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Receptor binding domain of infectious bronchitis virus pathogenic M41 strain.

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Contents:

Summary:

IBV (Infectious Bronchitis Virus) is a positive, RNA corona virus which predominantly causes disease in chickens (Gallus gallus) and causes great economical losses.

Earlier studies showed that the spike protein is responsible for receptor binding of the virus to host cells. The spike protein is a glycoprotein which consists of several domains: the ectodomain (outside the virus particle) a transmembrane domain (through the lipide layer) and a cytoplasmic domain (within the virus particle). Up till now the S1 part of the ecotodomain, (216 aminoacid residues to the N terminus of IBV), seems to be responsible for binding. S2 is believed to be responsible for fusion of the virus particle with the host cell.

In this study, we investigated the domain within S1 of M41 IBV which is responsible for attachment to the respiratory tissues. To do this we will recombine the S1 protein of two strains of IBV, Beaudette (a nonpathogenic laboratory strain) and M41 (a wild type pathogenic strain). We will test the binding capacity of this recombined protein on chicken tissue.

In conclusion, based on the result we obtained, our hypothesis: aminoacid residues relied on the S1 domain are important for binding to the respiratory tissue, could not be confirmed completely. Binding of IBV is not completely depending on the presence of one amino acid. It can be said that Beaudette gains partly binding capacity. And amino acid 38 does play an important role in IBV binding. It remains unclear why this recombined protein does bind to chicken CAM tissue but not to chicken trachea tissue.

Introduction:

1. Coronavirus characteristics:

Coronaviruses are single stranded positive RNA viruses. They have the longest known RNA genomes, variating from 30 to 32kb. Coronaviruses are 80-160nm in diameter and their nucleocapsid is 10-20nm.[12, 4] . The virus is enveloped. Coronaviruses are built out of several structural proteins: the nucleocapsid protein (N) which covers the RNA.[14] The membrane protein (M) and the envelop protein (E) are responsible for maturation of the virus particles.[14] The spike protein (S) is important for virus tropism and infection[3, 15, 28]. Coronaviruses that belong to group 2 also contain a hemmagglutinin esterase protein (HE). Apart from this big proteins, a lot of non structural proteins are discovered. Their function is however not clear[3, 15].

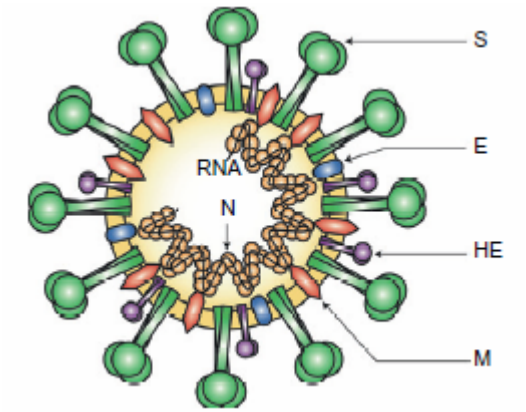


Figure 1[27]

Coronaviruses are seen in many animal species. Many cases of flu are induced by coronaviruses.[9] Especially the outbreak in November 2002 with "Severe Acute Respiratory Syndrome" SARS caused awareness when many people got sick and diseased due to this virus.[22,16]

Classification of nidoviruses.

Order	Family	Sub-Family	Genera	Representative Animal Species	Host and Tissue Tropism
Nidovirales	Coronaviridae	Coronavirinae	Alphacoronavirus	Transmissible gastroenteritis virus	Pigs (GI)
				Feline Coronavirus	Domestic cats (GI, Peritoneal)
				Bovine Coronavirus	Cattle (GI)
				Bat Coronavirus HKU2 and HKU8	Bats (Carrier)
			Betacoronavirus	Mouse Hepatitis Virus	Mice (Res, GI, Hep, CNS)
				Bat Coronavirus HKU9	Bats (Carrier)
				Severe Acute Respiratory Syndrome Coronavrus*	Palm Civets (Carrier) Bats (Carrier)
			Gammacoronavirus	Avian Infectious Bronchitis Virus	Chickens (Res, Neph, Rep)
				SW1 virus	Beluga Whale (Res, Hep)
		Torovirinae	Bafinivirus	White bream virus	Fish (GI, Hep)
			Torovirus	Breda virus (bovine torovirus)	Cattle (GI)
	Arteriviridae	-	Arterivirus	Porcine reproductive and respiratory syndrome virus	Pigs (Resp, Rep)
				Equine arteritis virus	Horses (Resp, Rep)
	Roniviridae	-	Okavirus	Yellow head virus	Crustaceans (prawns); cephalothorax

#: Same as the virus isolated in humans. (Abbreviations) GI: Gastrointestinal System, Resp: Respiratory System, Rep: Reproductive System, CNS: Central Nervous System, Neph: Nephrogenic System, Hep: Hepatic System.

Figure 2[12]

On veterinary domain there are several important diseases caused by a coronavirus like “winter dysentery” caused by Bovine Coronavirus which infects the intestinal tract[20], Feline Infectious Peritonitus (FIP) coronavirus in cats which causes a severe syndrome defined by severe peritonitis.[21] Porcine Reproductive and Respiratory Syndrome Virus (PRSSV) in pigs which causes severe respiratory symptoms especially in the lungs[18] and Infectious bronchitis virus IBV in chickens, which infects the respiratory tract. However not all coronaviruses cause severe disease, most of them are of veterinary importance due to the economical losses they evoke.[31]

It turns out to be difficult to vaccinate against coronaviruses.[5] When immunization occurs, it only lasts a short period of time and immunization to other strains of IBV do not occur.[2, 5]
Because coronaviruses are very susceptible to mutations, a large variation in coronaviruses exists. It seems that the S glycoprotein, which all coronaviruses possess, is responsible for the binding of the virus and therefore influences the immune response.

Although many coronaviruses are very similar, huge differences occur among different Spike proteins.[5] This difference in spike protein could explain why cross immunity cannot be obtained.[19]

2. Virus replication:

Virus replication takes place in the cytoplasm of the host cell. The coronavirus binds to the receptors of the target cells. When the virus contains a HE protein, this protein is involved in binding. For other coronaviruses this task rests with the spike protein. For IBV the spike protein is a 60-64K glycoprotein which binds to cilia and goblet cells in the epithelial cells of the trachea in chickens. The binding of IBV is α2,3-sialylated glycans dependent.[28] The virus then enters the cell by fusions with the plasma membrane, or forms an endosome.[14] This is not clarified yet. [12]

After the virus enters the cell, the viral RNA is released from the virus particle. Host ribosomes translate the mRNA from which the “replicase complex” is formed. This complex transcript the mRNA to form new genomic RNA but also forms a set of positive-sense-sub-genomicsized RNA’s. This occurs with the assistance of host proteins (see figure 3)

This positive-sense-sub-genomic sized RNA’s are translated by host ribosomes to form the construct proteins: S,E,M and N. The N protein wraps around the positive sense genomic RNA into a ribonucleocapsid, and with help of beta actin the virus particle is assembled. After maturating in the Golgi apparatus the virus exits the host cel by exocytosis.[31]

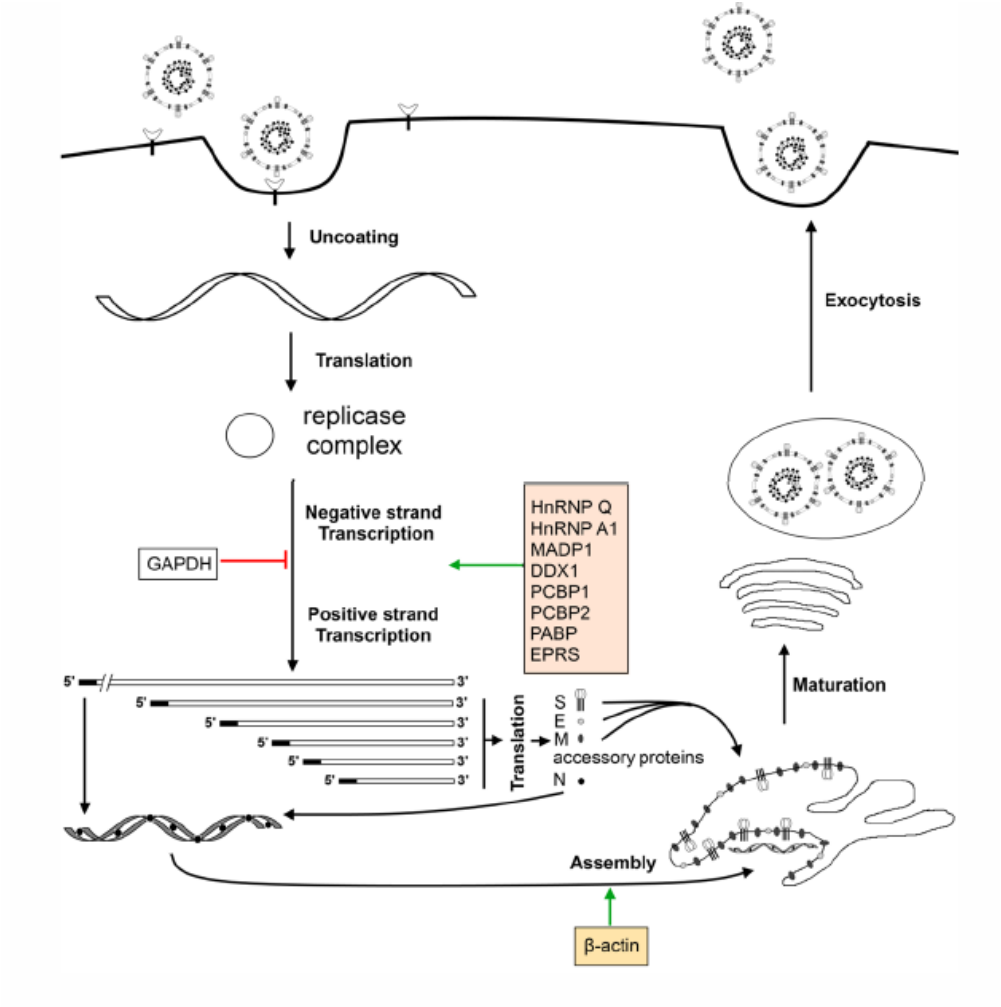


Figure 3[12]

3. IBV (Infectious Bronchitis Virus):

Avian Infectious bronchitis virus (IBV) is a 27,6 kb large, group 3 coronavirus belonging to the subfamily coronaviridae and the order nidovirales.[6] IBV primary infects the superficial respiratory tract. It binds to ciliated and mucus producing cells. The virus is mostly found in nose and trachea. After two days post infection the virus peaks and decreases after five to seven days post infection. [7] Also comparable titers are found in the lungs and air sacs. Through damage of the mucosa secondary infections can occur. The virus itself causes barely primary diseases. IBV causes great economical losses which makes it an important veterinary disease. Apart from the respiratory tract, virus particles can also be found in other epithelial cells like the kidney and parts of the digestive tract. IBV hardly ever cause clinical disease. However vaccines are available they trigger a poor immune response which results in a short period of immunization. Another problem is a lack of cross immunization.[5]

4. The spike protein

It turned out that the spike protein could predict the tropism of the coronavirus. Recombined spike protein of FIPV with the MHV coronavirus lead to the observation, that this recombined virus gained the ability to infect feline cells and simultaneously lost the ability to infect murine cells.[16] Also for different IBV strains the spike protein proofed to be of great importance relating to host tropism. Casais et al., 2003 discovered that IBV Beaudette virus with the spike protein of M41 gained the cell tropism of IBV M41.

This conclusion could explain why vaccinating for IBV was not that effective. At least evidence was put forward for this hypothesis when Casais et all 2004, put forward that the spike protein plays an important role in gaining immunity for the virus. They recombined IBV Beaudette strains with the M41 spike protein and tried to immune chickens by vaccinating them with Beaudette, M41 and the recombined virus. They discovered that the recombined virus gave a much better protection against M41 then vaccinating with only Beaudette. Only vaccinating with M41 gave 100% protection.

Spike protein not only seems to determinate the specificity of the virus, it also could relate the clinical symptoms to the place of binding.
Wickramasinghe et al., 2011 compared binding capacity of different IBV strains and the binding capacity of H5N1. It turned out that the cells which the virus binds to, correlate with the clinical symptoms that they cause. It turned out that non pathogenic IBV Beaudette also does not or hardly binds to chicken tissues in vivo. M41 binds mostly to ciliated cells and goblet cells in the trachea. In vivo this virus cause mainly a disease of the respiratory tract.

5. Aim of the study:

The purpose of this research is to fully understand the pathogenicity of IBV. With this information it would be possible to develop a better vaccine which would give a good protection against several strains of IBV (cross protection). This would be of great importance in the poultry sector, as it would decrease the economic losses caused by IBV.

In this study we concentrate on the binding capacity of IBV to chicken respiratory tissues. We want to know which amino acid is or which amino acids are responsible for binding of the virus to chicken tissues. Further terms for pathogenicity will not be discussed.
Hypothesis: aminoacid residues relied on the S1 domain are important for binding to the respiratory tissue.

In this study, we will investigate the pathogenicity of IBV by taking a closer look at the receptor binding site of IBV. To do this we recombined the S1 protein of two strains of IBV, Beaudette (a nonpathogenic laboratory strain) and M41 (a wild type pathogenic strain). We tested the binding capacity of this recombined protein on chicken tissue.

Materials en methods

1. Recombinant DNA Technology:

Two different proteins were constructed:
Beaudette S1 M41 aa 56,63,66,69
PCR1 on PCD5-Beaudette-S1
IBV-S1-dom#1 FW:gtcgcttccgtgctagca
IBV-S1-M41 aa 56-69 RV: ctggaaatgttcaccacgg
⇨ 122 nt

PCR 2 on pCD5-MBB-S1
-IBV-BS1-M41aa56-6-Fw: ccgtggtgaacatttccag
IBV-S1-dom#3 RV: atggatccggtacccctc
⇨ 1458 nt

Beaudette S1 M41 aa 19,38,43
PCR1 on PCD5-M41-S1
IBV-S1-dom#1 FW:gtcgcttccgtgctagca
IBV-S1-M41 aa 43 RV: ctggaaatgttcaccacgg
⇨ 100 nt

PCR 2 on pCD5-beaudette-S1
IBV-BS1-M41aa43-Fw: ccgtggtgaacatttccag
IBV-S1-dom#3 RV: atggatccggtacccctc
⇨ 1480 nt

PCR#3 on PCR fragment 1 and 2 (fragment#1:fragment#2 als 1:3)

PCR#4
IBV-S1-dom#1 FW:gtcgcttccgtgctagca
IBV-S1-dom#3 RV: atggatccggtacccctc
⇨ 1580 nt

After PCR#1 and PCR#3 electrophoresis was performed (1% gel with 1kb ladder) of the DNA to check if the size of the fragments was correct.

Master Mix:

Milli Q	36,5 µl
5x Phusion HF buffer	10 µl
10 mM DNTPs	1 µl
Primer FW	0,5 µl
Primer RW	0,5 µl
Phusion HF DNA polymerase	0,5 µl
Total volume	49 µl
Template DNA	1 µl
Final Volume	50 µl

PCR #1

		122nt		1460/1475nt	
		Temperature	Time	Temperature	Time
1	Initial denaturation	98 C	30”	98 C	30”
2	Denaturation	98 C	10”	98 C	10”
3	Annealing	64 C	30”	64 C	30”
4	Extension	72 C	30”	72 C	42”
	Step 2-4 10 cycles			Step 2-4 35 cycles	
5	Final extension	72 C	10’	72C	10’
6	Hold	4 C	hold	4 C	hold

PCR #2 (overlap PCR)

		Temperature	Time
1	Initial denaturation	98 C	30”
2	Denaturation	98 C	10”
3	Annealing	64 C	30”
4	Extension	72 C	42”
	Step 2-4 10 cycles		
5	Final extension	72 C	10’
6	Hold	4 C	hold

PCR #3

		Temperature	Time
1	Initial denaturation	98 C	30”
2	Denaturation	98 C	10”
3	Annealing	64 C	30”
4	Extension	72 C	42”
	Step 2-4 35 cycles		
5	Final extension	72 C	10’
6	Hold	4 C	hold

PCR#1 and #3 purification was performed with PCR purification kit (High Pure PCR cleanup micro kit, Roche). DNA was digested with restriction enzymes: NheI and BAMHI.
Vector: PCD5 digested with NheI and BAMHI.

	Digestion PCR#3	Digestion vector
Milli Q	18 µl	14,5 µl
10X Tang buf	4 µl	4 µl
Sample	10 µl	13,5 µl (741ng/ µl)
NHeI	4 µl	4 µl
BAMHI	4 µl	4 µl

After digestion of the vector, electrophoresis, the correct band was extracted from the gel using the High Pure PCR cleanup micro kit, Roche.

Purification of the digested PCR#3 product, DNA measured with nanodrop.
The vector was ligated with DNA sample, incubated overnight. At 4 C.
Ligation:

	1	2	3	4	Empt
Milli Q	12,5 µl	12,5 µl	11,7 µl	10,7 µl	15,7 µl
10XT4 DNA buf	2 µl	2 µl	2 µl	2 µl	2 µl
DNA insert	3,2 µl	3,2 µl	4 µl	5 µl	0 µl
Vector	1,3 µl	1,3 µl	1,3 µl	1,3 µl	1,3 µl
T4 DNA ligase	1 µl	1 µl	1 µl	1 µl	1 µl

Transformation of E.coli MC1061 with expression vector pCD5-Beaud S1 M41 aa 19, 38, 43_GCN4-ST2 and pCD5-Beaud S1 M41 aa 56, 63, 66, 69_GCN4-ST2 by electroporation.
1 µl of the ligation product was used and mixed with 20 µl MC1061 E Coli. It was incubated for 1'. Electrophoresis was done with the sample using BIO Rad Gene Pulser Cuvette, using 0.2 electrode gap, pkg. of 50.

10 µl glucose was mixed with 1ml LB broth. It was incubated for 1 h. After 30' 10µl of 0,1 µg/ml Tetracycline was added. After incubation the samples were centrifuged . 900 µl of the LB broth was removed and the pallet in was dissolved in 100 µl. 100 µl was spread on a LB agar plate, which was treated with antibiotics (10 µl tetracycline, 10µl Ampincilline and 80 µl H2O per plate). The plates were incubated overnight.

After incubation, 4 samples from each plate were taken and each colony was dissolved in 50 µl LB broth. In total we have 4x4 samples and 2 samples of the Empty vector.

A colony PCR was performed on each sample. pCD5_seq_Fw and pCD5_seq_RV_2685 as were used as primers.

Mastermix:

Milli Q	12,2 µl
10X Taq buffer	2 µl
10 mM DNTPs	0,4 µl
Primer FW	1 µl
Primer RW	1 µl
Taq DNA polymerase	0,5 µl
MGcl	1,2 µl
Total volume	18 µl
Template DNA	2 µl
Final Volume	20 µl

		Temperature	Time
1	Initial denaturation	95 C	4'
2	Denaturation	95 C	30"

3	Annealing	56 C	30”
4	Extension	72 C	90”
	Step 2-4 35 cycles		
5	Final extension	72 C	5’
6	Hold	15 C	hold

The PCR product was loaded on gel and the samples with a size of 15,80 kb were selected. This sample was put in a medium of 2ml LB broth and 1 µl Tetracycline en 1 µl Ampicilline and was grown for at least 16h. After purification with the Med prep purification kit the sample was sent for sequencing with Macrogen. If the sample had the correct construct we continued as followed:
50 µl of the miniprep sample was mixed with 100ml LB broth , 50 µl Ampicilline, 50µl Tetracycline. It was incubated for 16 hours and the DNA was purified with the Qiagen plasmid purification kit. And DNA was measured by nanodrop.

2. Cell culture:

Passage 20 of HEK293T cells was used. Cells were grown in T175 bottles. New flask were prepared 10^7 cells per flask. The next day the conference was 50-60%
5ml medium per flask was removed. For each sample was used: 300 µl PEI + 1000 µl medium -/- and 30 µl DNA (1 µg/ µl) + 1670 µl medium -/- mixed and incubated for 15’. After that 1500 µl was added to each flask (2 flasks per sample).
The next day the medium was discarded and replaced by transfection medium:
The cells were incubated for 6 days.

3. Protein expression:

On day 7 we removed the medium containing proteins and purified the proteins using strep-tag sepharose 50% suspension.

A western blot was performed to express proteins from the non-purified medium, the purified protein and the post purified medium.

Protein amounts were calculated with nanodrop.

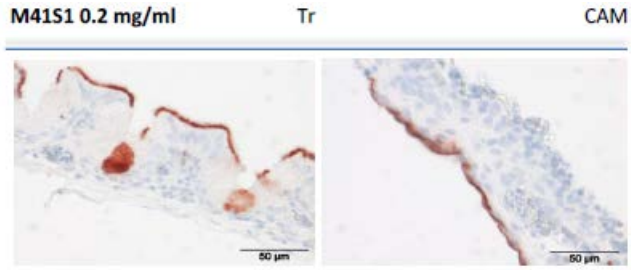
Purified proteins were dissolved in to similar concentrations.

Protein amount were controlled with westernblot.

4. Spike-histochemistry:

Purified proteins were tested on chicken (broiler) trachea and CAM tissue. Each protein was tested on both tissues. We used 0.2mg/ml Spike protein.

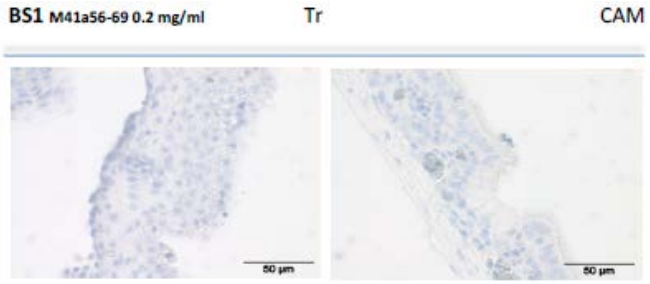
Results:



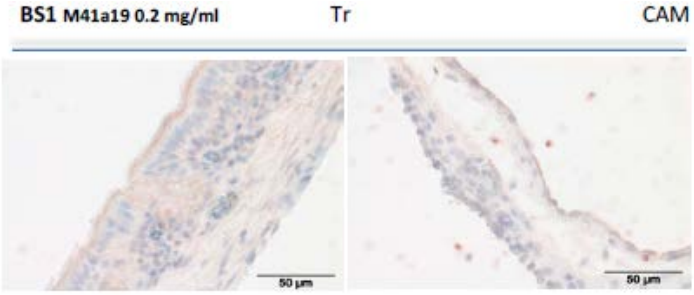
Figuur 4

M41S1 was used as a positive control. Spike Histo Chemistry shows clear binding to the epithelial cells and goblet cells of the trachea and to the chorio-allantoic membrane CAM cells. Visible by the red color.

The large construct Beaudette M41S amino acid 56, 63, 66, 69 did not show any binding to trachea or CAM cells.

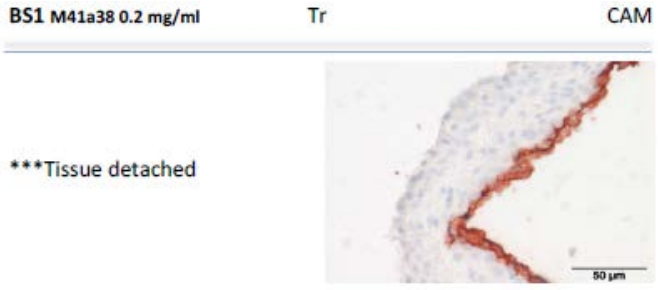


Figuur 5



Figuur 6

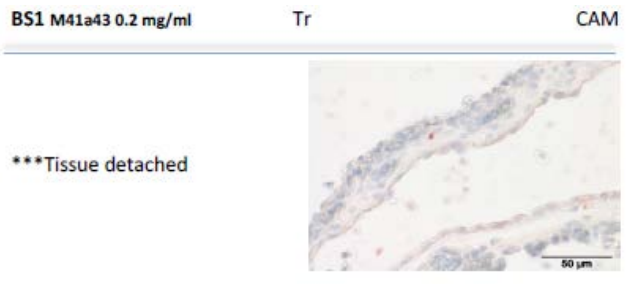
Beaudette M41S1 aa 19 was not unambiguous. A slightly pink color is seen all over the slide. It seems like the epithelial cells are more colored. The CAM tissue is less colored but in comparison to the construct in fig 5 its clearly more pink. When we repeated the SHC no binding could be detected in this construct and the results were clearly negative (data not shown).



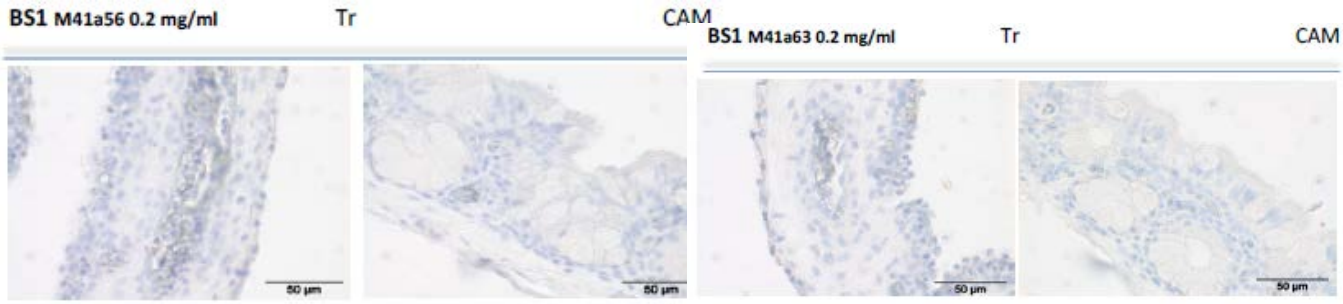
Figuur 7

The SHC of the construct Beaudette M41 S1 aa38 shows clear binding to the CAM cells. Due to tissue detachment, data of the trachea is not shown. In a repeated experiment no binding was seen on trachea tissue. CAM tissue was again positive for binding.

Beaudette S1 M41 aa43 seemed negative although a slightly pink color could be seen.



Figuur 8



Figuur 9

Figure 9 shows clearly negative results for the construct Beaudette S1 M41 aa56 and Beaudette S1 M41 aa 63. Also the other constructs were negative (data not shown).

Discussion

In addition to Materials and Methods it can be explained why we sometimes did not do exactly as the protocol prescribes (see attachment). For example, during SHC we used 0,2 mg/ml instead of 0,1mg/ml of protein. We did this because earlier attempts with less amount of protein did not give clear results. We wanted to be sure the amount of protein on the slides was sufficient enough. This could explain why some of the slides were more or less pink without a clear positive result.

Although we wanted to focus on the binding of S1 to respiratory tissue we also used CAM tissue along. We expected M41 S1 to bind to CAM tissue and Baudette not to. By using CAM along we had an extra positive/negative control. Also we could determent if the tropism of the constructs is comparable to either Baudette or M41.

In this study we repeated the SHC with proteins from the same transfection batch. It might be useful to repeat the experiments with proteins from a new transfection, in case any mistake happened in previous steps.

Although the S protein of IBV plays an important role in its tropism. It turns out that recombining the S protein of Beaudette with parts of M41 S1 does not result in gaining complete M41 tropism. By replacing amino acid 38 of Beaudette S1with amino acid 38 of M41 S1 the protein gains the ability to bind to CAM tissue but does not bind to Trachea tissue as M41 S1 does. It turns out that there are big differences between the tropism of M41 and Beaudette S1. Beaudette can grow in cultured cells where M41 does not. Beaudette however does not cause infection or disease in chicken, but can infect embryonated eggs.[26] In our experiment we discovered no binding of Beaudette S1 on CAM cells. This is in contrast with the observation that Beaudette is able to infect embroyonated eggs. This leads to the believe that it is possible for Beaudette to bind in a different way than M41.
Many theoretical explanations for this remarkable observation exist. Madu et all[18] observed that Beaudette has, apart from a sialic acid receptor, a heparine sulfate receptor in the S2 domain of the S protein, referred to as the Beaudette-specific motif, existing out of three amino acid changes identified in proximity to each other, which are specific for Beaudette and is not found in any other strains of IBV. They conclude that neither one of those receptors have great affinity with the attachment factors, and neither of them seems a critical receptor for binding. However it stays unclear which receptor would play a critical role in receptor binding of Beaudette.[13]
An other option could be that Beaudette binds differently depending on the environment the protein is in. It is possible that Beaudette can only bind to tissue, if no cleavage takes plays.[29] Other viruses like the FCoV seems to lose their ability to bind to tissue, when the S protein is cleaved.[23] It seems that for infection with Beaudette, cell to cell fusion needs to take place.[30] When the Spike is cleaved, cell to cell fusion does not occur and no infection is seen.[30] In our experiments we discovered that the S protein of Beaudette is cleaved twice. It is not yet clear what causes the protein to cleave. A possibility could be that pH changes during the process cause the Beaudette spike to cleave. An other reason could be that changes in the environment cause conformation changes which blocks the binding site of Beaudette. Studies showed that if Beaudette binding is indeed heparine sulfate dependent, cleavage of the protein could block this receptor from binding.[8] However blocking

experiments show that Beaudette binding can not be prevented by blocking with heparine, it is possible that this can not be compared with the situation in which heparine sulfate receptors would be absent.[17, 30, 10]
An other explanation could be that Beaudette binds to different cells in the embryonated egg apart from the CAM. It is shown in a study that two different strains of FCoV bind to different cells which gave an advantage to the more pathogenic strain.[23]
Finally due to the low affinity of Beaudette for several receptors it might be that the protein levels are too low to see any binding.
In summary this could explain why Beaudette can grow in embryonated eggs, but does not cause any visible binding to CAM tissue in our experiments. As mentioned above it could be possible that Beaudette needs more than one receptor to bind to, which receptor may not be present in our CAM tissue. It might be possible that due to cleavage Beaudettes binding site is not accessible, or that the amount of proteins incubated on the slides was not sufficient enough to detect any binding. Because of the low affinity of the receptor for its substrate, described above.
In answer to the question why Beaudette S1 M41 amino acid 38 binds only to CAM tissue, it might be possible that replacement of amino acid 38 causes a conformation change which evokes the exposure/activation of the Beaudette binding site, or maybe prevents the Spike from cleavage.[24] Maybe amino acid 38 is a part of the Beaudette-specific motif. This could mean that the binding we see in construct Beaudette M41 S1 amino acid 38 is not binding specific for M41 but in fact specific for Beaudette. This could mean that amino acid 38 is in fact of importance for Beaudette receptor binding instead of M41 receptor binding.
In conclusion, our hypothesis: The S1 protein of IBV M41 is responsible for binding of the virus to chicken tissue and it has 1 specific amino acid which is essential for its binding capacity is rejected. Binding of IBV is not completely depending on the presents of one amino acid. It can be said that Beaudette gains partly binding capacity. And amino acid 38 does play an important role in IBV binding. It remains unclear why this recombined protein does bind to CAM but not to trachea. And whether or not this says anything about M41 binding.
Therefor it would be interested to know if the different mutation constructs also are cleaved in different ways. Maybe this could tell us whether the way in which a protein is cleaved says something about its binding capacity.
Also of interest would be to know if the larger constructs Beaudette M41 aa 19,38,43 could bind to both trachea as CAM tissue.
Another option is to use mutations of M41 in Beaudette to see if it leads to a loss of binding capacity.
Finally it remains the question to what extend in vitro tests resemble the in vivo situation. Because proteins are very susceptible for change in pH and environment factors. This can cause conformation changes, which can keep the protein from binding. It also turned out that serially passaged virus strains in vitro could cause extended host range.[25,1] This suggest that mutation in binding sites can easily occur in vitro.

Acknowledgements:

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Attachments:

DNA Electrophoresis

Gel 1%:
0,5g agarose molecular biology grade eurogentec
50ml electrophorese buffer
Heat until it boils
Add 2,5 µl (1:20.000) Ethidium Bromide solution 100mg/ml in H2O (stigma)
1. Prepare gel 1%
2. Put comb in warm gel.
3. Let the gel to polymerize for 45minutes to 1 hour
4. Put the gel into biorad power pac 300 filled with buffer.
5. Remove comb
6. Prepare samples:
20 µl of PCR product
4 µl Thermo Scientific 6x DNA loading Dye
Marker:
10 µl Generuler 1kb Fermentas 0,5 µg/ µl
2 µl Thermo Scientific 6x DNA loading Dye
7. Load samples and marker
8. Run gel for 30' on 100V. using biorad power pac 300

Cell culture:

Materials:
-T175 flasks
-Lanza dmem medium with
- Trypsin 0,25%-EDTA (1x)
- PBS sterile

1. Grow cells in T175 till 90-100% confluent.
2. Remove medium from cells.
3. Wash cells twice with PBS
4. Add 2ml of trypsin and incubate till cells detach'
5. Knock flask on hand
6. Add DMEM+/+ (8 ml) and resuspend cells
7. Transfer cells to 50 ml tube.
8. Before transfection count cells and calculate for 10^7 cells per flask.
   Passaging use 1:30 dillution.
9. Add up to 25ml of DMEM+/+

Purification proteins using sapharose 50%:

1. The supernatant removed the cellular sediment after centrifugation is added with 1000µl of strep-tag sepharose 50 % suspension to 10ml of protein containing medium
2. Incubate at 40C overnight on a rotter
3. Centrifuge at 2000rpm for 10minutes

4. Pipette the supernatant carefully and transfer the beads to a 2ml appendorf
5. Wash x 3 times with PBS (2x 2000, 1x 3000 rpm)
6. Add elution buffer (¾ of the volume of the 50 % suspension)
7. Mix and keep for 5 minutes at RT
8. Centrifuge for 5 minutes at 2000rpm
9. Aliquot and store at -200C
Note: Avoid addition of protease inhibitors to purified proteins. Protease inhibitors hamper the binding of proteins to tissue in SHC

Western blot:

SDS-Page and Western blot (for IBV proteins with strep tag II) (by: I.N.Wickramasinghe)

Protein gel:
40% Acrylamide (Bio-Rad stored at 4 C)
Ammoniumpersulfate (APS, Bio- Rad stored at 4)
Dilute 0.1g in 1ml of distilled water for 10% working solution
TEMED (Bio-Rad stored at 4C)
0.5M Tris/HCl pH 6.8 (check for sediments before use)
1.5M Tris/HCl pH 8.8 (check for sediments before use)
10% SDS, pre warm at 37C if there is any crystallization

	Running gel	Stacking gel
	1 gel (7.5%)	1 gel (5%)
MilliQ (ml)	4.13	1.48
0.5M tris-HCL, pH 6.8 (ml)		0.63
1.5 tris-HCL, pH 8.8 (ml)	1.88	
40% acrylamide/bis (ml)	1.43	0.313
10% SDS (µl)	75	50
Mix well by rolling the tube manually		
10% APS (µl)	37.5	25
TEMED (µl)	3.75	2.5
Mix gently by rolling		

1. Prepare **7.5%** a gel/s according to the table
2. Pipette gently the mix into the assembled plates and overlay with 200ul of distilled water
3. Let the gel to polymerize for 45minutes to 1 hour
4. After polymerization, remove the over laid distilled water and pipette the stacking gel prepared according to the table
5. Carefully place the comb after filling the stacking gel on top of the running gel. Minimize making air bubbles in slots
6. Let the stacking gel to polymerize 10 to 15 minutes
7. Assemble the electrophoresis apparatus and place the gel in the electrophoresis buffer
8. preparation of the proteins
- Mix 10 µl of sample with 5 µl of laemmli buffer (freezer A) in hood.
- Heat at 95 C for 5 minutes, keep in ice

- Centrifuge for 1 minute at 3000 rtf
Note: type of proteins: 10ul/5ul of the supernatant of cells
Or 50 to 100ng of purified proteins dissolved in distilled water to make 10ul
9. Remove the comb and carefully load the proteins in to designated slots, including the protein marker.
10. Run the gel for 1-2 hours at 100V, RT, or until the dye runs out of the gel
11. Prepare the x1 blotting buffer and pour in to the blotting apparatus
12. Assembling the sandwich type blotting module, cut a piece of PVDF membrane to the size of the gel to start with
a. start from the black side of the holder
b. place the sponge first
c. then two whitman papers soaked in blot buffer
d. carefully remove the running gel from the plates and lay on the paper
e. role over the gel to remove air bubbles between paper and gel. Use a piece of pipette wetted with blot buffer for rolling
f. soak the PVDF membrane first in methanol and then in blot buffer, lay on top of the gel. Role over carefully
g. then repeat c. and role over
h. finally place a sponge and clamp the holder
13. place the holder in the blotting apparatus and cover with sufficient amount of buffer
14. blot for 1 hour at 100V or over night at 20V in cold room/in ice
15. take the membrane out of the blot and soak in methanol for 15 seconds
16. place the membrane carefully in a 50ml falcon tube with blotted surface facing into the interior
17. pour 10ml of distilled water in to the tube and wash for 2 minutes on the roller
18. prepare 3% milk powder (blocking buffer) and pour 10 ml in to the tube, incubate for 1 hour at RT on the roller
19. remove blocking buffer and wash as two quick washes followed by one 15 minutes wash and a quick wash with 0.1% tween PBS
20. Pour the blocking buffer containing strep-tactin HRPO (1:5000) in to the membrane, incubate for 1 hour at RT on the roller
21. Wash the membrane as described in step 19
22. Last wash with PBS without tween minimizes the back ground staining
23. take the membrane out and carefully place in a polyethene sac
24. pipette the pre mixed ECL detection reagents and seal the sac, roll over to spread the reagents evenly on the membrane
25. incubate for 5 minutes at RT
26. cut open one side and remove the reagents completely by rolling over
27. fix the sac with membrane in a x-ray cassette and expose for 5 seconds to 15 seconds
28. Develop the film to visualize protein bands

<u>Spike-histochemistry for IBV spike (S1) proteins as trimmers (by: I.N.Wickramasinghe)</u>

For formalin fixed paraffin embedded sections of adult chicken tissues
1. Deparaffinze and rehydrate sections on superfrost plus or poly-lysin coated glass in a series of xylene (5min.), 100% alcohol l(3min.), 96% alcohol (3min.), 70% (3min.)alcohol and distilled water (3 min 2x)

2. Antigen retrieval by boiling with citrate (pH 6) for 10 minutes in a microwave (90P) and keep at RT for 10 minutes
3. Rinse in PBS 3x 5 minutes
4. Inhibit endogenous peroxidase activity by immersing the slides in freshly prepared 1% hydrogen peroxide in methanol 30min at RT
5. Rinse in PBS/tween-20 3x 5 minutes
6. Treat the slides with goat serum (1:10 in PBS) 30min at RT
7. Premix spike protein (0.1mg/ml) and strep-tactin HRP 1:200 and incubate 30 minutes on ice (the amount of strep-tactin HRP has been optimized only for adult chicken tissues)
8. Incubate the sections with spike protein:strep-tactin HRP complex overnight at 40C
9. Rinse in PBS 3x 5 minutes
10. Incubate with AEC 15 minutes
11. Rinse in running tap water 5 minutes
12. Counter stain with Hematoxylin 30-40seconds
13. Rinse in running tap water 10 minutes
14. Mount with aquamount

Strep-tactin HRP lot: 1502-5017
The staining is different with different lots, new batches have to be optimized
The concentration should be adjusted according to the concentration of spike protein. Too much Strep-tactin HRP will give non specific staining in tissues

Buffers and solutions:

DNA electrophoresis buffer:
30,25g Tris
15,43g boorzuur
1,86g EDTA
10l H2O (distilled)

Stock Electrophoresis buffer (western blot) (5x)
5x stock)
7.5g Tris
36g Glycine
2.5g SDS
Fill to 500ml of distilled water

Working electrophoresis buffer (western blot) (1x)
100ml of x5 stock
400ml of distilled water

Stock blot buffer (western blot) (10x)
30.3g Tris
144g Glycine
Fill to 1L of distilled water

Working blot buffer (western blot) (1x)
100ml of 10x stock

200ml of Methanol
700ml of distilled water

Laemmili buffer
Contains:
0.188M Tris/HCl, pH 6.8
6% SDS
30% glycerol
15% 2-mercaptoethanol
0.03% bromophenol blue
For 100ml of laemmili buffer
Tris/HCl, pH 6.8 37.6ml
SDS 6g
Glycerol 34.5ml
2-mercaptoethanol 15ml
bromophenol blue 30mg
Distilled water 12.9ml

KCL detection reagents
0,5ml of A to 0,5ml of B
Mix and keep for 5 minutes at RT before using

Stock PBS x10 (with out ca2+ and Mg2+)
For 1l For 5l
80g NaCl 400g NaCl
2g KCl 10g KCl
14.4g Na2HPO4 72g Na2HPO4
2.4g KH2PO4 12g KH2PO4

PBS – tween 0.1%
Stock PBS 50ml
Distilled water 450ml
Tween- 20 500ul

Protein Markers and amounts being used for 1.0mm gel
- Pre stained protein marker (fermentas, at 4C) without strep tag II 5ul
- Unstained protein marker (Fermentas, at -20C) with the strep tag II 5ul
- Spectra multicolor broad range protein ladder (Fermentas, at -20C) 10ul

Citrate buffer (pH 6.0)
Citric acid 2,1g
Distilled water 1000ml
Mix to dissolve, adjust pH by adding 10N NaOH and store at RT

1% H2O2
2,85ml of 35% H2O2 in 100ml of absolute methanol

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