Localization of 9-O-Acetylated Sialic Acids in Bovine Respiratory and Gastrointestinal Tract using Influenza D HEF Protein

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Influenza D is a recently discovered virus, first collected in April of 2011 from nasal swabs of swine. It was soon discovered that the natural hosts of the virus were cattle, a species that is not susceptible to other subtypes of Influenza. Influenza D is closely related to Influenza C,both using the HEF-protein for attachment, in contrast to the HA protein Influenza A and Influenza B possess. The receptor the virus needs to enter the cell are 9-O-Acytelated Sialic Acids. To study the localization of the receptor of this new virus tissue micro arrays (TMAs) were made from the bovine respiratory tract and the bovine gastrointestinal tract. The initial attempts to produce HEF recombinantly were unsuccessful. Though unable to use IDV HEF to localize its receptor 9-O-Acetylated Sialic Acids, lectin stainings developed for Influenza A have been performed on the produced TMAs to visualize sialic acid presence. In performed lectin stainings mucus in both the bovine respiratory as gastrointestinal tract was found to contain contain 2,3- linked and 2,6-linked sialic acids. One of the liver sections tested positive for 2,6-linked sialic acids. A staining with an H1 Influenza A virus showed no attachment to bovine respiratory epithelium.

I. INTRODUCTION

In April of 2011 nasal swabs were collected from swine in Oklahoma, these swine were exhibiting influenza-like symptoms [10]. The strain found was provisionally named C/Oklahoma/1334/2011 (C/OK) (can also be found under D/swine/Oklahoma/1334/2011). However, when further analyzed, the segments of C/OK were only distantly related to other Influenza C (ICV) viruses, and no cross-reactivity on antibody was observed between C/OK and human ICV [10]. It was proposed that this was a new subtype of influenza circulating, Influenza D (from now on referred to as IDV).

IDV is a member of the Orthomyxoviridae family and a single strand, negative sense RNA-virus with 7 genomic segments. It shares less than 50% protein sequence with ICV, which is the influenza virus that is genetically the closest to IDV [8]. The virus has been found circulating in multiple locations, including France, Japan and the United States [5] [7] [11] [13]. While only identified as a new subspecies of influenza in 2011, the virus seems to have been circulating in the bovine population from at least 2004 [7].Cattle seem to be the natural host to IDV, but other species are susceptible to the virus [8]. Examples of other species that are susceptible are swine, ferrets [10], small ruminants [16] and humans [24].Turkey and chickens seem not to be susceptible to the virus [16].

The most vulnerable to the virus seem to be six to eight month old calves that were comingled, immunologically nave and recently weaned.The virus was more often isolated from clinically sick calves than from healthy calves [7]. In cattle, although the virus is often isolated from diseased animals, we cannot be sure the virus causes symptoms on its own or only in combination with other agents that play a role in Bovine Respiratory Disease Complex [8]. Research has shown that in controlled infected groups, the symptoms that can be found are mild respiratory ones. However, other important parameters such as heart rate, respiratory rate and rectal temperature did not differ from those in a control group [8].

While seemingly not causing any pathology on its own, it is implied that the virus also plays a role in Bovine Respiratory Disease Complex (from now on referred to as BRDC) [15], a disease that is of major economic importance in the United States [15]. In this disease complex viral infection can cause an animal to become more susceptible to secondary bacterial infection, making the outcomes of this disease very variable [14]. A study found that IDV was the only virus positively associated with clinical signs of BRDC [15], indicating a more important role for IDV.

Like Influenza C, IDV possesses a Hemagglutinin Esterase Fusion protein (HEF-protein) which is used for receptor binding, receptor destroying and membrane fusion activities [19]. The structure of this protein is known [19], as it is very similar to ICV HEF. It is also known it uses 9-0-Acetylated-Sia as its receptor to bind to host cells [19]. This study aims to determine where this receptor is expressed in different tissues and if expression has changed

between healthy and previously infected individuals.

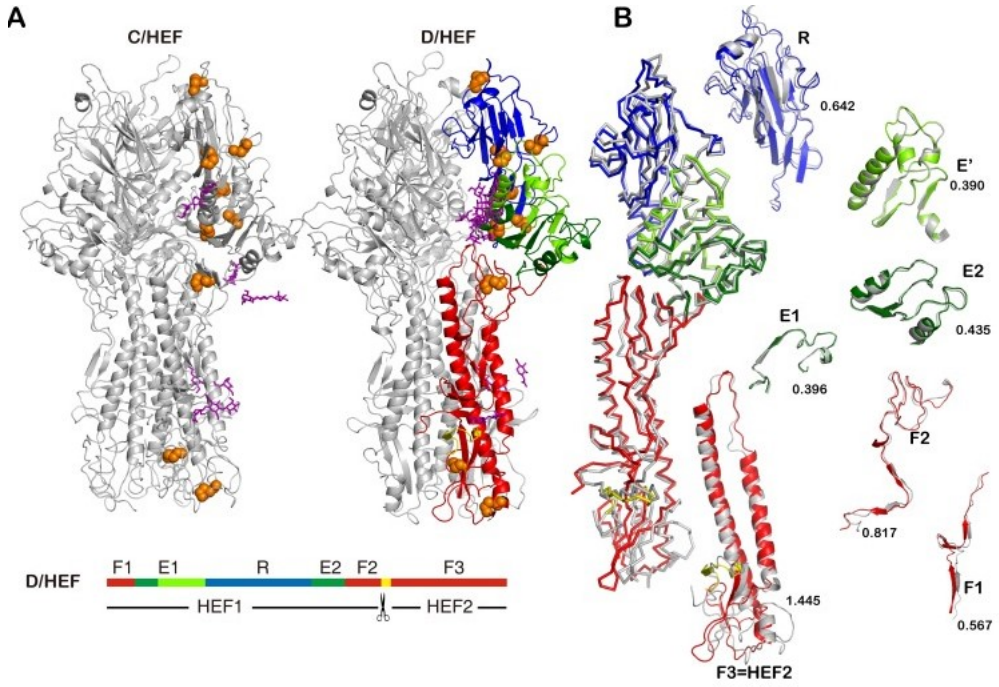

FIG. 1. Overall crystal structure of IDV HEF protein [19]

As can be seen in figure 1, the IDV HEF protein consists of a receptor binding domain, three fusion domains and esterase domains. The yellow domain in the picture is the fusion peptide. As can also be seen, it is similar to the ICV HEF. For this study, the ectodomain of the protein is most important, considering that this is the part that initiates contact with the receptor, as it is the only part of the protein in the extracellular space. For Influenza D, the ectodomain of the HEF protein is responsible for receptor detection, receptor binding and virus entry [19].

Besides Influenza D, no type of Influenza has been described in cattle, save for one strain of Influenza A [12]. Besides this article, very little is published regarding Influenza A in cattle. So, to test whether cattle brings only the receptor for Influenza D to expression or also the Influenza A, 2,6-linked and 2,3-linked sialic acid receptors in the bovine respiratory tract HA stainings will also be performed.

II. MATERIAL AND METHODS

A. HEF Production

TABLE I. Constructs Used

Amino Acid Sequence	Dimer or Trimer	GFP Tag present
HEF Complete	Trimer	No
1-605	Trimer	No
1-439	Trimer	No
HEF Complete	Dimer	Yes
1-605	Dimer	Yes
1-439	Dimer	Yes
HEF Complete	Trimer	Yes
1-605	Trimer	Yes
1-439	Trimer	Yes

The constructs used in this study can be found in table I. Those with a GFP tag were obtained from R.P. de Vries from the department of Molecular Pharmacy of Utrecht University.

Constructs in pCD5 expression vectors were transfected into HEK293T cells. Medium was removed from cells until there was 20mL left in the well plate. Next, the transfection mix was added. Cells were incubated over night at 37℃. The next day medium was changed to expression medium.

The next step was a western blot for protein visualization. Running gel was prepared by mixing components and leaving it for 30 minutes until polymerized. Water was then removed and stacking gel added. Then loading buffer was added to the sample and incubated for 3-5 minutes at 98℃, this was repeated three times. The gels were then put in the electrophoresis system and the tank was filled with 1x electrophoresis buffer. Wells were rinsed before 5-10 μL of the protein markers and the samples were loaded. Empty wells were filled with a marker. Electrophoresis was started at 80V and was increased to 120V after 20 minutes. Total running time was 1,5 hours.

After this the protein was blotted. Blotting buffer was prepared and poured into the apparatus. From the white side of the holder it was loaded with sponge, then two whatman papers soaked in blot buffer, then a PVDF membrane (first soaked in methanol and then in blot buffer). After this running gel was carefully removed from the plates and placed over the PVDF membrane. Air bubbles were carefully removed. Then another two whatman papers soaked in blot buffer were placed over it and then finally another sponge before the holder is clamped shut. The holder was then placed in the blotting apparatus and covered in sufficient buffer. The protein was blotted for one hour at 100V. After this the membrane was taken out and soaked in methanol for 15 seconds before being placed in a 50mL falcon tube with the blotted surface facing the interior. 10mL of distilled water was added to the tube and was used to wash for 2 minutes on the roller. 3% milk powder (blocking buffer) was prepared in PBS and 10mL of this mixture was placed into the tube. It was then incubated for 1 hour on the roller over night at 4 ℃. After this blocking buffer was removed and membrane was washed for 15 minutes in PBS-Tween, followed by two 5 minute washes in PBS-Tween. Blocking buffer containing strep-tactin HRPO was poured onto the membrane and incubated for 1 hour at RT on the roller. Antibody was then removed and membrane was washed for 15 minutes in PBS-Tween, followed by two 5 minute washes in PBS-Tween. ECL components were premixed and incubated for 5 minutes at room temperature (RT). Premixed ECL detection reagents were pipetted on a piece of foil. Membrane was placed on the ECL upside down and incubated for 5 minutes at RT. Membrane was placed protein side up in the Odyssey scanner and was scanned.

For those with a GFP tag visualization was attempted to discover if the actual protein was produced or not. For this, infected HEK293T cells (Human Embryonic Kidney Cells) were seeded into a 24 well plate using a 1:10 dilution and 0.5 mL per well. The following day the wells were covered with approximately 60% cells. The cells were then transfected. The next day all medium was removed from the cells. After this 1 mL medium was pipetted per well. The cells transfected with a GFP tag construct were visualized under a fluorescence microscope.

No usable protein was produced.

B. Tissue Micro Arrays

Tissues obtained from France were paraffinezed as normal. Hematoxyline and Eosin stainings were performed and the slides were judged by a pathologist (Nermin Caliscan), who marked the spots showing the best preserved sections of tissue. These sections were then punched out of the paraffized tissue and transferred to a 3D printed container, inserting the tissue sample on the spot allocated. When all of the tissue samples were in their allocated spots, fresh paraffine was added and left to cool overnight in the fridge. After this slides were sectioned using standardized techniques.This was done for bovine respiratory system and bovine gastrointestinal tract.

Complete layouts of the TMAs produced in this study can be found in the attachments.

C. Lectin Histochemistry

Paraffinized bovine trachea sections were deparaffinized, rehydrated and incubated 1% hydrogen peroxide to block endogenous peroxidase. After washing with PBS-Tween slides were then treated with Carbo-Free Blocking Solution (1:10 in MiliQ) for 60 minutes at RT. Antigen retrieval took place by keeping the slide for 10 minutes in pre-warmed citrate (pH 6.0). The trachea sections were incubated with biotinylated lectins (10ug/mL MaackiaAmurensis Lectin I (MAL1), 5ug/mL SambucusNigra Lectin (SNA) and MaackiaAmurensis Lectin II (MAL2)) for 30 minutes at RT. ABC was prepared 30 minutes before use and tissues sections were incubated with the freshly made ABC for 30 minutes at RT. Bovine trachea sections were incubated with AEC (3-amino-9-ethylcarbazole, Dako) for 15 minutes in the dark and counterstained with Hematoxylene.

D. Hemagglutinin Histochemistry

Paraffinized bovine respiratory TMAs were deparaffinized, rehydrated and incubated with 1% hydrogen peroxide to block endogenous peroxidase. Antigen retrieval took place by keeping the slides for 10 minutes in pre-warmed citrate (pH 6.0). After washing in PBS the slides were incubated overnight with 3% bovine serum albumin at 4 ℃. The next morning the respiratory TMAs were incubated with a mixture of HA protein, StepMAB-HRP and

Goast anti-Mouse IgG (H+L) for 90 minutes at room temperature. After washing with PBS the sections were incubated with AEC for 15 minutes at room temperature and finally counterstained with Hematoxylene.

The HA proteins used were CAL04 (humane influenza H1N1 California strain) and a mutant of CAL04 (mutated humane H1N1 to avian version of the California strain, from now on referred to as CAL04*) which switches its receptor preference from 2,6-sialic acid to 2,3-sialic acid. For the human trachea (positive control for CAL04) staining a concentration of 50 μg/mL was used and for the chicken trachea (positive control for CAL04*) 5μg/mL. For the bovine respiratory TMAs concentrations of 5 μg/mL and 50 μg/mL were used with CAL04 and 5 μg/mL was used with CAL04*.

III. RESULTS

A. Western Blot

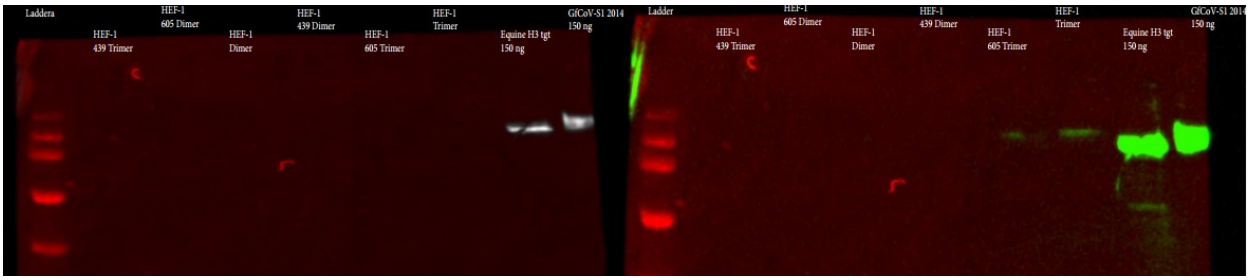

FIG. 2. Analysis of the recorded HEF production from HEK293T cells

In order to produce recombinant HEF proteins multiple constructs were transfected in HEK293T cells as described in figure 2. Culture supernatant was harvested 7 days post transfection and analysed by Western Blot using anti-StrepMAB antibodies.

On the western blot results none of the HEF construct protein is visible. Western blot was adequately preformed as can be seen by the positive control. Because none of the protein was visible under western blot, the next step was visualization of the GFP tag to see if actual production occurred and the problem was secretion from the cell, or if there was a problem in the production itself.

B. Visualization of HEF Production GFP Tag

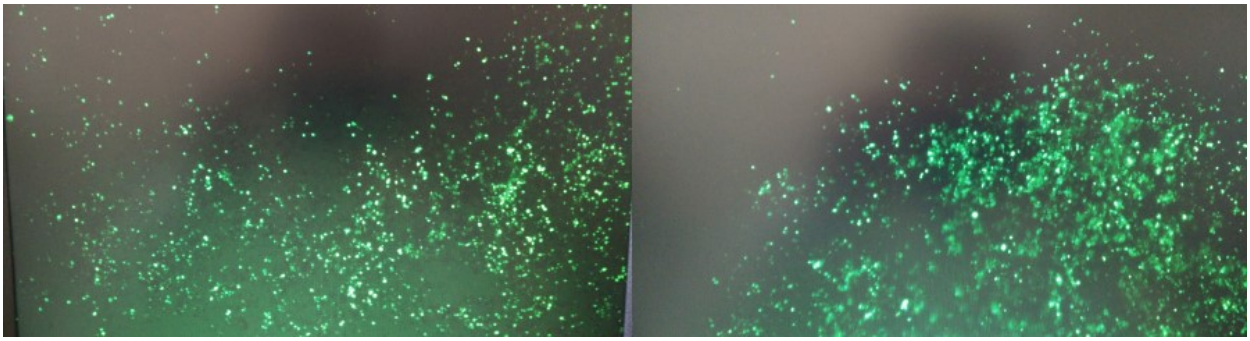

FIG. 3. Fluorescent visualization of GFP tag

The above figure shows the fluorescence visualization results of the 1-439 dimer with GFP tag and the 1-439 trimer with GFP tag, though all the constructs with a GFP tag as described in fig.2 have been visualized in this manner. As can be seen, the GFP tag has been visualized. With these results we concluded that at least the GFP is produced intracellular. The next step would be to collect the supernatant and to analyze it on SDS-page gel. Simultaneously, it would be favourable to lyse the cells (by freezing and thawing multiple times), resuspending the lysate with PBS, centrifuging it and analyzing the supernatant on Western Blot to see if the desired protein is produced.

C. Bovine Trachea Lectin Histochemistry

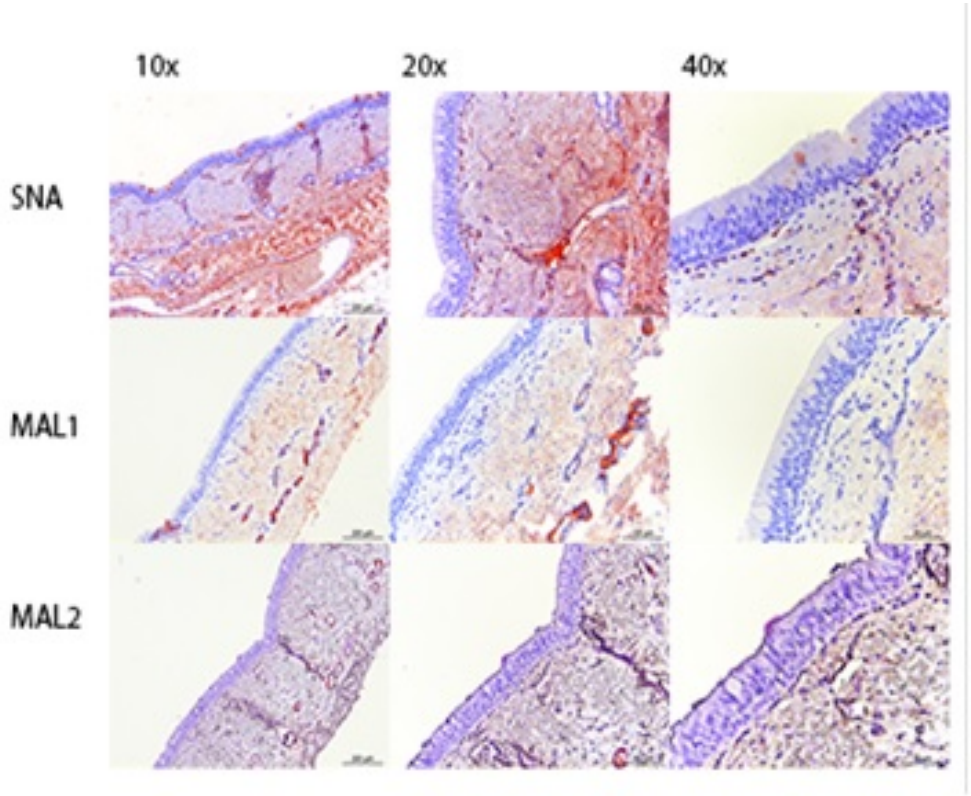

FIG. 4. Bovine trachea lectin staining results

To try and localize the 2,3-linked and 2,6-linked sialic acids on the bovine respiratory tract, SNA, MAL1 and MAL2 lectin stainings were performed.

Lectin histochemisty shows different degrees of staining for the different trachea sections present on the slide, as can be seen in figure4. One of the section shows no positive signal at all, while the other two show different intensities of signal in the goblet cells.

D. Bovine Respiratory TMA Lectin Histochemistry

Both SNA, MAL1 and MAL2 show very little staining in the bovine respiratory tract. For MAL1 and MAL2, this corresponds with the findings for the bovine trachea staining. For SNA however, which showed positive staining of the bovine trachea slides, it does not show the same intensity on the TMA. One of the lower trachea sections shows some very

faint staining in the goblet cells,however none of the tissue sections on the respiratory TMA showed positive staining.

E. Bovine Gastrointestincal TMA Lectin Histochemistry

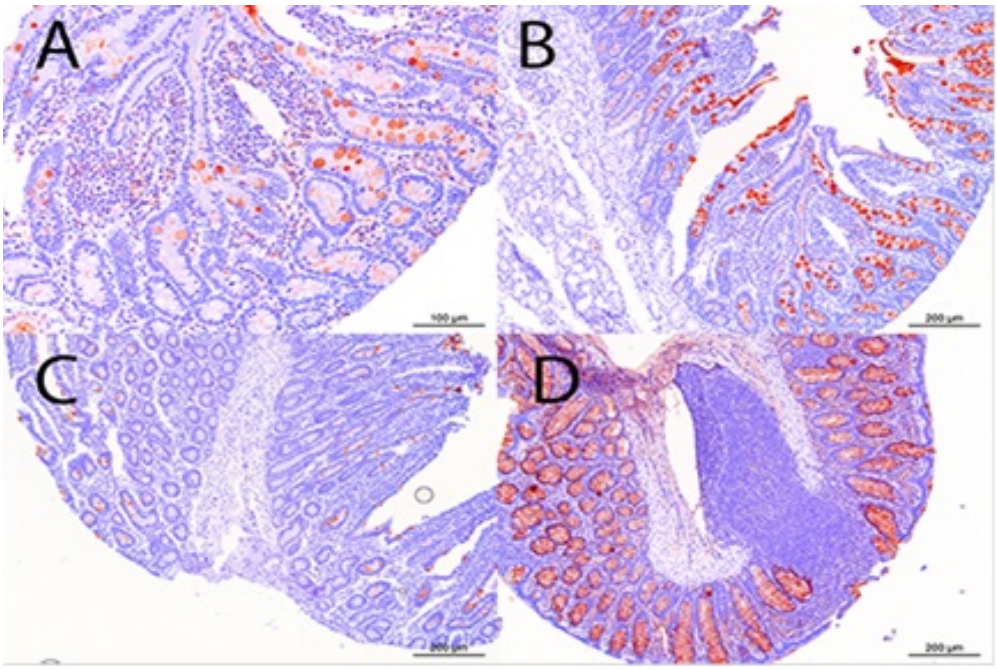

FIG. 5. Bovine respiratory lectin staining results.A: Bovine Duodenum SNA 20x, B: Bovine Duodenum MAL1 10x, C: Bovine Jejunum MAL1 10x, D: Bovine Colon MAL2 10x

After finding very little positive staining in the respiratory tract, the same staining was done on gastrointestinal tissue, as in other species this is one of the other locations these sialic acids are often present.

Lectin histochemistry for the bovine gastrointestinal tract shows some staining for SNA, MAL1 and MAL2. For SNA, all on the duodenum sections and one of the liver sections stain positive. For MAL1 all of the duodenum sections, 2 out of 3 of the jejunum sections and one of the ileum sections stain positive. For MAL2 2 of the jejunum sections show faint positives and all of the colon sections stain positive.

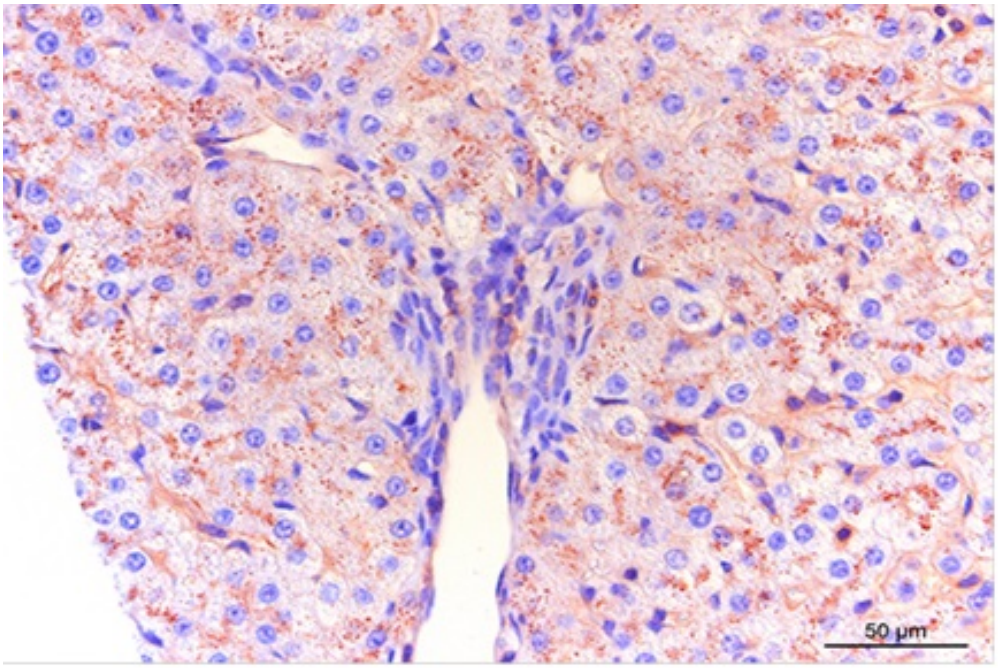

FIG. 6. SNA staining Bovine Liver Section 40x

For the one positive liver section one can clearly see granules in the same depth of field as the nuclei, as can be observed in figure 6. We also do not see any granules along the lining of the blood vessels. This would indicate that it is in fact a positive staining, and not background we see here.

F. Bovine Respiratory TMA Hemagglutinin Histochemistry

After lectin stainings a stainings gave mixed results a hemagglutinin staining using CAL04 (H1N1 humane influenza) and CAL04* (H1N1 mutant avian influenza) was performed.

Unfortunatly, tests done with the CAL04* protein gave no positive control, leaving these tests to be discarded.

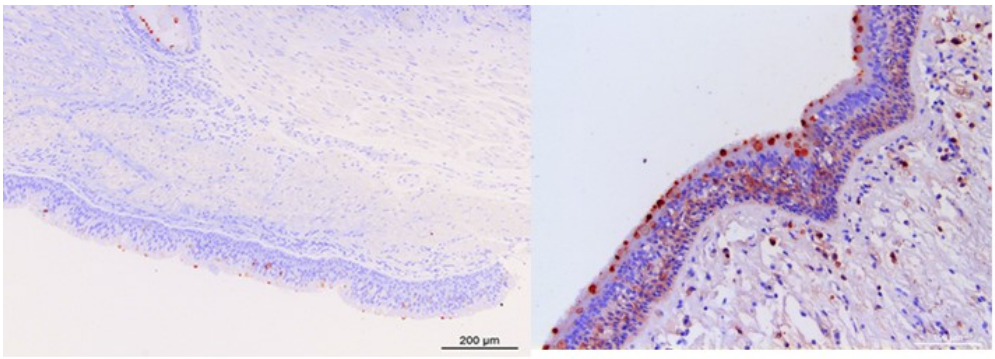

FIG. 7. staining humane trachea. Humane Trachea, CAL04, 10x (our staining) ; Humane Trachea CAL04 20x (previous staining)

As can be seen in figure 7, the amount of signal found is much less than expected. The second picture is the exact same staining, done with the same concentrations, only a few months earlier

The humane trachea control staining shows some positive results, so protocol was followed. However, none of the bovine respiratory TMAs were positive for this protein however, at either concentration.

IV. DISCUSSION AND CONCLUSION

In this study it was observed that the manner described manner to produce the HEF protein in a pCD5 vector and with HEK293T cells did not yield any usable protein. The most likely conclusion is that the protein, for some reason, remained intracellular. In the performed lectin stainings for 2,3-linked and 2,6-linked sialic acids positives were found in the trachea, duodenum, jejunum, ileum, colon and liver. The HA-staining with H1N1 HA protein yielded no positive results.

None of the HEF productions described in this paper produced protein. In the later constructs, those with a GFP tag showed production under fluorescent staining, however, none of the protein seemed to be able to leave the cell. A different vector was used than that described in other papers [19], this to be able to get the more complicated sugar chains and get as close to in vivo conditions as possible. These complicated sugar chains might have caused difficulty for the protein to get leave the cells, or something in the modifications might have caused it to change its conformation so it is no longer able to leave the cells. On the other hand, there are many things that can cause recombinant protein to not fold properly and therefore remain intracellular. A possible explanation for this is conformational stress which disables the recombinant protein to fold correctly [9].

The next step in the HEF production would be to try and purify the lysate to obtain usable protein. Another possibility might be to return to the wild type protein, as the mutations that were introduced after reading the paper by Song et al. [19] might cause to protein to either not fold properly or get stuck in the cell membrane.

A pCD5 vector was used instead of the previously described baculovirus vector [19] because this vector is able to express using mammalian cells, and the final product will therefore contain the more complicated sugar structures it would also contain in a field setting. This to keep possible alteration of receptor binding capabilities at a minimum.

In the bovine trachea staining differences were found between the animals in the expression of 2,6-linked sialic acids demonstrated by SNA lectin staining. However, in a similar

staining in pigs, no such difference was found [22].This might be because in these pigs epithelial cells are positive for 2,6 linked sialic acids, whereas in the bovine trachea observed only the goblet cells were positive. In cats however, a difference has been observed in the presence of 2,3-sialic acids in the alveoli, possibly because of breed difference [21] [23]. Since it is no longer possible to determine the breeds of the cattle from which the trachea samples originate because during the embedding process some of the tissue sections shifted, making us unable to be sure that every first tissue section comes from animal 1, such a breed difference might explain the difference between expression of 2,6-sialic acids in the trachea. In the TMA slides however no staining of the trachea was observed for SNA.

Another reason for the staining of the goblet cells in certain trachea sections might be that it are not the cells themselves that are showing up positive, but the mucus inside the cells. It has been demonstrated that there is a mucin population from the surface epithelium of the trachea that is rich in sialic acids [20].

The results from the lectin staining of the respiratory tract seem to support the hypothesis that it is not the cells themselves, but the mucus in those goblet cells that shows up as positive. In the gastrointestinal tract, as in the trachea, positives showed up exclusively in the goblet cells (with the exception of the single liver section that showed up as positive for SNA). This would also explain why in the same section, some of the goblet cells stain positive while others remain negative. It is known that paraffin coupes provide suboptimal results when studying mucus because part of the mucins are removed from the tissue during the paraffin-embedding clearing step [4], which in this experiment was done with xylene. This would explain why in some of the sections, only part of the goblet cells are positive.

It is known that like in the trachea, mucus in the colon and ileum, as well as other parts of the small intestine contains sialic acids [1]. The most likely explanation for the positive staining in goblet cells and the difference in cells in the same tissue sections is therefore that it is not the cells themselves, but the mucus that is still present that is giving the signal.

The problem with this theory is that not all the sections of the same organ stain similarly, nor do those of one animal show all positives while those from another animal remain

negative. If it truly was the mucus giving positive signal, it would be expected for the tissue sections containing said mucus to show consistent signal, which it does not. Small variations could be explained by the removal of mucins during the paraffin-embedding clearing step [4], but such a degree in variantion on the same slide would still be against expectations.

In the results one of the SNA stained liver sections showed a positive signal, while the other two remained negative. It is interesting to note that cattle are not known to be susceptible to influenza A infection, it is possible they might possess 2,6-linked sialic acids as a receptor as per this result. Another explanation coould be that liver cirrhosis can cause the concentration of free sialic acids to rise [3], however, this is not in line with our findings. For one, only the SNA staining showed up as positive on that particular section, the MAL1 and MAL2 remained negative. And also, the liver used did not show cirrhosis.

While cattle are not known to undergo Influenza A infection, it has been shown that in a clinical setting cattle can be infected with Influenza A virus, and even in the wild, seroconversion is possible [18]. For this reason, one would expect the necessary receptors to be present. However, this seems not to be the case on the tissue tested, with the exception of the single liver section that gave a positive signal for SNA. There is in fact a strain of Influenza A that comes from cattle (A/calf/Duschanbe/55/71), this is a H3N2 strain [12] [2]. The two positive sections of trachea containing 2,6-linked sialic acids might explain the infectiousness of this strain.

However, this study's experiment with CAL04 wild type (humane Influenza A virus), none of the bovine respiratory tract tissue sections stained positive. This supports the hypothesis that while the sialic acid present in the bovine respiratory tract contains some 2,6 linked sialic acids, the epithelium does not. While these results show that cattle do not possess the receptor necessary for infection with H1N1 wild type, this cannot be said for other strains, because the receptor configuration varies slightly. A further option to keep exploring the presence of 2,3 and 2,6 linked sialic acids in cattle would be to use the H3N2 strain described to circulate in cattle [2]. This is an old study however, and no similar results have been found recently.

The TMA slides, while a great option when looking at multiple tissue sections, does give some difficulties. During the staining process, some of the tissue sections seemed to move, making it impossible to get reliable results. This could result in a smaller sample size, influencing results. Another observed problem was that when sectioning the paraffin blocks, some of the first and last slides do not have all the tissue sections present. This could easily be remedied however, by making sure to use slides that come from a batch that is sure to have all tissues present.

Concerning the localization of 9-O-Acetylated Sialic Acids, one paper found positive staining in the bovine and swine trachea [6]. This would be in line with respiratory disease for Influenza D, as well as a possible factor in Bovine Respiratory Disease Complex. Future studies should be able to explore this theory futher.

For bovine coronavirus (from now on abbreviated to BCoV) both enteropathogenic and respiratory strains have been described [17]. Because this virus, like Influenza C and D, also uses 9-O-Acetylated sialic acids as its receptor it is interesting to look at this virus when looking at Influenza D. This virus seems to target epithelial cells in the respiratory and gastrointestinal tract [17], making it likely that in both those anatomical locations 9-O-Acytelated sialic acids are present. Influenza D, however, seems to only be a respiratory virus.A possible theory for this is that it needs a second protein for eventual infection, but this would need further studying.

V. ATTACHMENTS

TABLE II. Bovine Respiratory TMA Layout

Nasal Epithelium	Pharyngeal Epithelium	Upper Trachea	Primary Bronchus	Bulbus Olphactorius
Nasal Epithelium	Pharyngeal Epithelium	Upper Trachea	Primary Bronchus	Bulbus Olphactorius
Nasal Epithelium	Pharyngeal Epithelium	Upper Trachea	Primary Bronchus	Bulbus Olphactorius
Laryngeal Epithelium	Soft Palate	Lower Trachea	Bronchioli and Alveoli	Cornea
Laryngeal Epithelium	Soft Palate	Lower Trachea	Bronchioli and Alveoli	Cornea
Laryngeal Epithelium	Soft Palate	Lower Trachea	Bronchioli and Alveoli	Cornea

TABLE III. Bovine Gastrointestinal TMA Layout

Esophagus	Pancreas	Ileum	Stomach	Liver
Esophagus	Pancreas	Ileum	Stomach	Liver
	Pancreas	Ileum	Stomach	Liver
Duodenum	Jejunum	Colon	Spleen	Galbladder
Duodenum	Jejunum	Colon	Spleen	Galbladder
Duodenum	Jejunum	Colon	Spleen	

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