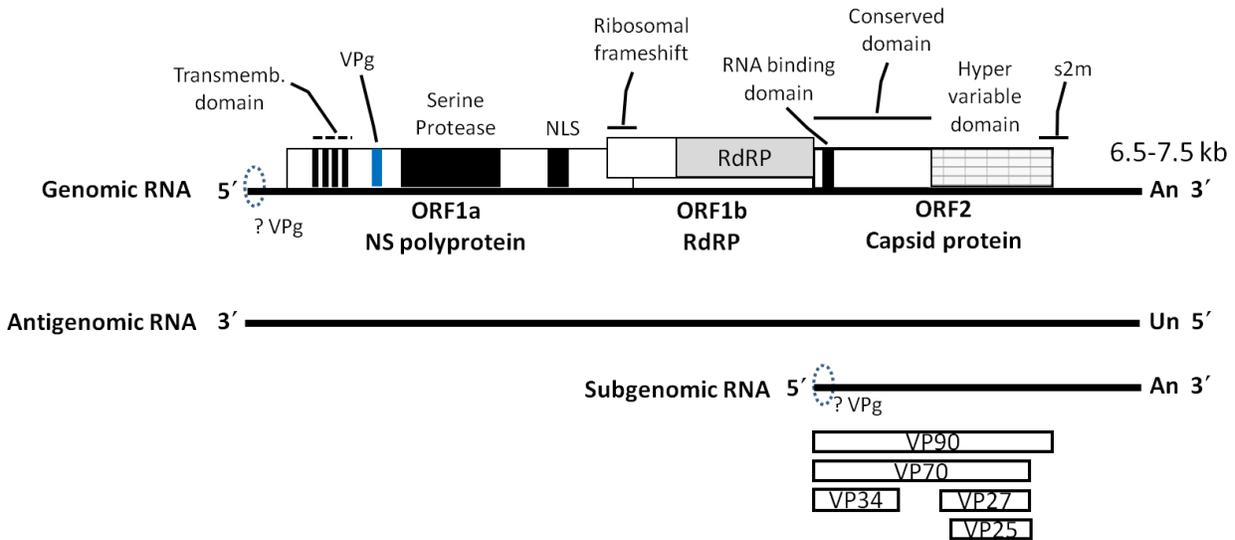
- Madeley, C. R., & Cosgrove, B. P. (1975). Letter: 28 nm particles in faeces in infantile gastroenteritis. *Lancet*, *2*, 451-452.
- Matsui, S. M., Kim, J. P., Greenberg, H. B., Young, L. M., Smith, L. S., Lewis, T. L., Herrmann, J. E., Blacklow, N. R., Dupuis, K., & Reyes, G. R. (1993). Cloning and characterization of human astrovirus immunoreactive epitopes. *Journal of Virology*, *67*, 1712-1715.
- Matsui, S. M., Kiang, D., Ginzton, N., Chew, T., & Geigenmüller-Gnirke, U. (2001). Molecular biology of astroviruses: selected highlights. In D. Chadwick & J. A. Goode (Eds.), *Gastroenteritis* (pp. 219-235). England: Novartis Foundation.
- McNulty, M. S., Connor, T. J., McNeilly, F., & McFerran, J. B. (1990). Biological characterisation of avian enteroviruses and enterovirus-like viruses. *Avian Pathology*, *19*, 75-87.
- Mendez, E., Fernandez-Luna, T., Lopez, S., Mendez-Toss, M., & Arias, C. F. (2002). Proteolytic processing of a serotype 8 human astrovirus ORF2 polyprotein. *Journal of Virology*, *76*, 7996-8002.
- Mendez, E., Salas-Ocampo, E., & Arias, C. F. (2004). Caspases mediate processing of the capsid precursor and cell release of human astroviruses. *Journal of Virology*, *78*, 8601-8608.
- Mendez, E., & Arias, C. F. (2007). Astroviruses. In D. M. Knipe & P. M. Howley (Eds.), *Fields Virology* (pp. 981-1000). Philadelphia: Lippincott Williams & Wilkins.
- Monroe, S. S., Stine, S. E., Gorelkin, L., Herrmann, J. E., Blacklow, N. R., & Glass, R. I. (1991). Temporal synthesis of proteins and RNAs during human astrovirus infection of cultured cells. *Journal of Virology*, *65*, 641-648.
- Monroe, S. S., Jiang, B., Stine, S. E., Koopmans, M., & Glass, R. I. (1993). Subgenomic RNA sequence of human astrovirus supports classification of Astroviridae as a new family of RNA viruses. *Journal of Virology*, *67*, 3611-3614.
- Montgomery, R. D., Boyle, C. R., Maslin, W. R., & Magee, D. L. (1997). Attempts to reproduce a runt/stunting-type syndrome using infectious agents isolated from affected Mississippi broilers. *Avian Diseases*, *41*, 80-92.
- Moser, L. A., Carter, M., & Schultz-Cherry, S. (2007). Astrovirus increases epithelial barrier permeability independently of viral replication. *Journal of Virology*, *81*, 11937-11945.
- Nighot, P. K., Moeser, A., Ali, R. A., Blikslager, A. T., & Koci, M. D. (2010). Astrovirus infection induces sodium malabsorption and redistributes sodium hydrogen exchanger expression. *Virology*, *401*, 146-154.
- Nili, H., Jahantigh, M., & Nazifi, S. (2007). Clinical observation, pathology, and serum biochemical changes in infectious stunting syndrome of broiler chickens. *Comparative Clinical Pathology*, *16*, 161-166.

- Oh, D., & Schreier, E. (2001). Molecular characterization of human astroviruses in Germany. *Archives of Virology*, *146*, 443-455.
- Olsen, D. E. (1977). Isolation of a reovirus-like agent from broiler chicks with diarrhea and stunting. Proceedings of *the 26th western poultry diseases conference* (pp. 131–139). Davis, California.
- Otto, P., Liebler-Tenorio, E. M., Elschner, M., Reetz, J., Lohren, U., & Diller, R. (2006). Detection of rotaviruses and intestinal lesions in broiler chicks from flocks with runting and stunting syndrome (RSS). *Avian Diseases*, *50*, 411-418.
- Pantin-Jackwood, M. J., Spackman, E., & Woolcock, P. R. (2006). Molecular characterization and typing of chicken and turkey astroviruses circulating in the United States: implications for diagnostics. *Avian Diseases*, *50*, 397-404.
- Pantin-Jackwood, M. J., Day, J. M., Jackwood, M. W., & 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.
- Pantin-Jackwood, M. J., Spackman, E., & Day, J. M. (2008b). Pathogenesis of type 2 turkey astroviruses with variant capsid genes in 2-day-old specific pathogen free poult. *Avian Pathology*, *37*, 193-201.
- Pantin-Jackwood, M. J., Strother, K. O., Mundt, E., Zsak, L., Day, J. M., & Spackman, E. (2011). Molecular characterization of avian astroviruses. *Archives of Virology*, *156*, 235-244.
- Parashar, U. D., & Glass, R. (2003). I, 1. Viral causes of gastroenteritis. In U. Desselberger & J. Gray (Eds.), *Viral Gastroenteritis* (pp. 9-21): Elsevier Science.
- Perry, R. W., Rowland, G. N., & Glisson, J. R. (1991). Poult malabsorption syndrome. I. Malabsorption in poult enteritis. *Avian Diseases*, *35*, 685-693.
- Rebel, J. M. J., Balk, F. R. M., Post, J., Van Hemert, S., Zekarias, B., & Stockhofe, N. (2006). Malabsorption syndrome in broilers. *World's Poultry Science Journal*, *62*, 17-30.
- Reuter, G., Pankovics, P., & Boros, A. (2011). Identification of a novel astrovirus in a domestic pig in Hungary. *Archives of Virology*, *156*, 125-128.
- Risco, C., Carrascosa, J. L., Pedregosa, A. M., Humphrey, C. D., & Sanchez-Fauquier, A. (1995). Ultrastructure of human astrovirus serotype 2. *Journal of General Virology*, *76* (Pt 8), 2075-2080.
- Schultz-Cherry, S., King, D. J., & Koci, M. D. (2001). Inactivation of an astrovirus associated with poult enteritis mortality syndrome. *Avian Diseases*, *45*, 76-82.

- Sebire, N. J., Malone, M., Shah, N., Anderson, G., Gaspar, H. B., & Cubitt, W. D. (2004). Pathology of astrovirus associated diarrhoea in a paediatric bone marrow transplant recipient. *J Clin Pathol*, *57*, 1001-1003.
- Sellers, H., Linneman, E., Icard, A. H., & Mundt, E. (2010). A purified recombinant baculovirus expressed capsid protein of a new astrovirus provides partial protection to runting-stunting syndrome in chickens. *Vaccine*, *28*, 1253-1263.
- Shirai, J., Obata, H., Nakamura, K., Furuta, K., Hihara, H., & Kawamura, H. (1990). Experimental infection in specific-pathogen-free chicks with avian reovirus and avian nephritis virus isolated from broiler chicks showing runting syndrome. *Avian Diseases*, *34*, 295-303.
- Shirai, J., Nakamura, K., Shinohara, K., & Kawamura, H. (1991). Pathogenicity and antigenicity of avian nephritis isolates. *Avian Diseases*, *35*, 49-54.
- Shirai, J., Tanimura, N., Uramoto, K., Narita, M., Nakamura, K., & Kawamura, H. (1992). Pathologically and serologically different avian nephritis virus isolates implicated in etiology of baby chick nephropathy. *Avian Diseases*, *36*, 369-377.
- Smart, I. J., Barr, D. A., Reece, R. L., Forsyth, W. M., & Ewing, I. (1988). Experimental reproduction of the runting-stunting syndrome of broiler chickens. *Avian Pathology*, *17*, 617-627.
- Smyth, J. A., Connor, T. J., McNeilly, F., Moffet, D. A., Calvert, V. M., & McNulty, M. S. (2007). Studies on the pathogenicity of enterovirus-like viruses in chickens. *Avian Pathology*, *36*, 119-126.
- Smyth, V. J., Jewhurst, H. L., Adair, B. M., & Todd, D. (2009). Detection of chicken astrovirus by reverse transcriptase-polymerase chain reaction. *Avian Pathology*, *38*, 293-299.
- Snodgrass, D. R., & Gray, E. W. (1977). Detection and transmission of 30 nm virus particles (astroviruses) in faeces of lambs with diarrhoea. *Archives of Virology*, *55*, 287-291.
- Songserm, T., Pol, J. M., van Roozelaar, D., Kok, G. L., Wagenaar, F., & ter Huurne, A. A. (2000). A comparative study of the pathogenesis of malabsorption syndrome in broilers. *Avian Diseases*, *44*, 556-567.
- Songserm, T., Engel, B., van Roozelaar, D. J., Kok, G. L., Pijpers, A., Pol, J. M., & ter Huurne, A. A. (2002a). Cellular immune response in the small intestine of two broiler chicken lines orally inoculated with malabsorption syndrome homogenates. *Veterinary Immunology and Immunopathology*, *85*, 51-62.
- Songserm, T., Zekarias, B., van Roozelaar, D. J., Kok, R. S., Pol, J. M., Pijpers, A. A., & ter Huurne, A. A. (2002b). Experimental reproduction of malabsorption syndrome with different combinations of reovirus, Escherichia coli, and treated homogenates obtained from broilers. *Avian Diseases*, *46*, 87-94.

- Strain, E., Kelley, L. A., Schultz-Cherry, S., Muse, S. V., & Koci, M. D. (2008). Genomic analysis of closely related astroviruses. *Journal of Virology*, *82*, 5099-5103.
- Takase, K., Uchimura, T., Yamamoto, M., & Yamada, S. (1994). Susceptibility of embryos and chicks, derived from immunized breeding hens, to avian nephritis virus. *Avian Pathology*, *23*, 117-125.
- Tang, Y., Murgia, A. M., & Saif, Y. M. (2005). Molecular characterization of the capsid gene of two serotypes of turkey astroviruses. *Avian Diseases*, *49*, 514-519.
- Todd, D., Smyth, V. J., Ball, N. W., Donnelly, B. M., Wylie, M., Knowles, N. J., & Adair, B. M. (2009a). Identification of chicken enterovirus-like viruses, duck hepatitis virus type 2 and duck hepatitis virus type 3 as astroviruses. *Avian Pathology*, *38*, 21-30.
- Todd, D., Wilkinson, D. S., Jewhurst, H. L., Wylie, M., Gordon, A. W., & Adair, B. M. (2009b). A seroprevalence investigation of chicken astrovirus infections. *Avian Pathology*, *38*, 301-309.
- van Hemert, F. J., Lukashov, V. V., & Berkhout, B. (2007). Different rates of (non-)synonymous mutations in astrovirus genes; correlation with gene function. *Virology*, *4*, 25.
- Vertommen, M., van Eck, J. H., Kouwenhoven, B., & Van Kol, N. (1980). Infectious stunting and leg weakness in broilers: I. Pathology and biochemical changes in blood plasma. *Avian Pathology*, *9*, 133-142.
- Wang, Q. H., Kakizawa, J., Wen, L. Y., Shimizu, M., Nishio, O., Fang, Z. Y., & Ushijima, H. (2001). Genetic analysis of the capsid region of astroviruses. *Journal of Medical Virology*, *64*, 245-255.
- Willard, M. D., Helman, G., Fradkin, J. M., Becker, T., Brown, R. M., Lewis, B. C., & Weeks, B. R. (2000). Intestinal crypt lesions associated with protein-losing enteropathy in the dog. *J Vet Intern Med*, *14*, 298-307.
- Willcocks, M. M., Ashton, N., Kurtz, J. B., Cubitt, W. D., & Carter, M. J. (1994). Cell culture adaptation of astrovirus involves a deletion. *Journal of Virology*, *68*, 6057-6058.
- Willcocks, M. M., Boxall, A. S., & Carter, M. J. (1999). Processing and intracellular location of human astrovirus non-structural proteins. *Journal of General Virology*, *80* ( Pt 10), 2607-2611.
- Yamaguchi, S., Imada, T., & Kawamura, H. (1979). Characterization of a picornavirus isolated from broiler chicks. *Avian Diseases*, *23*, 571-581.
- Zekarias, B., Songserm, T., Post, J., Kok, G. L., Pol, J. M., Engel, B., & ter, H. A. (2002). Development of organs and intestinal mucosa leukocytes in four broiler lines that differ in susceptibility to malabsorption syndrome. *Poultry Science*, *81*, 1283-1288.

Zekarias, B., Stockhofe-Zurwieden, N., Post, J., Balk, F., van Reenen, C., Gruys, E., & Rebel, J. M. (2005). The pathogenesis of and susceptibility to malabsorption syndrome in broilers is associated with heterophil influx into the intestinal mucosa and epithelial apoptosis. *Avian Pathology*, 34, 402-407.



**Figure 1.1.** Schematic depiction of astrovirus genomic organization. Specific regions of RNA motifs and representative encoding proteins were shown in the genomic RNA. The predicted transmembrane domains, VPg domain, serine protease encoding region, predicted nuclear localization signal (NLS), ribosomal frameshift structure, and the RNA dependent RNA polymerase (RdRp) were indicated in ORF1a and ORF1b encoding region. The predicted RNA binding domain composed of conserved basic amino acids, a highly conserved N-terminal domain, a hyper variable domain, and a stem-loop II like molecule (s2m) are located within ORF2. Representative ORF2 delineated protein products are described in the open boxes below the subgenomic RNA: full length ORF2 protein (VP90), intermediate protein (VP70), as well as the end products of protein processing (VP34, VP27, VP25). Note that a VPg-like domain in the virus genome has been detected, but the presence of VPg at the 5' end of genomic and subgenomic RNAs was not proven. Modified from Mendez *et al.* (2002), Geigenmüller *et al.* (2003), and Krishna (2005).

CHAPTER 2  
INVESTIGATION INTO THE ETIOLOGY OF RUNTING AND STUNTING SYNDROME IN  
CHICKENS<sup>1</sup>

---

<sup>1</sup> Kyung-Il Kang, Mohamed El-Gazzar, Holly S. Sellers, Fernanda Dorea, Susan M. Williams, Taejoong Kim, Stephen Collett, Egbert Mundt. 2012. *Avian Pathology*. 41:41-50.  
Reprinted here with permission of the publisher.

## Summary

Currently, the etiology of runting and stunting syndrome (RSS) in chickens is unknown. The impact of RSS on weight gain and microscopic lesions in immunological organs and the duodenum was investigated in 1-day-old commercial broilers at 12 days following exposure to RSS-contaminated litter. Furthermore, the presence of the viral nucleic acids of three astroviruses and one parvovirus was analyzed by *in situ* hybridization from days 1 through 5 post exposure. A 70% decrease in weight was observed in the RSS exposed group at the end of the experiments when compared with the unexposed controls. Lesions in the bursa of Fabricius and thymus were present in both groups but were significantly higher at the end of the study in the RSS-exposed group. In contrast, no significant difference in Harderian gland lesions was observed between the groups. Histological lesions in the duodenum were already present 24 h after exposure in the RSS-exposed group only, peaked at day 4 and declined until the end of the study. Results of the *in situ* hybridization studies clearly indicate replication of three astroviruses (chicken astrovirus, avian nephritis virus [ANV]-1, ANV-2) in the duodenum but not in other organs evaluated. Chicken astrovirus nucleic acids were detected on days 1 and 2 post exposure, while ANV-1 and ANV-2 nucleic acids were observed on several days during the period investigated. Surprisingly, no viral nucleic acid specific for the chicken parvovirus was observed. The results indicate that astroviruses probably play an important role during RSS due to the concurrence of viral RNA detection and lesions in the duodenum.

## Introduction

Runting and stunting syndrome (RSS) in chickens is a transmissible disease of uncertain etiology. RSS affects chickens early in life and is characterized by growth retardation, ruffled

feathers, and diarrhea, resulting in considerable economic losses especially in the commercial broiler industry. RSS was initially reported in the broiler industry during the 1970s and has since been described in various parts of the world (Olsen, 1977; Kouwenhoven *et al.*, 1978). Due to the absence of a known etiology, identification of the disease commonly relies on descriptive terms such as malabsorption syndrome, infectious stunting syndrome, broiler runting syndrome, and helicopter syndrome (Rebel *et al.*, 2006). Currently there is no effective commercial vaccine available for control of the disease, due primarily to the absence of known etiologic agents. One experimental vaccine, based on a recombinant baculovirus encoding for a new astrovirus capsid protein, has recently been described (Sellers *et al.*, 2010). Nonetheless, clinical signs and microscopic lesions of RSS have been reproduced experimentally using oral inoculation of filtered and non-filtered intestinal homogenate from affected chickens (Montgomery *et al.*, 1997; Songserm *et al.*, 2000; Songserm *et al.*, 2002a).

Histopathological changes in birds affected by RSS are primarily associated with lesions in the small intestine. A hallmark lesion observed in clinical and experimental cases of RSS is cystic enteropathy (Otto *et al.*, 2006; Sellers *et al.*, 2010). The cells lining the crypt degenerate and detach into the lumen, forming cystic crypts lined by flattened epithelial cells. The severity of clinical signs may depend on the extent of villous atrophy resulting from epithelial loss and decreased turnover. Nonetheless, the actual cause and pathogenesis of the intestinal lesions remains unclear; however, increased apoptosis along the lining of villi and crypt epithelial cells has been reported (Zekarias *et al.*, 2005). Approximately 2 to 3 weeks after experimental infection, recovery of the mucosal lining has been observed (Zekarias *et al.*, 2005).

Although bacteria and environmental factors may be associated as contributors to the development of disease (Otto *et al.*, 2006; Rebel *et al.*, 2006), small, round, non-enveloped

viruses have been suggested as the most probable etiologic agents based on electron microscopy and the ability to reproduce the disease using a chloroform-treated, bacteria-free filtrate (Sellers *et al.*, 2010). Over the years, reovirus (Songserm *et al.*, 2000), rotavirus (Otto *et al.*, 2006), parvovirus (Kisary *et al.*, 1984) and astrovirus (Baxendale & Mebatsion, 2004) have been detected or isolated from clinically affected chickens. Kisary *et al.* (1984) described the presence of parvovirus in guts of RSS-affected chickens. The parvovirus, called also ABU isolate, was purified by ultracentrifugation and was able to induce growth retardation in chickens (Kisary, 1985). On the contrary, broiler chickens experimentally infected with either a reovirus or the parvovirus ABU isolate did not show clinical signs specific for RSS (Decaesstecker, et al, 1986). The importance of parvovirus infection in turkeys and chickens for the occurrence of enteric diseases in both species was supported by recently published investigations in poultry flocks in Hungary (Pallade et al, 2011) but is still not fully understood, probably due to the lack of a pure virus isolate. On the other hand, infection with an enterovirus-like virus resulted in enteric disease (Decaesstecker *et al.*, 1986). The group of enterovirus-like viruses (ELV) is not well defined. In fact, prior to the year 2000, astroviruses were placed taxonomically with the ELV group (Imada *et al.*, 2000). Astroviruses are associated with gastrointestinal disease in many species of mammals and birds (reviewed in Koci & Schultz, 2002). In chickens with enteric disease, chicken astrovirus (Baxendale & Mebatsion, 2004) and avian nephritis virus (ANV) (Imada *et al.*, 2000) have been identified. Recently, the genome of a parvovirus from particle-associated nucleic acid was described and suggested as a possible poultry enteric disease agent (Zsak *et al.*, 2008; Day & Zsak, 2010). Using other techniques, different viruses have been detected in the intestines of affected birds. Reovirus antigen has been detected in the epithelial cells of the tip and middle regions of the intestinal villi in RSS-affected chickens (Songserm *et*

*al.*, 2000; Songserm *et al.*, 2002b). Similarly, in an *in situ* hybridization (ISH) study using astrovirus infected turkeys, astrovirus RNA was only detected in the villous epithelial cells in the intestine (Koci *et al.*, 2003). Furthermore, Pantin-Jackwood *et al.* (2008b) observed cells positive for a turkey astrovirus in the intestinal crypts of infected turkeys using ISH and immunohistochemistry methods.

The role of these viruses in RSS-affected chickens is still poorly understood. Moreover, the viruses may cause RSS in combination with other viruses or unknown factors. The target cell predilection as well as the pathogenicity of these viruses can also differ. To further complicate identification of the disease etiology, a steady increase in carcass condemnations for septicaemia-toxaemia was observed after the onset of the clinical RSS outbreak in late 2004 (Smith, 2008). While this suggests an association of RSS with immune suppression, it is not entirely clear which comes first. However, due to the extremely early onset of signs, it is possible that the agent(s) causing clinical RSS are themselves directly and profoundly immunosuppressive (Sklan, 2001). Due to the complexity of RSS and the possibility of multiple etiologies, developing treatment and control strategies are hindered. In previous studies, a novel chicken astrovirus was identified in RSS-affected chickens (Sellers *et al.*, 2010). The role of this astrovirus and other small round viruses may hold an important key to identification of the etiology/etiologies and improve our understanding of the pathogenesis of RSS. The aim of the present study was to evaluate the development of microscopic changes over time in the small intestine, the bursa of Fabricius, thymus, and Harderian gland in commercial broilers challenged with RSS in a previously described challenge model (Sellers *et al.*, 2010). Using riboprobes designed to hybridize to regions of ANV-1, ANV-2, chicken parvovirus and a novel chicken

astrovirus, tissues were examined for the presence of replicating virus using an *in situ* hybridization assay.

## **Material and Methods**

**Chickens.** Three hundred 1-day-old commercial broiler chicks obtained from a commercial flock were randomly separated into two experimental groups consisting of 150 birds each. The number of chickens was necessary to obtain a chicken density comparable to commercial production conditions. The birds came from the same company and had the same genetic background as described before (Sellers et al., 2010). Each group of chicks was placed into a separate 10 m<sup>2</sup> isolation house. Water and feed were provided *ad libitum*. One group was placed on fresh pine shavings which served as bedding material. For the RSS-challenged group, litter material obtained from the same local commercial broiler farm with a history of clinical RSS was used as previously described (Sellers *et al.*, 2010). Starting at 24 h following placement, five birds were randomly collected daily as a representative sample from each group. Birds were numbered (from 1 to 5) and weighed. After euthanizing the birds with CO<sub>2</sub>, the duodenal loop, thymus, bursa of Fabricius (BF), and Harderian glands were collected from each bird and placed separately, by individual bird, in 10% neutral buffered formalin. The container was labeled with the bird number, so that the collected tissues could be linked with the bird weight. The same procedure was repeated at 11 days post placement. At 12 days post placement, the experiment was terminated. At this time, all chickens were euthanized and 30 out of the remaining 95 chickens were weighed and samples collected.

**Treatment of the tissue samples.** A cross section of the duodenal loop, just above the tip of the pancreas including the ascending and descending sections of the loop, a section of the BF,

the thymic lobes and the Harderian gland were placed in 10% buffered formalin for 24 hours. The fixed tissues were embedded in paraffin blocks and labeled with the group identification number, age, and bird number. The paraffin-embedded blocks were consecutively cut into 4 µm thick sections for subsequent experiments. Sections placed on regular glass slides were stained with hematoxylin and eosin (H&E) for light microscopic examination. Sections for *in situ* hybridization were placed on Superfrost/Plus microscopic slides (Fisher Scientific).

**Evaluation of the microscopic lesions.** For the microscopic evaluation, the presence of cystic formation in the crypts of Lieberkühn (further designated as cystic lesions) in both parts of the duodenal loop was evaluated and the number of cystic lesions per bird was counted. For the primary immune organs (bursa of Fabricius and thymus) and the secondary immune organ (Harderian gland), the evaluation was performed by microscopic assessment of the lymphocytic population. Since it is difficult to evaluate lymphoid tissues in young birds less than 2 weeks of age due to the high dynamic of the development of the tissues, a subjective scoring system was based on comparing lymphocyte populations in the challenged group versus the control group on a daily basis. As the goal of the study is to gauge differences between the two groups, individuals with the largest lymphocyte population within the control group were considered the reference for the scoring system. The subjective microscopic evaluation of the tissues was expressed on a scale of 0 (normal lymphocyte population) to 3 (severely affected lymphocyte population). For a score of 0, there were no differences in the lymphocyte population versus the selected reference tissue. As reference tissue a sample of a control bird was chosen which showed the highest density of the lymphocyte population, thus was automatically scored as 0. For a score of 1, there was a subjectively mild difference (25%) in the lymphocyte population versus the selected reference tissue(s). For a score of 2, there was a subjectively moderate difference (50%) in the

lymphocyte population versus the selected reference tissue(s). For a score of 3, there was a subjectively severe difference (75%) in the lymphocyte population versus the selected reference tissue. It needs to be kept in mind that even with a high caution during the scoring it is highly subjective.

**Data analysis.** Weight measures, counts of cystic lesions in the cross section of duodenum and microscopic scoring of immune organs were entered into an Excel spreadsheet (Microsoft® Office Excel® 2007) and averages and standard deviations were calculated. The determination of significant differences between groups was calculated using the one-way analysis of variance (ANOVA) of summary data employing a free calculation tool available online (<http://danielsoper.com/>).

**Generation of riboprobes.** Since astroviruses (Koci & Schultz-Cherry, 2002; Baxendale & Mebatsion, 2004) and a parvovirus (Kisary *et al.*, 1984; Zsak *et al.*, 2008; Day & Zsak, 2010) have been identified as potentially playing a role in the etiology of RSS, sequences of these viruses were amplified by reverse transcription-polymerase chain reaction (RT-PCR) for astrovirus and PCR for parvovirus. The initial material used for the preparation of plasmids for the transcription of the riboprobes was gut material obtained from chickens exposed to the RSS-contaminated litter which have been taken at day 12. The gut material was homogenized with the FastPrep 24 by Bio101 (MP Biochemiclas, Solon, OH). The resulting homogenate was centrifuged at 13000 x g at 4C for 20 min. The supernatant was taken for either RNA purification using the High-Pure-RNA-Isolation-Kit (Roche, Applied-Science, Indianapolis, IN) or DNA purification using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). The extracted nucleic acids were used in the experiments described below. Oligonucleotides (see table 2.1) were designed based on sequences available in the NCBI database for ANV-1

(Genbank accession number AB033998), ANV-2 (AB046864), chickens astrovirus (JF414802) and chicken parvovirus (GU214704). The probes for ANV-1 and ANV-2 were located in the coding sequence of the capsid protein. The probe for the chicken parvovirus was located in the viral VP2 sequence, while the probe for the chicken astrovirus was located the ORF1a region of the virus. Appropriate restriction enzyme cleavage sites were introduced for linearization prior to the DNA dependent RNA polymerase reaction using either T7 or T3 phage polymerase. After RT-PCR or PCR, the appropriate cDNA fragments were separated on a 1% gel and gel purified using QIAquick Gel Extraction Kit (Qiagen, Maryland, USA). The purified PCR products were incubated with the appropriate restriction enzymes, gel purified again and ligated into the appropriately cleaved pBluescript® II Phagemid vector (Stratagene, La Jolla, CA, USA). Plasmids containing the expected inserts were selected by restriction enzyme analysis and those plasmids containing an insert were sequenced as described above. A plasmid containing the target sequence was transformed into Top10 F cells (Invitrogen) and plasmid DNA was prepared using the GeneJET™ Plasmid Miniprep Kit (Fermentas, Glen Burnie, MD, USA). The resulting purified plasmids were cleaved with the restriction enzyme EcoRI (ANV-1, ANV-2), or Xba I (chicken astrovirus), purified and subsequently transcribed *in vitro* using phage polymerase T3 (Applied Biosystems/Ambion, Austin TX, USA) to generate an antisense RNA of approximately 500 nucleotides in length. For sense probes, cDNA constructs for ANV-1, ANV-2, and the new chicken astrovirus were linearized with Sac I followed by *in vitro* transcription using T7 RNA-polymerase (Takara, Madison, WI, USA). For the parvovirus-specific sense probes one recombinant plasmid for each probe was generated due to the incompatibility of the T7 polymerase for the subsequent transcription reaction. The parvovirus-specific cDNA was amplified with the appropriate primer pair (ChPVpr-FP, ChPVpr-RP, see table 2.1). The

obtained PCR fragment was cleaved either with Hind III/SacII (sense probe) or Eco RI/Sac I (antisense probe) and ligated into the appropriately cleaved pBluescript® II Phagemid vector (Stratagene). The plasmids obtained were cleaved with either Hind III (sense probe) or Eco RI (antisense probe), purified and used for the T3 RNA-polymerase reaction. For the polymerase reaction, the DIG RNA Labeling Mix (Roche, Basel, Switzerland) was used in accordance with the manufacturer's instructions. Following the polymerase reaction, the plasmid DNA was degraded by adding 10 units of RNase free DNase I (Roche) and subsequently incubating for 60 min at 37°C. The reaction was stopped by the addition of 2 µl of 0.2 M EDTA (pH 8.0). The reaction products were purified using SigmaSpin™ Post-Reaction Clean-Up Columns (Sigma-Aldrich, St Louis, MO, USA). The presence of the synthesized RNA probe was evaluated by agarose gel electrophoresis (data not shown).

The riboprobe concentration was determined by comparison with a known amount of a DIG-labeled control RNA (Roche) in a dot blot assay as described by the manufacturer. The dilution factor for each RNA probe was determined to include 35 ng/ml of RNA into each hybridization procedure.

***In situ* hybridization using tissue samples of RSS-infected and control chickens.** The unstained tissue slides were first heated at 70°C for 10 min and deparaffinized in Citrosolv™ (Fisher Scientific, Pittsburgh, PA, USA). Slides were then air-dried thoroughly and tissue sections were rehydrated in 5 mM MgCl<sub>2</sub> in phosphate buffered saline (PBS) for 10 min. Before enzyme digestion, slides were treated in Tris-Glycine buffer (0.1 M glycine in 0.2 M Tris, pH 7.5) for 10 min at room temperature (RT) and then incubated with proteinase K (35 µg/ml) in proteinase K buffer (10 mM Tris, pH 7.5, 2 mM CaCl<sub>2</sub>) for 15 min at 37°C. The enzymatic reaction was stopped in the Tris-Glycine buffer. Pre-hybridization solution [5x saline-sodium

citrate buffer (SSC) containing 0.75 M NaCl, 0.075 M sodium citrate with 50% formamide, 5% blocking reagent (Roche), 0.1% N-lauroylsarcosine and 0.02% sodium dodecyl sulphate (SDS)] was added to sections for 30 min at 42°C. Seventy microliters of the hybridization solution, which consisted of the pre-hybridization solution containing the riboprobe (35 ng/μl), was applied directly onto the section and covered with a siliconized cover slip (HybriSlip™, Grade Bio-Labs). The hybridization was performed overnight at 42°C in a humidity chamber. The next day, coverslips were removed and slides were washed once at 50°C in 2x SSC (0.3 M NaCl, 0.03 M sodium citrate) with 1% SDS followed by one wash at 50°C with 1x SSC (0.15 M NaCl, 0.015 M sodium citrate) with 0.1% SDS and at RT with one wash in 1x SSC followed by one wash with 0.1X SSC (0.015 M NaCl, 0.0015 M sodium citrate) for 30 min each. After the washing steps, slides were treated in buffer I (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 10 min then incubated with a 300-fold diluted sheep anti-digoxigenin alkaline phosphatase-conjugated Fab<sub>2</sub>' (Roche) in Buffer I containing 1% fetal bovine serum (FBS) and incubated for 2 h at 37°C. After three washes with buffer I, the binding of the conjugate was visualized by adding a chromogen mixture [200 μl of NBT/BCIP stock solution (Roche) in 10 ml of 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl]. The development of the signal was allowed to progress for 45 to 60 minutes and was stopped by rinsing the slide in distilled water. Slides were lightly counterstained with Gill's hematoxylin and coverslipped with Permount™ (Fisher Scientific). The slides were evaluated under a light microscope.

**Functionality test for the parvovirus riboprobe.** Cells of the chicken fibroblast cell line DF-1 (Himly *et al.*, 1998) grown in Dulbecco's Modified Eagles's Medium with 4.5g/l glucose (DMEM-4.5, Thermo Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Mediatech, Manassas, VA, USA) were prepared in an 8-well-chamber slides

(Lab-Tek® II CC<sup>2</sup>™, Nunc) 12 hours before transfection. Using the *TransIT*-mRNA Transfection Kit (Mirus), cells were transfected with either DIG-labeled ChPV sense probe, or non labeled ChPV sense probe, or mock transfected as negative control following the instructions of the manufacturers. Four hours after transfection of the cells, the supernatant was removed, cells were washed once with PBS followed by incubation with 10% neutral buffered formalin containing 5% acetic acid for 30 min at RT. After fixation, cells were washed with PBS and stored in 70% ethanol at 4 °C until use. For *in situ* hybridization, cells were incubated with 5 mM MgCl<sub>2</sub> in PBS for 10 min and then in Tris-Glycine buffer (0.1 M glycine in 0.2 M Tris, pH 7.5) for 10 min. Cells were incubated with proteinase K (3.5 µg/ml) in proteinase K buffer (10 mM Tris, pH 7.5, 2 mM CaCl<sub>2</sub>) for 15 min at 37°C. The enzymatic reaction was stopped by a rinsing step using Tris-Glycine buffer. The hybridization with the DIG-labeled ChPV antisense probe was performed as described above.

## Results

**Cystic lesions in the small intestine were present 24 h after exposure.** Determination of the body weights indicated a severe challenge of the birds exposed to the RSS-contaminated litter (Figure 2.1A). The average of the bodyweights was significantly different ( $p < 0.05$ ) starting from day 3 after exposure. Five days following the start of the experiment, the body weight difference between the groups was approximately 50%. The difference in weight culminated at the end of the study where the median body weight of the chickens exposed to the RSS-contaminated litter was only 30% of the weight of the control group. The microscopic evaluation of the cross section of the duodenal loop revealed the presence of cystic lesions only in chickens that were exposed to RSS-contaminated litter (Figure 2.1B). No differences between control and

RSS-exposed birds were observed during the evaluation of the pancreatic tissues. The average lesion numbers were determined per group and by day. One striking finding was that cystic lesions were observed in four out of five birds examined as early as 24 h after placement. Interestingly, the lesions observed at this early time point already showed the structure previously described (Songserm *et al.*, 2000; Nili *et al.*, 2007). The lesions observed were characterized by dilatation of the crypts of Lieberkühn in addition to atrophy of the intestinal villi ranging from mild to moderate. Furthermore hyperplasia of the crypt region was observed. In the dilated crypt lesion, the epithelial cells were markedly flattened. The dilated crypt lumen occasionally contained cellular debris that was composed of degenerated cells and eosinophilic cellular debris. Starting with day 10 after exposure, affected crypts first became surrounded and later replaced by connective tissues. In addition, mineralization was occasionally observed within the lesions. The average number of cystic lesions increased until day 4 after placement. Four days following exposure (days 2, 3, 4, 7), lesions were observed in all five birds examined, while on the remaining days, lesions were observed in approximately 80% of the birds (see also Figure 2.3). The median number of lesions peaked at day 4 after exposure and declined until the end of the study. It needs to be mentioned that the variability in the numbers of lesions was extreme, as indicated by the minimum and maximum number of lesions per bird (Figure 2.1b). Furthermore, the data was analyzed to determine whether there was a correlation between number of lesions in the duodenal loop and the weight of the same bird (Figure 2.1c). The data of each bird for both values was shown side by side for day 4, 5, and 6 post inoculation (p.i.) since the highest lesion scores were observed at these time points (see also Figure 2.1b). It became obvious that no direct correlation was observed between these parameters, since birds with a comparatively low body weight showed a low number of lesions (day 5 p.i., bird 4) while in the

opposite case a bird with a comparatively high bodyweight showed a high number of lesions (day 5 p.i., Bird 5).

**Primary lymphoid organs were affected during infection.** In order to assay whether exposure to RSS-inducing litter caused changes in two of the primary lymphoid organs (bursa of Fabricius, thymus) and one secondary lymphoid organ (Harderian gland), microscopic evaluations were performed. The results obtained for the bursa of Fabricius showed that the scores for the RSS-litter-exposed group increased over the course of the experiment (Table 2.2). The scores of the bursa of Fabricius were statistically significantly different between the RSS-exposed group and the control group from day nine onwards. The changes observed in the thymus serving as the second primary lymphoid organ showed a similar trend (Table 2.2). Thymus sections of chickens exposed to the contaminated litter presented a significantly higher score from day 6 through day 12 after placement, in comparison with the control group which was placed on fresh shavings. One exception was observed on day 8 after placement where no significant difference in the average lesion scores between both groups was observed. In contrast, the evaluation of the Harderian gland as a representative for a secondary lymphoid organ resulted in no detectable differences between both groups in respect to microscopic tissue changes (Table 2.2) except for day 6 after placement where significant differences were observed.

**Sequence comparison of the probe nucleotide sequences.** The length of the amplified sequence from the intestinal content of RSS-infected chickens encoding for parts of the viral capsid protein for the ANV-1 probe was 516 nucleotides (nt) and for the ANV-2 was 521 nt. The homology of these nucleotide sequences to each other was 46 %. The similarity to the published ANV-1 capsid encoding sequences (AB033998) was 84 % while the ANV-2 sequence showed a

similarity of 89% to a published ANV-2 sequence (AB046864). Nucleotide sequence similarities to other poultry astroviruses [turkey astrovirus 1 (EU143848), turkey astrovirus 2 (EU143843), duck astrovirus (NC\_012437), chicken astrovirus (JF414802)] were below 30%. The sequence used for the probe for the new chicken astrovirus was also obtained from the intestinal sample. The sequence was located in the region of the new chicken astrovirus encoding for the non-structural protein. A NCBI Genbank Blastn search did locate any similar sequences, and a direct comparison with the appropriate nucleotide sequences for ANV-1 (NC\_003790), turkey astrovirus 1 (EU143848) and turkey astrovirus 2 (EU143843), and the duck astrovirus (NC\_012437) showed a similarity of below 20%. The probe for the amplified parvovirus sequence showed an 88% identity to a recently published chicken parvovirus sequence (Day & Zsak, 2010).

**In situ hybridization revealed astroviruses as agents for RSS.** The *in situ* hybridization (ISH) was performed on all tissues which included the bursa of Fabricius, thymus, Harderian gland, and the cross-section of the duodenum including the tip of the pancreas. Samples from day 1 through day 5 were analyzed. One major problem with the investigations was that viruses of interest were not isolated in cell culture and thus the availability of a positive control for the ISH to show specificity was limited. To address this problem from the very beginning, all probes were designed that they would have the approximate same length (ANV-1: 516 nt; ANV-2: 521 nt; chicken astrovirus: 450 nt; chicken parvovirus: 511 nt). Due to the sequence composition, the GC content was approximately 45% and very similar in all probes. For the adjustment of the ISH conditions, DF1 cells were transfected with positive sense transcripts of each virus (ANV-1, ANV-2, chicken astrovirus, and chicken parvovirus) and cross tested for specificity with each probe. In addition, the antisense-oriented transcripts were also

transfected and probed with the sense probes. The adjustment factor for the specificity was the temperature during the washing procedure, probably due to the GC content and the similar length of probes. Interestingly, for all probes the same temperature could be used without losing specificity. Since the study was designed so that each sample could be traced to the appropriate chicken, association of ISH signals to appropriate changes observed microscopically could be evaluated. The bursa of Fabricius, thymus, tip of the pancreas, and Harderian gland showed no ISH signals, neither those samples obtained from the group which was exposed to the RSS-contaminated litter nor the tissue samples obtained from the negative control group. This indicated that neither one of the three astroviruses nor the chicken parvovirus replicated in these organs. A different result was observed when the samples representing the duodenal loop were investigated with the probes specific for chicken astrovirus, ANV-1, and ANV-2. The sense probes for the astroviruses showed no signals that could be appreciated as positive on slides of two chickens which were positive on day 1 after placement (data not shown). All antisense probes specific for each of the astroviruses showed a positive signal in a number of samples. This indicated a high specificity of the antisense ISH probes with the respective virus. Furthermore, none of the samples investigated from the negative control group showed a positive ISH signal from any of the birds that were taken on day 1 and 3 after placement regardless of whether the sense or antisense probe was used. The general signature of the astrovirus-specific antisense probes was very similar (Figure 2.2). The majority of positive signals were observed along the villous epithelial layer although occasionally a few rare signals were seen in the lamina propria as irregular particles. The signals in the villous epithelial cells were localized in the cytoplasm, indicating the expected cytoplasmic replication of the virus. Interestingly, no matter whether tissues contained the dilated crypt lesions, the signals for astroviruses were present

neither in the crypt epithelial cells nor in the adjacent tissues of the crypt. The signals were clearly distinguishable, which indicated an intense virus replication. No difference in the strength of the signal among the three different astroviruses was observed. The pattern of the staining might be important for the dynamics of the disease. Although the lesions are already shown in figure 2.1b, the association between lesions and presence of ISH signals is shown in figure 2.3. The signal for ANV-1 was observed in one chicken at day 1, all five chickens at day 3, no chickens at day 4, and four out of five chickens at day 5. The signal specific for the ANV-2 probe was scattered throughout the investigated time points. Two chickens were positive at day 1 and 3 after exposure to the litter while at days 2, 4, and 5 after exposure, one chicken out of the five investigated chickens showed a positive signal. The chronological presence of viral RNA for the new chicken astrovirus was noted. Four out of the five birds evaluated showed a positive signal 24 h after exposure to the RSS-contaminated litter and only one chicken at day 2. None of the investigated sections showed any signal from day 3 through day 5 after exposure. It needs to be mentioned that the sections were cut consecutively from the paraffin embedded blocks and on an individual basis. The initial concern was that the probes specific for ANV-1 and ANV-2 might cross-react although the nucleotide sequence similarity was only 46% (see above). However the data obtained show that although Bird 1 and 2 at day 1 after exposure showed ISH signals for the ANV-2 probe in a high number of cells, hybridization with the ANV-1 probe remained negative. A similar result was observed with Bird 5 on day 4. The ISH with the ANV-2 probe was positive in a high number of cells but negative for the ANV-1 ISH probe. The opposite result was present in Bird 3 and 5 on day 5 where the ISH probe for the ANV-1 was positive in a high number of cells while the ISH for the ANV-2 was negative. A very similar result was observed for the ISH probe of the new chicken astrovirus, where no reaction was

observed beyond day 2 following exposure while positive signals were observed with both of the other probes (ANV-1 and 2). The comparison of all three ISH signals on a single bird basis demonstrated that some birds were positive for two viruses at the same time regardless of the strength of the signal. In addition, it was also observed that the presence of cystic lesions was not necessarily related to the presence of viral RNA as was observed for Bird 4 24 h after exposure.

The use of both sense and antisense ISH probes specific for parvovirus resulted in no signal in any samples at any time points investigated. Since this result was not expected, experiments were performed to evaluate whether the anti sense parvovirus probe was functional. Thus, DF1 cells were left untransfected or were transfected with either the labeled sense probe to verify the reaction conditions or were transfected with the unlabeled sense probe and probed with the labeled antisense probe as shown in Figure 2.3B. The results showed clearly that the antisense parvovirus probe was able to bind to the sense parvovirus cRNA. This result was an additional strong indicator that no parvovirus was replicating in the duodenal loop of the investigated intestines.

## **Discussion**

Although the etiology for RSS remains unknown, early investigations revealed that RSS has probably a viral etiology (Decaesstecker *et al.*, 1986; Smart *et al.*, 1988). These observations have been supported by experiments using either filtered intestinal content (Nili *et al.*, 2007) or chloroform treated, filtered intestinal content (Sellers *et al.*, 2010). The latter supported the assumption that the viruses causing RSS are non-enveloped. Initially, reovirus was believed to be the major causative agent for RSS since these viruses have been identified in RSS-affected chickens (Rekik *et al.*, 1987; Kouwenhoven *et al.*, 1988; van Loon *et al.*, 2001). In addition,

intestinal lesions typical for RSS have been reported in specific pathogen free (SPF) chickens after infection with reovirus of enteric origin (Shirai *et al.*, 1990; Goodwin *et al.*, 1993; Songserm *et al.*, 2003). In contrast, neutralization of reovirus from infective homogenates or vaccination of breeder hens against reovirus did not reduce the severity of RSS (Eidson *et al.*, 1985). Also viruses from a variety of different virus families (*Adenoviridae*, *Parvoviridae*, *Togaviridae*) have been associated with RSS (Kouwenhoven *et al.*, 1978; McNulty *et al.*, 1984; Zsak *et al.*, 2008). However, the exact etiology of the disease has not been proven to date but more than one agent has been proposed to be involved in this disease syndrome (Rebel *et al.*, 2006). Despite viral multiplication in experimentally inoculated birds, no clinical signs or growth retardation were observed in SPF and broiler chickens infected with a reovirus or parvovirus, but abnormal feces and reduction in weight gains were observed after infection with the field materials and entero-like viruses (Decaesstecker *et al.*, 1986). Otto *et al.* (2006) described a correlation between the presence of cystic lesions in the intestine and the presence of rotavirus. Furthermore it has been described that infection of SPF leghorn chickens with a chicken astrovirus resulted in mild diarrhea and some distention of the small intestine (Baxendale & Mebatsion, 2004).

In order to investigate the etiology of RSS, the investigations described here focused on three members of the *Astroviridae* family and a chicken parvovirus. In addition, the possibility of an immuno-compromising component associated with the disease was investigated. The data clearly show that the RSS challenge was severe as indicated by the dramatic difference in weight gain and presence of cystic lesions in the small intestine. The difference in weight at day 12 p.i. in about 70% of the RSS-litter exposed group was stronger than described before (50%) in a very similar challenge model (Sellers *et al.*, 2010). In both cases, the number of birds with cystic

lesions at day 12 p.i. was very similar. In this study, 22 out of 30 birds showed cystic lesions compared with 20 out of 30 birds in the study previously described (Sellers et al., 2010). This indicated that the presence of either cystic lesions in the duodenum or decreased weight gain can not be used as a standalone indicator for RSS, but the presence of lesions and a decreased weight gain can be used as a strong indicator for RSS when a certain number of chickens are included in the study. The latter holds true since chickens with a comparable higher weight showed a high number of lesions while birds with a low weight showed a low number of lesions (see also Figure 2.1c). Another reason might be that the load of the infection agent(s) was much higher. But since the exact cause for RSS is unknown it can not be investigated. Interestingly, the average of the cystic lesions were detected in the small intestine in 80% of the RSS group as early as 24 h following exposure and peaked at day 4 after exposure but declined until the end of the study. The dynamics for the presence of the lesions was described previously by Smart *et al.* (1988) where the first cystic intestinal lesions were observed as early as 3 days after inoculation due to the sampling schedule used in that study. It is possible that lesions in this study were present at an earlier time point. The immediate presence of cystic lesions in the intestine followed by lymphocytic depletion in the immune organs 5 to 6 days later is probably not a direct result of any of the four viruses targeted in this study. The absence of ISH signals in the immune organ supports this notion. These observations indicate rather that RSS-contaminated litter from poultry houses harbors other pathogens that cause lesions in the bursa of Fabricius and the thymus of the chicken. This was not surprising and has been shown before (Reece *et al.*, 1984; Montgomery *et al.*, 1997; Nili *et al.*, 2007). Another possibility is that an organ is involved which regulates the growth of chickens, such as pituitary gland that synthesizes the growth hormone. But to test this hypothesis, another set of experiments needs to be performed. Also

other organs could be included, such as the kidney and liver, since these organs are important for growth and therefore have the potential for involvement in the disease complex.

The absence of ISH signals using both parvovirus probes provided critical evidence that parvovirus, although described to be present in RSS cases (Zsak *et al.*, 2008), may not play an important role in the etiology of the disease. Although Kisary *et al.* (1984) described the presence of parvovirus in guts of chickens that suffered from RSS and showed later that the obtained parvovirus ABU isolate was able to induce growth retardation in chickens (Kisary, 1985), other experiments in broiler chickens using the same ABU isolate could not confirm the results (Decaesstecker *et al.*, 1986) since neither weight depression nor lesions in the intestines were observed. Additional evidence has been reported that implies chicken parvoviruses might play an important role in enteric diseases in poultry since parvoviruses were detected in most cases with an enteric disease in chickens and turkeys (Pallade *et al.*, 2011). Due to the lack of a virus isolate and induction of the disease complex with such isolate, however, the evidence presented to date is only circumstantial (Kisary, 1985, Pallade *et al.*, 2011, Zsak *et al.*, 2008). As described here, the presence of signals for three members of the family *Astroviridae* (a new chicken astrovirus, ANV-1, ANV-2) may provide additional *in vivo* support that members of this virus family play an important role for the induction of the disease. Some known avian astroviruses have previously been detected by RT-PCR in materials obtained from RSS-affected chickens (Pantin-Jackwood *et al.*, 2008a; Smyth *et al.*, 2009; Spackman *et al.*, 2010; Pantin-Jackwood *et al.*, 2011).

We showed for the first time, a physical presence of astroviruses at locations in the intestine where RSS-associated lesions were also observed. Initially, we were concerned that ISH probes for ANV-1 and ANV-2 might cross-react with the viral RNA of the other ANV, but the

results showed that while ISH signals in the consecutive intestinal sections were present for ANV-1, the next section was negative for both chicken astrovirus and ANV-2, respectively. Although cystic lesions were present soon after exposure to the RSS-contaminated chicken litter, the ISH signals observed were limited to the epithelial cells in the intestine. The results of this study implied that the lesions in the intestine were caused by another viral agent or that cystic enteropathy is coincidentally present but not associated with the outcome of the disease. This hypothesis is supported by the early observation of cystic enteropathy at 24 h after exposure but a lack of ISH staining in the crypts. On the other hand, experimental evidence using an recombinant astrovirus capsid-based vaccine indicated that the new chicken astrovirus might play an role in this disease (Sellers *et al.*, 2010). Thus, further experiments are necessary and already under way to clarify these remaining questions.

## References

- Baxendale, W., & Mebatsion, T. (2004). The isolation and characterisation of astroviruses from chickens. *Avian Pathology*, *33*, 364-370.
- Day, J. M., & Zsak, L. (2010). Determination and analysis of the full-length chicken parvovirus genome. *Virology*, *399*, 59-64.
- Decaesstecker, M., Charlier, G., & Meulemans, G. (1986). Significance of parvoviruses, enterovirus-like viruses and reoviruses in the aetiology of the chicken malabsorption syndrome. *Avian Pathology*, *15*, 769-782.
- Eidson, C. S., Kleven, S. H., & Fletcher, O. J. (1985). Performance of broiler progeny of breeder flocks vaccinated with inactivated oil emulsion malabsorption syndrome virus vaccine. *Poultry Science*, *64*, 2081-2086.
- Goodwin, M. A., Davis, J. F., & Player, E. C. (1993). Reovirus-associated enteritis in Georgia broiler chicks. *Avian Diseases*, *37*, 229-233.
- Himly, M., Foster, D. N., Bottoli, I., Iacovoni, J. S., & Vogt, P. K. (1998). The DF-1 chicken fibroblast cell line: transformation induced by diverse oncogenes and cell death resulting from infection by avian leukosis viruses. *Virology*, *248*, 295-304.

- Imada, T., Yamaguchi, S., Mase, M., Tsukamoto, K., Kubo, M., & Morooka, A. (2000). Avian nephritis virus (ANV) as a new member of the family Astroviridae and construction of infectious ANV cDNA. *Journal of Virology*, *74*, 8487-8493.
- Kisary, J. (1985). Experimental infection of chicken embryos and day-old chickens with parvovirus of chicken origin. *Avian Pathology*, *14*, 1-7.
- Kisary, J., Nagy, B., & Bitay, Z. (1984). Presence of parvoviruses in the intestine of chickens showing stunting syndrome. *Avian Pathology*, *13*, 339-343.
- Koci, M. D., & Schultz-Cherry, S. (2002). Avian astroviruses. *Avian Pathology*, *31*, 213-227.
- Koci, M. D., Moser, L. A., Kelley, L. A., Larsen, D., Brown, C. C., & Schultz-Cherry, S. (2003). Astrovirus induces diarrhea in the absence of inflammation and cell death. *Journal of Virology*, *77*, 11798-11808.
- Kouwenhoven, B., Davelaar, F. G., & Van Walsum, J. (1978). Infectious proventriculitis causing runting in broilers. *Avian Pathology*, *7*, 183-187.
- Kouwenhoven, B., Vertommen, M., & Goren, E. (1988). Investigations into the role of reovirus in the malabsorption syndrome. *Avian Pathology*, *17*, 879-892.
- McNulty, M. S., Allan, G. M., Connor, T. J., McFerran, J. B., & McCracken, R. M. (1984). An entero-like virus associated with the runting syndrome in broiler chickens. *Avian Pathology*, *13*, 429-439.
- Montgomery, R. D., Boyle, C. R., Maslin, W. R., & Magee, D. L. (1997). Attempts to reproduce a runting/stunting-type syndrome using infectious agents isolated from affected Mississippi broilers. *Avian Diseases*, *41*, 80-92.
- Nili, H., Jahantigh, M., & Nazifi, S. (2007). Clinical observation, pathology, and serum biochemical changes in infectious stunting syndrome of broiler chickens. *Comparative Clinical Pathology*, *16*, 161-166.
- Olsen, D. E. (1977). Isolation of a reovirus-like agent from broiler chicks with diarrhea and stunting. *Proceedings of the 26th western poultry diseases conference* (pp. 131-139). Davis, California.
- Otto, P., Liebler-Tenorio, E. M., Elschner, M., Reetz, J., Lohren, U., & Diller, R. (2006). Detection of rotaviruses and intestinal lesions in broiler chicks from flocks with runting and stunting syndrome (RSS). *Avian Diseases*, *50*, 411-418.
- Palade E. A, Kisary, J., Benyeda, Z., Mándoki, M., Balka, G., Jakab, C., Végh, B., Demeter, Z., & Rusvai M. (2011). Naturally occurring parvoviral infection in Hungarian broiler flocks. *Avian Pathology*. *40*,191-197.

- Pantin-Jackwood, M. J., Day, J. M., Jackwood, M. W., & 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.
- Pantin-Jackwood, M. J., Spackman, E., & Day, J. M. (2008b). Pathogenesis of type 2 turkey astroviruses with variant capsid genes in 2-day-old specific pathogen free poult. *Avian Pathology*, *37*, 193-201.
- Pantin-Jackwood, M. J., Strother, K. O., Mundt, E., Zsak, L., Day, J. M., & Spackman, E. (2011). Molecular characterization of avian astroviruses. *Archives of Virology*, *156*, 235-244.
- Rebel, J. M. J., Balk, F. R. M., Post, J., Van Hemert, S., Zekarias, B., & Stockhofe, N. (2006). Malabsorption syndrome in broilers. *World's Poultry Science Journal*, *62*, 17-30.
- Reece, R. L., Hooper, P. T., Tate, S. H., Beddome, V. D., Forsyth, W. M., Scott, P. C., & Barr, D. A. (1984). Field, clinical and pathological observations of a runting and stunting syndrome in broilers. *Veterinary Record*, *115*, 483-485.
- Rekik, M. R., Silim, A., & Bernier, G. (1987). Experimental infection of broiler chickens using different serotypes of reovirus from malabsorption syndrome cases. Proceedings of *the 36th western poultry diseases conference* (pp. 146–148). Davis, California.
- Sellers, H., Linneman, E., Icard, A. H., & Mundt, E. (2010). A purified recombinant baculovirus expressed capsid protein of a new astrovirus provides partial protection to runting-stunting syndrome in chickens. *Vaccine*, *28*, 1253-1263.
- Shirai, J., Obata, H., Nakamura, K., Furuta, K., Hihara, H., & Kawamura, H. (1990). Experimental infection in specific-pathogen-free chicks with avian reovirus and avian nephritis virus isolated from broiler chicks showing runting syndrome. *Avian Diseases*, *34*, 295-303.
- Sklan, D. (2001). Development of the digestive tract of poultry. *World's Poultry Science Journal*, *57*, 415-428.
- Smart, I. J., Barr, D. A., Reece, R. L., Forsyth, W. M., & Ewing, I. (1988). Experimental reproduction of the runting-stunting syndrome of broiler chickens. *Avian Pathology*, *17*, 617-627.
- Smith, J. A. (2008). Runting/Stunting/Cystic Enteritis Syndrome. Available online at <http://www.thepoultrysite.com/articles/1114/runting-stunting-cystic-enteritis-syndrome> (accessed Mar 24 2011).
- Smyth, V. J., Jewhurst, H. L., Adair, B. M., & Todd, D. (2009). Detection of chicken astrovirus by reverse transcriptase-polymerase chain reaction. *Avian Pathology*, *38*, 293-299.

- Songserm, T., Pol, J. M., van Roozelaar, D., Kok, G. L., Wagenaar, F., & ter Huurne, A. A. (2000). A comparative study of the pathogenesis of malabsorption syndrome in broilers. *Avian Diseases*, *44*, 556-567.
- Songserm, T., Engel, B., van Roozelaar, D. J., Kok, G. L., Pijpers, A., Pol, J. M., & ter Huurne, A. A. (2002a). Cellular immune response in the small intestine of two broiler chicken lines orally inoculated with malabsorption syndrome homogenates. *Veterinary Immunology and Immunopathology*, *85*, 51-62.
- Songserm, T., Zekarias, B., van Roozelaar, D. J., Kok, R. S., Pol, J. M., Pijpers, A. A., & ter Huurne, A. A. (2002b). Experimental reproduction of malabsorption syndrome with different combinations of reovirus, *Escherichia coli*, and treated homogenates obtained from broilers. *Avian Diseases*, *46*, 87-94.
- Songserm, T., van Roozelaar, D., Kant, A., Pol, J., Pijpers, A., & ter Huurne, A. (2003). Enteropathogenicity of Dutch and German avian reoviruses in SPF white leghorn chickens and broilers. *Veterinary Record*, *34*, 285-295.
- Spackman, E., Day, J. M., & Pantin-Jackwood, M. J. (2010). Astrovirus, reovirus, and rotavirus concomitant infection causes decreased weight gain in broad-breasted white poults. *Avian Diseases*, *54*, 16-21.
- van Loon, A. A., Koopman, H. C., Kosman, W., Mumczur, J., Szeleszczuk, O., Karpinska, E., Kosowska, G., & Lutticken, D. (2001). Isolation of a new serotype of avian reovirus associated with malabsorption syndrome in chickens. *Veterinary Quarterly*, *23*, 129-133.
- Zekarias, B., Stockhofe-Zurwieden, N., Post, J., Balk, F., van Reenen, C., Gruys, E., & Rebel, J. M. (2005). The pathogenesis of and susceptibility to malabsorption syndrome in broilers is associated with heterophil influx into the intestinal mucosa and epithelial apoptosis. *Avian Pathology*, *34*, 402-407.
- Zsak, L., Strother, K. O., & Kisary, J. (2008). Partial genome sequence analysis of parvoviruses associated with enteric disease in poultry. *Avian Pathology*, *37*, 435-441.

**Table 2.1.** Oligonucleotides used amplification of the sequences for the RNA probes.

Name	Sequence	RE site <sup>a</sup>	Promoter <sup>b</sup>	Probe orientation
ANV1pr-FP	gg <b>GAATTC</b> TTACAACCCAAAACCTGGGC <sup>c</sup>	EcoRI	T3	antisense
ANV1pr-RP	gg <b>GAGCTC</b> GGGAGTATAGGGTCTTCAGATGG	Sac I	T7	sense
ANV2pr-FP	gg <b>GAATTC</b> GGACCATTGTGGCAGATCGAAGC	EcoRI	T3	antisense
ANV2pr-RP	gg <b>GAGCTC</b> GGTGCTGAACCAGTACCTGGC	Sac I	T7	sense
CAstVpr-FP	gg <b>TCTAGA</b> TTCTTGTCTAAAGTTATAACAGGAACAAAGAT	Xba I	T3	antisense
CAstVpr-RP	gg <b>GAGCTC</b> GGGCTTTTGGATTGGTAGAATCCTTC	Sac I	T7	sense
ChPVpr-FP	gg <b>CCGCGGGAATTC</b> GGCAACACTAACGGACAACACG	EcoRI	T3	antisense
ChPVpr-RP	gg <b>AAGCTT</b> GAGCTCGGAAAAACAAATGTAGTTTCCC	HindIII	T3	sense

<sup>a</sup> The restriction enzyme used for the linearization of the plasmid. The sequence is bold typed in the primer sequence.

<sup>b</sup> Phage promoter used for the transcription of the viral cDNA for the preparation of the digoxigenin-UTP labeled cRNA probe.

<sup>c</sup> Sequence of the oligonucleotide used for reverse transcription- polymerase chain reaction [avian nephritis virus (ANV) 1, ANV2, chicken astrovirus (CAstV)] or polymerase chain reaction [chicken parvovirus (ChPV)].

Virus specific sequences are shown in upper letter code and a small clamp sequence is shown in lower letter code. The restriction enzyme cleavage sites used for linearization were underlined.

**Table 2.2.** Average scores of microscopic changes in bursa of Fabricius, thymus, and Harderian gland.

Organ	Group	Day after placement											
		1	2	3	4	5	6	7	8	9	10	11	12
Bursa of Fabricius	Con	1.4 <sup>a</sup> (5) <sup>b</sup>	0.8 (5)	1 (4)	0.6 (5)	0.8 (5)	0.6 (5)	0.4 (4)	0.2 (5)	0.2 (5)	0 (5)	0.4 (4)	0.2 (29)
	RSS	1.4 (5)	1 (5)	0.6 (5)	0.8 (5)	0.6 (5)	1.5 (5)	1.25 (5)	1.2 (5)	2.2 (5)	2.2 (5)	2.5 (5)	1.72 (29)
		ns*	0.339	0.187	0.539	0.664	0.126	0.132	0.094	<b>0.005</b>	<b>&lt;0.001</b>	<b>0.005</b>	<b>&lt;0.001</b>
Thymus	Con	0‡ (5)	0 (5)	0 (5)	0 (5)	0 (5)	0 (5)	0 (5)	0.4 (4)	0 (5)	0 (5)	0 (5)	0 (30)
	RSS	0 (5)	0 (5)	0.2 (5)	0.4 (5)	1.2 (5)	2.25 (4)	2 (4)	1.4 (5)	2 (5)	2.4 (5)	2.6 (5)	1.4 (29)
		ns	ns	0.339	0.136	0.073	<b>0.001</b>	<b>0.001</b>	0.114	<b>0.006</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>
Harderian gland	Con	1.6 (5)	0.75 (4)	0.75 (4)	0 (1)	0.75 (4)	1.2 (5)	0.8 (5)	1.4 (5)	1.4 (5)	0.8 (5)	1 (4)	1.19 (26)
	RSS	1.4 (5)	1 (5)	1.6 (5)	1.2 (5)	0.75 (4)	0 (3)	1 (4)	1 (1)	1 (4)	0.8 (5)	0.75 (4)	1 (28)
		0.765	0.649	0.069	0.257	ns	<b>0.004</b>	0.648	0.506	0.402	ns	0.356	0.061

<sup>a</sup>Average of the scores evaluated by microscopic examination.

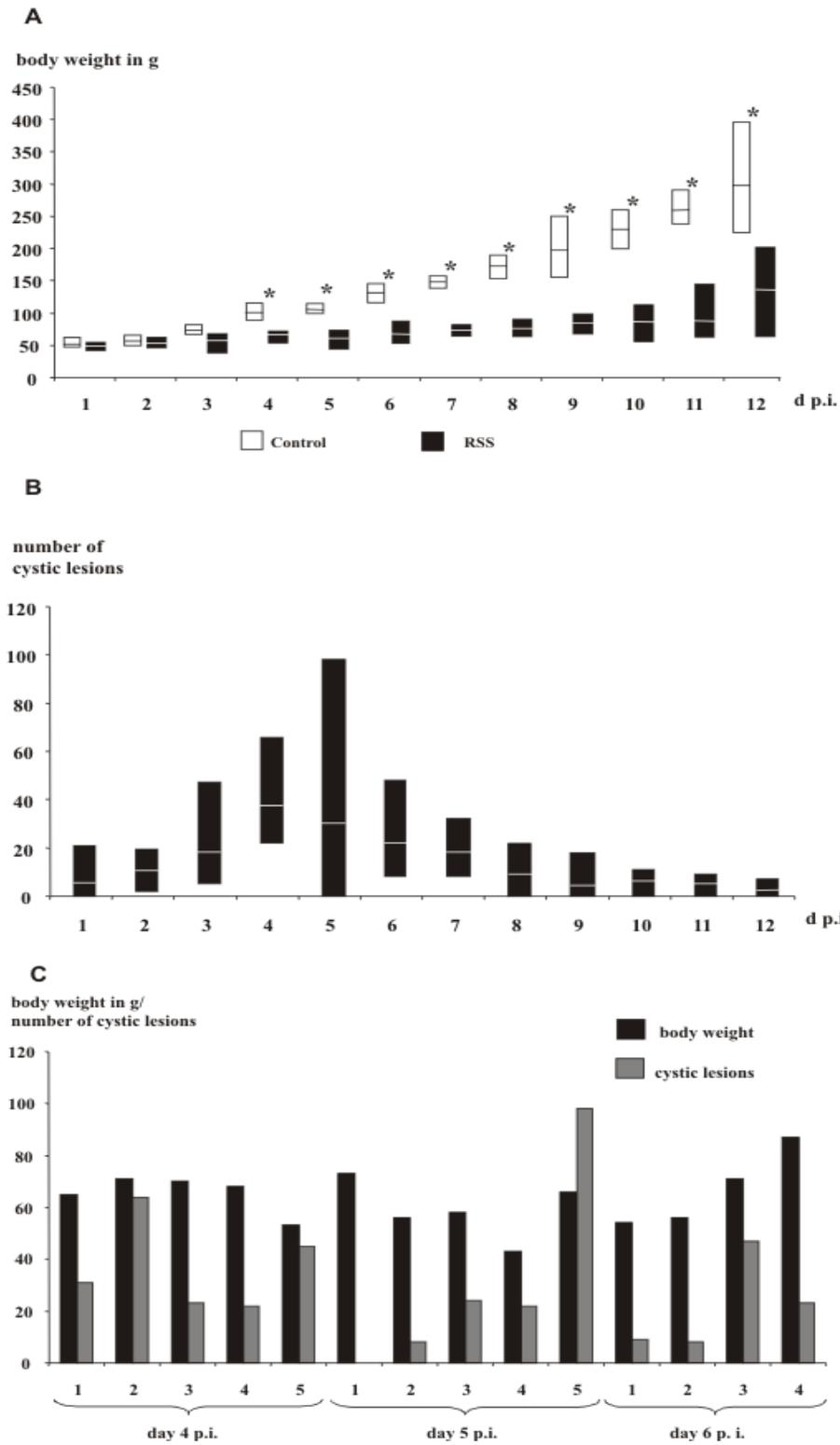
<sup>b</sup>Number of animals evaluated is listed in brackets.

\**P*-value of the comparison between the control group (Con) and challenge group (RSS) using On-Way-Anova test from summary data. Statistically significant differences (< 0.05) were bold typed.

ns, Insufficient differences to allow comparison.

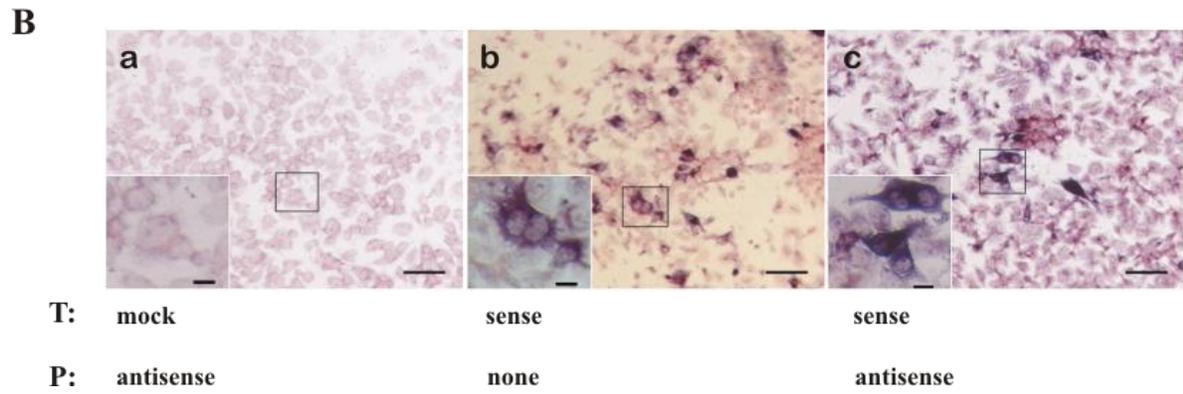
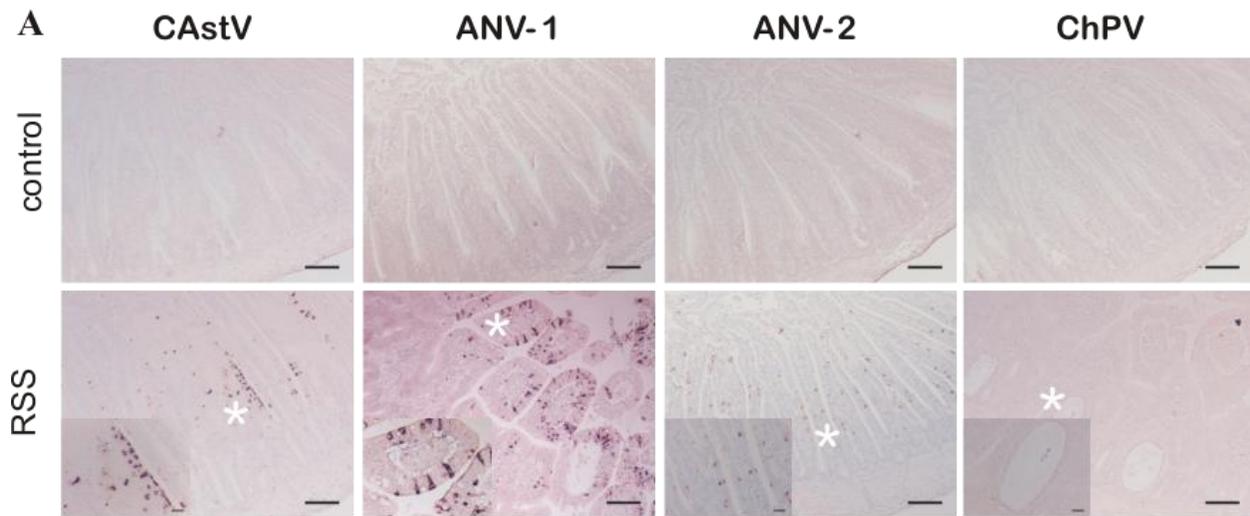
**Figure 2.1.** Weight gain and presence of cystic lesions in RSS-affected chicken. Commercial broiler chickens were exposed either to RSS-contaminated litter (RSS) or to fresh wood shavings (control). **(A)** Five chickens at day 1 through day 11 and thirty chickens at day 12 after infection (day p.i.) were euthanized and the body weights were determined. The average bodyweights for each day is shown as a white line in the RSS related box and black line in the white box representing the control chickens. In addition, the recorded minimum and maximum weights within the group were shown. Statistic significant different values between control birds and RSS-exposed birds were marked by an asterisk. **(B)** The cross section of each duodenal loop was assessed for the presence of cystic lesions for each chicken as described under (A). The average value for the number of cystic lesions is shown for each day in the box as white line. The minimum and maximum observed numbers of lesions were indicated. **(C)** The body weight and number of cystic lesions in the duodenal loop for each chicken at day 4, 5, and 6 after exposure to the RSS-contaminated litter was shown. The body weight and cystic lesions were plotted both on the y-axis.

Figure 2.1.



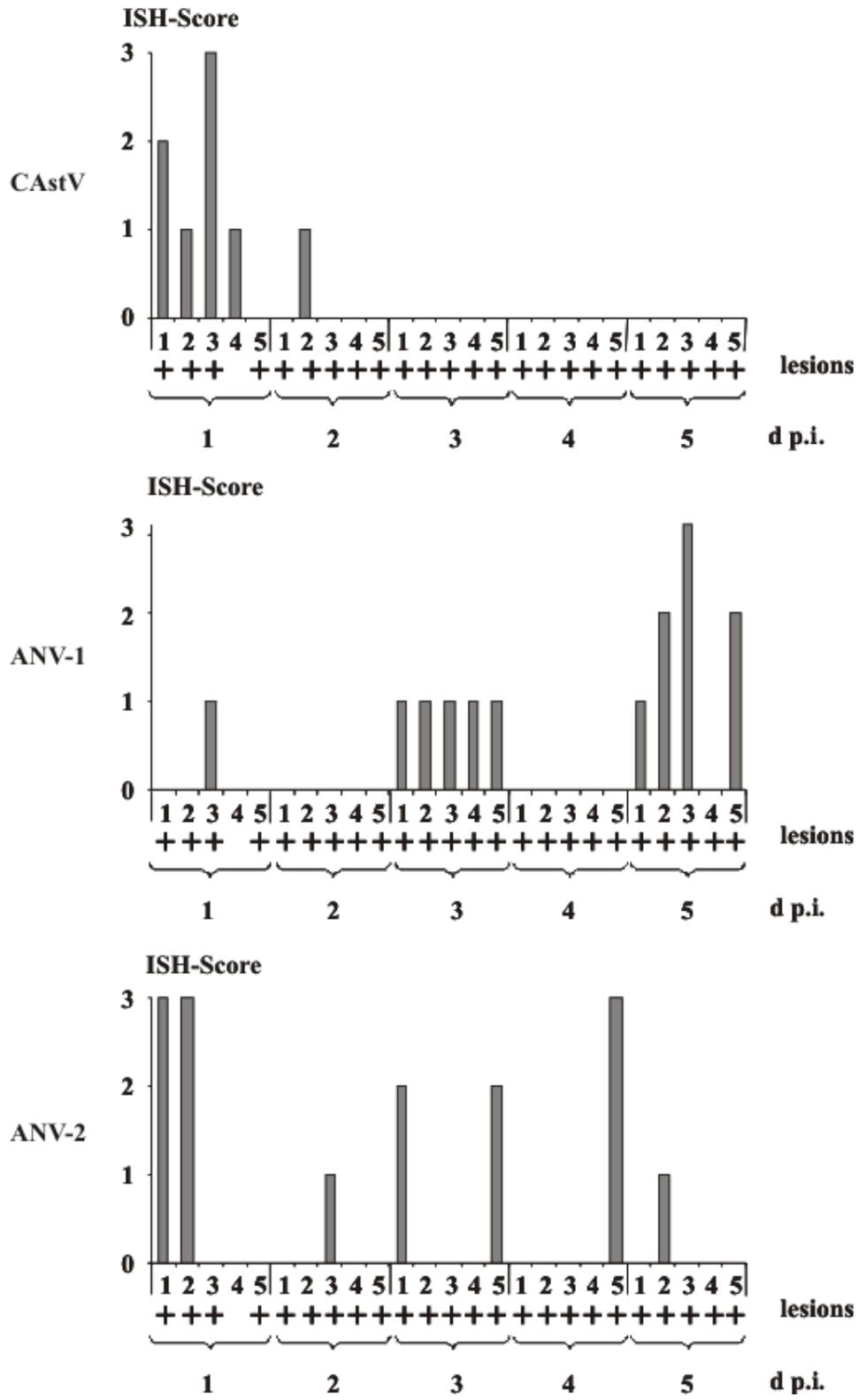
**Figure 2.2.** Astrovirus RNA was detected in epithelial cells of RSS-exposed chicken. (A) Sections from the duodenal loop of commercial broiler chickens exposed either to RSS-contaminated litter (RSS) or chickens exposed to fresh wood shavings (control) were exposed to antisense DIG-labeled riboprobes specific to a chicken astrovirus (CAstV), avian nephritis virus 1 (ANV-1), avian nephritis virus 2 (ANV-2), and a chicken parvovirus (ChPV). Selected areas (asterisks) of RSS infected chickens are shown as inset. The bar in the picture of the lower magnification represents 100  $\mu\text{m}$  and 20  $\mu\text{m}$  in the inset. (B) DF-1 cells were either mock transfected (a), or transfected with a DIG-labeled sense cRNA riboprobe (b), or transfected with unlabeled sense riboprobe (c). The fixed cells of (a) and (c) were hybridized with DIG-labeled antisense riboprobe. The NBT/BCIP reagents were applied to all three samples for colorimetric detection of the DIG-labeled cRNA probe. Bar: 50  $\mu\text{m}$ . Bar in the inset with the higher magnification: 20  $\mu\text{m}$ . Transfection (T), Probe (P).

**Figure 2.2.**



**Figure 2.3.** Presence of viral RNA specific for astroviruses during exposure to RSS-contaminated litter. Sections from the duodenal loop of commercial broiler chickens exposed either to RSS-contaminated litter (RSS) or to fresh wood shavings (control) were exposed to antisense DIG-labeled riboprobes specific to a chicken astrovirus (CAstV), avian nephritis virus 1 (ANV-1), avian nephritis virus 2 (ANV-2) were scored for the presence of an *in situ* hybridization signal (ISH) on an individual basis during the first five days after exposure (day p.i.). The presence of cystic lesions per cross-section is indicated (+). The ISH score was estimated on basis of following scale: score of 0 was the equivalent of no signals; score of 1, < 5 signals per high-power field; score of 2, 5-15 signals per high-power field; score of 3, > 15 signals per high-power field.

Figure 2.3.



### CHAPTER 3

## DETERMINATION OF THE FULL LENGTH SEQUENCE OF A CHICKEN ASTROVIRUS SUGGESTS A DIFFERENT REPLICATION MECHANISM<sup>2</sup>

---

<sup>2</sup> Kyung-il Kang , Alan H. Icard, Erich Linnemann, Holly S. Sellers and Egbert Mundt. 2012.  
*Virus Genes*. 44:45-50.

Reprinted here with permission of the publisher.

## Summary

The genomic RNA of a novel chicken astrovirus was determined. The full length sequence is 7520 nucleotides and encodes three open reading frames (1a, 1b, 2) for three proteins. The genomic organization was similar to other astroviruses with two exceptions. The open reading frame of the RNA-dependent RNA polymerase contains its own start codon which is different from other astroviruses described to date, providing evidence for a replication mechanism different than what has previously been described for astroviruses. Furthermore, the stem-loop structure located at the potential ribosomal frameshift signal described for other astroviruses has been shown to be a hairpin structure for the novel chicken astrovirus. Phylogenetic analysis of the full length sequence revealed that this chicken astrovirus formed a branch independent from other astroviruses, indicating that this astrovirus is significantly different from astroviruses described to date.

## Introduction

The determination of the full length sequences obtained from environmental samples will enhance the understanding of the biologic diversity of our world. To understand the steps taken during viral evolution, determination of full length sequences are valuable since only with their availability can mechanisms, such as recombination between viral genomes, be fully understood. Viruses belonging to the family *Astroviridae* have a non-enveloped capsid which contains a positive sense, ssRNA genome [1]. The viruses belong to a large group of small viruses with a diameter of approximately 28–30 nm. The genome length varies between 6.8 and 7.9 kb irrespective of the species of isolation. The genome encodes for three proteins, the nonstructural polyprotein (NS polyprotein), the RNAdependent RNA polymerase (RdRp), and the capsid

protein [2]. The NS polyprotein and the capsid protein are each encoded by an individual open reading frame (ORF), ORF1a and ORF2, while the RdRP (ORF1b) has been reported to be expressed via a ribosome shift mechanism [2] as a fusion protein to the NS protein [3]. Astroviruses have been isolated worldwide from several mammals (humans, cats, pigs, sheep, bat) as well as birds (ducks, chickens, turkeys) and are associated in general with gastroenteric diseases. While in mammals astroviruses mainly cause diarrhea, in birds astroviruses are associated with wider spectrum of diseases, including diarrhea, hepatitis, and nephritis. One of the diseases in poultry associated with astrovirus is the runting and stunting syndrome (RSS) in chickens. RSS is a transmissible disease of uncertain etiology. RSS affects chickens early in life and is characterized by growth retardation, ruffled feathers, and diarrhea resulting in considerable economic losses especially in commercial broiler production. The syndrome is also known as malabsorption syndrome, infectious stunting syndrome, broiler runting syndrome, and helicopter syndrome [4]. Currently, there is no effective licensed vaccine against the disease, mainly because of the absence of known etiologic agent/agents. One experimental vaccine, based on a recombinant baculovirus encoding for a novel astrovirus capsid protein, was recently described [5]. Clinical and pathological signs of RSS have been experimentally reproduced using oral inoculation of filtered and non-filtered intestinal homogenates from RSS affected chickens [5–8]. Based on preliminary sequence data of the capsid protein, obtained from gut samples collected from chickens experimentally exposed to RSS-contaminated litter, the full length sequence of astrovirus was determined. Comparisons to published astrovirus sequences indicated that the virus which harbors this genome belongs to a chicken astrovirus not previously described.

## **Material and Methods**

### **Generation of material for sequence determination**

One-day-old commercial broiler chickens were exposed to chicken litter transported from a commercial farm with chickens exhibiting RSS to a research isolation house, as previously described [5]. Chickens from this study were euthanized with CO<sub>2</sub> and the small intestine was harvested and homogenized with sterile phosphate buffered saline (PBS) at a 1:3 ratio (w/v) in a blender. The resulting homogenate was centrifuged at 3500 x g for 20 min at 4°C. The supernatant obtained was centrifuged a second time at 16000 x g for 20 min at 4°C, followed by a sequential filtration through a 0.45 µm and subsequently through a 0.22 µm filter (Whatman, Florham Park, NJ, USA). The filtrate was treated with chloroform and used for RNA purification using the High Pure RNA Isolation-Kit (Roche, Diagnostics GmbH, Mannheim, Germany). RNA was stored at -80°C until use.

### **Determination of the sequence of a novel chicken astrovirus from gut samples**

RNA isolated and purified from the gut homogenate described above was used for 5'-rapid amplification of cDNA using the 5' RACE System, Version 2.0 (Invitrogen, Carlsbad, CA, USA). The first primer used for the initial cDNA synthesis was located inside the open reading frame (ORF) of the capsid protein from a previously reported chicken astrovirus [5]. The subsequent PCR was performed with a nested astrovirus-specific primer and the anchor primer from the 5' RACE System. The RT-PCR fragment obtained was gel eluted and purified using the QIAquick Gel Extraction Kit (Qiagen Sciences, Md, USA) and cloned into the pCR2.1 plasmid using the TOPO TA cloning kit (Invitrogen) and transformed into competent *E. coli*. The recombinant plasmids obtained were sequenced using the BigDye Terminator v3.1 Cycle

Sequencing kit (Applied Biosystems, Foster City, CA, USA). Based on the new sequence obtained, two new astrovirus-specific oligonucleotides were delineated and used for the next 5'-RACE amplification. One was used for the initial cDNA synthesis while the second oligonucleotide was used as nested primer for the subsequent PCR. Using the primer walking approach, the full length sequence was determined. To determine the extreme 5' end of the viral genome, different 5' RACE reactions were performed as described by Mundt and Müller [9]. Briefly, for the determination of the first 5'-end nucleotide, the deoxynucleotide tailing reaction was performed using either dCTP or dGTP. The subsequent PCR was appropriately performed with either the anchor primer (dCTP-tailing) or a poly-C primer (dGTP tailing). The information regarding the primer sequences is available upon request from the corresponding author. Since the primer walking procedure was performed on a non-defined mixture present in the gut, the full length sequence was confirmed by amplification of overlapping 1 kb fragments using oligonucleotides delineated from the previously determined sequence. The RT-PCR fragments obtained were cloned and at least 3 plasmids were sequenced in both directions, obtaining a 6-fold coverage of the sequence.

### **Multiple alignment and sequence analysis**

Sequence data were analyzed using the DNASTar Lasergene 8 software package (DNASTAR Inc, Madison, WI, USA) for sequence alignments and *in silico* translation to amino acid sequences. Phylogenic analysis was performed using the MEGA-4.1 software [10], available as freeware online (<http://www.megasoftware.net/mega4/mega41.html>). The RNA secondary structure was determined using the RNA secondary structure prediction software available online ([http://www.genebee.msu.su/services/rna2\\_reduced.html](http://www.genebee.msu.su/services/rna2_reduced.html)).

## **Results and discussion**

### **Determination of the full length sequence of a novel chicken astrovirus**

The determination of the full length sequence, using the method of primer walking, resulted in RT-PCR fragments between 400 and 1200 bp in length. The determination of the extreme 5'-end was performed as previously described [9]. The full length sequence of the virus genome is 7520 nucleotides (Genbank accession number JF414802), not including the poly-A tail sequence. A schematic of the viral genome is shown in Fig. 3.1. The 5' - and 3'-noncoding regions were determined with 21 and 282 nucleotides, respectively. The data correlated with full length sequences from other avian astroviruses where the 5' NCR was also a short sequence from 10 nt (Turkey astrovirus [1], [11]) to 23 nt (Duck astrovirus, [12]). The 3' NCR was also comparable in length to other bird astroviruses, with a range between 192 nt for turkey astrovirus 2 [13] and 305 nt (ANV1, [14]). The alignment of the nucleotide sequences showed that the first five nucleotides (CCGAA), located at the 5' end, were highly conserved between all bird astroviruses (Fig. 3.1). In addition, this sequence motif was also observed in close proximity to the start codon for the ORF2, likely encoding the viral capsid protein. This feature has also been described for the duck astrovirus [12] and turkey astrovirus 1 [11] and 2 [13]. In contrast, this motif was absent upstream of the proposed start codon for the capsid protein of ANV1 [14]. Furthermore, turkey astroviruses, duck astrovirus, and the chicken astrovirus described in this article shared six homologous nucleotides at the very 3' end (Fig. 3.1). Interestingly, when the ANV 1 sequence was also taken into consideration in the comparison of the 3' end, the last three nucleotides were highly conserved between all astroviruses analyzed. The genome of the novel chicken astrovirus encodes three open reading frames (ORF), one protein each (Fig. 3.2) and

follows the principal genomic structure for an astrovirus [2]. The first ORF (ORF1a) encodes for a protein of 1139 amino acids (aa), while the second ORF (ORF1b) encodes for 519 aa. ORF1a encodes for the NS polyprotein and ORF1b encodes for the viral RNA dependent RNA-polymerase (RdRp) as previously proposed [2]. The third ORF (ORF2) encodes, with 743 aa, the viral capsid protein (see also [5]). ORF1a and ORF1b are located in an overlapping position, while ORF2 is downstream from the ORF1b. Despite genomic similarities to other astroviruses described to date, slight differences were identified within the novel chicken astrovirus. Although there is a potential ribosomal frameshift signal, consisting of a heptanucleotide (5'-AAAA AAC-3'), previously described [2], the ORF1b contains its own start codon which makes this, by definition, a true ORF (Fig. 3.2). In addition, the proposed typical stem-loop structure was not present in the sequence determined, but rather a sequence was present in the proposed region which may form a strong hairpin structure with no possibility of forming a pseudo knot structure as proposed earlier [2]. The importance of this stem-loop structure is not clearly understood since changes in a model system in the structure did not abolish the expression of a pseudo ORF1a-ORF1b fusion protein but decreased the efficacy [3]. On the other hand, deletion of the ribosomal frameshift signal sequence and also only a point mutation within the ribosomal frameshift signal sequence abolished the translation of the fusion protein in this model system [3], thus this sequence likely plays a central role for the translation of the fusion protein. Based on the data described in this article, it is possible that the ORF1b encodes the RdRp in the classical mode containing a start and stop codon. The possibility exists that in case of the nucleotide sequence here described for the chicken astrovirus, either a ribosomal scanning at the viral RNA with initiation at the start codon of ORF1b, or that the ribosomal unit dissociates at the ribosomal frameshift signal sequence and reinitiates at the methionine of ORF1b or there is a

ORF1b mRNA transcribed by an unknown mechanism. The latter possibility is rather unlikely due to the nature of the ORF1b encoding protein, the RdRp.

### **Analysis of the full length sequence with other Astroviruses**

The nucleotide sequence obtained from the chicken astrovirus was compared in a phylogenetic analysis with other astroviruses using full length sequences. To this end, full length astrovirus sequences from several species [turkey astrovirus 1 (Y15936), turkey astrovirus 2 (EU143843), duck astrovirus 1 (NC012437), avian nephritis virus 1 (NC003790), bat astrovirus (EU847155), human astrovirus VA1 (FJ973620), mink astrovirus (GU985458), ovine astrovirus (NC002469)] were included in this analysis (Fig. 3.3). The sequences were aligned using the ClustalW program (<http://www.ebi.ac.uk/Tools/msa/clustalw2>) and the multiple alignment obtained was analyzed using the program MEGA4.1. The neighbor-joining method and the minimum-evolution method were applied using 1000 replicates. The results of the neighbor-joining method clearly show that the novel sequence was significantly different (bootstrap value of 100) from other astrovirus sequences, including those described for ducks, turkeys, and chicken (Fig. 3.3) regardless of the algorithm used for the phylogenetic analysis. To further analyze the relatedness of this virus to other astroviruses, the amino acid sequences of all three in vitro translated ORFs were compared with published sequences (Table 3.1) using the pBlast search option in the NCBI database with one exception—the partial amino acid sequence for the ORF1a protein for a previously published chicken astrovirus [15] was taken from the publication and compared using the DNASTAR program package since this sequence is not available in the NCBI database. A 100% identity with a 99 aa partial ORF1a sequence was observed which has been described for a chicken astrovirus isolated in Europe [15]. A high similarity (99–89%) was

observed with partial sequences of the capsid protein of previously described chicken astrovirus sequences obtained from US field samples [16]. Interestingly, the overall amino acid sequences indicated that the most similar relative to the novel chicken astrovirus was a recently described duck astrovirus which caused a fatal hepatitis in ducklings [12], followed by turkey astrovirus 2 [13, 17] and 1 [11]. A turkey astrovirus 3 capsid protein sequence showed a 38% identity [17]. Surprisingly, the deduced amino acid sequences of all three proteins of the novel chicken astrovirus similarly showed a low similarity to the corresponding sequences of ANV1 [14] and to the capsid protein sequence of ANV2 in addition to the expected lack of similarity observed with the mammalian astroviruses, such as ovine, mink, human, and bat astrovirus (see Table 3.1). This data indicates the high degree of variability between astroviruses isolated from the same species. In addition, the similarity to the RdRp amino acid sequence was always higher likely due its nature as a functional enzyme responsible for the replication of the virus genome. RdRp sequences appear over-represented in the NCBI database, likely due to their highly conserved nature, compared to the few sequences available for the remaining regions of the genome. This region also serves as a target for the development of diagnostic tools [18–20]. Determination of the full length sequences of viral genomes will certainly provide the basis for a better understanding of the biology of particular virus. Data obtained in this study support the need to determine the full length sequence of novel viruses since due only to the availability of the full length sequence was evidence found to suggest that this particular astrovirus may employ a different replication strategy than what has been described for other astroviruses. Based on the findings, experiments to isolate the virus in cell culture and to generate antisera against the proteins encoded by ORF1a and ORF1b are under way to determine the proposed alternate mechanism for astrovirus replication.

### Acknowledgement:

This work was supported by the US Poultry and Egg Association's Harold E. Ford Foundation grant #F036, the Georgia Poultry Federation, and the University of Georgia Research Foundation.

### References

1. E. Mendez, C.F. Arias, in *Fields Virology*, ed. By D.M. Knipe, P.M. Howley (Lippincott Williams & Wilkins, Philadelphia, 2007), pp. 981-1000.
2. B. Jiang, S.S. Monroe, E.V. Koonin, S.E. Stine, R.I. Glass, *Proc. Natl. Acad. Sci. USA* 90, 10539-10543 (1993).
3. B. Marczinke, A.J. Bloys, T.D.K Brown, M.W. Willcocks, M. J. Carter, I. Brierley, J. *Viol.* 68, 5588-5595 (1994).
4. J.M.J. Rebel, F.R.M. Balk, J. Post, S. Van Hemert, B. Zekarias, N. Stockhofe, *World Poultry Sci. J.* 62, 17-30 (2006).
5. H. Sellers, E. Linneman, A.H. Icard, E. Mundt, *Vaccine* 28, 1253-1263 (2010).
6. R.D. Montgomery, C.R. Boyle, W.R. Maslin, D.L. Magee, *Avian Dis.* 41, 80-92 (1997).
7. T. Songserm, J.M. Pol, D. van Roozelaar, G.L. Kok, F. Wagenaar, A.A. ter Huurne, *Avian Dis.* 44, 556-567 (2000).
8. T. Songserm, B. Zekarias, D.J. van Roozelaar, R.S. Kok, J.M. Pol, A.A. Pijpers, A.A. ter Huurne, *Avian Dis.* 46, 87-94 (2002).
9. E. Mundt, H. Muller, *Virology* 209, 10-18 (1995).
10. K. Tamura, J. Dudley, M. Nei, S. Kumar, *Mol. Biol. Evol.* 24, 1596-1599 (2007).
11. C.M. Jonassen, T.O. Jonassen, B. Grinde, *J. Gen. Virol.* 79, 715-718 (1998).
12. Y. Fu, M. Pan, X. Wang, Y. Xu, X. Xie, N.J. Knowles, H. Yang, D. Zhang, *J. Gen. Virol.* 90, 1104-1108 (2009).
13. E. Strain, L.A. Kelley, S. Schultz-Cherry, S.V. Muse, M.D. Koci, *J. Virol.* 82, 5099-5103 (2008).

14. T. Imada, S. Yamaguchi, M. Mase, K. Tsukamoto, M. Kubo, A. Morooka, *J. Virol.* 74, 8487-8493 (2000).
15. W. Baxendale, T. Mebatsion, *Avian Pathol.* 33, 364-370 (2004).
16. M.J. Pantin-Jackwood, K.O. Strother, E. Mundt, L. Zsak, J.M. Day, E. Spackman, *Arch. Virol.* 156, 235-244 (2011).
17. Y. Tang, A.M. Murgia, Y.M. Saif, *Avian Dis.* 49, 514-519 (2005).
18. M.J. Pantin-Jackwood, J.M. Day, M.W. Jackwood, E. Spackman, *Avian Dis.* 52, 235-244 (2008).
19. V.J. Smyth, H.L. Jewhurst, B.M. Adair, D. Todd, *Avian Pathol.* 38, 293-299 (2009).
20. D. Todd, J. Trudgett, F. McNeilly, N. McBride, B. Donnelly, V.J. Smyth, H.L. Jewhurst, B.M. Adair, *Avian Pathol.* 39, 207-213 (2010).

**Table 3.1.** Similarities of amino acid sequences of different astrovirus to sequences of the new chicken astrovirus.

Different astroviruses	Chicken astrovirus		
	ORFa <sup>a</sup> (1139 aa)	ORF1b (519 aa)	ORF2 (743 aa)
Chicken Astrovirus <sup>b</sup>	N/A <sup>c</sup>	N/A	99%-89% <sup>d</sup>
Chicken Astrovirus <sup>e</sup>	100%	N/A	N/A
Duck astrovirus 1	60% (YP002728001)	71% (ADB79812)	41% (YP002728003)
Turkey astrovirus 1	37% (Y15936)	56% (Y15936)	34% (Y15936)
Turkey astrovirus 2	46% (ABX46564)	71% (AAF60952)	44% (ABX46566)
Turkey astrovirus 3	NA	NA	38 % (AAV37187.1)
Avian nephritis virus 1	26% (NP620617)	55% (BAA92848)	36% (NP620618)
Avian nephritis virus 2	NA	NA	35% (p) (ADQ43319.1)
Avian nephritis virus 3	49% (ACR56309)	NA	NA
Mink astrovirus	26% (ADR65075)	33% (GU985458)	32% (NP795336)
Human astrovirus VA1	24% (ADR65075)	43% (AAY84778)	34% (ACX85476)
Ovine astrovirus	23% (NP059945)	34% (NC002469)	32% (NP059946)
Bat astrovirus	NA	38% (EU847155)	32% (ACN88712)

<sup>a</sup> Number of amino acid in the encoded by the appropriate open reading frame

<sup>b</sup> Sequences published by Patin-Jackwood et al. (2011)

<sup>c</sup> no sequences have been found in the database (not applicable: NA)

<sup>d</sup> Percent homology to the amino acid (aa) sequence of the new chicken Astrovirus. The number in the brackets below the percentage represents the genbank accession number.

<sup>e</sup> Sequence published by Mebatsion and Baxendale (2004)

**5' -NCR**

Chicken Astrovirus	CCGAAAGCGAGGGUGUGGGCGAUG
Turkey Astrovirus 1	CCGAAAGUGUUGUCGGGGCGAUG
Turkey Astrovirus 2	CCGAAAGUGUUGUCGGGGCGAUG
Duck Astrovirus	CCGAAGUGGGCGAGUUGGGGCCAUG
ANV1	CCGAAUAGAUGGGAUG
	*****

**3' -NCR**

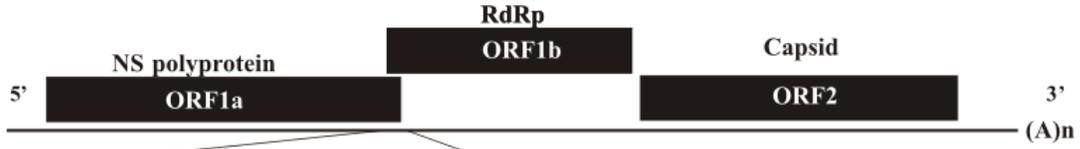
Chicken Astrovirus	AAUUAUUUUAAUUGGCAUUU (A) n
Turkey Astrovirus 1	UGAAAUUUUUUAUUAGCAUUU (A) n
Turkey Astrovirus 2	UAAAAUUUUUUAUUAGCAUUU (A) n
Duck Astrovirus	AGUAGAUUCAAUUGGCAUUU (A) n
	*      **    *****
ANV1	UUUUGAAUUGGCUAACUUUU (A) n
	+      +++

**Figure. 3.1.** Conserved nucleotides in the noncoding regions of bird astroviruses. The 5'- and 3'-noncoding regions (NCR) of the new chicken astrovirus (described in this paper, JF414802), turkey astrovirus 1 and 2 (19), duck astrovirus (18), and avian nephritis virus 1 (ANV1, 17) were aligned. Highly conserved nucleotides in the 5'-NCR were marked by an asterisk. The highly conserved nucleotides in the 3'-NCR between chicken astrovirus, both turkey astroviruses, and the duck astrovirus were marked by an asterisk while the highly conserved nucleotides between all analyzed sequences of the 3'-NCR were marked by a plus sign. The poly-A sequence at the 3'-NCR was labeled as (A)n.

**Figure. 3.2.** Schematic of the genomic organization of the new chicken astrovirus. **a.** The position of the open reading frames encoding for the nonstructural (NS) polyprotein, RNA-dependent RNA polymerase (RdRp), and the capsid protein (Capsid) are shown. The viral RNA associated poly-A tail [(A)<sub>n</sub>] is shown. **b.** The heptanucleotide sequences (chicken and duck astrovirus) and octanucleotide sequences (turkey astrovirus 1 and 2) serving as the proposed “shifty” sequence as part of the potential ribosomal frameshift signal (9) were highlighted by bold type letters. The noncoding region for the NS protein is marked by an asterisk. The location of the methionine marked in single letter code, likely serving as start amino acid for the RdRP of the chicken astrovirus, is highlighted bold typed. **c.** The secondary structure for the proposed region of the potential ribosomal frameshift signal is shown for the chicken astrovirus (JF414802) and human astrovirus 2 (L13745). The heptanucleotide sequence was highlighted by asterisks and the proposed hairpin structure (Chicken Astrovirus) and stem-loop structure (Human Astrovirus 2) was marked by a bracket. The nucleotide numbers shown in the structures is in accordance to the numbering in the sequence published in the Genbank.

Figure. 3.2.

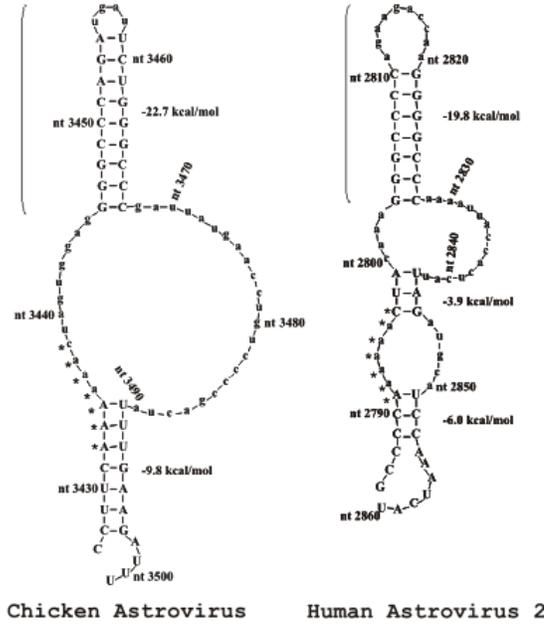
a

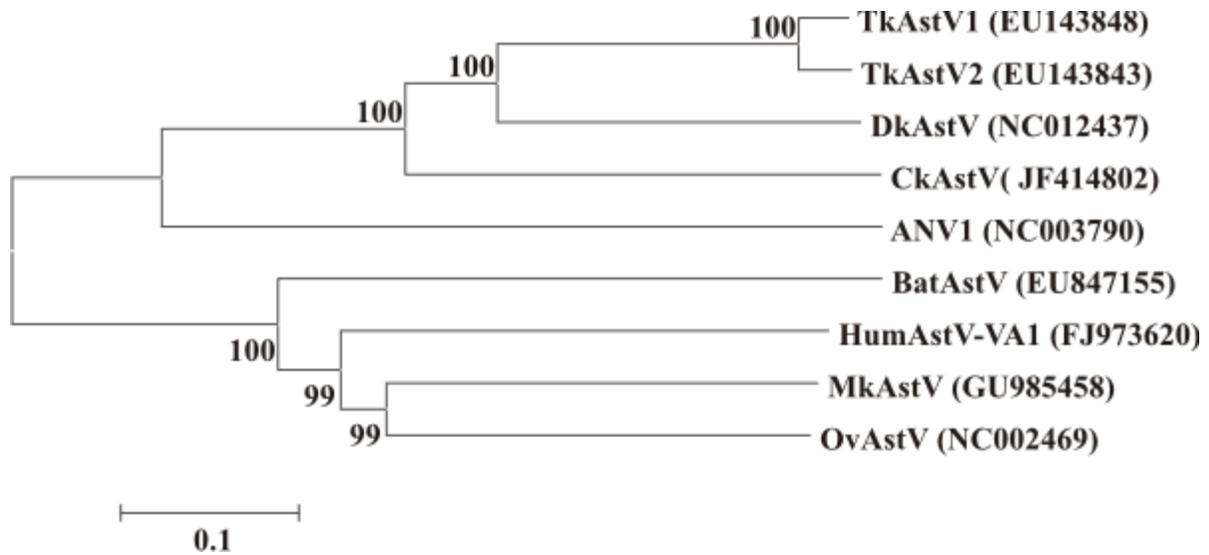


b

	<b>Chicken Astrovirus</b>
	GAGUGAAUGCCCUCC <b>AAAAAAC</b> UAGUGGAG
<b>RdRp</b>	<b>M</b> P S K K L V E
<b>NS polyprotein</b>	S E C P P K N *
	<b>Duck Astrovirus</b>
	UUGUUC <b>AAAAAAC</b> UAGAUGAGGGGCCUGAA
<b>RdRp</b>	K K L D E G P E
<b>NS polyprotein</b>	C S K N *
	<b>Turkey Astrovirus 1</b>
	AGUGCC <b>AAAAAAC</b> UAGUAGAGGGGCCUGU
<b>RdRp</b>	V P K K L V E G P V
<b>NS polyprotein</b>	E C E K N *
	<b>Turkey Astrovirus 2</b>
	AGAGUGUC <b>AAAAAAC</b> UAGUAGAGGGGCC
<b>RdRp</b>	V S K K L V E G P
<b>NS polyprotein</b>	E C Q K N *

c





**Figure 3.3.** The sequence of the new chicken astrovirus forms a new branch. The full length sequences of published full length sequences of turkey astrovirus (TkAstV) 1 and 2, duck astrovirus (DkAstV), chicken astrovirus (CkAstV), avian nephritis virus 1(ANV1), bat astrovirus (BatAstV), human astrovirus (HumAstV), mink astrovirus (MkAstV), and ovine astrovirus (OvAstV). The NCBI genbank accession number was shown in brackets. The phylogenetic tree of a neighbor-joining method is shown performed with 1000 replications. The bootstrap values are shown at the branch knobs.

CHAPTER 4

ISOLATION AND CHARACTERIZATION OF A CHICKEN ASTROVIRUS - AN  
ETIOLOGICAL AGENT OF RUNTING STUNTING SYNDROME IN BROILER  
CHICKENS<sup>3</sup>

---

<sup>3</sup> Kyung-il Kang , Alan H. Icard, Vijay Durairaj, Erich Linnemann, Holly S. Sellers and Egbert Mundt. To be submitted to PLoS Pathogens.

## **Abstract**

Despite descriptions of runting stunting syndrome (RSS) in broiler chickens dating back over 40 years, the etiology is unknown. A chicken astrovirus (CkAstV) from the gut content of chickens affected with runting stunting syndrome, characterized by retarded growth and cystic lesions in the small intestine of young chickens, has been isolated in the LMH liver cell line. The in vitro characterization showed that the virus replicated in cell culture to high titers while a large number of viral particles remained cell associated. Infection of susceptible one-day-old broiler chickens with the isolated virus revealed that the virus initially replicated in cells of the gut villi but later in cells of the crypt of Lieberkühn following infection. During the first passage in broiler chickens, the virus isolate did not induce retarded growth or cystic lesions in the small intestine. The serial chicken-to-chicken passage of the virus resulted in an increased virulence. Decreased weight gain due to retarded growth and presence of cystic lesions in the small intestine, using filtered material from the 5<sup>th</sup> chicken passage, showed that the isolated CkAstV caused typical signs of RSS in chickens. The data obtained indicate that the isolated virus has the potential to cause RSS in broiler chickens and can be regarded as an etiological agent of the disease. The analysis of the full length sequences of the isolated CkAstV and of the CkAstV obtained after the 5<sup>th</sup> chicken passage revealed only four exchanges in the amino acids of the two viral proteins. Whether or not the amino acid exchanges might have caused the change in phenotype needs to be analyzed by future experiments employing reverse genetics.

## **Introduction**

Astroviruses are small, round, non-enveloped viruses with a positive-sense, single-stranded RNA genome, and belong to the virus family, *Astroviridae*. Virus particles, 28-30 nm in

diameter, with a star-like shape, can be observed by electron microscopy (Madeley & Cosgrove, 1975). Astroviruses have been isolated from feces in a wide variety of animals (e.g. including humans, cats, cattle, deer, dogs, ducks, mice, pigs, sheep, mink, turkeys, chickens, bats, cheetahs, guinea fowl, rats and marine mammals) and the identification was mostly associated with gastroenteritis in young individuals (Bosch et al., 2011). In addition, a fatal hepatitis in ducklings was also associated with astroviruses (Fu et al, 2009). Astroviruses isolated from birds belong to the genus *Avastrovirus* and include viruses isolated mainly from chickens, turkeys, and ducks. Astroviruses, specifically, avian nephritis virus 1, isolated from chickens were initially grouped in the family *Picornaviridae*, but after determination of the full length genome sequence, designated as a member of the *Astroviridae* family (Imada et al, 2000). Based on sequence data, the existence of an avian nephritis virus 2 (Pantin-Jackwood et al, 2011), as well as, avian nephritis virus 3 have been reported (deWit et al., 2011). In addition to the chicken astroviruses, three subtypes of turkey astrovirus have been described. Recently, another astrovirus was described from the intestines of chickens (Kang et al, 2012b) affected with runting and stunting syndrome.

While numerous partial sequences of avian astroviruses are available in gene sequence repositories fewer full length sequences have been determined. The first full length nucleotide sequence of a chicken astrovirus was reported for ANV 1 (Imada et al, 2000). Two additional full length genome sequences for chicken astroviruses have since been deposited in Genbank (Kang et al, 2012b, Zhao et al, 2011). Also, full length sequences for avian astroviruses from ducks, pigeon and turkeys have been published.

The concept of the general genome organization of astroviruses, 5'-noncoding region-ORF1a/ORF1b-ORF2-3'-noncoding region-poly(a) tail, holds true, thus far, for all published full

length sequences. ORF 1a encodes for a protease containing a protease a 3C motif and ORF 1b encodes for the RNA-dependent RNA polymerase (RdRp) which was identified due to the presence of amino acid sequence motifs typical for nucleic acid polymerases (Carter and Willcocks, 1996.) ORF 2 is the coding sequence for the capsid protein likely translated from a subviral messenger RNA (Lewis et al, 1994, Monroe et al, 1993). The proposed replication mechanism involves a frameshift slippery sequence in the overlap region between ORF1a and ORF1b. It is thought that this leads to the synthesis of an ORF1a/1b fusion polyprotein (Jiang et al., 1993; Lewis et al., 1994; Lewis & Matsui, 1996). Kang et al. (2012b) recently proposed a different translation mechanism for a newly described chicken astrovirus where the translation initiation for RdRp occurs at the existing start codon of ORF1a.

The detection of chicken astroviruses in the intestines of chickens with or without disease has been frequently described (Day et al, 2007, de Wit et al, 2011, Pantin-Jackwood et al., 2011, Smyth et al, 2010, Canelli et al, 2012). The characterization of these viruses is mostly deterred by the fact that they can rarely be adapted to infect cell cultures. Recently a novel chicken astrovirus was reported in broiler chickens (Kang et al, 2012a). This virus replicated intensely in the duodenum, but to a very low extent in the kidneys. Baxendale and Mebatsion (2004) previously reported the isolation and characterization of another chicken astrovirus from broilers exhibiting RSS-like problems, but sequence data is very limited so determination of similarities or differences between the chicken astroviruses is not possible. Smyth et al (2012) described the comparison of 26 amino acid (aa) sequences of astroviruses tentatively named as chicken astroviruses to differentiate them from avian nephritis viruses. A relatively high diversity within the capsid sequences were found to exist between chicken astroviruses, but even more divergence was observed when compared to avian nephritis virus 1. Baxendale and Mebatsion

(2004) and de Wit et al. (2011) described the isolation of chicken astroviruses in cell culture and embryonated eggs, respectively. One chicken astrovirus caused mild clinical signs (Baxendale and Mebatsion, 2004) while de Wit et al (2011) described clinical signs and death after infection of SPF chickens. Thus, the ability to cause disease varies between the isolates and more research is needed to identify common markers for virulence.

The involvement of astroviruses in enteric diseases in mammals and birds has been described. An enteric disease of broiler chickens is runting stunting syndrome (RSS) and while its description dates back over 40 years, the etiology is unknown. RSS is characterized by retarded growth of young broiler chickens and is associated with a mild diarrhea. In clinical cases and experimental infections, consistent microscopic lesions were observed and characterized as a cystic enteropathy in the crypts of Lieberkühn located in the small intestine (Otto et al., 2006; Rebel et al, 2006, Nilli et al, 2007, Sellers et al., 2010). Based on the induction of clinical signs and microscopic lesions following administration of filtered gut content (Nilli et al, 2007, Sellers et al, 2010), a viral etiology is almost certain. Several viruses have been described as etiologic agents for RSS: rotavirus (Otto et al, 2006), chicken astrovirus (Baxendale and Mebatsion, 2004, Kang et al, 2012a), and parvovirus (Kisary et al, 1994).

Here we describe the isolation of a chicken astrovirus in cell culture and its full length sequence. The pathogenesis of this isolate was evaluated for its ability to induce clinical signs and microscopic lesions, compared to those described for RSS, in commercial broiler chickens.

## **Material and Methods**

### **Cells**

The following cell lines were used for isolation of a chicken astrovirus: Madin Darby canine kidney cells (MDCK, CRL-2285, ATCC, Manassas, VA), DF1, a chicken fibroblastoid cell line (CRL-12203, ATCC), Vero cells (CRL-1587; ATCC), and LMH, a chicken hepatocellular carcinoma epithelial cell line (CRL-2117, ATCC). The cells listed above were grown in Dulbecco's modified Eagles' medium with 4.5 g/liter glucose (DMEM-4.5; Thermo Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Mediatech, Manassas, VA). A quail muscle cell line (QM-7; RIE 466; Collection of Cell Lines in Veterinary Medicine [CCLV], Insel Riems, Germany) was also used and propagated in a mixture of equal parts of minimal essential medium (MEM; Invitrogen, Carlsbad, CA) with Earle's balanced salt solution and MEM with Hanks' balanced salt solution (Invitrogen, Carlsbad, CA), supplemented with 10% FBS. All cells, excluding the LMH cells were cultivated in a humidified incubator at 37°C with 5% CO<sub>2</sub>. LMH cells were cultivated in a humidified incubator at 39°C with 5% CO<sub>2</sub>. The insect cell line of *Spodoptera frugiperda* (Sf9; Invitrogen, Carlsbad, CA) was cultivated in serum-free SFX-Insect medium (Thermo Scientific, Waltham, MA) at 28°C.

### **Generation of a rabbit antiserum specific for the capsid protein of a new chicken astrovirus**

In order to generate necessary diagnostic tools for studies on the recently described chickens astrovirus (Sellers et al, 2010, Kang et al, 2012b), the recombinant capsid protein of a chicken astrovirus, expressed in a baculovirus system, was purified (Sellers et al, 2010) and used for immunization of a rabbit at the Polyclonal Antibody Production Service facility (University of Georgia, Athens, GA). The resulting serum was named r-anti-CkAstV serum.

To test the reactivity of the rabbit serum, r-anti-CkAstV, LMH cells grown in T25 tissue culture flasks were infected with the isolated chicken astrovirus, CkAstV-p5 (see below), at a multiplicity of infection (MOI) of 1. Noninfected LMH cells were used as a negative control. Three days after infection, the cells were trypsinized, resuspended in serum-containing DMEM-4.5 and sedimented at 700x g for 5 min. In addition, Sf 9 cells grown in T25 flask were either infected with the recombinant baculovirus encoding for the capsid protein of a chicken astrovirus (Seller et al, 2010) or left uninfected as a control. Five days after infection, the cells were scraped into the medium and sedimented at 700x g for 5 min. For all cell pellets obtained (Sf9 and LMH cells), supernatants were discarded and the cells were washed once in phosphate buffered saline (PBS). After a repeated centrifugation step, the cell pellet was resuspended first in 300 ml PBS and then 300 µl of 2x Laemmli buffer [4% sodium dodecylsulphate (SDS), 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 Tris HCL, Laemmli, 1970]. The lysate was heated at 95°C for 2 min, centrifuged for 5 min at 13,000x g and the supernatants were transferred into a 1.5 ml reaction tube. For Western blot analysis, the lysates were separated on an SDS-12% polyacrylamide gel and blotted onto a nitrocellulose membrane. After blocking with a 5% nonfat dry milk solution in TBST ( 150 mM NaCl, 10 mM Tris-HCl, 0.05% Tween 20, pH 8.0), the membrane was incubated with r-anti-CkAstV serum or an HRP-conjugated anti-6x His-tag monoclonal antibody (Genscript, Piscataway, NJ, USA). For the detection of r-anti-CkAstV antibody binding, goat anti-rabbit HRP-conjugated antibodies (Sigma-Aldrich, St Louis, MO, USA) were used. The binding of the antibodies was visualized using the chemiluminescent substrate, Immobilon Western (Millipore, Billerica, MA) and Gel Logic 2200 (Carestream Health, New Haven, CT).

### **Virus isolation from gut material of chickens affected with runting stunting syndrome**

The material used for virus isolation was the same as was used for the infection experiments described earlier (Sellers et al., 2010) where significant weight differences and clinical signs of RSS were observed. The starting material was prepared from homogenized gut material and stored at -80°C. Fifty milliliters of the homogenate was thawed and centrifuged at 4000x g at 4°C for 30 min to remove cellular debris and gut contents. The supernatant obtained was centrifuged again at 16,000x g at 4°C for 20 min and then treated with chloroform to further remove cellular components and enveloped viruses. The supernatant obtained after this treatment was filtered through a 0.45 micron syringe filter followed by filtration through a 0.22 micron syringe filter (Whatman, Florham Park, NJ, USA). The filtrate was incubated with chicken reovirus (ck-reovirus) and chicken rotavirus (ck-rotavirus) antiserum from chickens (Charles River SPAFAS, Wilmington, MA, USA) in a 1:1:1 ratio for 60 min at 37°C to neutralize the respective viruses. One milliliter of the final material was used for passage in cell cultures (MDCK, DF1, QM7, Vero, LMH) propagated in T25 cell culture flasks grown to 80% confluency and incubated for five days. The cell culture supernatant was obtained after centrifugation at 2000x g for 10 min, aliquoted, and stored at -80°C. One milliliter from each passage of each cell line was used for a subsequent passage up to passage 4. Cell cultures grown in 24-well tissue culture plates were inoculated with material obtained from passage 4. At several time points after inoculation (24h, 48h, 72h, 96 h), cells were fixed with ethanol, air dried then incubated with r-anti-CkAstV serum and goat anti-rabbit FITC-conjugated antibodies (Jackson Immunoresearch, West Grove, PA) for the detection of specific immunofluorescence. In parallel, the cell cultures were also incubated with ck-rotavirus antiserum or ck-reovirus antiserum followed by incubation with goat anti-chicken FITC-conjugated antibodies (Jackson

Immunoresearch, West Grove, PA). The immunofluorescence was evaluated using a Carl Zeiss Axiovert 40 CFL inverted microscope.

### **Determination of virus titers and growth kinetics in LMH cells**

A cell suspension of LMH cells, with a density of  $3 \times 10^5$  cells/ml, was prepared in DMEM-4.5 containing 10% FBS. In a 24 well tissue-culture-plate, 900  $\mu$ l of serum free DMEM medium was added to eight wells. In the first well, 100  $\mu$ l of the virus suspension was added (dilution  $10^{-1}$ ) followed by tenfold serial dilutions up to  $10^{-8}$ . One hundredmicroliter of the diluted virus from each dilution was added into each of four wells of a 96 well tissue-culture-plate along with 100  $\mu$ l of the LMH cell suspension ( $3 \times 10^5$  cells/ml) using a multichannel pipette. The 96 well tissue-culture plate was incubated for three day at 39°C. The supernatant was removed and cells were rinsed once with phosphate-buffered saline (PBS) and fixed with ice cold 96% ethanol for 10 min at room temperature. The cells were air-dried and incubated to a 1:100 dilution of r-anti-ckAstV serum for 30 min, rinsed three times with PBS and incubated with goat anti-rabbit FITC conjugated antibodies diluted 1:300. After a 30 min incubation, the cells were rinsed three times with PBS and overlaid with 50  $\mu$ l of an anti-fading solution containing 1.25 % (w/v) 1,4-diazabicyclo[2.2.2]octane (DABCO, Sigma-Aldrich, St Louis, MO, USA) in PBS. Tissue cultures were evaluated for specific fluorescence using a Carl Zeiss Axiovert 40 CFL inverted microscope. The viral titer was determined at the virus dilution where 50% of the tissue culture wells were positive for infectious virus ( $TCID_{50}/100 \mu$ l) as calculated by the method of Reed and Muench (1938).

Replication kinetics were evaluated in LMH cells grown in 24-well tissue culture plates and infected with a multiplicity of infection (MOI) of 1. To this end, the cells were infected with

250 µl of virus containing medium and incubated for 60 min at 39°C. Next, the virus containing supernatant was removed, cells rinsed once with serum-containing medium, and finally overlaid with 1 ml cell culture medium. At several time points after infection, cells were scraped into the supernatant, the suspension removed and stored at -80°C until determination of the TCID<sub>50</sub>. In further experiments, the ratio between cell associated virus and virus released into the supernatant was investigated. For this experiment, the cells were infected as described above, except that after the removal of the supernatant, 1 ml of cell culture medium was added and the cell culture plates were frozen and thawed three times. Both the cell culture supernatants and the medium obtained after the freeze/thaw cycles were stored at -80°C and the TCID<sub>50</sub> for each sample was determined.

### **Pathogenicity in chickens of the chicken astrovirus isolated in cell culture**

To determine the pathogenic potential of the fifth passage of the chicken astrovirus isolated in cell culture (CkAstV-p5), thirty-eight one-day-old chickens were distributed into three experimental groups: CkAstV-p5, RSS (positive control), and a negative control. The CkAstV-p5 (n=10) group was inoculated orally with 300 µl of CkAstV-p5 at 10<sup>6.3</sup> TCID<sub>50</sub>/ml. The RSS (n=10) group was inoculated orally with 300 µl of the RSS gut homogenate (Sellers et al, 2010, Kang et al, 2012a) to serve as the RSS positive control, and the chickens in the control group (n=10) were left untreated. All chicken experiments described were performed in HEPA filtered Horsfall-Bauer isolation units with forced air positive pressure. Water and feed was provided *ad libitum*. Five and 12 days after infection (pi), five birds were humanely euthanized. Each bird was weighed, and the duodenal loop was harvested, fixed in neutral buffered formalin and examined for microcopic lesions and by *in situ* hybridization.

Based on the results obtained from the first animal experiment, a short-term study was performed to monitor presence of virus, weight gain, and microcopic lesions in the duodenal loop during earlier time periods. Forty, one-day-old, chickens were inoculated orally with 300 µl of CkAstV-p5. Five chickens were randomly selected at 6, 12, 18, 24, 48, 72, 96, and 120 hours pi. At each time point, chickens were weighed, and the duodenal loop was taken for microscopic examination and *in situ* hybridization. An equal number of hatch mates were inoculated with 300 µl of cell culture media to serve as controls.

### **Serial passage of the cell culture isolated chicken astrovirus in chickens**

#### *Experiment 1*

The aim of this experiment was to investigate whether a change in pathotype and/or genotype of CkAstV-p5 occurred during serial passage in broiler chickens. To this end, one-day-old commercial broiler chickens were used, and ten chickens were placed per group. In the first passage experiment, chickens were orally inoculated with 300 µl of CkAstV-p5 at  $10^{6.3}$  TCID<sub>50</sub>/ml. One group of the hatch mates was inoculated with the cell culture media to serve as negative control. On day 5 pi, the birds were humanely euthanized and weighed individually. The duodenal loop was harvested, and a section of each tissue was fixed in neutral buffered formalin, and processed for microscopic examination. The remaining sections of the small intestines of each group were combined, mixed at a 1:1 ratio (v/v) with virus transport media [VTM, 500 ml of minimum essential medium (MT-15-010-CV, Mediatech, Manassas, VA) containing 0.15% sodium bicarbonate, 7.5 mM HEPES buffer, 250 µg/ml gentamicin, 1 µg/ml fungizone, and 1% heat inactivated horse serum] and homogenized with a sterile stainless steel blender. The resulting homogenate was centrifuged at 1500x g for 20 min at 4°C. The

supernatant (further designated as unfiltered gut homogenate) obtained was centrifuged a second time at 16000x g for 20 min at 4°C, followed by a sequential filtration through a 0.45 µm then a 0.22 µm filter (Whatman, Florham Park, NJ, USA). Aliquots were stored at -70°C until use for the next passage. Filtered homogenates from CkAstV-p5 chicken passages 2 through 5 were inoculated orally into chickens to make the next passage. Hatch mates at each passage were inoculated with cell culture media to serve as controls. The virus recovered from the fifth chicken passage was identified as CkAstV-p5-Ckp5. For passage 6, chickens were allocated into 5 groups. One group of chickens was inoculated orally with filtered homogenate from passage 5 negative control chickens, the second group of chickens was inoculated with unfiltered homogenate from the same passage 5 negative controls, the third group of chickens was inoculated with filtered homogenate from the CkAstV-p5-Ckp5 group and the fourth group of chickens was inoculated with unfiltered homogenate from the CkAstV-p5-Ckp5 group. The fifth group of chickens was inoculated with cell culture media to serve as negative controls. On day 5 pi, the same protocols were conducted as described for the initial passage.

### *Experiment 2*

The next passage experiment in chickens was performed to determine if the serial passage of gut material from negative controls and CkAstV-p5 would induce differences in weight gain. Again, ten one-day-old commercial broiler chickens were inoculated orally with 300 µl of CkAstV-p5. A second group of ten hatch mates was inoculated with the cell culture media to serve as negative control. At day 5 pi, duodenal loop homogenates from each of the passage 1 groups (i.e., CkAstV-p5-Ckp1 and negative control-p1) were prepared. For the second passage, four groups of chickens were placed and inoculated orally as follows: group 1: filtered gut homogenate from the negative control of passage 1, group 2: unfiltered gut homogenate from

the negative control of passage 1, group 3: filtered gut homogenate from CkAstV-p5-Ckp1) passage 1, and group 4: unfiltered gut homogenate from CkAstV-p5-Ckp1 passage 1. On day 5 pi of passage 2, the intestinal samples were collected from each group and processed by group treatment. In detail, intestines from birds inoculated with filtered homogenate were processed to obtain a filtered homogenate, while intestines from the unfiltered homogenate were prepared as unfiltered homogenate. Accordingly, continued passage of these four groups as described for the first passage was performed until passage 5. On the fifth day of each passage, the birds were euthanized and body weights measured. The scheme of the experiments is depicted in figure 4.5A.

#### **Detection of chicken astrovirus RNA by RT-PCR**

For each animal experiment, RT-PCR was performed in one-day-old hatchmates in parallel to investigate presence or absence of chicken astrovirus RNA. Homogenates from the duodenal loops of each group were incubated at 95°C for 10 min and subsequently used for RNA extraction with the Qiagen RNeasy plus mini kit (Qiagen, Valencia, CA). The SuperScript® One-Step RT-PCR with Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) was used following the manufacturer's instructions. Primers were designed to amplify a 428 nt cDNA of the capsid protein coding region using oligonucleotides ASTCAP-DIAFP (GATAAGGCTG GGCCGCAGAAGAAGAGG) and ASTCAP-DIARP (ACAAATTTAACAACACACC GCTG) that were delineated from the CkAstV sequence described in a previous study (NCBI Genbank accession number JF414802). The amplified products were separated on a 1.5 % gel.

## **Evaluation of the microscopic lesions**

The paraffin-embedded blocks were consecutively cut into 4  $\mu\text{m}$  thick sections for subsequent microscopic evaluation. Sections placed on regular glass slides were stained with hematoxylin and eosin (H&E) for light microscopic examination while sections for *in situ* hybridization were placed on Superfrost/Plus microscopic slides (Fisher Scientific). A portion of descending and ascending loops of the duodenum and the pancreas were prepared for microscopic evaluation. The crypt of Lieberkühn in the duodenum was evaluated, and the number of cystic lesions per section was counted in the both loops of duodenal sections. The location of ISH signals and the microscopic lesions were compared for each tissue using consecutively cut tissue sections.

## ***In situ* hybridization (ISH)**

The RNA probe for the CkAstV was generated as previously described (Kang *et al.*, 2011a). Tissue sections on unstained slides were first incubated at 70°C for 10 min and deparaffinized in Citrosolv<sup>TM</sup> (Fisher Scientific, Pittsburgh, PA, USA). Slides were thoroughly air-dried and tissue sections were rehydrated in 5 mM MgCl<sub>2</sub> in PBS for 10 min, rinsed in Tris-Glycine buffer (0.1 M glycine in 0.2 M Tris, pH 7.5) for 10 min at room temperature, and incubated with proteinase K (35  $\mu\text{g}/\text{ml}$ ) dissolved in proteinase K buffer (10 mM Tris, pH 7.5, 2 mM CaCl<sub>2</sub>) for 15 min at 37°C. The enzymatic reaction was stopped with the Tris-Glycine buffer. Pre-hybridization solution [5x saline-sodium citrate buffer (SSC) 0.75 M NaCl, 0.075 M sodium citrate) containing 50% formamide, 5% blocking reagent (Roche), 0.1% N-lauroylsarcosine and 0.02% sodium dodecyl sulphate (SDS)] was added to sections for 30 min at 42°C. Seventy microliters of the hybridization solution, which consisted of the pre-hybridization

solution containing the riboprobe (35 ng/μl), was applied directly onto the section and covered with a siliconized cover slip (HybriSlip™, Grade Bio-Labs). The hybridization was performed overnight at 42°C in a humidity chamber. The next day, cover slips were removed and the slides were washed as follows: once at 50°C in 2x SSC (0.3 M NaCl, 0.03 M sodium citrate) with 1% SDS followed by one wash at 50°C with 1x SSC (0.15 M NaCl, 0.015 M sodium citrate) with 0.1% SDS and once in 1x SSC at room temperature (RT) followed by one wash with 0.1x SSC (0.015 M NaCl, 0.0015 M sodium citrate) for 30 min each. After the washing steps, slides were treated in buffer I (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 10 min followed by incubation with a 300-fold dilution of sheep anti-digoxigenin alkaline phosphatase - conjugated Fab<sub>2</sub>' (Roche) in buffer I containing 1% FBS for 2 h at 37°C. After three washes with buffer I, the binding of the conjugate was visualized by adding a chromogen containing mixture [200 μl of NBT/BCIP stock solution (Roche) in 10 ml 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl]. The development of the signal was allowed to progress for 45 to 60 min and was stopped by rinsing the slides in distilled water. Slides were lightly counterstained with Gill's hematoxylin, coverslipped with Permount™ (Fisher Scientific) and evaluated under a light microscope.

### **Determination of the full length sequences of the isolated chicken astrovirus before and after passage in chickens**

For the determination of the full length sequence of the chicken astrovirus isolate, either the cell culture supernatant from passage 5 (CkAstV-p5) or filtered gut material obtained after five consecutive passages of CkAstVp5 in chickens (CkAstV-p5-Ckp5) was used. Either cell culture supernatant (CkAstVp5) or gut material (CkAstVp5-Ckp5) was centrifuged, filtered and chloroform treated, as described above, and used for RNA isolation using High-Pure-RNA-

Isolation-Kit (Roche, Diagnostics GmbH, Mannheim, Germany). Based on the sequence obtained for a new chicken astrovirus from gut samples of RSS-affected chickens (Kang et al, 2012b), several pairs of oligonucleotides were delineated to amplify approximately 800 bp cDNA fragments. The primer sequences can be obtained from the corresponding author upon request. RT-PCR was performed using the SuperScript™ One-Step RT-PCR with Platinum® *Taq* (Invitrogen, Carlsbad, CA). The extreme 5'-end of the virus genome was determined using the 5' RACE System, Version 2.0 (Invitrogen, Carlsbad, CA, USA) as previously described (Kang et al, 2012b). The cDNA fragments obtained were separated on a 1% agarose gel, purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into the pCR2.1 Topo TA plasmid using the Topo TA cloning kit (Invitrogen). Three recombinant plasmids for each cDNA fragment were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) in both directions to obtain a six-fold coverage for each nucleotide.

### **Multiple alignment and sequence analysis**

Sequence data was analyzed using the DNASTar Lasergene 8 software package (DNASTAR Inc, Madison, WI, USA) for multiple alignments and *in silico* translation.

### **Data analysis**

Mean body weights between groups were compared by SigmaStat® (SigmaStat for Windows, Jandel Scientific, San Rafael, CA). Differences between two groups were conducted by t-test, and results between more than two groups were analyzed using one-way analysis of variance followed by Fisher LSD for all pairwise multiple comparison.

## **Results**

### **Isolation of an astrovirus from gut material from RSS-affected chickens**

Based on the findings that a new chicken astrovirus might play a role in the etiology of RSS (Sellers et al, 2010, Kang et al, 2012a, Kang et al, 2012b), experiments were performed to isolate the astrovirus in several cell lines since isolation of viruses which replicate mainly in the gut is still not very well established for many disease causing agents. The presence of reoviruses and rotaviruses were expected in the gut of chickens, thus the gut filtrate was incubated with virus specific sera to neutralize both viruses. To determine if the sera used to neutralize the chicken reoviruses and rotaviruses contained antibodies capable of neutralizing the new astrovirus, an ELISA was performed as previously described (Sellers et al., 2010) using the recombinant capsid protein of the chicken astrovirus as antigen. The investigated serum samples had OD values below the threshold of 0.2 and were regarded as negative for antibodies to the recombinant chicken astrovirus antigen. The inoculated cells were examined daily for the presence of a cytopathic effect (CPE) as compared to appropriate negative control cells. Supernatants from each passage were stored at -80°C. Only inoculated LMH cells showed CPE beginning at passage three. A CPE became visible after 72 h and was characterized by small round cells. Forty eight hours later, the CPE was 100%. A subsequent fourth passage was performed in a T175 tissue culture flask of cultured LMH cells. The supernatant obtained served as the virus stock, was aliquoted and stored at -80°C (CkAstV-p4). One 100 µl aliquot was used for a fifth passage in a T175 tissue culture flask containing LMH cells. Five days after infection, the cells were frozen overnight at -80°C, thawed and then centrifuged at 2000x g for 10 min. The supernatant was filtered through a 450 nm syringe filter, aliquoted and stored at -80°C. This

virus stock (CkAstV-p5) served as inoculation material for subsequent experiments. To characterize the CPE-causing agent, LMH cells cultured in 8-well-chamber slides were infected with a 1:100 dilution of the supernatant and fixed with ethanol 24 hours after infection for indirect immunofluorescence using r-anti-CkAstV serum, ck-anti-reovirus serum, and ck-anti-rotavirus serum along with the appropriate FITC labeled species-specific conjugates. Uninfected cells were used as negative controls. The immunofluorescence was only positive when the infected cells were incubated with the r-anti-CkAstV serum (Figure 4.1A). The uninfected negative controls, as well as infected cells incubated with the ck-anti-reovirus and ck-anti-rotavirus serum, did not show specific fluorescence (data not shown). In order to determine whether the CPE-causing agent was indeed a chicken astrovirus, 100  $\mu$ l of the original supernatant as well as, ten-fold dilutions of the supernatant up to  $10^{-8}$ , were incubated in a virus neutralization experiment with either the r-anti-CkAstV serum or with serum obtained from the same rabbit prior to immunization for 60 min at 37°C. Each sample was inoculated into a T25 tissue culture flask containing LMH cells and incubated for five days and followed by two subsequent passages. CPE was observed in all tissue culture flasks up to a dilution of  $10^{-6}$  in flasks containing the diluted virus incubated with the pre-immune rabbit serum. In contrast, virus dilutions  $10^{-4}$  up to  $10^{-8}$  incubated with r-anti-CkAstV serum showed no CPE and were also negative in an indirect immunofluorescence assay, using the r-anti-CkAstV serum, subsequently performed, indicating a specific neutralization. In order to support the assumption that indeed only a chicken astrovirus was present in the preparation, several PCRs and RT-PCRs specific for chicken reovirus, chicken rotavirus, infectious bursal disease virus, Newcastle disease virus, avian encephalomyelitis virus, infectious bronchitis virus, chicken adenovirus, reticuloendotheliosis virus, chicken anemia virus, Marek's disease virus, infectious

laryngotracheitis virus and fowlpox virus were performed along with appropriate positive controls for each virus. The investigations were performed at the diagnostic virology laboratory at the Poultry Diagnostic and Research Center (College of Veterinary Medicine, University of Georgia, Athens, GA, USA) using primer pairs designed to detect a broad range of subtypes within each virus analyzed. In addition, oligonucleotides were used for RT-PCR previously described for the amplification of cDNA for the ANV1, ANV2, and chicken astrovirus riboprobes or PCR for a chicken parvovirus (Kang et al, 2012a). Only the primer pair CAstVpr-FP/CAstVpr-FP, specific for the chicken astrovirus, revealed a cDNA fragment of the appropriate size indicating that only the chicken astrovirus was likely present in the cell culture supernatant after virus isolation.

In addition, Western blot analysis was performed for two purposes 1) to test if the rabbit serum (r-anti-CkAstV) recognized other viral antigens possibly present in CkAstV-p5 infected LMH cells and 2) to test for recognition of the capsid protein of CkAstV-p5 in infected LMH cells with the appropriate size of the calculated molecular weight of 98 kDa (Figure 4.1B). The results showed that the only protein detected by the rabbit serum had an apparent molecular weight of approximately 95-100 kDa which was in the expected range. Since it was the only protein detected in the LMH cells, it was likely that the rabbit serum was specific in the immunofluorescent detection of CkAstV-p5 infected LMH cells. In addition, the single protein detected in infected LMH cells was the same size as the recombinant capsid protein from infected Sf9 cells. The results from the Western blot and immunofluorescence assay along with the results of molecular detection methods (RT-PCR, PCR) are strong indicators that r-anti-CkAstV recognized only one virus and that this virus is indeed a chicken astrovirus.

### **Characterization of the chicken astrovirus in cell culture**

The 5<sup>th</sup> passage of the chicken astrovirus (CkAstV-p5) was determined to be  $10^{5.3}$  TCID<sub>50</sub>/100  $\mu$ l. Based on this titer, growth kinetics were performed using  $10^2$  TCID<sub>50</sub> per well. The data showed that virus titer increased at 48 h after infection and reached the highest titer at 120 h after infection which remained at this level until the end of the study (Figure 4.1C). Furthermore, the cell association of the virus was investigated (Figure 4.1D). The analysis indicated that one hour after adding the virus to the cell culture (time point 0 h after infection), the virus was primarily associated with the cells, whereas, only a few infectious viruses were present in the supernatant. This was followed by the viral eclipse since intracellular and extracellular virus was almost undetectable. The analysis of the following time points revealed a constant increase in viral titers in both cells and supernatants. Based on these results, it appears that one replication cycle was complete between 12 h and 24 h after infection. Furthermore, it became clear that at least 50% of the newly produced viruses remained cell associated and might be of interest when a vaccine is produced.

### **The chicken astrovirus isolate replicated in the duodenum of infected broiler chickens**

The next sets of experiments were designed to determine whether or not the virus was able to infect broiler chickens. Broiler chickens have been used as a model for RSS (Sellers et al, 2010, Kang et al, 2012a, Kang et al, 2012b) and served as the model for the astrovirus infection studies. Chickens were infected with either CkAstV-p5, gut content from RSS-affected chickens (RSS, positive control), or left untreated (control). Five days and 12 days after infection, five chickens from each group were humanely euthanized. Body weights, presence of cystic lesions and presence of viral RNA in the duodenum was evaluated (Figure 4.2). The analysis of the body

weights revealed only the chickens inoculated with the gut content from RSS-affected chickens showed a significant weight difference at day 5 and 12 after infection (Figure 4.2A). Similar results were obtained when the presence of cystic lesions in the crypt region of the duodenal loop were evaluated (Figure 4.2B). On day 5 p.i., one cystic lesion was observed in one chicken from the control group and similarly on day 12 from one chicken in the CkAstV-p5 infected group. In contrast, four out of five chickens at day 5 p.i. and one chicken at day 12 p.i. showed cystic lesions after infection with the RSS material. The presence and quantity of chicken astrovirus RNA was investigated by ISH (Figure 4.2D). Interestingly, RNA was detected in both groups of infected chickens only at day 5 p.i. and to a similar extent but in none of the control chickens at any time point. Surprisingly, the majority of RNA positive cells were in crypt epithelial cells in both RSS and CkAstV inoculated groups. However, a few scattered signals specific for the presence of RNA were also observed along the villi, regardless of the material used for infection. The estimated ratio of ISH signals between crypt epithelial cells and gut villi was 95% and 5%, respectively, and indicated that a chicken astrovirus replicated in the crypt region in both groups (Figure 4.2C). This result was confusing since cystic lesions in a significant number of chickens at day 5 p.i. were only observed in the RSS group but not in the group of chickens infected with the chicken astrovirus alone. Thus the link between replication of the investigated CkAstV-p5 and cystic lesions in the duodenum, as observed in RSS-affected chickens, was not obvious at this time point and additional experiments needed to be performed.

#### **Location of chicken astrovirus RNA changed in the gut during time of infection.**

Prompted from results obtained in the previous experiment, the presence of chicken astrovirus replication was investigated during the first five days after infection (Figure 4.3).

Mean body weights showed that significant differences were only observed at 72 h p.i. ( $p < 0.05$ ) which is not sufficient to claim a difference in weight gain during the course of the study (Figure 4.3A). Cystic lesions in the crypt region were observed in the duodenum of one chicken in the control group in one bird each at 18 h and 24 h pi. In the group of chickens infected with the CkAstV-p5, one chicken at 72 h p.i. showed two cystic lesions in the duodenal crypt region. More interesting was the dynamic of the presence of chicken astrovirus RNA during the course of the experiments (Figure 4.3B and C). During the first 12 h p.i., viral RNA was exclusively detected in cells located within the villi. The location of staining changed at 18 h p.i. onwards where initially some ISH signals were still detected in cells located within the villi as well as in the crypt region. Within 48 h after infection, viral RNA was exclusively observed in the crypt epithelial cells. In addition, ISH signals were detected in the dilated cystic lesions at 72 h p.i.. Although the association between cystic lesions and presence of virus replication was not observed in every inoculated birds, there is likely a connection between both events and needs to be investigated.

#### **Back passage of chicken astrovirus isolate in chickens induced weight depression and cystic lesions in the crypt region.**

Based on the assumption that the isolated virus was attenuated during cell culture passage, an experiment was performed by passing the chicken astrovirus isolate in broiler chickens using filtered material for the consecutive passages (Figure 4.4). The differences in weight gain between control chickens and infected chickens were not significantly different during passage 1, 2, and 3 (Figure 4.4A). However, body weights obtained during passage 4 were significantly different ( $p < 0.05$ ) and became even more pronounced during passage 5 with a

17% difference in weight ( $p < 0.001$ ). To investigate the role of bacteria in differences between filtered and unfiltered gut material, a 6<sup>th</sup> passage was made from passage 5. Significant differences were observed in both groups infected with the material containing the chicken astrovirus, regardless whether the material was filtered or not, indicating that the chicken astrovirus probably played a major role for the phenotype observed (Figure 4.4B). The presence of cystic lesions in the duodenum along with the presence of viral RNA as indicated by the presence of ISH signals was evaluated on day 5 (Figure 4.4C). Viral RNA was detected in tissues from infected chickens at all passage levels; however not in every infected chicken. This is in agreement with the previous experiment evaluating the presence of RNA sequentially during the first five days chickens (see figure 4.3B). The number of chickens showing lesions also increased during passaging from 2 out of 10 in the 1<sup>st</sup> passage to 4 out of 10 (5<sup>th</sup> passage) and 6 out of 10 (6<sup>th</sup> passage). The presence of lesions observed during the 6<sup>th</sup> passage in the group that received filtered gut content from the 5<sup>th</sup> passage of control chickens is likely unrelated to the chicken astrovirus since no ISH signal was observed in all three group representing the controls (uninfected chickens from the negative controls and chickens inoculated with either filtered or unfiltered gut content from the 5<sup>th</sup> passage control chickens). In addition, the presence or absence of infectious virus was evaluated in the gut of infected chickens. The RT-PCR for chicken astrovirus RNA was negative for all chickens in the negative control groups, as well as, the three groups representing the negative controls during the 6<sup>th</sup> passage. The gut content from the infected chickens was positive by RT-PCR in all groups infected with virus-containing gut content while the negative controls remained negative. From each passage, the gut content was filtered and the TCID<sub>50</sub> was determined (Figure 4.4D). The determination can only serve as an estimation since there was variation between the gut samples due to the sampling during

necropsy. The TCID<sub>50</sub> of CkAstVp5 used for infection was 10<sup>6.3</sup>/ml. The titer in the gut samples was between 10<sup>3.8</sup>/ml (passage 2) to 10<sup>5.9</sup>/ml (passage 6). Since the determination of the virus titer was performed by indirect immunofluorescence, it can be concluded that indeed the chicken astrovirus was quantitated. Cystic lesions in the crypt region of the duodenal loop (Figure 4.4E) in chickens infected with the chicken astrovirus and in subsequent passages showed a similar pattern as observed after infection with non-filtered gut material (Sellers et al, 2010).

### **Differences in weight gain were independent of bacterial presence during passage of the chicken astrovirus**

The previous experiment showed that the passage of chicken astrovirus caused differences in weight gain. The next experiment was designed to determine whether or not the cause of the weight differences were due to other agents present in the gut of the broiler chickens since the broiler chickens came from a commercial source. To obtain a deeper insight, the gut contents of control chickens inoculated with cell culture medium and chickens infected with CkAstV-p5 were passaged in parallel as filtered and unfiltered gut contents (see also Figure 4.5A). The weights were measured at day 5 pi and the results are summarized in figure 4.5B. A significant difference was observed between chickens infected with Ck-AstVp5 and control chickens ( $p < 0.05$ ) as early as passage 1. During the second passage, a similar picture was observed although chickens belonging to the control group (inoculated with unfiltered gut content) also showed a decreased weight. Environmental monitoring of the isolation units revealed a drop in temperature occurred during the second night of the experiment and can be traced back to the weight suppression observed. During the remaining passages (passage 3 -5), in the absence of temperature variation, a significant difference in body weight was observed

between control and CkAstV-p5 passaged groups. No significant differences were observed within groups of the control chickens and groups of CkAstV-p5 infected chickens, regardless of whether the gut content was filtered or not. Also the presence of chicken astrovirus RNA, as determined by RT-PCR, was detected only in chicken astrovirus passaged groups. The viral titers were also determined and ranged between  $10^{4.5}$  TCID<sub>50</sub>/ml (passage 2, unfiltered group) and  $10^{5.75}$  TCID<sub>50</sub>/ml (passage 5, unfiltered group), thus a relatively uniform range of virus titers.

### **Analysis of the nucleotide as amino acid sequences**

The nucleotide, as well as amino acid sequences of a new chicken astrovirus present in the gut of RSS-infected chickens (CkAstV-Gut) were recently published (Kang et al, 2012b). The sequence of this virus served as the basis for the full length sequence determination for the virus isolated in cell culture (CkAstV-p5) following the fifth passage in chickens (CkAstV-p5-Ckp5). The nucleotide sequences of CkAstV-p5 and CkAstV-p5-Ckp5 were determined to be the same length (7499 nucleotides without poly-A tail sequence) and were 21 nucleotides shorter than the nucleotide sequence of the original CkAstV-Gut. The 21 nucleotide difference was caused by a deletion of six amino acids within the coding region of the capsid protein (ORF2) and a six nucleotide deletion in the 3'-noncoding region (Figure 4.6A-C). In addition, within the ORF2 coding region of CkAstV-Gut, one amino acid was deleted compared to CkAstV-p5 and CkAstV-p5-Ckp5. The overall homology of the nucleotide sequences between CkAstV-Gut and CkAstV-p5 was 88.2%, while the homology between CkAstV-p5 and CkAstV-p5-Ckp5 was 99.8%. The analysis of the aa sequences of the single ORFs (ORF1a encodes for the nonstructural polyprotein, ORF1b encodes for the RNA dependent RNA polymerase, ORF2 encodes for the capsid protein) are shown in figure 4.6. The highest number of exchanges

between CkAstV-p5 and CkAstV-Gut was observed in ORF2 (113 aa), followed by the ORF1a (35 aa) and ORF1b (9 aa) which resulted in a homology of 84.8%, 96.9%, and 98.3% given the total length of 743 aa (ORF2), 1139 aa (ORF1a), and 519 aa (ORF1b) respectively. Interestingly, most amino acid exchanges within the ORF2 encoding for the capsid protein were observed in the C-terminal third of the protein. Furthermore, the exchanges between CkAstV-p5 and CkAstV-p5-Ckp5 were analyzed. As previously mentioned, only a few nucleotide exchanges occurred during the passage and resulted in an almost identical nucleotide sequence with a homology of 99.8%. The comparison of the aa sequences revealed three aa exchanges in ORF1a (A6V, V45A, S50T) and one aa exchange in ORF2 (F371Y) which indicated a stable virus during passage from chicken-to-chicken (Figure 4.6D). Interestingly, two of the aa exchanges observed in ORF1a (V45A, S50T) and the one aa exchange in ORF2 (F371Y) were amino acids present in the aa sequence of the CkAstV-Gut.

## **Discussion**

The isolation of a chicken virus in an *in vitro* system, such as embryonated eggs or cell culture, depends on many factors and cannot be predictable. The advantage of using cell culture is that verification of virus replication can be observed by cytopathic effect or, in the absence of cytopathic effect, confirmed by other tests when appropriate diagnostic tools are available. However, few diagnostic tools for detection of avian enteric viruses are readily available. As shown in our study, the isolated CkAstV-p5 infected and subsequently replicated only in one cell line evaluated, the liver derived LMH cell. The propagation of CkAstV in LMH cells was previously described (Baxendale and Mebatsion, 2004, Smyth et al, 2012, de Wit, 2011) and seemed to be a suitable host cell for isolation of CkAstV from intestinal contents. In contrast to

isolation of astrovirus from bovine, swine and some human origin samples (Aroonprasert et al., 1989, Taylor et al, 1997, Indik et al., 2006), trypsin was not necessary for isolation of the CkAstV described here. Isolation of some human astroviruses, from clinical samples, has been successful in the absence of trypsin (Taylor et al, 1997). Production of a recombinant protein targeting a structural viral protein allowed the generation of a virus specific antiserum in a heterologous host and a means of direct identification of the targeted virus. The availability of the rabbit serum (r-anti-CkAstV), previously generated, was very helpful for the detection of the CkAstV-p5 by immunofluorescence. The serum was generated against a recombinant capsid protein of CkAstV in an SPF rabbit (Sellers et al., 2010) and thus cross reactivity with other chicken pathogens was unlikely. Verification of the specific reactivity was confirmed by Western blot of CkAstV-p5 infected LMH cells and a single band, representing the capsid protein of CkAstV-p5, was observed. In addition, the serum was also able to neutralize 100 TCID<sub>50</sub> of the CkAstV-p5 up to a dilution of 2<sup>-13</sup> (data not shown), indicating its specificity for the isolated virus. It would be interesting to determine whether or not the r-anti-CkAstV serum was also able to neutralize another CkAstV, isolated in LMH cells, (Baxendale and Mebatsion, 2004, Smyth et al, 2012, de Wit, 2011) to determine the antigenic relatedness between the different CkAstVs. Replication kinetics of CkAstV-p5 in LMH cells showed that infectious virus was not efficiently released into the cell culture supernatant since more virus particles remained cell associated compared to virus present in the supernatant. This would be an important factor to consider for virus production if the virus would become a candidate for a vaccine.

Initial infection experiments in chickens resulted in the absence of clinical signs and no significant cystic lesions in the crypts of Lieberkühn region. However, the virus replication was detected by ISH in the crypt region. Since this crypt epithelial cells are thought to migrate toward

intestinal villi, the dynamics of virus replication in the gut was investigated. The virus was first detected in the villi region of the duodenum, then in both villi and crypt regions, and later only in the crypt region. This finding indicates that initially, cells of the villi region were susceptible but became refractory to infection with CkAstV-p5. The virus was able to spread to and replicate in the crypt region. This may be one explanation for the ability of the virus to replicate in cell culture in the absence of trypsin. The absence of cystic lesions and the presence of virus replication in the crypt region of the duodenum was puzzling. It appeared as though the virus was attenuated after virus isolation in cell culture and the serial passage in chickens may reverse the attenuated phenotype. Indeed, the experiments showed that initial infection caused neither significant numbers of cystic lesions, nor significant differences in weight between infected broiler chickens and the controls. However, following five passages, both cystic lesions and a significant difference in weight was observed. Passage of both bacterial/viral and only viral material for a 6<sup>th</sup> passage proved the difference was indeed caused by the viral load. In both cases, the phenotype was reproduced, thus the role of bacterial microflora in the chicks was not necessary. Data obtained from the serial passaged gut content, from both negative controls and CkAst-p5 infected chickens, supported the finding that indeed the isolated CkAstV-p5 might be at least one causative agent for RSS. Since the CkAstV-p5 isolate was tested by both molecular (PCR, RT-PCR), as well as, serological tests for a number of chicken pathogens and found to be negative, it is likely that the virus described here is a pathogen capable of inducing RSS. Although different viruses associated with RSS (Otto et al, 2006, Bányai et al, 2011, Zsak et al, 2008, Palade et al, 2011) have been described, virus isolation of the appropriate virus and reproduction of the clinical RSS and cystic lesions in the duodenum has not been shown. Indirect evidence that the CkAstV-p5 might be a primary causative agent was provided by Sellers et al.,

(2010) when vaccination of broiler breeders with the recombinant capsid protein, of a closely related CkAstV, mitigated the outcome of RSS in the offspring, likely due to the presence of CkAstV-specific antibodies.

Interestingly, when the nucleotide and amino acid sequences of CkAstV-p5 were compared to the recently published CkAstV (Kang et al, 2011b), analysis revealed that the two viruses were not the same virus even though the source for both viruses was the same. This indicated that at least two different CkAstVs were present in the original sample. Interestingly, the r-anti-CkAstV serum neutralized the CkAstV-p5 isolate even though the amino acid sequence of the recombinant protein was based on the sequence for the chicken astrovirus from the gut. This indicated that the neutralizing antibody-inducing epitopes are either located primarily in the N-terminal region of the capsid protein, where the highest homology was observed, or that the neutralizing antibody-inducing epitopes were still present even though a divergence between the capsid proteins of both viruses was identified. Other findings were also very interesting. The cell culture adapted CkAstV-p5 contained several deletions in the capsid encoding sequence compared to the sequence of the CkAstV from the gut. Also, the 3'-noncoding region of CkAstV-p5 was six nucleotides shorter. Whether these deletions influence the replication of CkAstV-p5 in cell culture needs to be elucidated by reverse genetics (see below). A similar phenotype was described for a human astrovirus where a 45 nucleotide deletion within the viral genome was also observed after adaptation to cell culture. In addition, during the back passage of CkAstV-p5 in broiler chickens, amino acid sequences reverted from the sequence observed in the CkAstV-p5 (ORF 1a: V45, S50; ORF2: F371) to those observed in the astrovirus sequences obtained from the gut samples (ORF 1a: A45, T50; ORF2: Y371). Since these were almost the only aa exchanges observed during back passage of Ck-AstV-p5, it will be

interesting to determine whether or not aa exchanges influence the disease outcome, since clinical signs of RSS were reproduced during the back passage of the virus. To this end, a reverse genetics system needs to be established for CkAstV, as previously described (Imada et al, 2000) for ANV1, to analyze the importance of the differences observed between the each virus and improve our biological understanding of CkAstV.

**Acknowledgement.** This study was supported by the Office of Vice President for Research (University of Georgia), the Georgia Poultry Federation, the Georgia Research Alliance, and US Poultry and Egg Association.

## References

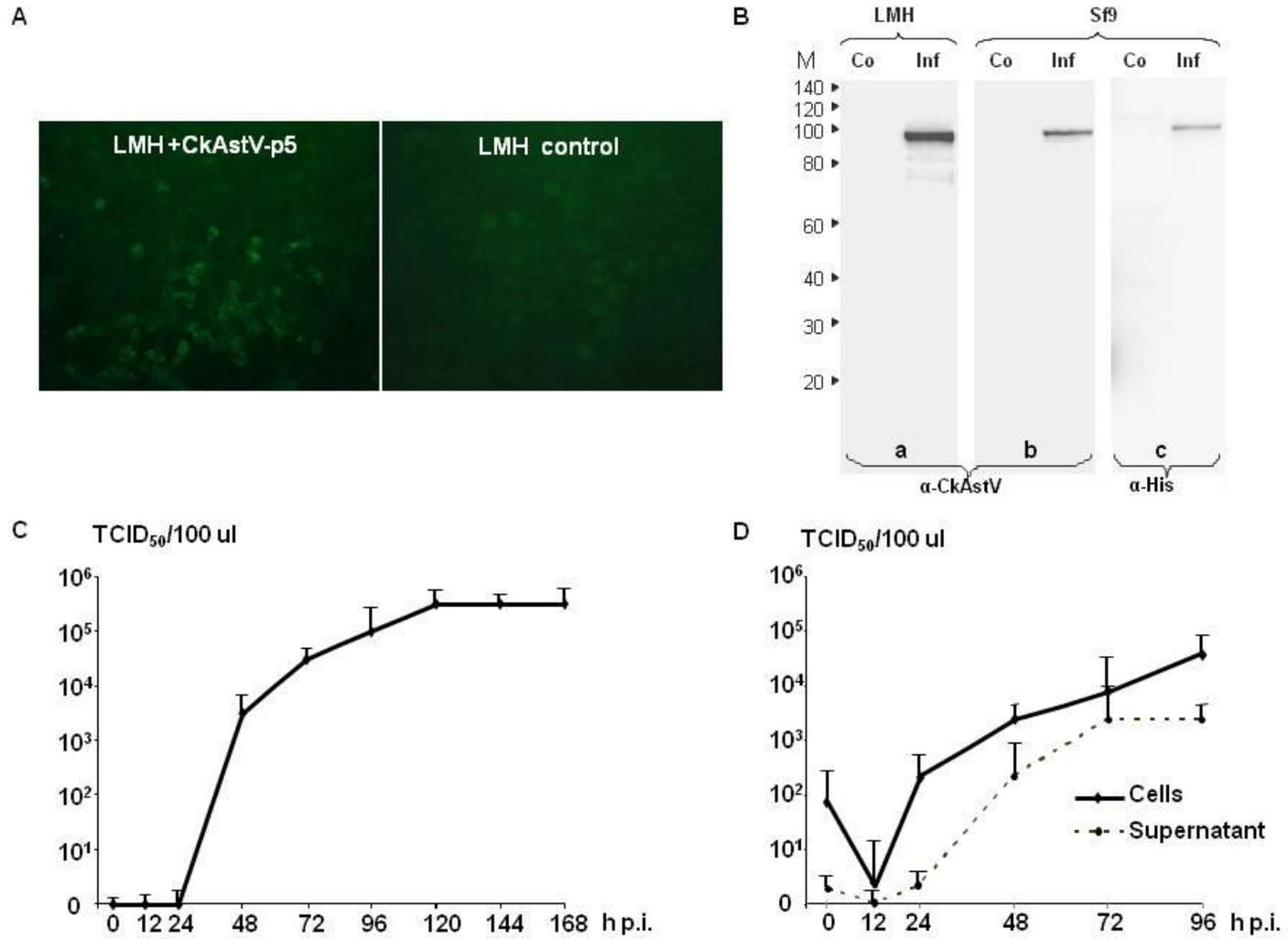
- Aroonprasert D, Fagerland JA, Kelso NE, Zheng S, Woode GN. (1989) Cultivation and partial characterization of bovine astrovirus. *Vet Microbiol.* Feb;19(2):113-25.
- Bányai K, Dandár E, Dorsey KM, Mató T, Palya V. (2011). The genomic constellation of a novel avian orthoreovirus strain associated with runting-stunting syndrome in broilers. *Virus Genes.* 42:82-89.
- Baxendale W, and Mebatsion T. (2004). The isolation and characterisation of astroviruses from chickens. *Avian Pathol.* 33:364-70.
- Bosch, A., Guix, S., Krishna, N.K., Méndez, E., Monroe, S.S., Pantin-Jackwood, M. and Schultz-Cherry, S. (2011). *Astroviridae*. pp 953-959 In: *Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses.* Ed: King, A.M.Q., Adams, M.J., Carstens, E.B. and Lefkowitz, E.J. San Diego: Elsevier.
- Canelli E, Cordioli P, Barbieri I, Catella A, Pennelli D, Ceruti R, Moreno A, Lavazza A. (2012). Astroviruses as causative agents of poultry enteritis: genetic characterization and longitudinal studies on field conditions. *Avian Dis.* 56:173-182.
- Carter, M. J., and M. M. Willcocks. 1996. The molecular biology of astroviruses. *Arch. Virol. Suppl.* 12:277–285.

- Day JM, Spackman E, Pantin-Jackwood M. (2007). A multiplex RT-PCR test for the differential identification of turkey astrovirus type 1, turkey astrovirus type 2, chicken astrovirus, avian nephritis virus, and avian rotavirus. *Avian Dis.* 51:681-684.
- de Wit JJ, Dam GB, de Laar JM, Biermann Y, Verstegen I, Edens F, Schrier CC. (2011). Detection and characterization of a new astrovirus in chicken and turkeys with enteric and locomotion disorders. *Avian Pathol.* 40:453-461.
- Fu Y, Pan M, Wang X, Xu Y, Xie X, Knowles NJ, Yang H, Zhang D. (2009). Complete sequence of a duck astrovirus associated with fatal hepatitis in ducklings. *J Gen Virol.* 90:1104-1108.
- Imada T, Yamaguchi S, Mase M, Tsukamoto K, Kubo M, Morooka A. (2000). Avian nephritis virus (ANV) as a new member of the family Astroviridae and construction of infectious ANV cDNA. *J Virol.* 74:8487-8493.
- Indik S, Valíček L, Smíd B, Dvoráková H, Rodák L. (2006). Isolation and partial characterization of a novel porcine astrovirus. *Vet Microbiol.* 117:276-283.
- Jiang, B., S.S. Monroe, E.V. Koonin, S.E. Stine, R.I. Glass (1993) *Proc. Natl. Acad. Sci. USA* 90, 10539-10543.
- Kang KI, El-Gazzar M, Sellers HS, Dorea F, Williams SM, Kim T, Collett S, Mundt E. (2012a). Investigation into the aetiology of runting and stunting syndrome in chickens. *Avian Pathology*, 41, 41-50.
- Kang KI, Icard AH, Linnemann E, Sellers HS, Mundt E. (2012b). Determination of the full length sequence of a chicken astrovirus suggests a different replication mechanism. *Virus Genes.* 44:45-50.
- Kisary, J., Nagy, B., & Bitay, Z. (1984). Presence of parvoviruses in the intestine of chickens showing stunting syndrome. *Avian Pathology*, 13, 339-343.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
- Lewis, T. L., H. B. Greenberg, J. E. Herrmann, L. S. Smith, and S. M. Matsui. (1994). Analysis of astrovirus serotype 1 RNA, identification of the viral RNA-dependent RNA polymerase motif, and expression of a viral structural protein. *J. Virol.* 68:77-83.
- Lewis, T. L. & Matsui, S. M. (1996). Astrovirus ribosomal frameshifting in an infection-transfection transient expression system. *J Virol* 70, 2869-2875.
- Madeley, C.R. & Cosgrove, B.P. (1975). Letter: 28 nm particles in faeces in infantile gastroenteritis. *Lancet*, 2, 451-452.

- Monroe, S. S., B. Jiang, S. E. Stine, M. Koopmans, and R. I. Glass. 1993. Subgenomic RNA sequence of human astrovirus supports classification of Astroviridae as a new family of RNA viruses. *J. Virol.* 67:3611–3614.
- Nili, H., Jahantigh, M. & Nazifi, S. (2007). Clinical observation, pathology, and serum biochemical changes in infectious stunting syndrome of broiler chickens. *Comparative Clinical Pathology*, 16, 161-166.
- Otto, P., Liebler-Tenorio, E.M., Elschner, M., Reetz, J., Lohren, U. & Diller, R. (2006). Detection of rotaviruses and intestinal lesions in broiler chicks from flocks with runting and stunting syndrome (RSS). *Avian Diseases*, 50, 411-418.
- Palade EA, Kisary J, Benyeda Z, Mándoki M, Balka G, Jakab C, Végh B, Demeter Z, Rusvai M. (2011). Naturally occurring parvoviral infection in Hungarian broiler flocks. *Avian Pathol.* 40:191-197.
- Pantin-Jackwood MJ, Strother KO, Mundt E, Zsak L, Day JM, Spackman E. (2011). Molecular characterization of avian astroviruses. *Arch Virol.* 156:235-244.
- Rebel, J. M. J., Balk, F. R. M., Post, J., Van Hemert, S., Zekarias, B. & Stockhofe, N. (2006). Malabsorption syndrome in broilers. *World's Poultry Science Journal*, 62, 17-30.
- Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Epidemiol.* 27:493-497.
- Sellers, H., Linneman, E., Icard, A.H. & Mundt, E. (2010). A purified recombinant baculovirus expressed capsid protein of a new astrovirus provides partial protection to runting-stunting syndrome in chickens. *Vaccine*, 28, 1253-1263.
- Smyth VJ, Jewhurst HL, Wilkinson DS, Adair BM, Gordon AW, Todd D. (2010). Development and evaluation of real-time TaqMan® RT-PCR assays for the detection of avian nephritis virus and chicken astrovirus in chickens. *Avian Pathol.* 39:467-474.
- Smyth VJ, Todd D, Trudgett J, Lee A & Welsh MD. (2012). Capsid protein sequence diversity of chicken astrovirus, *Avian Pathology*, 41:2, 151-159.
- Taylor MB, Grabow WO, Cubitt WD. (1997). Propagation of human astrovirus in the PLC/PRF/5 hepatoma cell line. *J Virol Methods.* 1997 Aug;67(1):13-8.
- Willcocks MM, Ashton N, Kurtz JB, Cubitt WD, Carter MJ. (1994). Cell culture adaptation of astrovirus involves a deletion. *J Virol.* 68:6057-6058.
- Zhao,W., Hua,X.G., Yuan,L., Cui,L., Shan,T.L., Dai,X.Q., Zhu,A.L.,Yu,Y., Zhu,C.X. and Yang,Z.B. (2011). Sequence analyses of the representative Chinese-prevalent strain of avian nephritis virus in healthy chicken flocks. *Avian Dis.* 55, 65-69.
- Zsak L, Strother KO, Kisary J. (2008). Partial genome sequence analysis of parvoviruses associated with enteric disease in poultry. *Avian Pathol.* 37:435-441.

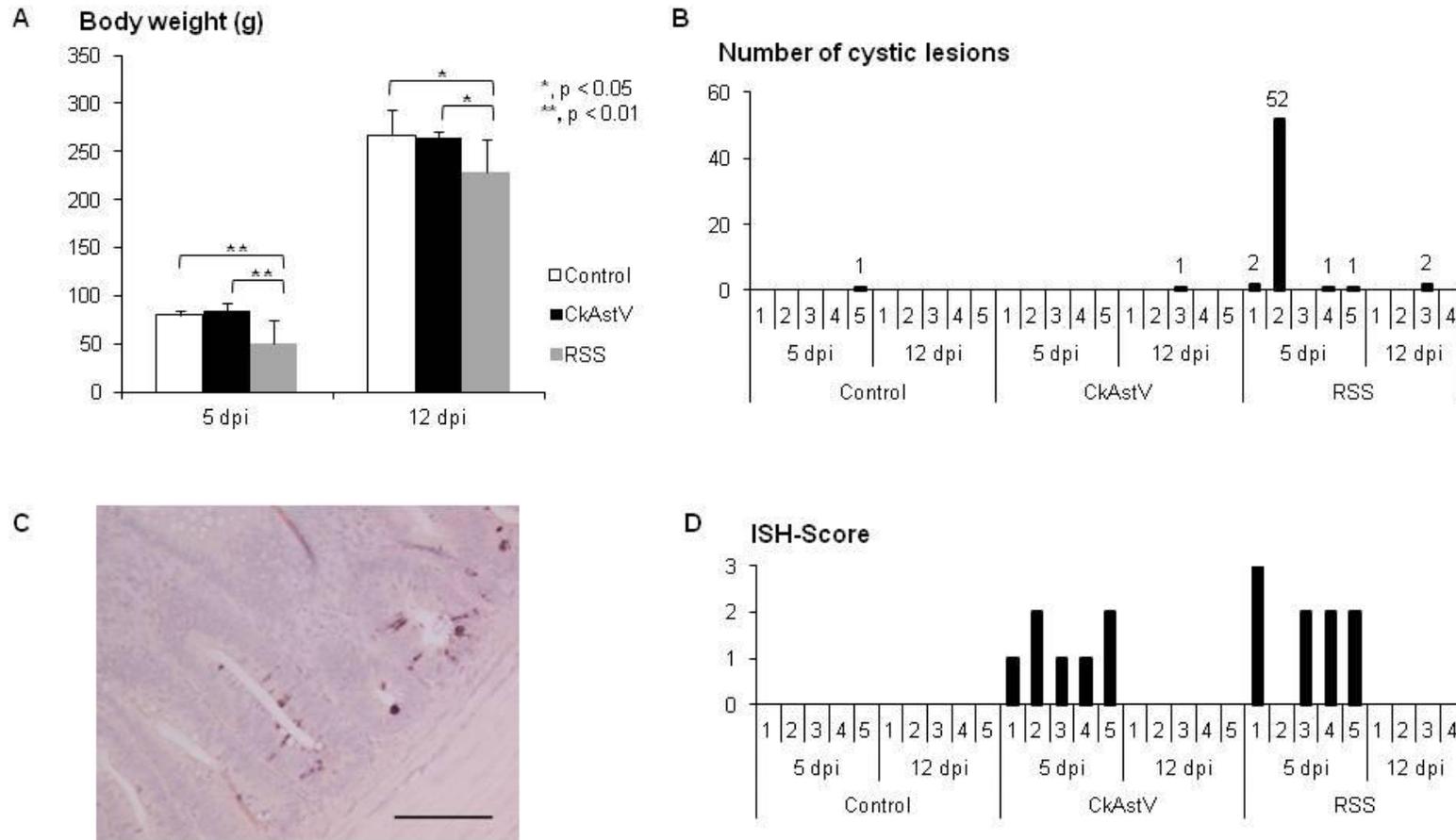
**Figure 4.1.** Characterization of a chicken astrovirus in cell culture. (A) LMH cells were infected with CkAstV-p5 and fixed with ice-cold ethanol 24 h after infection. Indirect immunofluorescence was performed using r-anti-ckAstV serum (diluted 1:100) and goat-anti rabbit FITC-conjugated antibodies (1:400). (B) Western blot analysis of protein samples of i) LMH cell either infected (inf) with CkAstV-p5 or not (co) ii) Sf 9 cells infected (inf) with a recombinant baculovirus encoding for the capsid protein of a chicken astrovirus (Sellers et al., 2010) or not infected cells (co). The membranes were incubated either with the rabbit serum r-anti-CkAstV (a and b, dilution 1:10000) or the HRP-conjugated anti 6x His monoclonal antibody (c, dilution 1:5000). A ladder (M) for protein molecular weights (Bionexus 20kDa dual color prestained protein marker) was indicated on the left part of the gel -(C) LMH cells cultured in a 24 well tissue culture plate were infected with CkAstV-p5 at 100 TCID<sub>50</sub>/well. Supernatants and cells were removed at the indicated time points and the TCID<sub>50</sub>/100 µl was determined. The diamonds represent the average of three independent studies and the standard deviation is shown by bars. (D) The virus titers were determined individually at the indicated time points after infection (h pi) for the supernatant and LMH cells infected with 100 TCID<sub>50</sub>/well of CkAstV-p5. The diamonds represent the average of three independent studies and the standard deviation was shown as bars at each time point.

**Figure 4.1.**



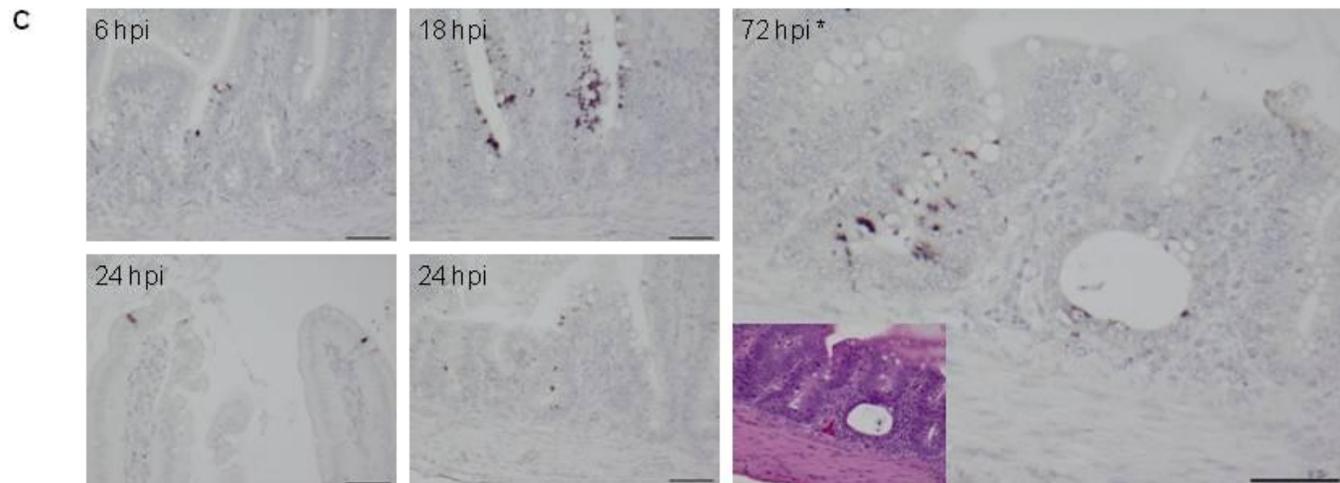
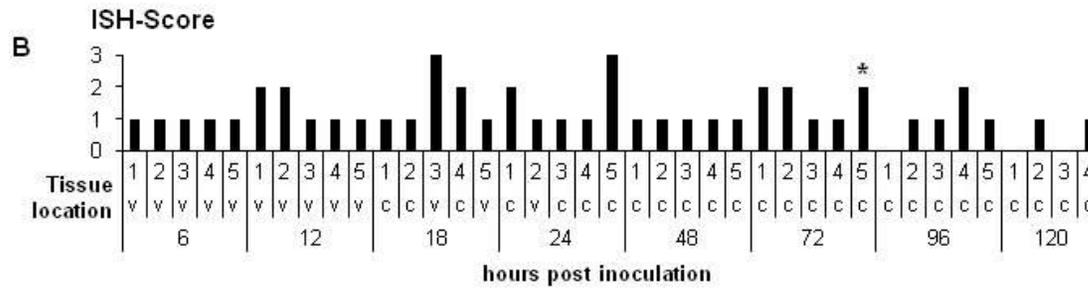
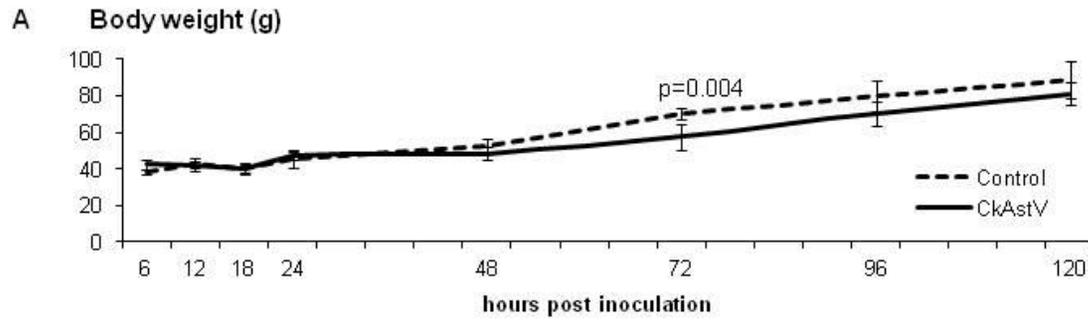
**Figure 4.2.** Infection of broiler chickens with a chicken astrovirus resulted in viral replication in the crypt region of the duodenum. Ten one-day-old broiler chickens were infected with either gut content of RSS-affected chickens (RSS) or a chicken astrovirus isolated in cell culture (CkAstV). One group of chickens was not inoculated and served as a negative control. The body weight (A), the number of cystic lesion in the duodenal loop (B), and the presence of viral RNA as detected by in situ hybridization (ISH, C) was determined from five chickens each at day 5 and 12 after infection (d pi). The presence of viral RNA was documented by ISH (C) of a chicken infected with the chicken astrovirus. The ISH score was estimated based on the following scale: 0 no signals; 1 = five signals per high-power field; 2 = five to 15 signals per high-power field; 3 > 15 signals per high-power field.

**Figure 4.2.**



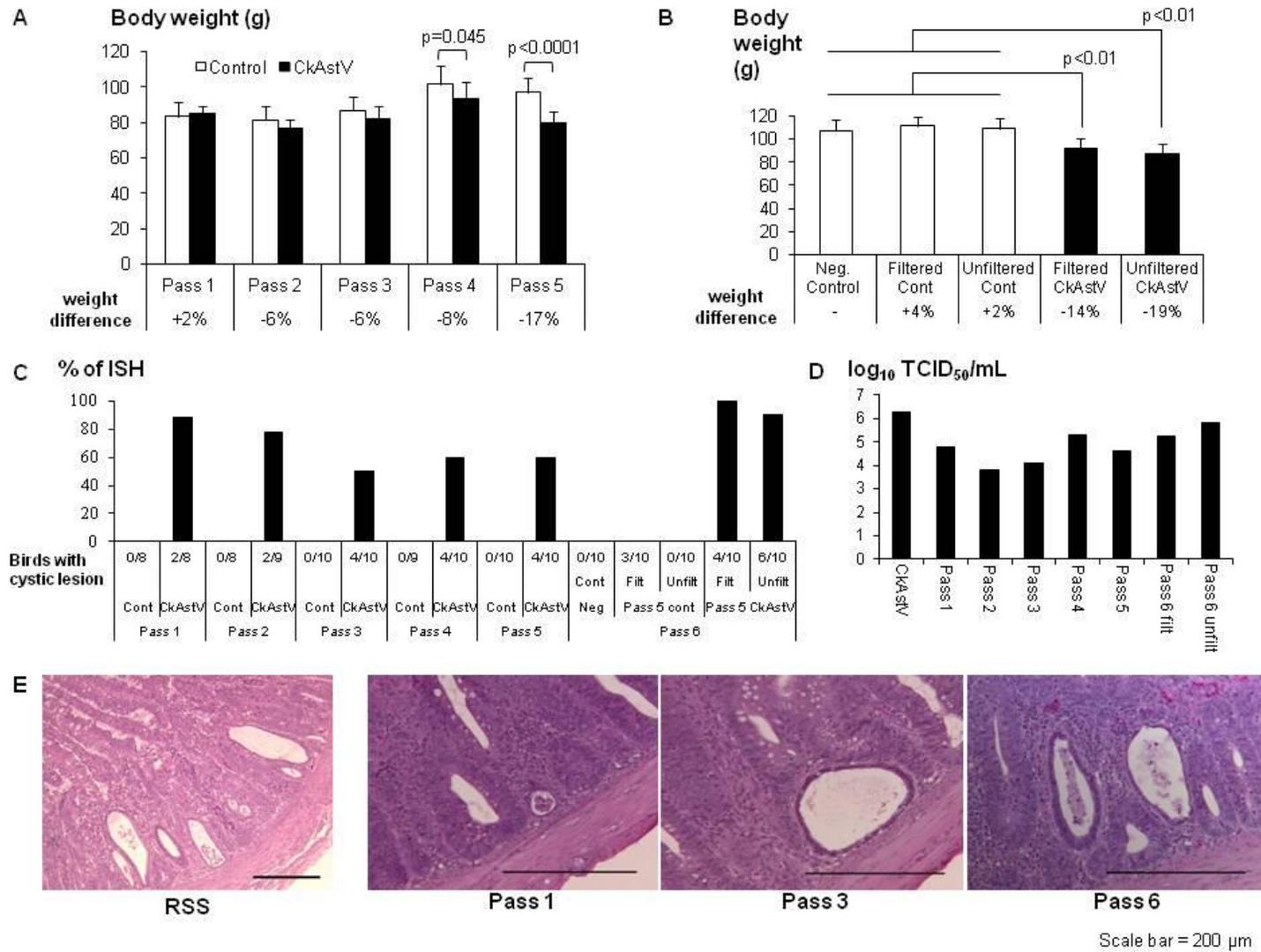
**Figure 4.3.** Dynamics of viral RNA in the duodenum of chickens infected with a chicken astrovirus. Forty one-day-old broiler chickens were infected with either a chicken astrovirus (CkAstV) or were not infected (control). Five birds from each group were euthanized at the given hours after infection (h p.i.). In addition to the body weights (A), the number of cystic lesions and the presence of viral RNA as detected by in situ hybridization (B) in the duodenal loop were determined. Location of the ISH signals was differentiated between the gut villi (v) and the crypt region (c). The presence of ISH signals were documented as an example for four time points after infection (C). The inset in the 72 h p.i. represented an H&E stained section and showed a lesion in the crypt region of the duodenum. The ISH score was based on the following scale: 0 no signals; 1 = five signals per high-power field; 2 = five to 15 signals per high-power field; 3 > 15 signals per high-power field.

**Figure 4.3.**



**Figure 4.4.** Serial passage of a chicken astrovirus in broiler chickens resulted in an increased weight retardation. Ten one-day-old broiler chickens were infected with either a chicken astrovirus (CkAstV) or were not infected (control). The filtered gut content from the initial chicken astrovirus infected chickens were serially passaged (pass) until passage 5. At day 5 after infection, the body weights (A), presence of viral RNA as detected by in situ hybridization (ISH) and the number of chickens with cystic lesions in the duodenum (C) were determined. The percentage of difference in the average weight between the groups of each passage was shown as well as the p value for each passage (A). During a subsequent 6<sup>th</sup> passage (B), in ten one-day-old broiler chickens per group, gut content of chicken astrovirus infected chickens and mock-infected chickens (cont) from passage 5 either filtered or unfiltered, was used for oral inoculation. Ten chickens were not inoculated and served as additional negative controls (Neg control). The average body weight for each group was shown and significant weight differences ( $p < 0.001$ ) compared with the negative control group were marked by an asterisk. The number of birds which showed ISH signals (as a percent of total number of birds) and the number of birds which showed cystic lesions in the duodenal sections after the 6<sup>th</sup> passage, were indicated in C. (E) Examples of cystic lesions in the crypt region of the duodenum during chicken astrovirus passage 1, passage 3, and 6 was shown. For comparison, cystic lesions are shown after infection with gut material of RSS-affected chickens.

**Figure 4.4**

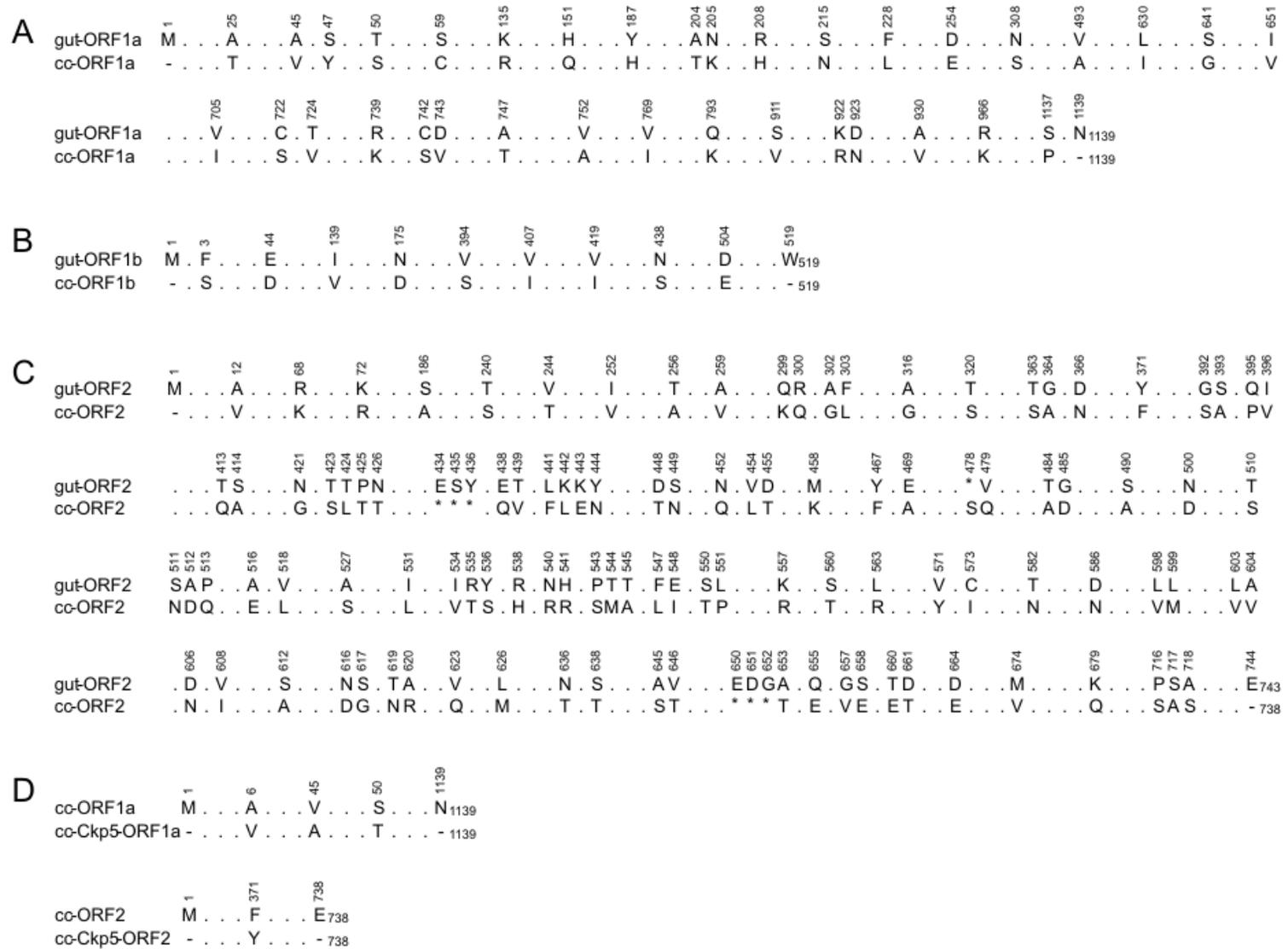


**Figure 4.5.** Presence of bacteria during serial passage of a chicken astrovirus in broiler chickens did not influence weight retardation. (A) Schematic diagram of the passages (pass) of gut content (GC) obtained from broiler chickens infected with chicken astrovirus (Ck-AstVp5) or mock-infected with cell culture medium (CC-medium). The gut content was either filtered (GCF) or left unfiltered (GCUF) prior inoculation for the subsequent passage. Passage 5 was the final passage (end). (B) The average body weights of each group of chickens at day 5 after inoculation were shown. For the first passage (pass 1) the chickens were inoculated either with cell culture medium or with CkAstV-p5. The subsequent passage groups (passage 2 to 5) included the passage of filtered and unfiltered gut content from chickens which have been initially inoculated with cell culture medium (Cont filt, Cont unfilt). The remaining groups were used to passage the filtered and unfiltered gut content from chickens which initially have been infected with CkAstV-p5 (CkAstV filt, CkAstV unfilt). Significant differences ( $p < 0.05$ ,  $p < 0.001$ ) between groups were indicated by asterisks above the bars.



**Figure 4.6.** Comparison of amino acid sequences of chicken astrovirus. The amino acids sequence of (A) the nonstructural polyprotein (ORF1a), (B) the RNA dependent RNA polymerase (ORF1b), and (C) the capsid protein (ORF2) of the recently described full length sequence of a chicken astrovirus from the gut of RSS-affected chickens (gut) (NCBI Genbank accession number JF414802) and a chicken astrovirus which has been isolated in cell culture (cc) were compared. Due to the length of the sequences only amino acid sequences were shown which are different except for the first (methionine) and last amino acid of each protein. Dashes represent single identical amino acids while dots represent stretches of identical amino acid sequences of varying lengths. Asterisks represent amino acids not present in the corresponding sequence. (D) The amino acid sequences of CkAstV-p5 (cc) and the same virus following five passages in chickens (cc-Ckp5) were compared. Only sequences which were different in the nonstructural protein (ORF1a) and capsid protein (ORF2) were shown. Identical amino acid sequences were marked by dashes.

**Figure 4.6**



## CHAPTER 5

### DISCUSSION & CONCLUSION

Runting stunting syndrome is a transmissible disease with no effective vaccines or intervention strategies are currently available for control of the disease. This is primarily due to the lack of knowledge about the etiologic agent of the disease complex. Recently, a novel chicken astrovirus (CkAstV) was identified as a possible RSS pathogen, but the pathogenicity of the virus as well as its mechanism in RSS-affected chickens was poorly understood. The objective of this study was to characterize a novel CkAstV as a possible causal agent of RSS and evaluate its role in the pathogenesis of the disease.

#### **Localization of etiologic viruses in the affected tissues by ISH**

Viral sequences belonging to the families *Astroviridae* and *Parvoviridae* were indicated in a metagenomic analysis of gut content from RSS-affected chickens. However, little was known about the biological implications of these viruses in RSS-affected chickens; therefore, investigation of their etiological role in RSS was first conducted using *in situ* hybridization (ISH) to identify the presence of nucleic acids in affected tissues. Riboprobes were designed to specifically hybridize to regions of avian nephritis virus 1 (ANV-1), ANV-2, a novel CkAstV, and a chicken parvovirus (CkParV). ISH revealed the presence of viral nucleic acids for only the astroviruses (ANV-1, ANV-2, CkAstV) in the intestines of RSS-affected chickens. On the contrary, CkParV nucleic acids were not detected in any of the tissues investigated. In addition, ISH signals for ANV-1, ANV-2, and CkAstV suggested early replication of the virus, as well as,

co-infections with multiple astroviruses in RSS-affected chickens. The results indicated the presence of the astroviruses in RSS-affected chickens, thus suggesting that astroviruses are closely involved in the etiology of RSS. However, the signals for astroviruses were limited to the epithelial cells of the intestinal villi during the initial study. In subsequent experiments using the same RSS infectious materials, the new CkAstV was primarily detected in crypt epithelial cells. Furthermore, the viral signals were detected even in the cystic crypt lesions that were characteristically found in the intestines of RSS-affected chickens. Therefore, it is very probable that the CkAstV was closely involved in the lesion formation, implying that this virus may play an important role in RSS pathogenesis.

### **Sequence analysis of the novel CkAstV**

Determination of the viral sequence was essential to determine its similarity to other astroviruses. In this study, the full length genomic RNA sequence of the new CkAstV was determined from intestinal homogenates of RSS-affected chickens. The full length sequence revealed that the virus belonged to the genus *Avastrovirus* and shared the same genomic organization with other members in the genus especially for the identical sequences of the extreme ends of the 5'- and 3'-noncoding regions. Nevertheless, the new CkAstV was significantly different from all other avian astroviruses published so far based on comparisons of the full length nucleotide sequence of the genome and amino acid sequences of the single proteins. Additionally, a different replication mechanism of the new CkAstV was indicated for the expression of the viral RNA dependent RNA polymerase (RdRp) by the presence of its own start codon and a strong hairpin structure rather than the proposed typical stem-loop structure. Therefore, the classical mechanism that uses a start codon and a stop codon in the given ORF

was suggested instead of a programmed -1 ribosomal frameshift mechanism which has been postulated for the expression of the RdRp for other *Astroviridae*.

### **Characterization of a novel CkAstV isolated in cell culture in RSS pathogenicity**

RSS can be reproduced by inoculating young chickens with filtered intestinal homogenates from RSS-affected chickens indicating a viral etiology. To our knowledge, however, none of the infectious agents described to date have successfully reproduced growth retardation and hallmark pathological lesions in the gut observed in the clinical disease. We recently isolated a new CkAstV in a cell culture system from RSS-affected chickens which enabled us to investigate the pathogenic potential of this virus.

In this study, when chickens were inoculated with the isolated CkAstV, the CkAstV replicated in the crypt epithelial cells and was present in the crypt lesions as detected by ISH. This may also support the finding that the CkAstV is directly associated with the formation of lesions characteristic for RSS. This was consistent with the ISH findings in tissues from RSS-affected chickens. Interestingly, it appears the virus favors actively dividing immature intestinal cells rather than differentiated mature counterparts as indicated by its limited detection in the crypt epithelial cells after day 3 p.i.. In addition, at day 12 p.i., the viral RNA was no longer detected in the affected intestines by ISH. Accordingly, the results imply that the CkAstV was replicating in chickens during the first few days of life.

The initial infection of chickens with the new CkAstV isolate did not induce the severe growth retardation and pathologic lesions in the duodenum as observed in a clinical case of RSS. We hypothesized that the virus did not induce the expected degree of pathogenicity due to either the absence of a secondary factor present during the clinical manifestation or a genetic

modification of the virus which might have occurred during the isolation and subsequent passage in cell culture. To this end, passage of the cell culture isolated CkAstV in chickens was conducted in an effort to evaluate possible restoration of virulence. Using intestinal homogenates from each subsequent passage in chickens, the virus was passaged successfully from chicken to chicken, as detected by ISH, virus isolation and titration. Most importantly, the body weight retardation and the lesion formation were repeatedly induced in each CkAstV-passaged group, as observed in RSS infected chickens. Moreover, filtered gut contents were sufficient to induce a comparable degree of the clinical and pathological signs of RSS, implying the restoration of virulence of CkAstV through passage in chickens. In order to investigate a genomic variation affecting the viral pathogenicity, comparison of the complete genomic sequence of the chicken astrovirus was evaluated from three full-length sequences of the CkAstV from 3 independent sources: RSS-affected gut content before virus isolation, CkAstV isolated in cell culture, and gut content after passage in chickens of the cell culture isolated CkAstV. Sequence differences were mainly observed between CkAstV cell culture isolate and CkAstV obtained directly from RSS-affected chickens. The majority of mutations, including several insertions/deletions occurred in ORF2, which encodes for the viral capsid protein. It is possible that important genetic information regarding pathogenicity was lost during adaptive processes in the cell culture system. Since minor genetic modifications of CkAstV may affect its pathogenicity, future studies to evaluate the sequence modification will be valuable. Interestingly, neither additional insertions/deletions nor significant amino acid variations occurred during passage of the CkAstV isolate in chickens. Thus, correlation of clinical outcomes to the CkAstV mutations identified needs to be evaluated in subsequent experiments utilizing a reverse genetics approach.

In conclusion, the involvement of the new CkAstV in RSS was strongly supported by the findings observed in our studies. Furthermore, the studies presented new insights into the role of CkAstV during the pathogenesis of RSS. It was concluded that the new CkAstV is a highly probable causal agent for RSS in chickens. These findings may be fundamental in developing effective prevention methods for RSS, as well as, understanding the pathogenesis of the family *Astroviridae*.