

Institute of Virology
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**Comparative analysis of current infectious bronchitis virus isolates
in primary cell culture systems**

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Jo

My parents, Husband and sons

(Ahmed & Mohammed)

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List of publications and presentations

Publications

1- Abd El Rahman S.; El-Kenawy A.A.; Neumann U.; Herrler G. and Winter C. (2009): Comparative analysis of the sialic acid binding activity and the tropism for the respiratory epithelium of four different strains of avian infectious bronchitis virus. *Avian Pathology*. **38**(1): 41- 45.

2- Abd El Rahman S.; Winter C.; El-Kenawy A.A.; Neumann U.; and Herrler G. (2010): Differential sensitivity of well-differentiated avian respiratory epithelial cells to infection by different strains of infectious bronchitis virus. *J Virol*. **84**(17): 8949-52.

3- Abd El Rahman S.; Winter C.; Neumann U.; and Herrler G.

Establishment of chicken oviduct explant cultures to analyze infection of differentiated oviduct epithelial cells by infectious bronchitis virus *in vitro*.

(In preparation)

Presentations

1- Importance of sialic acid for the infection by IBV (2007).

Christine Winter, Sahar Abd El Rahman, Georg Herrler and Ulrich Neumann.

The 3rd European congress of virology, Nürnberg, Germany, September 1st–5th, 2007, proceeding pp. 81.

2- Importance of sialic acid for the infection of the tracheal epithelium by different strains of infectious bronchitis virus (2008).

Sahar Abd El Rahman, Christine Winter, Ulrich Neumann and Georg Herrler.

The XIth international nidovirus symposium, Oxford, United Kingdom, June 22nd–27th, 2008, proceeding pp. 56.

3- Importance of sialic acid for the infection of the tracheal epithelium by different strains of infectious bronchitis virus (2009).

Abd El Rahman S, Neumann U, Georg H and Winter C.

The VIth international symposium on avian corona- and pneumoviruses and complicating pathogens. Rauschholzhausen, Germany, 14th–17th June, 2009, proceeding pp.100–107.

4- Importance of Sialic acid for the infection of the chicken tracheal and bronchial epithelium by different strains of infectious bronchitis virus (2010).

Sahar Abd El Rahman, Christine Winter, Ali El Kenawy, Ulrich Neumann, and Georg Herrler.

The 4th European congress of virology, como lake, Italy, 7th – 11th April, 2010, proceeding pp. 180.

5- The role of sialic acids for the infection of different primary avian cell culture by different strains of infectious bronchitis virus (2010).

Sahar Abd El Rahman, Christine Winter, Ali El Kenawy, Ulrich Neumann and Georg Herrler.

The 9th international symposium on positive stranded RNA viruses, Atlanta Georgia, USA, 17th–21st, June, 2010, proceeding pp. 42.

6- Wo bindet das Virus der Infektiösen Bronchitis des Huhnes? Neue Untersuchungsergebnisse (2010).

Christine Winter, Sahar Abd El Rahman, Ulrich Neumann, und Georg Herrler.

The 78th Expert meeting of poultry diseases, DVG, Hannover 6th–7th May, 2010, proceeding pp. 8-9.

List of abbreviations

APN	Aminopeptidase N
Ark.	Arkansas
Bd	Beaudette
BCoV	Bovine coronavirus
CCoV	Canine coronavirus
CEK	Chicken embryo kidney
COE	Chicken oviduct explant
Conn.	Connecticut
Cy3.	Indocarbocyanine
D1466	Dutch isolates
DAPI	4',6'-Diamidino-2-phenylindol
d.p.i.	days post infection
ELISA	Enzyme-linked immunosorbent assay
E-Protein	Envelope protein
et al.	Et alii
FCoV	Feline coronavirus
Fig	Figure
FITC	Fluoresceine isothiocyanate
h.	Hours
H120	IBV isolate from Holland
HCoV	Human coronavirus
HE	Hemagglutinin-esterase protein
HEV	Haemagglutinating encephalomyelitis virus
HI	Hemagglutination inhibition
IB	Infectious bronchitis
IBV	Infectious bronchitis virus
ICVT	International Committee for Virus Taxonomy
KDa	Kilodalton

List of abbreviations

MAA II	<i>Maackia amurensis</i> agglutinin
Mass.	Massachusetts
MHV	Mouse hepatitis virus
min.	Minutes
ml	Milliliter
mm	Millimetre
M.O.I.	Multiplicity of infection
M-Protein	Membrane protein
mRNAs	Messenger RNA
mU	milli-unit
Neu5AC	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
Neu4,5Ac ₂	N-acetyl-4-O-acetylneuraminic acid
Neu5,9 Ac ₂	N-acetyl-9-O-acetylneuraminic acid
nm	Nanometer
N-Protein	Nucleoprotein
PBS	Phosphate buffered saline
PCLS	Precision-cut lung slices
PCR	Polymerase chain reaction
RT- PCR	Reverse transcriptase PCR
pfu/ring	Plaque-forming unit per ring
Ph	Potential Hydrogenii
RBD	Receptor binding domain
RCoV-SDAV	Rat sialodacryoadenitis coronavirus
RNA	Ribo nucleic acid
RNP	Ribonucleoproteins
rt-PCR	Reverse transcriptase PCR
SARS-CoV	Coronavirus associated with severe acute respiratory syndrome
SNA	<i>Sambucus nigra</i> agglutinin
SPF	Specific pathogen free

List of abbreviations

S-Protein	Spike protein
S1-Protein	Spike protein subunit 1
S2-Protein	Spike protein subunit 2
TCoV	Turkey coronavirus
TGEV	Porcine transmissible gastroenteritis virus
TOCs	Tracheal organ cultures
UK	United Kingdom
UK/167/84	United Kingdom isolate
um	micrometer
USA	United states of America
VN	Virus neutralisation test

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1 General introduction

1.1 Infectious Bronchitis Virus (IBV)

1.1.1 Taxonomy

Avian *infectious bronchitis virus* (IBV) belongs to the order *Nidovirales* which comprises the families, *Arteriviridae* and *Coronaviridae* (CAVANAGH 1997); an additional family designated *Roniviridae* has been added in 2003 (GONZALEZ et al. 2003). *Coronaviridae* comprises two genera, *Coronavirus* and *Torovirus* which have similarities in the organization and expression of the genome but differences in the shape of the virion and the size of the genome (CAVANAGH and HORZINEK 1993). Members of the genus *Coronavirus* are divided into three groups based on antigenic relationship and sequence similarity (Table 1). Infectious bronchitis virus belongs to group 3. Coronavirus taxonomy has been updated in 2009 by the International Committee for Virus Taxonomy (ICVT) which subdivided this family into two subfamilies (*Coronavirinae* and *Torovirinae*). The former subfamily comprises three genera, *Alphacoronavirus*, *Betacoronavirus*, and *Gammacoronavirus*; avian coronaviruses belong to the latter genus (ICVT, 2009).

The designation ``Nidovirales`` has been adapted from the Latin term "Nidus" for nest; it was chosen because of the characteristic strategy of replication by members of this order, which includes the generation of an extensive 3' co-terminal nested set of mRNAs from which the 3' proximal region of the polycistronic genome is expressed. Coronavirus transcripts contain not only 3' co-terminal sequence portion but also a common 5' leader sequence of about 65–100 nucleotides, which is derived from the 5' end of the genome (SPAAN et al. 1982; LAI et al. 1982&1983).

The name of ``corona`` points to the characteristic shape of this group of viruses which are surrounded by a structure which - when observed under the electron microscope - resembles that of the solar corona. The corona-like structure is due to the spike protein (S) which forms large (20 nm), club-shaped, heavily glycosylated surface projections. Coronaviruses are enveloped, pleomorphic in shape, with a mean diameter of approximately 120 nm.

The genome consists of single-stranded RNA with positive orientation (CAVANAGH 1995; LAI and CAVANAGH 1997; WEISS and NAVAS-MARTIN 2005).

Table 1. Members of the three genera of the subfamily *Coronavirinae*

<i>Alphacoronavirus</i>	<i>Betacoronavirus</i>	<i>Gammacoronavirus</i>
TGEV ^a	BCoV	IBV
FCoV	HCoV-OC43	TCoV
CCoV	SARS-CoV	
HCoV-229E	MHV	
	RCoV-SDAV	

^aThe abbreviations indicate the following viruses: TGEV: porcine transmissible gastroenteritis virus; FCoV: feline coronavirus; CCoV: canine coronavirus; HCoV: human coronavirus; BCoV: bovine coronavirus; HEV: haemagglutinating encephalomyelitis virus; MHV: mouse hepatitis virus; RCoV-SDAV: rat sialodacryoadenitis coronavirus; SARS-CoV: coronavirus associated with severe acute respiratory syndrome; IBV: avian infectious bronchitis virus; TCoV: turkey coronavirus.

The coronavirus genome is considered to be the largest among all RNA viruses, as it comprises about 30,000 nucleotides. It is associated with the nucleocapsid protein and is surrounded by a lipid envelope. Several proteins are incorporated into the viral membrane: envelope protein (E), spike protein (S), membrane protein (M), and in some betacoronaviruses, the haemagglutinin-esterase protein (HE). Each viral protein has its own function either alone or in combination with other viral proteins; for example, the M and E proteins induce a budding process and thus play an important role during morphogenesis (Fig.1).

IBV is defined as the coronavirus of the domestic fowl, although it infects also animals of other avian species like turkeys, pheasants (CAVANAGH et al. 2002), pigeons (QIAN et al. 2006), domestic peafowl and teal (LIU et al. 2005).

It was the first coronavirus to be isolated in chicken embryos by Beaudette and Hanson (1937). Tissue tropism is an important aspect of IBV as this virus causes respiratory disease and reproductive disorders and also replicates at many non-respiratory epithelial surfaces, where it may cause cytopathogenic effects e.g. kidney, intestine, female reproductive system (SEVOIAN and LEVINE 1957) and male gonads (BOLTZ et al. 2004).

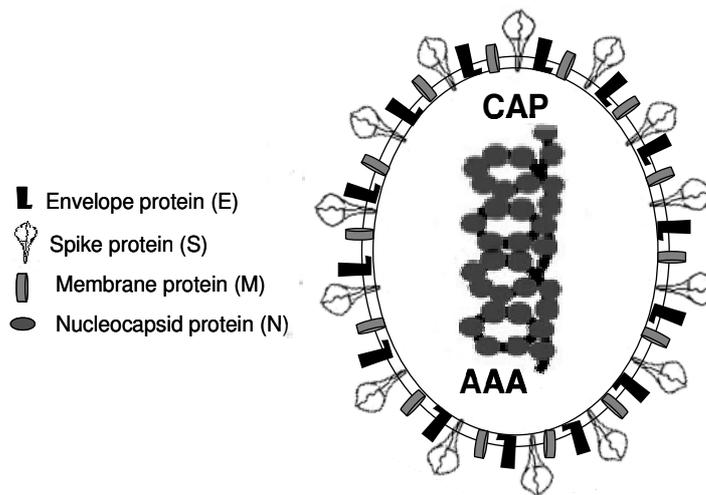


Fig 1 Schematic drawing of an avian coronavirus particle (IBV).

Strains of the virus vary in the severity of the pathogenicity in non-respiratory organs; some strains are highly nephropathogenic while others are not. Replication in the enteric epithelium does not result in clinical symptoms although virions are present in faecal excretions (CAVANAGH 2007).

1.1.2 Viral structural proteins

Four structural proteins designated S, M, E and N are integral parts of all coronaviruses:

- N - nucleoprotein : surrounds and protects the viral RNA genome.
- E - envelope protein : plays an important role for virus assembly.
- M - membrane protein : is an integral membrane protein.

- S - spike glycoprotein : mediates virus attachment and contains neutralising epitopes.

The N protein

The nucleoprotein (N) protein is a basic, phosphorylated protein that consists of 409 amino acids; it has a highly conserved region between residues 238 and 293 (HOGUE and BRIAN 1986; WILBUR et al. 1986; WILLIAMS et al. 1992). The N protein plays a role in the morphogenesis and replication of the virus as it directly binds the viral genomic RNA and forms a helical ribonucleoprotein complex (RNP) (DAVIES et al. 1981). It also plays an important role in the viral replication especially in transcription of subgenomic RNA, in translation (MASTERS and STURMAN 1990; TAHARA et al. 1998). The N protein together with the M protein forms the inner core of the virus (RISCO et al. 1996; ESCOR et al. 2001).

The E Protein

The envelope (E) protein is a minor virion component. Because of its small size (ca. 10 kDa) and the very low abundance relative to the M, N, and S proteins, it was relative late recognized as a viral structural protein (LIU and ENGLIS 1991; GODET et al. 1992; YU et al. 1994). E proteins are well conserved within each of the three groups of coronaviruses, but they show very limited homology across the different groups. All E proteins have the same general structure: a short hydrophilic amino terminus (7 to 9 amino acids), preceding a large (21 to 29 amino acids) hydrophobic region, followed by a large hydrophilic carboxy terminus making up one-half to two-thirds of the mass of the molecule (LIU and ENGLIS 1991). The E protein has an ion channel activity that is important for virus entry and assembly (XING et al. 2009).

The M protein

The membrane (M) protein is a multi-spanning, highly abundant membrane component that is important for maintaining the shape of the envelope. It has a small N-terminal domain which is exposed on the surface of the virion, or in the lumen of the endoplasmic reticulum (XING et al. 2009). Although the M proteins are quite divergent across the three groups of coronaviruses, they are moderately well

conserved within each coronavirus group. The region of the M protein that is most conserved among all coronaviruses is a segment of approximately 25 residues (DEN BOON et al. 1991). The M protein plays an important role not only in the budding process but also in determining the site of budding as it is the only envelope glycoprotein greatly required for budding of the virion, and its intracellular distribution correlates with the site of budding (TOOZE et al. 1984). The M and E proteins are the only two viral proteins sufficient to form "virus-like particles" (BOS et al. 1996; VENNEMA et al. 1996; KIM et al. 1997).

The S protein

The surface or spike (S) protein is about 20 nm in length and the bulbous end is about 10 nm wide, in a dimeric or trimeric form and of different molecular weights between 170-220 KDa. Infectious bronchitis virus has the S protein in a cleaved form, with two subunits, the amino-terminal S1 and the carboxy-terminal S2 protein. The cleavage site is usually associated with several pairs of basic amino acids. For example, Arg-Arg-Ser-Arg-Arg is a common S1-S2 connecting peptide of IBV (CAVANAGH et al. 1992). For IBV, the two subunits comprise a little over 500 and 600 amino acids, thus the S protein of IBV is the shortest spike protein among all coronaviruses (about 1160 amino acids). The bulbous head of the S protein is believed to be made up largely by the S1 subunit. The S protein is anchored in the membrane by the carboxyterminal portion of S2. The S protein has different functions: (i) attachment of the virus to the receptor through the S1 subunit; (ii) fusion of the viral membrane with cellular membranes via the S2 subunit (CASAIS et al. 2003; CAVANAGH 2007) and (iii) interaction with the transmembrane region of the M protein. The S protein of IBV also plays an important role in the immunogenesis via its major immunogen. The S1 protein has a molecular weight of ~90 kDa and 28–29 glycosylation sites (CAVANAGH et al. 1992). It contains epitopes that can induce the generation of specific antibodies capable of neutralizing the virus and inhibiting haemagglutination (CAVANAGH et al. 1986; IGNJATOVIC and GALLI 1994). In addition, the S1 protein N-terminus plays an important role in tissue tropism and the degree of virulence of the virus (CAVANAGH et al. 1992; KWON and JACKWOOD

1995). Therefore, this protein is important for strategies to control IB and for understanding the mechanism of IBV evolution.

The location of the receptor binding domain (RBD) within S1 is not known for IBV and is different from that of other coronaviruses. The S protein is the major target of the protective immune response elicited by a coronavirus infection.

The haemagglutinin-esterase (HE) protein

An additional viral envelope protein has been found in several members of the betacoronaviruses designated HE protein which is absent from alpha and gammacoronaviruses. It has an acetylerase activity as described for bovine coronavirus (BCoV), haemagglutinating encephalomyelitis virus and mouse hepatitis virus (MHV) (VLASAK et al. 1988b; YOKOMORI et al. 1989; SCHULTZE et al. 1991). The presence of such an activity in coronaviruses became evident when VLASAK and co-workers found that BCoV and HCoV-OC43 resemble influenza C viruses in their interactions with erythrocytes (VLASAK et al. 1988a). Following the haemagglutination reaction, bound virions eluted from the erythrocytes rendering the cells resistant to subsequent agglutination by either of the two coronaviruses or by influenza C virus. This suggested that BCoV, human coronavirus (HCoV-OC43) and influenza C virus have a receptor-destroying enzyme in common. For influenza C virus, it has been demonstrated that this enzyme is a *sialate 9-O-acetylerase* (HERRLER et al. 1985). The same enzyme activity was detected in BCoV (VLASAK et al. 1988b).

1.1.3 Variation of IBV

There are many IBV serotypes as defined by virus neutralization tests (COOK et al. 1999; CANANAGH 2001). The number of serotypes is expected to increase in the future due to the appearance of new variant strains. The large spike glycoprotein (S) comprises two subunits a carboxy-terminal S2 and the amino-terminal S1. The amino acid identity between different IBV strains is high, usually $\geq 90\%$ when the S2 portions are compared, whereas this identity is usually lower when the S1 subunits are compared, with most IBV serotypes differing from each other by 20 to 25%

(ADZHAR et al. 1997; KINGHAM et al. 2000). Sometimes differences may be as high as 50% (CAVANAGH et al. 1997; GELB et al. 1997).

Generally, it is assumed that isolates with less than 89% similarity in this S1 subunit belong to different serotypes. An exception is the strains Conn 46 and Fla 18288 which have a similarity of 96% and belong to different serotypes, indicating that only a few changes in the right place are required to change the serotype (Fig 2). So, vaccination of bird flocks by one serotype protects the flocks poorly against infections by viruses of heterologous serotypes, which in turn reduces the efficacy of vaccination and makes it more difficult to control IBV infections (CAVANAGH and NAGI 2003).

1.1.4 Replication of the virus

The attachment of IBV to target cells is mediated by the surface protein S. Host cell receptors have been identified for several coronaviruses but for others they are still unknown. For IBV, sialic acid serves as an attachment factor (WINTER et al. 2008), but it is not known whether virus entry requires subsequent interaction with a receptor protein. Following binding, the fusion of viral and plasma membranes is necessary to allow the entry of the nucleocapsid into the cytoplasm (MATSUYAMA and TAGUCHI 2002; ZELUS et al. 2003). Virus-specific RNAs and proteins are synthesized probably entirely in the cytoplasm (LEIBOWITZ et al. 1981; WIHLELMSEN et al. 1981).

A set of nested subgenomic mRNAs, also genomic RNAs are transcribed by the replicase complex. While the actual mechanism of synthesis of mRNAs is not well understood, it is currently believed that subgenomic negative-strand RNAs serve as templates for mRNA (BRIAN et al. 1994). The replicase carries out “discontinuous transcription” by connecting body and leader sequences in subgenomic RNAs and also in recombination events which occur at high frequency during coronavirus replication. New virions are assembled by budding into intracellular membranes and are released from the cells probably through vesicles by cellular secretory mechanisms.

1.2 Infectious Bronchitis (IB)

1.2.1 Definition and economic losses

Infectious bronchitis is a highly contagious and infectious disease of poultry, poses a major threat to the poultry industry and was first reported in 1931 in North Dakota, USA, as a novel respiratory disease (SCHALK and HAWN 1931). Infectious bronchitis infection affects the growth rate of broilers as well as egg production in layers and breeders. Broilers may perform badly due to poor feed conversion and reduced weight gain (CAVANAGH et al. 1999). Secondary infections by bacteria, such as *E. coli* or *O. rhinotracheale* may bring about increased condemnation at the processing plant, especially when infection occurs in the last weeks prior to slaughter (CAVANAGH and NAGI 2003). Infectious bronchitis can also affect the reproductive system of the bird, the clinical expressions depending mainly on the age of the bird at the time of infection. Animals infected in the first days of life by a very virulent IB virus may suffer from permanent damage of the oviduct. At the time of maturation; they may look like normal hens but produce no eggs (false layers) thus contributing to the economic losses (BROADFOOT et al. 1954; SEVOIAN and LEVINE 1957; MCDUGALI 1968). When infection affects birds at the time of laying, the economic losses are due to drops in production and poor quality eggs. Production often does not return to pre-infection levels. In breeders the hatchability rate may also be negatively affected.

1.2.2 Viral epidemiology

Infectious bronchitis virus is distributed worldwide. All ages of chicken can be infected. Although it is generally accepted that chickens are the most important natural hosts, IBV or closely related coronaviruses have also been isolated from other species such as turkeys, pheasants, quails and partridges. Several different serotypes can co-circulate in the same area at the same time (CAVANAGH 2001). Some are found worldwide, others have a more restricted geographical distribution. For example, some are found only in Europe; others only in the USA. The morbidity

rate is extremely high and the mortality rate depends on the age of the chickens when infected, and the presence of secondary invading organisms such as *E. coli*.

1.2.3 Pathogenesis

Initially, the upper respiratory tract cells are infected by IBV, preferably, the ciliated and mucus producing cells (PURCELL and CLARKE 1972; JONES and JORDAN 1972). Infection is characterized by the loss of the protective cells lining trachea and sinuses. Within three days of the infection, virus titres are maximal in the nose and trachea and remain for other two to five days (HOFSTAD and YODER 1966; AMBALI and JONES 1990). Similar virus titres occur in the lungs and air sacs which appear clinically as small areas of pneumonia, although IBV is not considered to cause pneumonia. After a brief viraemia, the virus can be detected in other non-respiratory organs such as the kidneys (causing minor and major nephritis), the reproductive tract (RAJ and JONES 1997), and many parts of the alimentary tract-oesophagus, proventriculus, duodenum, jejunum, bursa of fabricius, caecal tonsils, rectum and cloaca (AMBALI and JONES 1990), and as recently shown, in the gonads of male chickens (BOLTZ et al. 2004). The incubation period is relatively short (18-36 hours), with the disease spreading through an entire flock within one or two days. The IB virus is spread horizontally by aerosol transmission (sneezing), through contaminated organic material, drinking water, and equipment. So far, vertical transmission (from the hen to their progeny through the egg) has not been shown to be important. However, surface contamination of eggs with the IB virus is a possible way by which the virus can be spread in hatcheries or egg packing stations (JONES and AMBALI 1987).

1.2.4 Clinical and post mortem signs

The first recognized and most characteristic signs of IBV are the respiratory signs, hence the designation infectious bronchitis. However, the pathogenicity of the virus for the oviduct in very young chicks or birds in production is often more important.

The following symptoms may be seen:

- Young chickens are depressed and huddle under the heat source.

- Respiratory signs - gasping, coughing, tracheal rales and nasal discharge (CAVANGH and NAGI 2003).
- Birds in lay have a marked drop in egg production and an increased number of poor quality eggs may be produced either internally or externally (misshaped, non pigmented, thin albumen, soft shell eggs) (BROADFOOT et al. 1954)
- The hatchability rate of the eggs can be severely reduced.
- When the kidneys are affected, increased water intake, depression, scouring and wet litter.

Post mortally, lesions are found in the respiratory tract and urogenital tract. Renal damage associated with different IB strains is an increasingly important feature of IB infections, especially in broilers.

- Serous, catarrhal, or caseous exudates in the trachea, nasal passages and sinuses (CAVANAGH 1983)
- Cloudy air sacs which may contain yellow caseous exudates
- Caseous plug may be found in the trachea
- Swollen, pale kidneys, with distended tubules and ureters containing urate crystals in nephropathogenic cases
- Pneumonia as a secondary sign due to mucus accumulation and obstruction of the trachea.
- Fluid yolk material may be found in the abdomen of birds in production (egg peritonitis)
- Degeneration of the ovary and swollen oviducts.

1.2.5 Diagnosis of Infectious Bronchitis

Isolation and identification of the causative agent is required for a definitive diagnosis of IB. The disease can be diagnosed by:

1- clinical and post mortem signs.

2- laboratory tests used for either viral proteins (antigens), viral antibodies against IBV, or viral genome detection. For monitoring vaccination results, interval serum samples can be tested for viral antibodies. Different serological tests can be used for viral antibody detection e.g. virus neutralisation test (VN), haemagglutination

inhibition test (HI), enzyme-linked immunosorbant assay (ELISA). Detection of IBV using RT-PCR exhibited higher sensitivity than virus isolation and can be used as a rapid diagnostic method in the field (RAMNEEK et al. 2005) as well as in serotype differentiation and epidemiological studies.

1.2.6 Vaccination and viral serotypes

1.2.6.1 Vaccination

The RNA-dependent RNA-polymerase present in most RNA viruses and also in IBV, required for copying of the viral genome, is mainly responsible for the extremely high mutation rate. This enzyme does not have proofreading capability. So, when a mistake in copying the genome is made, the enzyme cannot go back and fix it. This high mutation rate creates a diverse population of virus particles that allows IBV to quickly adapt to selection pressures such as host immune responses (antibodies and T-cells; CAVANAGH 2001). This adaptation is evident clinically as variant viruses or emerging new serotypes of the virus. The control of IBV by vaccination is hampered because of the appearance of new emerging serotypes, which are only weakly affected by vaccination against the original serotypes. Although homologous live vaccines for IB are better than heterologous vaccines in controlling the disease, it is still recommended to develop the vaccines from different local strains to control IB (LIN et al. 2005). The most common serotypes of IBV in the USA are Arkansas, Connecticut, Delaware, and Massachusetts. However, when those IBV types undergo changes, also designated genetic drift, it can result in considerable variation away from the original serotype. The Arkansas type viruses appear to be quite prone to genetic drift resulting in viruses designated Arkansas-like viruses. If the extent of genetic changes, which can accumulate over time, reaches a critical point, the Arkansas vaccine strain (Ark-DPI) may no longer provide sufficient protection against the Arkansas-like viruses. In addition to genetic drift, genetic shift can lead to a dramatic change resulting in a unique variant virus. Such variant viruses are defined as previously unrecognized IBV types that are clearly different from the known IBV serotypes. Genetic shift usually results from recombination between two different parent viruses (that infected the same cell) to produce a distinct progeny virus.

Recombination events occur by a template switching mechanism along conserved regions of the viral genome between two different viruses (CAVANAGH et al. 1992a; KOTTIER et al. 1995; ESTEVEZ et al. 2003; GELB et al. 2005). The resulting virus is a hybrid of the two parental viruses that, under the right conditions, can break through immunity induced by the common vaccine types.

IBV continues to be one of the major pathogens of chickens throughout Europe. In fact, it is probably endemic in all regions where poultry are reared intensively. Despite the availability of high quality vaccines, IB continues to be a problem in most regions of Europe. The prevalence of IBV variants in Europe has been recognised as a problem at least since the 1960s.

1.2.6.2 Important serotypes in Europe.

Infectious bronchitis (IB) is still a significant problem in the poultry industry in Europe. Recently, several authors have reported many different serotype isolates. A survey conducted in UK, France, Holland, Germany, and Spain revealed that the most commonly found genotypes were 4/91 and Massachusetts (Mass), accounting for 34% and 24% of the studied isolates, respectively. The next most frequently detected genotypes were Italy-02 (It-02), QX, D274 and Arkansas (WORTHINGTON et al. 2004; WORTHINGTON and JONES 2006).

Infectious bronchitis virus variant 4/91

The IBV variant 4/91 was firstly reported in Britain in the early 1990s (ADZHAR 1997; CAVANAGH et al. 1998a). It was associated with outbreaks of respiratory diseases and rapidly spread displacing the D274 serotype that has been dominant in the 1980s (ADZHAR 1997; CAVANAGH et al. 1998). Currently, 4/91 also named 793/B and CR88 is one of the most common IBV serotypes in Europe. Its appearance and spread resulted in great economic losses. This variant has continued to be a major pathogen throughout Europe and also in other parts of the world although it is controlled by a live vaccine, usually followed in older birds by the use of an inactivated vaccine.

Infectious bronchitis virus variant Italy 02

The sequence analysis of this IBV strain isolated in the late 1990's in Italy was studied closely and designated at 2002 as "Italy 02". Later on this isolate became widespread in Europe. In the early 2000s, in a study carried out by Worthington and co-workers using the RT-PCR technique, the authors found that Italy-02 was one of the most predominant genotypes not only in Italy but also in many other countries (WORTHINGGTON et al. 2004). The studies so far performed have simply detected the virus genome, few virus isolates have been obtained and so it has not been proven that this IBV strain is pathogenic in chickens in the field.

In a recent survey (WORTHINGGTON et al. 2008) "Italy 02" in Spain continues to be the dominant type detected (DOLZ et al. 2006).

Infectious bronchitis virus variant (QX)

The QX serotype was first isolated in China in 1996 from birds with proventriculitis (YU DONG et al. 1998), spread rapidly to several provinces in China (YU et al. 2001; LIU et al. 2006) then to the European region of Russia (BOCHKOV et al. 2006). In 2005, the QX serotype was isolated in the European continent (BEATO et al. 2005; LANDMAN et al. 2005; ZANELLA et al. 2006) and spread rapidly to become the most wide spread serotype of non-vaccine origin (WORTHINGTON et al. 2008). In addition to the respiratory lesions, the QX-like serotype has been associated with proventriculitis (YU DONG et al. 1998), severe kidney damage (LIU and KONG 2004; BEATO et al. 2005; ZANELLA et al. 2006; WORTHINGTON et al. 2008) and false-layer syndrome (LANDMAN et al. 2005) as well as severe egg production problems. Respiratory signs have been reported in broilers older than four weeks of age. In birds in production, the problems are characterized by a low production rate with peak levels reaching 30% to 55% in apparently healthy flocks. Some of the birds show a peculiar stance (penguin-like) and a pendulous abdomen. These cases were associated with earlier outbreaks of nephropathogenic infectious bronchitis that had occurred in 2003 in broilers and pullets (layer and broiler rearing pullets) from which a variant IB virus, designated D388 by the Animal Health Service in Deventer, The Netherlands, was isolated. Using a PCR test, it was demonstrated that this isolate can be classified with 99% genotypic agreement as a Chinese isolate designated

“QX” and this is the name by which this IB variant is now known (BEATO et al. 2005 and WORTHINGTON et al. 2008). This original isolate was similar to a Chinese isolate known as QX. When this isolate was inoculated into either SPF or commercial birds it was found to be nephropathogenic and could also be detected in the oviducts of the infected birds.

This IB virus has continued to be a cause of major disease problem in poultry flocks in some parts of Europe, being associated with nephritis and so-called “false layers” as a result of early infection with this virus. In a recent survey using the RT-PCR technique (WORTHINGTON et al. 2008), the QX IBV variant has been detected frequently in The Netherlands, Belgium, Germany and France.

1.2.6.3 IBV in Egypt

Isolates related to Massachusetts, D3128, D274, D-08880, 4/91 and the novel genotype Egypt/Beni-Suef/01 have been isolated from different poultry farms in Egypt (SHEBL et al. 1986; EL KADY et al. 1989; ABDEL-MONEIM et al. 2002; SULTAN et al. 2004). The commonly used IBV vaccines are derived from the Massachusetts serotypes.

1.3 Receptor binding of coronaviruses

Attachment of coronaviruses to the cell surface is mediated by the S protein through its interaction with receptors protruding from the plasma membrane into the surrounding environment. For several viruses, specific proteins have been shown to serve as receptors (Table 2). Alphacoronaviruses attach to aminopeptidase N (APN). Some members have a species specific binding behaviour, e.g. porcine APN is only recognized by TGEV (DELMAS et al. 1992), and human APN is only used as a receptor by HCoV-229 E (YEAGER et al. 1992), but Feline APN serves as a receptor for feline coronaviruses, TGEV and HCoV-229 (TRESNAN et al. 1996). MHV interacts with CEACAM1, a cell adhesion molecule within the carcinoembryonic antigen family of proteins (DVEKSLER 1991). SARS-CoV recognizes angiotensin-converting enzyme 2 (LI et al. 2003). For several members of betacoronaviruses (BCoV, HCoV-OC43, and HEV) 9-O-acetylated sialic acid serves as a receptor determinant (Table 2). Furthermore, TGEV preferentially recognizes α 2-3-linked sia

Table 2. Binding activity of Coronaviruses^a

Virus binding to	TGEV	BCoV	HCoV-OC43	MHV	IBV	SARS Cov
a- Sialic acid	(Neu5Gc, Neu5Ac)	(Neu5, 9 Ac ₂)		(Neu5,9 Ac ₂ , Neu4,5 Ac ₂)	(Neu5 AC)	—
b- Protein receptor:	(Aminopeptidase N)	—		(CEACAM1)	—	ACE2

^aCoronaviruses may use specific proteins as receptor or recognize type of sialic acid as a receptor determinant on cell surface components. Sialic acids in brackets indicate that the respective derivative of neuraminic acid (Neu 5Ac: N-acetylneuraminic acid; Neu5Gc:N-glycolylneuraminic acid; Neu4,5Ac₂: N-acetyl-4-O-acetylneuraminic acid; Neu5,9 Ac₂:N-acetyl-9-O-acetylneuraminic acid) is not required or has not been shown to be required for infection of cultured cells (SCHWEGMANN-WESSELS and HERRLER 2006).

acid (SCHULTZE et al. 1993; KREMPL and HERRLER 2001). The importance of the sialic acid binding activity for the pathogenicity of IBV is not known.

1.4 Sialic acid

Sialic acid is a generic term for the *N*- or *O*-substituted derivatives of neuraminic acid a monosaccharide with a nine-carbon backbone (AJIT and SCHAUER 2008). The most common member of this group is N-acetylneuraminic acid (Neu5Ac). Sialic acids are found widely distributed in animal tissues and to a lesser extent in other species ranging from plants and fungi to yeasts and bacteria, mostly in glycoproteins and glycolipids (gangliosides). The amino group generally bears either an acetyl or glycolyl group but other modifications have been described. The hydroxyl substituents may vary considerably: acetyl, lactyl, methyl, sulfate, and phosphate groups have been found (SCHAUER 2000).

The role of sialic acids for many coronaviruses has been studied long time ago. Vlasak and colleagues observed that BCoV, HCoV-OC43 have the same behaviour like Influenza C virus in agglutination of erythrocytes. They reported that they all have the same common feature, a receptor-destroying enzyme which renders erythrocytes resistant to agglutination. Many researches started to study the importance of sialic acids for the members of coronaviruses, which show different affinities for binding to sialic acid. Transmissible gastroenteritis virus (TGEV) also has a sialic acid binding activity besides binding to a protein receptor. Infectious bronchitis virus also has a sialic acid binding activity (WINTER et al. 2006), but in addition it may interact with a defined protein receptor (SCHWEGMANN and HERRLER 2006).

There are some similarities in the sialic acid binding activity of TGEV as a representative of alphacoronaviruses and the gammacoronaviruses avian infectious bronchitis virus (IBV). Bingham and co-workers reported in 1975 that some IBV strains were able to agglutinate erythrocytes (BINGHAM et al. 1975). Similar to TGEV, IBV requires pre-treatment with neuraminidase for efficient haemagglutinating activity.

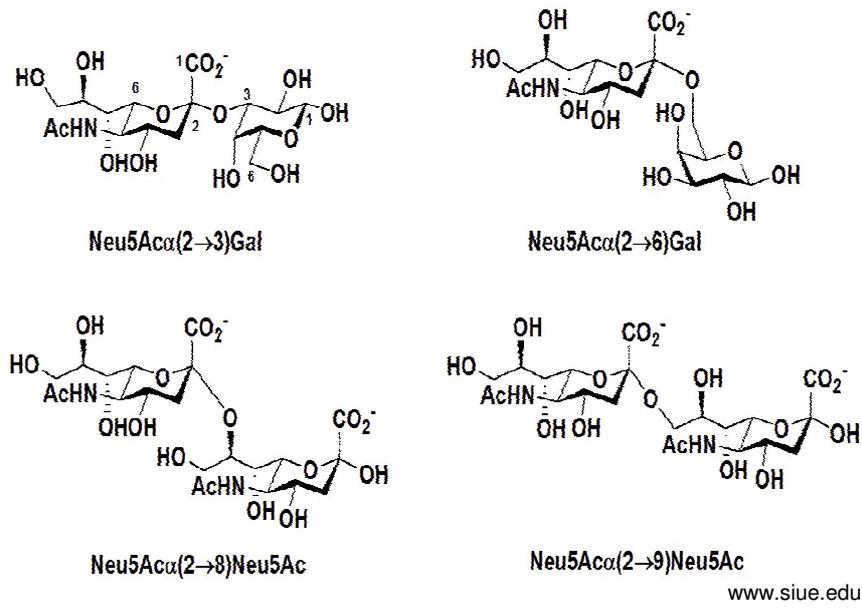


Fig 2 The chemical composition of *N*-acetyl-neuraminic acid (Sialic acids).

2 Aim of the study

The aim of this study is to compare the infection of four IBV strains (QX, 4/91, Italy02 and Beaudette) in different primary avian cell culture systems. In addition, the expression of the receptor determinant of IBV, alpha2,3-linked sialic acid (WINTER et al. 2006) is analyzed to correlate sialic acid expression with sensitivity of the cells to infection. The primary cell cultures used in this study reflect the main target organs during an IBV infection in the chicken, the respiratory tract, the kidneys and the reproductive tract (SEVOIAN and LEVINE 1957). For the respiratory tract tracheal organ cultures (TOCs) and precision-cut lung slices (PCLS) are used to cover the upper and lower respiratory tract. Primary chicken embryo kidney cells (CEK) are used to analyze infection of cells derived from the renal system, and chicken oviduct explants (COE) from immature chicken are used in this study to represent cells of the reproductive system. The advantage of tissue cultures like TOCs, PCLS and COE is that, they comprise cells in their *in vivo* arrangement, which can be used under *in vitro* conditions. Thus, the use of these cell culture systems helps to reduce the number of animal experiments and therefore is a contribution to animal welfare. The cell culture methods of TOCs and CEK are well established and have been used for infection analysis of IBV before. The method of PCLS has to be adapted from the mammalian to the chicken lung and the organ culture of COE has to be established in this study.

The role of alpha2,3-linked sialic acid in the initiation of an IBV infection has already been investigated using cell cultures and TOCs with three strains of IBV (WINTER et al. 2008), In this study we extended the analysis using three field strains (4/91, Italy02 and QX) which cause many problems in the poultry industry in Europe and other parts of the world. In CEK and TOCs, pre-treatment of the tissues with neuraminidase prior to IBV infection will elucidate the dependence of these strains on the presence of sialic acid. Detection of the cells infected by IBV within TOCs and PCLS by immunofluorescence will allow identify the primary target cells of these IBV strains and lectin staining will visualize the sialic acid expression of these cells. The adaptation of PCLS and COE to the chicken will open the way for further studies on

other viruses affecting the respiratory tract or the reproductive system, e.g. paramyxoviruses and influenza viruses.

3. Chapter 1

Comparative analysis of the sialic acid binding activity and the tropism for the respiratory epithelium of four different strains of avian infectious bronchitis virus

3 Chapter 1

Comparative analysis of the sialic acid binding activity and the tropism for the respiratory epithelium of four different strains of avian infectious bronchitis virus

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Running title: Comparison of sialic acid binding of IBV strains

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Abstract

Avian infectious bronchitis virus (IBV) is a major pathogen in commercial poultry flocks. We recently demonstrated that sialic acid serves as a receptor determinant for IBV on the tracheal epithelium. Here we compared the IBV strains Beaudette, 4/91, Italy02, and QX for their sialic acid-binding properties. We demonstrate that sialic acid binding is important for the infection of primary chicken kidney cells and the tracheal epithelium by all four strains. There were only slight differences between the four strains, indicating the universal usage of sialic acids as receptor determinants by IBV. In addition, we analysed the primary target cells in the respiratory epithelium of the four different strains and found all of them infected ciliated and goblet cells.

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4. Chapter 2

Differential Sensitivity of Well-Differentiated Avian Respiratory Epithelial Cells to Infection by Different Strains of Infectious Bronchitis Virus

4 Chapter 2

Differential Sensitivity of Well-Differentiated Avian Respiratory Epithelial Cells to Infection by Different Strains of Infectious Bronchitis Virus

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Running title: infection of the bronchial epithelium by IBV

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Keywords:

Infectious bronchitis virus, coronavirus, precision-cut lung slices, pathogenicity, bronchial epithelium

Abstract

Infectious bronchitis virus (IBV) is an avian coronavirus affecting the respiratory tract of chickens. To analyze IBV infection of the lower respiratory tract, we applied a technique that uses precision-cut lung slices (PCLSs). This method allows infection of bronchial cells within their natural tissue composition under *in vitro* conditions. We demonstrate that the IBV strains 4/91, Italy02 and QX infect ciliated and mucus-producing cells of the bronchial epithelium, whereas cells of the parabronchial tissue are resistant to infection. This is the first study, using PCLSs of chicken origin, to analyze virus infection. PCLSs should also be a valuable tool for investigation of other respiratory pathogens such as avian influenza viruses.

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5. Chapter 3

**Establishment of chicken oviduct explant cultures to
analyze infection of differentiated oviduct epithelial cells by
infectious bronchitis virus *in vitro*.**

5 Chapter 3

Establishment of chicken oviduct explant cultures to analyze infection of differentiated oviduct epithelial cells by infectious bronchitis virus *in vitro*.

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(In preparation)

Abstract

Infectious bronchitis virus (IBV), an avian coronavirus, replicates not only in the respiratory epithelium, but also in other tissues e.g. kidney, intestine, gonads and the oviduct. Especially infection of the reproductive system has a great economic impact on the poultry industry. Here, we established a culture system for cells from the oviduct, which can be considered as a main target organ of IBV infections. Our method allows virus infection of cells within their natural tissue composition under *in vitro* conditions. Virus antigen was detected already at 8 hours post-infection in most parts of the oviduct. Lectin staining revealed that the sensitive epithelial cells express alpha2, 3-linked sialic acid. This is the first study, using chicken oviduct epithelial cell explants to study viral infection and sialic acid distribution.

Keywords:

IBV, chicken oviduct epithelial cells, sialic acid, QX strain

Although IBV has been classified according to its pathogenicity as either respiratory or nephropathogenic or mixed pathogenic (IGNJATOVIC et al. 2002), the role of IBV in the reproductive system cannot be ignored as it not only causes reduction in egg production and egg quality (SEVOIAN and LEVINE 1957), but also has an effect on the oviduct maturation in young animals, being responsible for the appearance of false layers in the affected flocks (CRINION and HOFSTAD 1971; JONES and JORDAN 1972; McDOUGALI 1968). The effects on the reproductive system extend also to male gonads retarding the fertility (BLOTZ et al. 2004)

The chicken oviduct is divided into five parts with respect to their different functions during egg formation: infundibulum, magnum, isthmus, uterus and vagina. The infundibulum part, the place of fertilization, has also a role in secretory function during egg formation (AITKEN 1971). In the magnum the albumin is secreted and in the isthmus the cuticle is formed. The uterus forms the shell gland and the vaginal part is responsible for the formation of the outer shell cuticle and possibly for the shell pigments. Because of these important functions of each part, infection by IBV might cause disorders of the reproductive system like watery albumin, miss-shaped eggshells and wrong pigmentation. Although some IBV strains showed differences in their virulence for the oviduct (CRINION and HOFSTAD 1971), they probably all have the property to infect the epithelial cells of the oviduct (DHINAKA and JONES 1997). IBV infection of reproductive systems usually takes several days post-infection to be evident by the histopathological changes of the oviduct; usually it cannot be detected before 10 days p.i. (SEVOIAN and LEVINE 1957). Histopathological changes have been reported in experimentally infected chicken by several serotypes of IBV in all

parts of the oviduct, (CHOUSALKAR et al. 2007). This makes clear, why a method of oviduct tissue culture has great advantages for the analysis of IBV infections.

Animal experiments to investigate infections of the reproductive system are time-consuming, expensive and animal welfare aspects have to be considered.

In this preliminary work, we collected the oviduct from 18 weeks old SPF chicken. At this time point, the different parts of the oviduct could be easily identified. Mid-parts from the segments infundibulum, magnum, isthmus and vagina were selected and cut manually into thin rings of approximately 5 mm thick slices. The uterus portion was discarded, as no rings could be cut from this tissue. The rings were immersed in eDulb medium in 24 wells-plastic plates and carefully washed to remove the oviduct fluids. They were kept in an incubator at 37°C and embedded in eDulb medium. The viability of the rings was monitored by observing the ciliary activity under a light microscope and by a live and dead staining which showed that almost all cells of the chicken oviduct epithelial cell lining the oviduct were alive one day after preparation (Fig. 3).

To analyze the cells within COEC rings for their sensitivity to IBV infection, four rings of each part, each in a well of a 24-well plastic plate, were infected by the QX strain applying an inoculum of 1 ml (10^5 PFU/ml). After incubation for eight hours at 37 °C, COE explants were frozen in liquid nitrogen, cryosections were prepared and stained with antibodies to visualize antigen by indirect immunofluorescence microscopy. For detection of IBV antigen, a monoclonal anti N protein antibody was used. Infected cells were detected in rings of infundibulum, magnum and vagina (Fig. 4).

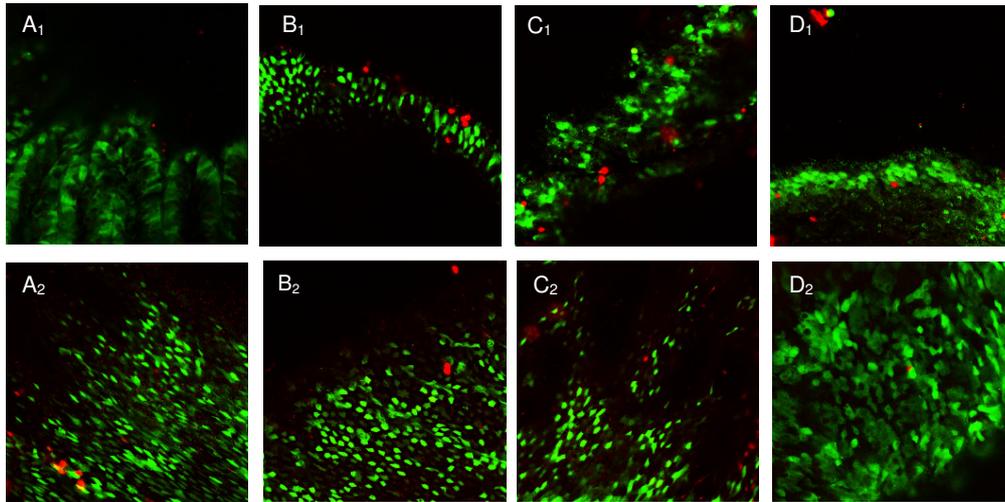


Fig.3 Viability of COE: Live and dead staining showed that the majority of the oviduct epithelial cells are alive (green staining) one day after preparation. Only few dead cells (red) are detectable. The apical epithelial cells of infundibulum (A₁), magnum (B₁), isthmus (C₁) and vagina (D₁) respectively, and the basal cells of infundibulum (A₂), magnum (B₂), isthmus (C₂) and vagina (D₂) respectively.

Having shown that chicken oviduct explants are suitable for infection studies, we also analyzed the samples for sialic acid expression. Staining of COE cryosections with MAII lectin indicated that alpha 2,3-linked sialic acid is abundantly expressed on the surface of the epithelial cells of infundibulum and magnum, and at lower amounts on cells of isthmus and vagina (Fig. 5). Future work has to establish whether the different parts show different sensitivity to infection by IBV. This preliminary data

show that this system of oviduct explants should be a valuable tool to investigate IBV infections and to analyze sialic acid expression on the epithelial cells of the oviduct.

This system will be interesting also for studies with other avian viruses infecting the oviduct

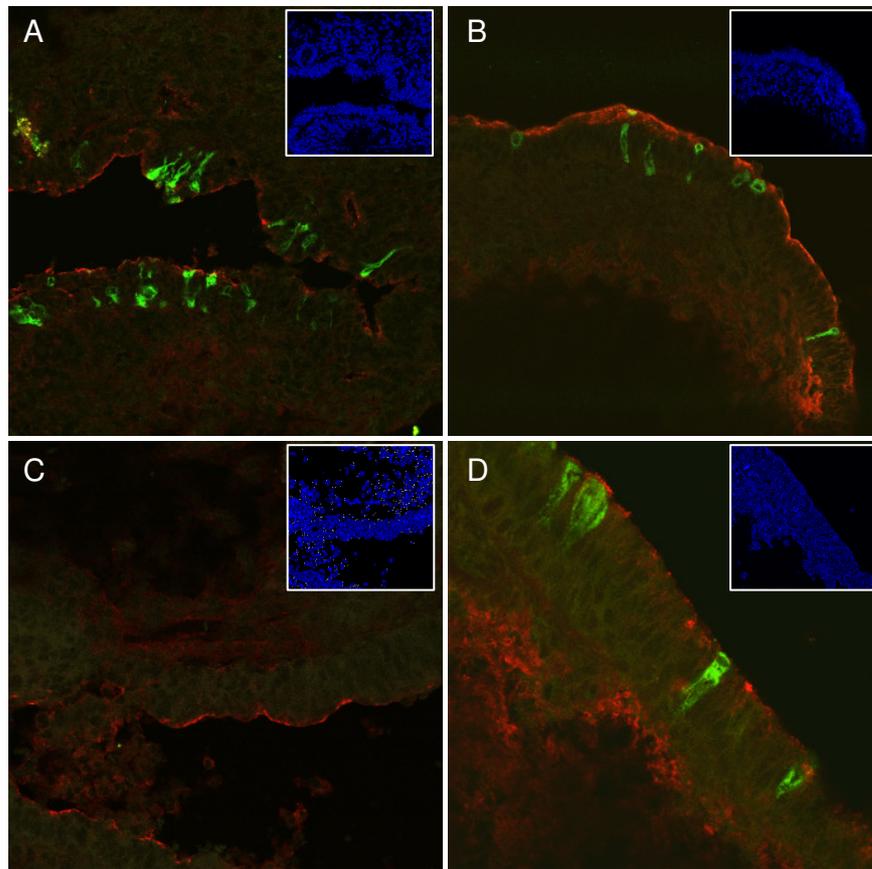


Fig.4 Infection of COE by the QX strain of IBV. Immunostaining showed the presence of viral antigen (green color) in infected epithelial cells of infundibulum (A), magnum (B), isthmus (C) and vagina (D). The nuclei were stained by DAPI (blue).

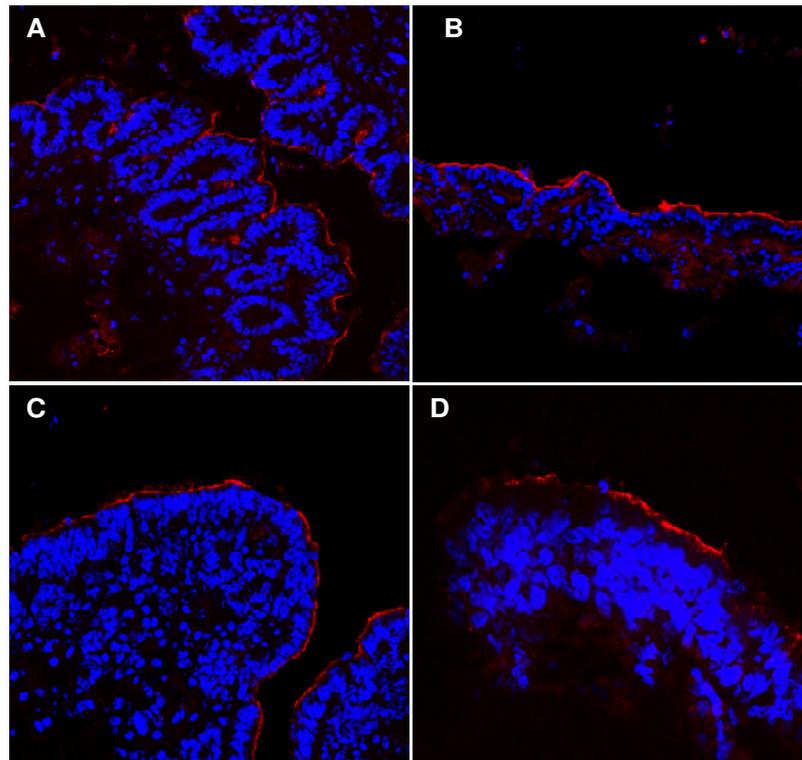


Fig.5 Sialic acid expression in COE. Lectin staining with MAA II (red) shows that alpha2,3-linked sialic acid is expressed in infundibulum (A) and magnum (B), isthmus (C) and vagina (D), the nuclei were stained by DAPI (blue).

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6 General discussion

Although vaccination programmes are used all over the world to control IBV infection, the economic losses within the poultry industry are still great. The reason for this problem is the continuous emerging of new viral variants which cannot be kept under control by vaccination, because they differ serologically from the vaccine strains. To understand the pathogenic potential of these variants, more information about their replication properties are required. Analyzing the receptors for IBV is important to understand the first steps of the replication cycle. In this study three recent field strains and a control strain were compared for their sialic acid binding property and for their primary target cells in the respiratory and reproductive tract. Furthermore, the sialic acid expression on these cells was analyzed using different cell culture systems.

6.1 Importance of the sialic acid binding property of different IBV strains

Coronaviruses are restricted in host range and tissue tropism (MCINTOSH 1990). It is already known, that IBV uses sialic acid as a receptor determinant (WINTER et al. 2008). This has been shown with the strains Beaudette, M41 and B1648. Among them, only the Beaudette strain has an extended species tropism in cell culture. Recently it has been suggested that the broader tropism may be related to the ability of this virus to use heparan sulfates as an additional attachment factor (MADU et al. 2007) which might facilitate its replication in non-avian cell cultures. Other strains of IBV can be propagated only in primary avian cells. Here, recent IBV isolates were compared for the ability to initiate infection in different primary cell culture systems and to use sialic acid as a receptor determinant. This comparison is of special interest, because many different serotypes of IBV exist which show an extremely high variation in parts of their spike sequences. As the binding site for sialic acid on the S protein has not yet been identified, a prediction about differences among IBV variants in their ability to use sialic acid as a receptor determinant cannot be made. If one compares IBV with other viruses that use sialoglycans as receptors, e.g. influenza viruses, it is noticeable that the latter viruses possess a receptor-destroying

enzyme, which helps to permeate the sialic acid-rich mucus layer on the trachea and bronchi. This enzyme also facilitates the release of virions from infected cells by desialylation and thus inactivation of the receptors which might prevent virus spreading. In the case of IBV, the lower affinity for sialic acid that has been demonstrated with different IBV strains may help to avoid this problem (WINTER et al. 2006). Another coronavirus which has a sialic acid binding property and lacks a receptor-destroying enzyme is the transmissible gastroenteritis virus (TGEV). TGEV uses the attachment to sialic acid as an additional binding activity, which helps the virus to infect cells under unfavourable conditions, i.e. in the intestine. The functional receptor of TGEV is porcine aminopeptidase N (DELMAS et al. 1992). The presence of the protein receptor on cultured cells is sufficient to allow infection. However, the sialic acid binding activity is required for the virus to be enteropathogenic (KREMPL et al. 1997). For IBV it is still unknown if there are other attachment factors which are necessary downstream of the binding to sialoglycoconjugates.

6.1.1 Relevance of sialic acids for infection of primary chicken embryo kidney cells (CEK)

CEK cells are a well established primary cell culture system which is widely used for propagation and titration of IBV strains. As many IBV strains have a predilection for the kidneys, primary kidney cells were used in this study to analyze the importance of sialic acid for viral entry. All strains used, Beaudette, Italy02, 4/91 and QX are able to infect primary kidney cells. And with all strains a clear reduction in the number of plaques was observed after pre-treatment of the cells with neuraminidase to remove sialic acids (see chapter 1). This finding indicates that not only laboratory strains or vaccine strains but also field strains are dependent on the presence of sialic acids on the cell surface to initiate an infection. The number of plaques was reduced with all strains by about 50%. The only exception was strain Italy 02; here, the reduction of plaques was about 75%. This may be explained by a lower affinity of the Italy 02 spike protein to sialoglycoconjugates on the surface of kidney cells. These results raise the question why the reduction of plaque numbers does not reach 100%, when the enzyme treatment removes the essential binding partners from the cell surface.

One possible explanation is, that the neuraminidase does not cleave all sialic acids from the surface; there may still be some receptor determinants left after the enzyme treatment. Italy 02 cannot utilize these remaining sialic acids in the same efficiency compared to the other strains. Probably, this strain has a weaker affinity for sialoglycoconjugates. One should also take into account, that the duration of the infection time, 24 hours, to enable plaque formation, allows already the new synthesis of sialoglycans by the cell, which can restore some receptors on the surface. Another explanation for the partial inhibition of infection by neuraminidase is that there may be an additional binding partner different from sialic acid, which IBV strains can utilize. Interestingly, the strain Beaudette behaves in a similar way like 4/91 and QX, even though it has been postulated that this strain has an additional binding property to heparan sulfates, which might explain its broader tropism on cell cultures (MADU et al. 2007). IBV may resemble TGEV, i.e. sialic acids may be used for primary attachment to cells, but subsequent interaction with a protein receptor may be required for entry into cells. This receptor may allow and may even be sufficient for infection but binding to sialic acid may increase the efficiency of infection. Whereas aminopeptidase has been identified as a receptor for TGEV, no such receptor is known so far for IBV. The presence of such a receptor would explain the restriction of most IBV strains to avian cells.

6.1.2 Importance of sialic acids for infection of tracheal organ cultures

Tracheal organ cells cultures (TOCs) are a well-established culture system for cells of the upper respiratory tract of chicken. It is simple and can be easily handled. It is mainly used for the propagation, titration, and diagnostics of avian viruses that cannot be grown in permanent cell lines. However, Winter et al. (2008) have shown that TOCs are a valuable tool to study infection of IBV in respiratory epithelial cells. These authors showed the importance of sialic acids for infection of the tracheal epithelial cells for the strains Beaudette, M41 and B1648 (WINTER et al. 2008). In this work, recent field strains were compared with the model strain Beaudette in the TOC system. All strains infected the tracheal epithelial cells, as indicated by the

induction of ciliostasis (see chapter 1). Strain QX appeared to be the most virulent strain in the TOC cells.

Complete ciliostasis (destruction of all ciliated cells) was observed already at the third day post-infection. This observation is in accordance with the results obtained in precision-cut lung slices (chapter 2) and with studies of experimentally infected chicken in which QX showed a great affinity to the respiratory system (BENYEDA et al. 2009). With Beaudette and 4/91, the time of complete ciliostasis in TOCs was determined to be at five days post infection and with Italy02 even the experimental time of five days was not sufficient to reach complete death of all epithelial cells. As discussed above, a feasible explanation is that Italy 02 has a lower binding affinity to sialoglycoconjugates on the tracheal epithelial surface, which may result in a lower number of infected cells.

When the TOCs had been pre-treated with neuraminidase to remove sialic acids from the apical surface of the cells, a delay in the onset of ciliostasis was achieved with all strains. The protection of the epithelium by this treatment was obvious when the ciliary activity was observed. The ciliary activity of TOCs can help to study the virus pathogenicity through observation and evaluation of the percentage of ciliary movement. Interestingly, even with the highly pathogenic QX isolate the epithelium showed after five days a residual ciliary activity of about 50% after enzymatic pre-treatment. For the other strains, this protective effect was even more pronounced. This effect demonstrates clearly that after removal of the receptor determinants, infection of the epithelial cells is strongly impaired. The reason why the epithelium could not be protected completely (maintenance of 100 % ciliary activity) may have the same reasons as discussed above. I: incomplete removal of sialic acids, II: restoration of sialic acids, III: Existence of a receptor that is not altered by neuraminidase treatment. Anyway, this result shows impressively the dependence of all strains on the presence of sialic acids on the tracheal epithelial surface.

6.2 Role of the susceptibility of cells in target organs for an IBV infection

There is still much to determine about the pathogenesis of IBV. It is of great interest to understand more about the course of infection. One question of interest is, which cells are highly permissive in the target organs and get first infected, when the virus enters the organ.

6.2.1 Target cells in tracheal organ cultures

In cryosections of infected TOCs, viral antigen was detected in two types of epithelial cells, in ciliated and goblet cells (see chapter 1). This was observed with all four strains analyzed here and has also been described for other lab or vaccine strains (WINTER et al. 2006 & 2008; SHEN et al. 2010). Therefore, the tropism for ciliated cells and mucus-producing cells may be a characteristic feature of all IBV strains. Interestingly in a recent publication, Shen et al. (2010) not only confirmed these results with two Taiwanese strains but they also showed that basal cells of the respiratory epithelium are resistant to infection. These data raise the question how the virus spreads from the trachea to other organs. Infection of the bronchi can occur via horizontal spread, but if the virus wants to get access to other target organs like, the kidneys and gonads/oviduct, it must leave the airways. Whether the virus gets to the blood vessels to spread via viraemia as reported by JONES and JORDAN (1972) or by another kind of strategy, this is still a matter of speculation.

6.2.2 Target cells in precision-cut lung slices

Precision-cut lung slices have so far mainly been used for pharmacological studies and have been described for several mammalian species. Recently, Goris et al. (2009) have shown that this technique can be adapted to the bovine lung and that it is a valuable tool to analyze viral infections in cells of the lower respiratory tract (GORIS et al. 2009).

To adapt this method to the chicken, embryonic lungs were used as organ source. In this way an organ culture was obtained, that comprises all structures of the avian lung, e.g. the bronchial and parabronchial areas. In immunofluorescent analysis, it was observed that infection occurs only in the bronchial epithelium. The cells of the parabronchial tissue were resistant to IBV infection. Only small areas at the edge of

the slice showed viral antigen by the immunofluorescence analysis, but this can be explained by the slight destruction of the cells in this area which impairs the integrity of the tissue and thus enables the virus to establish an infection. All strains, Italy 02, 4/91 and QX showed the same tropism for the bronchial epithelium (see chapter 2). Also the target cells within the epithelium did not differ between the different strains. As already observed in the trachea, they infected, ciliated and goblet cells. One can assume from these results that the pathogenesis of an IBV infection in the chicken is directly linked to the high susceptibility of these cell types to an IBV infection. The typical respiratory symptoms like gasping, coughing, tracheal rales and nasal discharge and the appearance of bronchitis without pneumonia, can be explained by the destruction of the ciliated and goblet cells in the tracheal and bronchial epithelium.

When the number of cells infected by the different strains was compared, it was noted that the QX isolate had a higher affinity to the bronchial epithelial cells than the strains 4/91 and Italy 02 (chapter 2). When the same amount of virus (10^5 pfu) was added to the cultures, more cells were infected by QX than by the other two viruses. When the amount of virus in the inoculum was diluted 10 fold, viral antigen of the QX strain was readily detected by fluorescence microscopy but not in the case of the other two viruses. This leads to the conclusion that the QX strain is more efficient in infecting the epithelial cells compared to 4/91 and Italy 02. This result is in accordance with the results obtained with TOCs. As discussed above, the QX strain was the fastest of the analyzed strains to induce complete ciliostasis in TOCs (chapter 2). Whether this is due to a stronger binding of QX to the sialic acid receptors or due to recognition of other binding partners on the cell surface remains unclear. It might also be, that factors downstream of viral attachment during the replication are responsible for this result. This effect that we have described *in vitro* can also be observed *in vivo*, as described by Benyeda and co-workers (2009). These authors found the QX strain to grow to higher titres in infected chicken and to cause more severe lesions. This shows the value of PCLS and TOC cultures to make predictions about the infection *in vivo*.

6.3 Distribution of sialic acid on target cells for IBV

To corroborate the finding that all of the analyzed IBV isolates use sialic acids on the epithelial cell surface as a receptor for a primary attachment to host cells, and to investigate the most prominent types of terminal sugars, lectin stainings were performed.

Staining with the lectin MAA II from *Maackia amurensis* revealed that alpha2,3-linked sialic acid is the predominant terminal sugar expressed on the surface of the chicken epithelial cells of trachea and bronchi. This is consistent with the findings reported by others (WAN and PEREZ 2006; WINTER et al. 2008; PILLAI and LEE 2010) and explains why different chicken respiratory viruses that use sialic acids as a binding partner like influenza A virus and IBV, both show a preference for alpha2,3-linked sialic acid.

The results of lectin stainings of the lung tissue helps to understand the different susceptibility of the epithelial cells of bronchi and parabronchi. Binding of MAII was only detectable on the surface of bronchial epithelial cells, whereas no binding of MAII was observed in the area of parabronchi. From our results we propose that an essential factor that determines the resistance of parabronchial cells to IBV infection is the lack of alpha2,3-linked sialic acids on the cell surface.

Staining of the lung tissue with the lectin *Sambucus nigra* agglutinin shows that on the bronchial epithelium hardly any alpha2,6-linked sialic acids are expressed. This result is in contrast to the result of Pillai and Lee (2010) who found high amounts of alpha2,6-linked sialic acids on the surface of chicken bronchi. This contradiction can be explained by the different techniques used in their study compared to this work. They used paraffin-embedded sections instead of cryosections. One cannot exclude that the paraffin embedding causes slight modifications of surface antigens. Furthermore, these authors used a lectin from a different supplier which might have a varying specificity. As it has been shown by Winter and co-workers (2008) that IBV uses alpha 2,3 linked sialic acids as receptor determinant, the question whether or not alpha2,6-linked sialic acids are expressed on IBV sensitive cells is not so relevant for IBV infections. However, this feature is of great interest for the research concerning current avian influenza viruses. In this context, it should be noted that our

results on the predominance of alpha2,3-linked sialic acid are in agreement with the report by Wan and Perez (2006).

An interesting result that was obtained in both TOCs and PCLS was that the binding of MAII was always greatly reduced after infection with either of the IBV strains. This finding raises the question whether the reduction in the expression of sialic acids is a consequence of the infection. One possible explanation for this phenomenon is that spike proteins of viral particles bind to the sialic acids and thus interfere with the binding of the lectin. It may also be that, after infection, the cells are able to down-regulate the expression of sialic acids to avoid over-infection. The reduction of sialoglycoconjugates on the surface of infected cells may also be an effect of receptor internalization after endocytosis of viral particles, as it has been described that IBV entry into cells is dependent on low pH suggesting endocytosis as entry strategy (CHU et al. 2006). However, it appears as if the reduction of sialic acids on the cell surface following infection affects the complete epithelium not only the infected cells. Thus, one can also speculate that there is a mechanism that allows down-regulation of sialic acids not only in infected cells but also in neighbouring cells, probably as a strategy of the host to impede viral infection.

The impact of this phenomenon for an IBV infection is not clear. As IBV lacks a receptor-destroying enzyme there is – in contrast to influenza viruses – no straightforward explanation for the disappearance of sialic acids from the cell surface. Whatever the mechanism for this finding may be, down-regulation of sialic acids may be an explanation for the effect of viral interference when two IBV serotypes are used for vaccination of the same animal (WINTERFIELD and FADLY 1975).

6.4 Infection in the chicken respiratory tract by IBV

Taken together, one can describe the early infection of chickens by IBV as follows: The virus enters the bird via the oro-nasal route. Further downstream it reaches the tracheal lumen. Its relative low affinity for sialic acids (when compared to influenza and Sendai virus (WINTER et al. 2006) may allow the virus to permeate the mucus barrier. On the surface of the epithelium IBV has access to ciliated and goblet cells,

both expressing alpha2,3-linked sialic acids. Whether an additional receptor is required for the virus to enter the cell remains to be established by future work.

After infection of differentiated cells in the epithelium, it most likely comes to a down-regulation of sialic acids on the surface of the epithelial cells. This phenomenon is not yet understood. It may have been developed by the host as a defence mechanism to avoid new infections.

On the other hand, this reduction of receptors may also be an advantage for the virus. Due to the lack of a receptor-destroying enzyme, IBV virions may facilitate the release from infected cells by down-regulation of sialic acids. These newly released viral particles may spread along the trachea into the bronchi leading to the typical respiratory symptoms of an IBV infection. How the virus spreads from the respiratory tract into other organs is not clear. The virus could penetrate the epithelium and reach the lamina propria with access to immune cells and blood vessels and could thus spread via viremia (JONES and JORDAN 1972). On the other hand, a viral spread from the luminal side of trachea and bronchi may also be considered. The virus has access from the main bronchi to the airsacs and airsacculitis is a common symptom of an IBV infection. So, when infection of the airsacs occurs, from there the virus may enter the abdominal cavity by penetrating the airsac. In direct proximity of the saccus abdominalis, there are the kidneys located and on the ventral side of the kidneys there are in very close proximity the gonads and the infundibulum of the fallopian tube. Via the infundibulum IBV particles might reach the epithelium of the oviduct from the luminal side. The close proximity of the main target organs of IBV within the abdominal cavity makes a spread via the abdominal airsac a feasible scenario. Future work has to test this hypothesis.

6.5 Infection in chicken oviduct epithelial cells by IBV

Infection of the reproductive system by IBV causes many economic losses due to reduction in the egg production and egg quality and to the appearance of false layers in the infected flocks (SEVOIAN and LEVINE 1957). Next to the analysis of IBV infections in cell culture systems of the respiratory tract, infection studies in a cell culture system of the oviduct was a big demand. Cutting and culturing thin rings of

the different parts of the oviduct proved to be sufficient to analyse IBV infections. Using immature chicken at an age of 18 weeks as organ donors turned out to provide cultures that were suitable to investigate IBV infection. Some studies have been investigating the infection by IBV in the oviduct of hormone-treated animals (PRADHAN et al. 1983; RAJ and JONES 1997) or in experimentally infected chicken (CHOUSALKAR and ROBERTS 2007; BENYEDA et al. 2009; CHOUSALKAR et al. 2009). The culture system described in this work has several advantages: 1) It does not require animal experiments, 2) The immature oviduct reflects the situation of IBV damages before the onset of lay. 3) No hormonal side effects.

In first infection studies with this system, the IBV strain QX was used. By immunofluorescence staining, we could detect infected cells in most parts of the oviduct: in infundibulum, magnum and vagina. Further experiments have to confirm these results. The QX strain is of special interest for infection studies of the oviduct as this strain is highly pathogenic for the reproductive tract, leading to cystic dilatation of the oviduct which is a prominent feature that has been related to the QX strain (BENYEDA et al. 2009). In first experiments analyzing the sialic acid expression of the oviduct epithelial cells, we found that all the analyzed sections of the oviduct including the infundibulum, magnum, isthmus and vagina showed positive staining for alpha2,3-linked sialic acid. This finding is in agreement with the results obtained by Pillai and Lee. These authors stained the oviduct of layers with MAA (PILLAI and LEE 2010). The preliminary results obtained with the chicken oviduct explant system demonstrate its intrinsic value for the investigation of IBV infections and for the analysis of sialic acid expression on the epithelial cells of the oviduct. This system will be interesting also for studies with other avian viruses infecting the oviduct.

7 Summary

Sahar Abd El Rahman (2010)

Comparative analysis of current infectious bronchitis virus isolates in primary cell culture systems

Avian infectious bronchitis virus (IBV) is the causing agent of a highly contagious disease with a major economic impact on the poultry industry. It is characterised clinically by respiratory, renal and reproductive manifestations. Despite various vaccination protocols, IBV still plays a role in poultry flocks, mostly because of the appearance of new variant strains which are not neutralized by antibodies induced by available vaccines.

Viral entry into host cells is mediated by binding of the viral glycoprotein S to a receptor on the cell surface. Alpha 2,3 linked sialic acid has been reported to play an important role as a receptor determinant for IBV. Here, a comparative study of current field strains, 4/91, Italy 02 and QX has been carried out to investigate their dependence of sialic acid for infection in different primary cell culture systems. To reflect the main target organs of an IBV infection in chicken, the following tissue cultures were used in this study: a) primary chicken embryo kidney cells, b) chicken tracheal organ cell cultures (TOCs), c) chicken precision-cut lung slices (PCLS) and d) chicken oviduct explants (COE).

Removal of sialic acids from the surface of the target cells by treating the cells with the enzyme neuraminidase affected the infection of all analyzed IBV strains. In primary chicken kidney cells, a plaque reduction test revealed that desialylation reduced the number of plaques with all strains. Infection of TOCs by different IBV isolates results in ciliostasis, which can be observed under a light microscope. In TOCs treated with neuraminidase prior to infection, a prolonged ciliary activity was observed. These results indicate that sialic acids play an important role for the infection of all analysed IBV strains.

In addition to the dependence of the IBV strains on sialic acid, the primary target cells in the epithelium of trachea and bronchi were identified. Immunofluorescence analysis of infected TOCs and PCLS revealed that ciliated and goblet cells are sensitive to infection by all strains analysed. No viral antigen was detected in cells of the parabronchi. Staining of the sensitive cells with the lectin MAAII, to detect alpha 2,3-linked sialic acids, showed that this linkage type of sialic acid is abundantly expressed on the target cells. Interestingly, the amount of sialic acids on the cell surface detectable by MAAII was reduced after infection by the different IBV strains in the trachea and also in the bronchi.

First infection experiments in chicken oviduct explants show, that these tissue cultures can be infected by IBV and a lectin staining revealed, that alpha2,3-linked sialic acids are expressed on the oviduct epithelial cells. Future work will compare the infection by IBV in different parts of the oviduct and will analyze the expression of sialic acids.

In this study, we have established two culture systems for well-differentiated epithelial cells, PCLS and COE, which promise to be valuable tools in the future to analyse the infection of the respiratory tract and oviduct by IBV and other avian viruses.

8 Zusammenfassung

Sahar Abd El Rahman (2010)

Vergleichende Analyse von aktuellen Stämmen des infektiösen Bronchitis-Virus in primären Zellkultur-Systemen.

Das aviäre infektiöse Bronchitis-Virus (IBV) ist der Erreger einer hochkontagiösen Erkrankung, welche eine große Bedrohung für die Geflügelindustrie darstellt. Diese Erkrankung wird klinisch durch Manifestationen im Respirationstrakt, in den Nieren und im Legeapparat charakterisiert. Trotz bestehender Impfprogramme spielen Infektionen mit IBV in den Geflügelbeständen noch immer eine große Rolle, vor allem durch das Auftreten von neuen Virusvarianten, gegen die die vorhandenen Impfstoffe nicht schützen.

Der Viruseintritt in Wirtszellen wird über die Bindung des viralen Glykoproteins S an einen Rezeptor auf der Zelloberfläche vermittelt. Die Bedeutung von alpha2,3-gebundenen Sialinsäuren als Rezeptordeterminante ist bereits beschrieben worden. In dieser Arbeit wurde eine vergleichende Studie über die aktuellen Feldisolate 4/91, Italy 02 und QX durchgeführt, um ihre Abhängigkeit von Sialinsäuren in primären Zellkultursystemen zu untersuchen. Um die Hauptzielorgane einer IBV-Infektion im Huhn abzudecken, wurden folgende Gewebekulturen verwendet: a) primäre Hühnerembryo-Nierenzellen, b) Trachealringkulturen, c) Lungen-Präzisionsschnitte und d) Hühner-Legedarm-Explantate. Das Entfernen der Sialinsäuren von der Zelloberfläche durch die Behandlung der Zielzellen mit dem Enzym Neuraminidase, führte dazu, dass alle in die Untersuchung einbezogenen Stämme in ihrer Infektion beeinträchtigt waren. Ein Plaque-Reduktionstest in primären Hühnerembryo-Nierenzellen ergab, dass nach einer Neuraminidase-Behandlung die Anzahl der Plaques bei allen Stämmen vermindert war. Infektionen von Trachealringen durch verschiedene IBV-Stämme verursachen eine Ziliostase, die im Lichtmikroskop deutlich beobachtet werden kann. In Trachealringen, die mit Neuraminidase vorbehandelt wurden, konnte eine verlängerte Zilienaktivität beobachtet werden.

Diese Ergebnisse zeigen, dass Sialinsäuren eine wichtige Rolle für die Infektion aller untersuchten IBV-Stämme spielen.

Zusätzlich zur Abhängigkeit der Virusinfektion von Sialinsäuren wurden die frühen Zielzellen im Tracheal- bzw. Bronchialepithel identifiziert. Die Immunfluoreszenz-Analyse von infizierten Trachealringen und Lungenschnitten ergab, dass sowohl zilienträgende Zellen als auch Becherzellen empfänglich sind für eine Infektion durch alle verwendeten Stämme. In den Zellen der Parabronchi konnte kein Virusantigen nachgewiesen werden. Eine Färbung der empfänglichen Zellen mit dem Lektin von *Maackia amurensis* II (MAAII) zum Nachweis alpha2,3-gebundener Sialinsäuren ergab, dass diese Art der Verknüpfung der Sialinsäuren auf den Zielzellen vorherrscht. Interessanterweise waren die Sialinsäuren, die von MAAII detektiert werden können, nach einer Infektion durch die verschiedenen IBV-Isolate auf der Zelloberfläche reduziert. Dies wurde sowohl in der Trachea als auch in den Bronchi beobachtet. Erste Infektionsexperimente in Legedarm-Explantaten von Hühnern zeigen, dass diese Gewebekulturen von IBV infiziert werden können. Eine Lektinfärbung macht deutlich dass alpha2,3-gebundene Sialinsäuren auf den Epithelzellen des Legedarms exprimiert werden. In künftigen Arbeiten soll die Infektion verschiedener Bereiche des Legedarms durch IBV vergleichend untersucht und die Expression von Sialinsäuren genauer analysiert werden.

In dieser Arbeit wurden zwei Kultursysteme für enddifferenzierte Epithelzellen, PCLS und COE, etabliert, die sich bei künftigen Arbeiten als interessante Hilfsmittel erweisen werden, um die Infektion des Respirationstrakts bzw. des Ovidukts durch IBV und andere aviäre Viren zu untersuchen.

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Declaration

I herewith declare that I autonomously carried out the PhD-thesis entitled:

Comparative analysis of current infectious bronchitis virus isolates in primary cell culture systems

I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.

I conducted the project at the following institutions:

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The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affirm the above statements to be complete and true to the best of my knowledge.

Sahar Abd ElRahamn