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Mimicking the passage of avian influenza viruses through the gastrointestinal tract of chickens

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ABSTRACT

In contrast to human influenza viruses that replicate in the respiratory tract and are airborne transmitted, avian viruses also replicate in gut epithelial cells and are transmitted via the fecal-oral route. On this route, the virus is exposed to destructive fluids of the digestive tract, which are acidic and contain the proteases pepsin (gizzard) or chymotrypsin and trypsin (intestine). Only the latter enzyme activates virus by cleaving hemagglutinin (HA) into HA_1 and HA_2 subunits.

We mimicked the passage of viruses through the gastrointestinal tract by treating them with digestive fluids from chicken and determined titers and integrity of HA by western-blot. Gizzard fluid completely inactivated virions and degrades HA even at a high dilution, but only if the pH was kept acidic. If the fluid is diluted with neutral buffer (mimicking virus uptake with seawater) particles were more resistant. Virions containing an uncleaved HA were even activated suggesting that gastric juice contains a trypsin-like protease. Undiluted intestinal fluid inactivated particles and destroyed HA, but diluted fluid activated virions. A virus isolated from the duck´s intestine is more tolerant against intestinal fluid compared to fowl plague virus suggesting that the former is better adapted to grow in the intestine. We also demonstrate that influenza viruses replicate to high titers in a novel chicken epithelial gut cell line. While viruses with a monobasic HA cleavage site require addition of trypsin, these cells effectively process HA with a polybasic cleavage site, which could be blocked with an inhibitor of the cellular furin protease.

1. Introduction

Human and mammalian influenza viruses replicate in epithelial cells of the respiratory tract and are transmitted via aerosols and droplets between individuals. In contrast, avian viruses preferentially replicate in epithelial cells of the intestine, are excreted in high concentrations in feces and are transmitted via the fecal-oral route. This is the main mode of transmission for influenza viruses in their natural reservoir, aquatic birds, where these viruses usually do not cause an apparent disease (Hinshaw et al., 1979; van Dijk et al., 2018; Webster et al., 1992, 1978). Influenza viruses occasionally spread from wild waterfowl to humans, other mammals and most frequently to poultry, where they can acquire a higher pathogenicity.

Based on their virulence in chickens, avian influenza viruses are classified into two pathotypes: low pathogenic avian influenza viruses (LPAIV) and highly pathogenic avian influenza viruses (HPAIV). Replication of the first is (usually) restricted to the portal of entry, i.e. the respiratory or digestive tract, while highly pathogenic strains cause a systemic infection often with a fatal outcome.

One important determinant of pathogenicity is the main glycoprotein of the virus, the hemagglutinin (HA). HA facilitates attachment of the virus particles to sialic-acid containing receptors and catalyzes the subsequent fusion of viral and cellular membranes. This process is well characterized and mediated by conformational changes triggered by exposure to mildly acidic pH. In order to do so, HA must be cleaved into the N-terminal HA_1 subunit, which carries the receptor binding site and the antigenic epitopes, and the C-terminal HA_2 subunit which anchors the protein to the membrane and also contains another hydrophobic sequence. This fusion peptide is instrumental for membrane fusion since it is exposed at the molecule's surface and inserts into the cellular membrane after triggering by low pH (Skehel and Wiley, 2000).

HA's cleavability is determined by the amino acids preceding the fusion peptide, i.e. at the C-terminus of HA_1. Low pathogenic avian viruses and non-avian influenza viruses possess a single arginine residue, whereas highly pathogenic H5 and H7 viruses contain several basic amino acids at the cleavage site. The expression of a suitable cellular enzyme determines whether infectious viruses are released by a certain cell type. A polybasic motif is recognized by ubiquitous

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proteases, such as furin and PC5/PC6 (Stieneke-Grober et al., 1992), and infection with a virus having an HA with a polybasic cleavage site leads to systemic infections. In contrast, HA with a monobasic cleavage site is processed by proteases present only in epithelial cells and hence viral replication is locally restricted. Several of these enzymes have now been identified in respiratory epithelia of humans, cattle and swine (Bottcher-Friebertshauser et al., 2013). However, essentially nothing is known about whether epithelial cells of the avian gut release infectious virus particles and cleave HA with a monobasic and/or polybasic cleavage site, which is mainly due to the lack of a suitable cell culture system.

Influenza viruses with uncleaved HA can be activated by trypsin *in vitro* (Klenk et al., 1975; Lazarowitz and Choppin, 1975), which cleaves the same peptide bond as cellular HA-activating enzymes (Garten et al., 1981). Other proteolytic enzymes, such as chymotrypsin, also process HA into HA_1 and HA_2 (Klenk et al., 1977; Lazarowitz and Choppin, 1975), but at a site within the fusion peptide and hence virus infectivity is not activated (Garten et al., 1981). Cleavage of HA is essential for infectivity, but viruses having a cleaved HA are rapidly and irreversibly inactivated at mildly acidic pH between pH 5.2 and pH 6, depending on the HA subtype. (Mair et al., 2013; Russier et al., 2016).

For an fecal-oral route of transmission, viruses must withstand the supposedly destructive fluids of the avian digestive tract. After passage through the crop, an enlarged part of the esophagus, which has a slightly acidic pH (5.5–6.0) and thus might already inactivate virus particles, ingested material is delivered to the bipartite stomach. The first part is the proventriculus, a rod-shaped tube, that secrets hydrochloric acid and pepsinogen. The low pH of 2 autocatalytically activates pepsinogen to generate pepsin, a protease with an acidic pH optimum. The second part of the stomach, the more voluminous gizzard (ventriculus) contains several layers of muscles and can grind the food with previously swallowed stones and pass it back to the proventriculus, and *vice versa*. The average pH of the gizzard content is ~3.5, but (depending on the diet) measurements vary between 1.6 and 4.4 (Beasley et al., 2015; Svihus, 2011). The retention time of food in the proventriculus and gizzard has been estimated to be between 30 min and two hours before the content is released into the small intestine. In the duodenum the milieu changes, the pH is neutralized to ~6.5, which inactivates pepsin. Instead, the proteases trypsin and chymotrypsin (among many other enzymes) are secreted by the pancreas and released into the duodenum (Pubols, 1990). After passage through the small and large intestine, the feces leave birds through the cloaca, the posterior orifice that serves as the only opening for the digestive, reproductive, and urinary tract.

Here we mimicked the virus passage through the gastrointestinal tract by incubating virus particles with stomach and gut fluids obtained from chickens. We quantified virus inactivation using plaque assays and integrity of viral HA protein by western blotting. Furthermore, we investigated virus replication in a new and unique gut epithelial cell line from chickens and investigated whether HA with monobasic and/or polybasic cleavage site is properly processed as an essential requirement for production of infectious virus particles.

2. Methods

2.1. Cell culture, virus preparations and anti-HA antibodies

8E11 cells were previously prepared from specific pathogen-free 18-day old embryos of white Leghorn hens (Witek, 2013) and are available at the DSMZ (German Collection of Microorganisms and Cell Cultures GmbH) repository with the number MM-CHIC CLONE 8E11. The cells were previously used for *Campylobacter jejuni* and parasite invasion assays (John et al., 2017; Kim et al., 2019), but never tested for replication of influenza virus. 8E11 and Madin Darby canine kidney (MDCK II) cells were grown in DMEM (Dulbecco's modification of Eagle's medium, PAN, Aidenbach, Germany) supplemented with 10% FCS (fetal calf serum, Perbio, Bonn, Germany) and penicillin [100 U/ml]/streptomycin [100 µg/ml] at 37 °C and 5% CO_2. Mutant 1 (M1) of the highly pathogenic strain A/FPV/Rostock/1934 (H7N1) was used in the experiments, that contains the sequence PSKGR instead of PSKKRKKR at the C-terminus of HA_1 (Wagner et al., 2013). This mutation creates a low pathogenic strain suitable for working in a BSL2 laboratory. In other experiments we used the avian virus A/duck/Bavaria/1/77 (H1N1) that was isolated from the cloacal swab of an adult mallard duck (*Arias platyrhynchos*) in Southern Germany (Ottis and Bachmann, 1980) and the A/WSN/1933 (H1N1) strain that was originally isolated from a human patient.

To create virus preparations with a cleaved HA, MDCKII or 8E11 cells were infected with FPV-M1 or A/duck/Bavaria/1/77 at a multiplicity of infection (MOI) of 0.0001. After 1 h, the supernatant was replaced by fresh DMEM containing 0.1% FCS and TPCK-trypsin at a final concentration of 2 µg/ml and cell cultures were further incubated at 37 °C until most cells were dead (usually within 2 days). To create a virus preparation with uncleaved HA in MDCKII or 8E11, cells were infected with an MOI of 1 and incubated in DMEM with 0.1%FCS but without TPCK-trypsin for 12 h. The cell supernatant was removed, cleared (10 min at 5000xg), viruses were pelleted at 29,000 rpm for 2 h in a SW 28 rotor, dissolved in 100 µl TNE buffer (50 mm Tris–HCl pH 7.4, 100 mm NaCl, 0.1 mm EDTA) and stored in aliquots at −80 °C. Both viruses grown in the absence of trypsin exhibit only a small amount of cleaved HA and had a titer of $\sim 2 \times 10^6$ to 2×10^7 plaque forming units/ml (pfu/ml), which increased (depending on the preparation) 100–1000 fold after incubation with trypsin.

The HA_2 specific antiserum was prepared using the gel-purified HA_2 subunit of FPV as an antigen for immunization of rabbits.

2.2. Plasmids and transfection of cells

The full-length HA genes from FPV wildtype (wt) or M1 with either polybasic or monobasic cleavage site was amplified from the plasmid pHH21 using the primers 5'CCGCTCGAGATGAACACTCAAATCCTGG3′ and anti-sense 5'GAAGATCTTTATATACAAATAGTGCACCGC3′, digested with XhoI and BglII and cloned into the corresponding sites of the expression vector pCAGGS. 4 µg plasmid DNA was transfected with TurboFect into 8E11 cells as described by the manufacturer (Thermo Fisher Scientific, Carlsbad, United States). 24 h post transfection, cells were washed with ice-cold PBS, collected in 1.5 ml Eppendorf tubes and pelleted at 5000xg for 10 min. Cell pellets were lysed in 100 µl 1% NP-40 in PBS for 20 min on ice. Lysates were cleared (10 min at 5000xg) and an aliquot (20 µl) of the supernatant was analyzed by western blotting. For the furin inhibition experiments, the peptidomimetic drug MI-701 (added from a 2 mM stock solution dissolved in H_2O) was added 1 h after transfection to get final concentrations of 25 to 150 µM.

2.3. Virus incubation with gizzard or gut fluid, with acidic pH and trypsin

Six and two VALO specific-pathogen-free (SPF) chickens (VALO BioMedia GmbH, Osterholz-Scharmbeck, Germany) were housed for 20 weeks under SPF conditions and humanely euthanized prior to the isolation of intestinal organs. The animal work was approved by the governmental agency, the Landesamt für Gesundheit und Soziales (LAGeSo) in Berlin, Germany (approval number T0245/14, approval date 23 October 2014). Termini were ligated at the beginning and the end of the gizzard and at the end of the small intestine (duodenum). Fluids from the gizzard (~10 ml) and intestine (~6 ml) were collected, combined and passed through 0.2 µm filters. The pH of the combined gizzard fluids and gut fluids was measured with pH test strips to be 3.5 and 6.5, respectively. Aliquots of both fluids were stored at −80 °C until further use. Serial dilutions of both fluids were made with citric acid-Na_2HPO_4 buffer (0.1 M citrate acid, 0.2 M Na_2HPO_4) adjusted to pH 3.5, 6.5 and 7.0, respectively. In some experiments, bovine serum albumin solution (BSA, PAN, Aidenbach, Germany) were added to a

final concentration of 1 µg/µl.

In each experiment, 10 µl concentrated virus (~5×10^6 pfu) was incubated with 60 µl undiluted or diluted gizzard or gut fluid at 42 °C for 20 min. Incubation at various pH values was performed with 10 µl concentrated virus in 60 µl citric acid-Na_2HPO_4 buffer adjusted to pH 3, 4, 5, 6 or 7 at 42 °C for 20 min. In other experiments 10 µl concentrated virus was incubated with 60 µl TPCK-treated trypsin (T1426, Sigma-Aldrich, Taufkirchen, Germany) dissolved in PBS at final concentrations of 2000 µg/ml, 200 µg/ml, 20 µg/ml or 2 µg/ml.

After the incubation, 10 µl was removed from each sample, reducing SDS-PAGE buffer (10 µl) was added, samples were boiled and 20 µl was used for western blot. The remaining 60 µl were immediately diluted with infection medium (1 ml, see 2.5.) to neutralize the sample and virus titers were determined by plaque assay as described in 2.5.

2.4. Evaluation of virus growth in 8E11 chicken and MDCK II cells

To assess growth kinetics, cell monolayers of 8E11 and MDCKII were infected with FPV M1 or WSN virus at an MOI of 0.00005. After 1 h adsorption, cells were washed with PBS, the supernatant was replaced by fresh DMEM without or with TPCK-trypsin (2 µg/ml). The supernatant was harvested after a defined incubation time, cleared of debris (10 min at 5000 × g) and stored at −80 °C.

2.5. Plaque- and HA-assay

Virus samples were diluted with 1 ml infection medium (DMEM with 0.1% FCS, 0.2% BSA, dissolved in H_2O), penicillin [100 U/ml]/streptomycin [100 µg/ml]. To activate virus particles with uncleaved HA, infection medium in some experiments also contained 2 µg/ml TPCK-trypsin.

Plaque assays were performed on MDCK II cells in six well plates. Cells were infected with serial 10-fold dilutions of virus, incubated for 1 h at 37 °C, washed with PBS and overlaid with 1.25% Avicel (FMC BioPolymer), 1% $NaHCO_3$, 0.1% FCS, 0.2% BSA (dissolved in H_2O) and 2 µg/ml TPCK-trypsin. After incubation for 48 h at 37 °C the cell cultures were fixed with 4% PFA, cells were stained with 0.1% crystal violet, and the plaques were counted.

In the case where trypsin was not present in the infection medium, it was also excluded from the overlay medium. Instead, 5 h post infection, a second layer of overlay medium containing 2 µg/ml TPCK-trypsin was added to activate progeny virus and allow plaque formation.

HA assays were performed in 96 well U-bottom microwell plates using 50 µl PBS, 50 µl serially diluted virus samples and 50 µl 1% chicken red blood cells. The results were recorded after 30 min of incubation at room temperature.

2.6. SDS-PAGE and western-blot

After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide, gels were blotted onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Freiburg, Germany). After blocking of membranes (blocking solution: 5% skim milk powder in PBS with 0.1% Tween-20 (PBST)) for 1 h at room temperature, anti HA_2 antibodies (diluted 1:2000 in blocking solution) were applied overnight at 4 °C. After washing (3 × 10 min with PBST), horseradish peroxidase-coupled secondary antibody (anti-rabbit, Sigma-Aldrich, Taufkirchen, Germany, 1:5000) was applied for 1 h at room temperature. After washing, signals were detected by chemiluminescence using the ECLplus reagent (Pierce/Thermo, Bonn, Germany) and a Fusion SL camera system (Peqlab, Erlangen, Germany).

3. Results

3.1. Virus incubation with gizzard fluid

To simulate the conditions that influenza encounters during a fecal-oral infection in birds, we collected fluids from the gizzard and the proximal part of the duodenum from six chickens. The samples exhibited a pH of 3.5 and 6.5, respectively, which is in agreement with published studies (Beasley et al., 2015). To determine their effect on infectivity of an avian influenza virus, we first used the fowl plaque virus (A/FPV/Rostock/34, (H7N1)) M1 mutant, which contains a deletion of three and an exchange of one basic amino acid(s) in the polybasic cleavage site of HA, i.e. PSKKRKKR is mutated to PSKGR. Unlike FPV with a polybasic cleavage site, FPV M1 shows low pathogenicity in chicken and requires trypsin for growth in cell culture (Wagner et al., 2013). For the following experiments, we concentrated FPV M1 by ultracentrifugation from the supernatant of cells, which were infected at a high MOI and incubated for ~12 h in the absence of trypsin. Western blotting with HA_2 specific antisera showed that the virus preparations contained mainly uncleaved HA_0 and plaque assays revealed a titer of ~8×10^5 pfu/ml. After incubation with a low concentration of trypsin, HA was completely cleaved and virus titers increased to 5×10^8 pfu/ml, i.e. around 600 fold (Fig. 1a), which is in agreement with published studies (Klenk et al., 1975; Lazarowitz and Choppin, 1975).

In a first set of experiments, we serially diluted the gizzard fluid with buffer adjusted to pH 3.5 to keep the pH acidic and incubated 60 µl of each dilution with 10 µl of the virus preparation (~5×10^6 infectious particles after trypsin activation) at 42 °C (the body temperature of birds) for 20 min, which is less than the minimal retention time of food in the gizzard. This was thought to mimic virus uptake with food, which might transiently neutralize the pH in the stomach, but the gastric secretory response to eating rapidly stimulates release of gastric acid to re-acidify the stomach fluid (Martinsen et al., 2005). Samples were then neutralized with medium and treated with trypsin to activate HA. However, using a plaque assay, we were not able to detect a single infectious virus particle, even at the highest dilution of 1:1000 (Fig. 1a).

The destructive effect of the gizzard fluid on virus infectivity might be due to its acidic pH. It is well documented that influenza viruses containing a cleaved HA are inactivated at slightly acidic pH between 5.2 and 6. This acid treatment triggers HA to undergo an irreversible conformational change that catalyzes membrane fusion (Mair et al., 2013; Russier et al., 2016). Since uncleaved HA is not able to perform this conformational change we asked whether it is more stable under acidic conditions. 5×10^6 infectious particles of FPV M1 were incubated with buffer adjusted to a pH between 3 and 7. No plaques were detected after incubation at pH 3 and pH 4. At pH 5, infectivity was greatly reduced by 90% to 5×10^5 pfu, but was only marginally affected at pH 6 (Fig. 1b). Thus, virus particles with uncleaved HA are more stable as particles harboring a cleaved HA, as described before (Scholtissek, 1985), but are (almost) completely inactivated at the pH of the chicken gizzard.

The gizzard fluid is not only acidic, but also contains the protease pepsin. We therefore tested the integrity of virus particles from the incubation with gizzard fluid by western blotting using antibodies against the HA_2 subunit. Undiluted gizzard fluid almost completely degrades HA, only one band with a molecular weight lower than authentic HA_2 remains. At intermediate dilution (1:10, 1:30) two bands running above and below the 36 kDa size marker were detected. Only at a high dilution of 1:100 and 1:1000 HA remains mainly undigested (Fig.1c). We repeated this experiment with combined gizzard fluids from two other chickens and added the protein albumin to a high final concentration of 1 µg/µl to better mimic virus uptake together with food. However, even at a dilution of 1:1000 no virus infectivity remains (not shown), and degradation of HA was only marginally retarded by the presence of albumin (Fig. 1d).

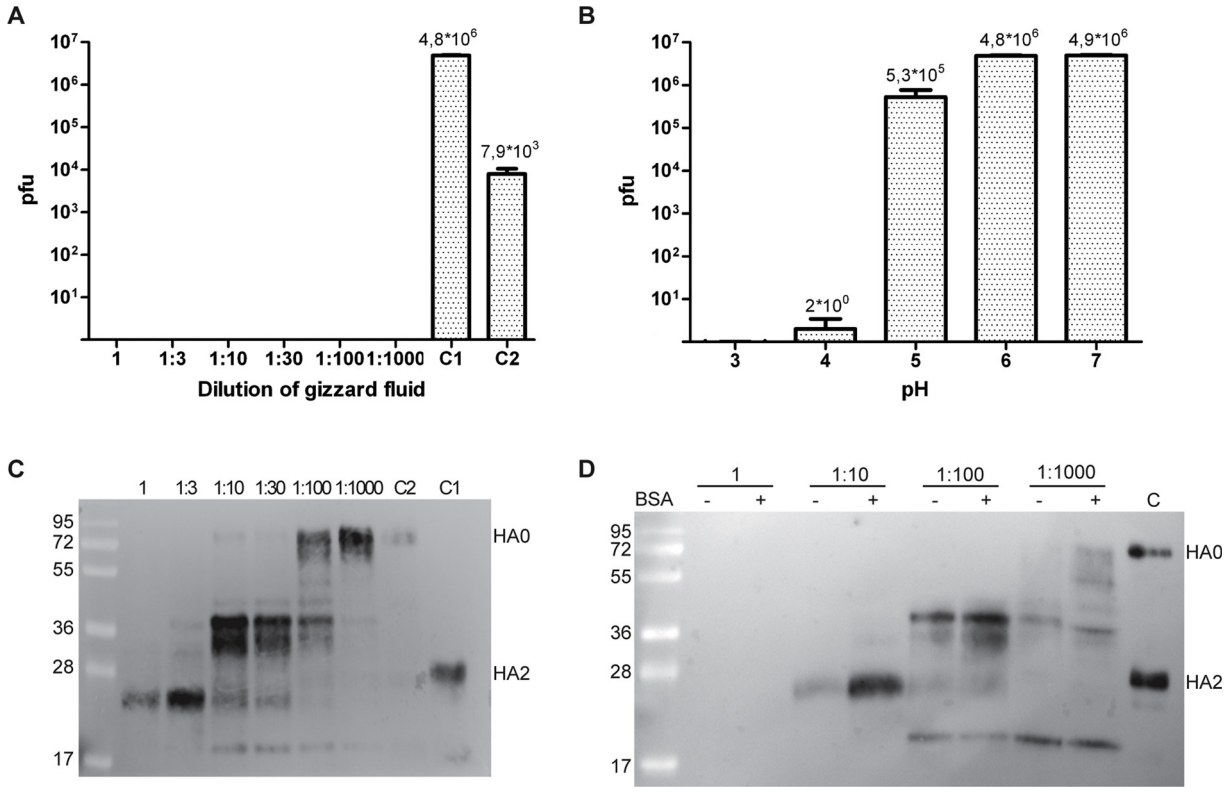


Fig. 1. Incubation of influenza virus FPV with gizzard fluid diluted with acidic buffer.
A. The avian influenza fowl plague virus M1 containing HA with a monobasic cleavage site was grown in the absence of trypsin and pelleted from cell culture supernatants. 10 µl of the preparation was incubated for 20 min with 60 µl of gizzard fluid serially diluted with buffer adjusted to pH 3.5 and then activated with trypsin (2 µg/ml). Viral titers were determined with a plaque assay. C1: 10 µl virus activated with trypsin. C2: 10 µl of the virus preparation without trypsin treatment. The mean of three different incubations including standard deviation is shown.
B. 10 µl of the virus preparation was incubated for 20 min with buffer adjusted to pH values between 3 and 7 and the plaque titer was determined.
C. Aliquots of the samples from A were subjected to SDS-PAGE and western blot using antisera against the HA_2 subunit. Controls: virus particles with cleaved (C1) and uncleaved HA (C2) as size marker for the SDS-PAGE mobility of HA0 and HA_2, respectively. Numbers indicate the size (kDa) of molecular weight markers.
D. FPV M1 was incubated with gizzard fluid serially diluted with buffer adjusted to pH 3.5 and in the absence (−) or presence (+) of BSA (1 µg/µl) for 20 min. Samples were then subjected to SDS-PAGE and western blot using antisera against the HA_2 subunit. C: virus particles with partially cleaved HA as size marker for the SDS-PAGE mobility of HA0 and HA_2. No virus infectivity was detected if an aliquot of each sample was analyzed in the plaque assay.

In order to mimic virus uptake by drinking water, the fluid was serially diluted with buffer adjusted to pH 7. In this set of experiments, we did not treat virus particles with trypsin after incubation with the gizzard fluid to determine whether it might contain a protease that activates HA. Similar to the previous experiments, undiluted gizzard fluid completely inactivates virus particles (Fig. 2a). However, the result from the incubations with diluted fluid is quite different. At a dilution of 1:3, virus titers were reduced by only ~50%, i.e. from 1×10^4 to 5×10^3. At higher dilutions (1:10, 1:30 and 1:100), the infectivity of the virus preparation even increased, from 1×10^4 to $\sim 3 \times 10^6$ and thus to a similar extent than treatment with trypsin suggesting that the gizzard fluid contains a trypsin-like protease.

This assumption is corroborated by western blotting using antibodies against the HA_2 subunit (Fig. 2b). In contrast to gizzard fluid diluted with acidic pH, the HA-band running below the 36 kDa size marker does not appear. Instead, various bands with the size of HA_2 (and smaller) appear that are highly similar to the bands obtained by trypsin-treatment. A possible explanation would be that pepsin, the main protease of the gizzard, which has a pH optimum at ~2 but becomes inactive at pH 5, does not digest if the fluid is diluted with neutral buffer. Instead, another unknown trypsin-like protease with a more neutral pH optimum activates HA.

Next, we performed the same experiment with a low-pathogenic avian virus (A/duck/Bavaria/1977, (H1N1)) that was isolated from cloacal swabs of a mallard duck (Ottis and Bachmann, 1980) and thus might be better adapted to replication in the gastro-intestinal tract and to fecal-oral transmission between birds compared to the FPV M1 mutant. We generated a virus preparation in the absence of trypsin; upon trypsin treatment virus titers increased 1000 fold, from $\sim 2 \times 10^3$ to 2×10^6. Undiluted gizzard fluid completely inactivated A/duck/77, but gizzard fluid diluted with neutral buffer activates viral infectivity at all dilutions, almost to the same extent as trypsin treatment (Fig. 2c). Thus, the duck virus is apparently better adapted to infect via the gastro-intestinal tract compared to FPV M1 because it is completely activated at lower dilutions of the gizzard fluid. Since no antibody is available that reacts with this H1 subtype HA we could not determine whether HA is properly processed.

3.2. Virus growth and HA cleavage in a new chicken intestinal epithelial cell line

We then asked whether influenza virus replicates in epithelial cells from the chicken intestine and whether the cells properly process HA with monobasic and/or polybasic cleavage sites as one prerequisite for the release of infectious virus particles. We used a recently established and to our knowledge the only available chicken enterocyte cell line (Witek, 2013). The avian origin and the epithelial phenotype were confirmed using marker-specific antibodies against catenin (data not shown) and E-cadherin, villin and cytokeratin (W. Rudy, MicroMol, personal communication). To investigate virus growth, the CHIC 8E11 cells were infected at a low MOI (0.00005) with FPV M1 in the absence or presence of trypsin and HA- and plaque-titers were determined

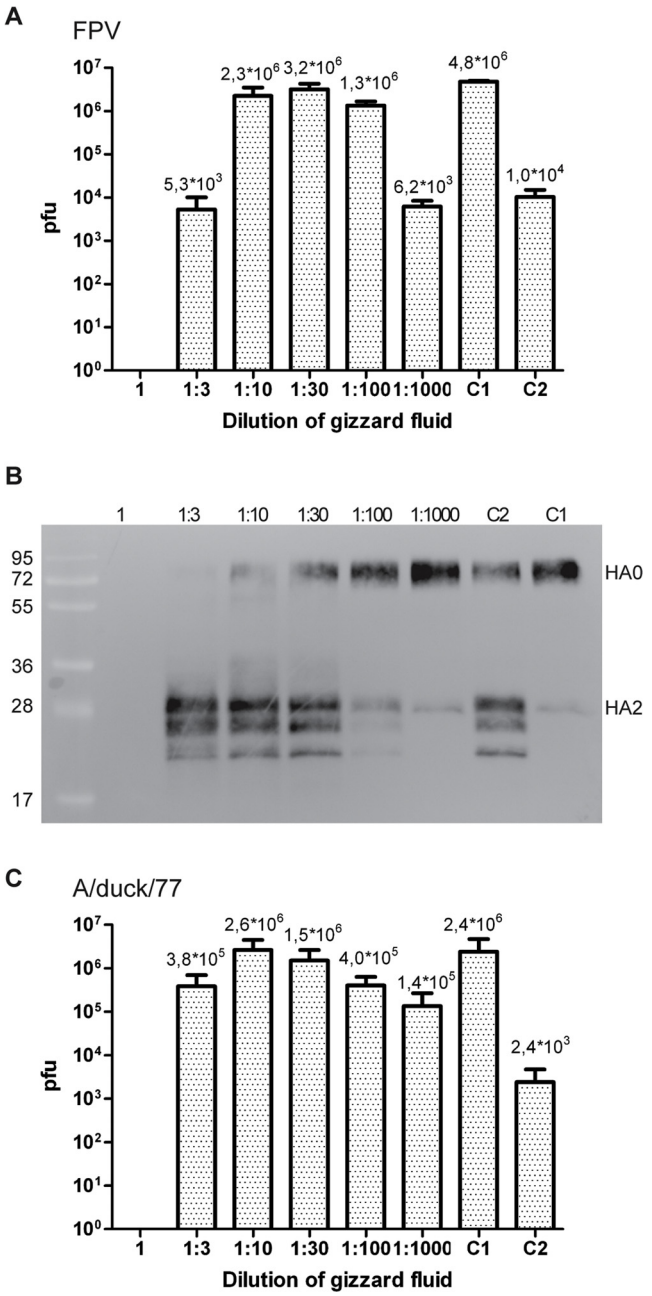


Fig. 2. Incubation of FPV and A/duck/77 with gizzard fluid diluted with neutral buffer.

A. FPV M1 (10 µl) was incubated for 20 min with 60 µl gizzard fluid serially diluted with buffer adjusted to pH 6.5. In contrast to experiments shown in Fig. 1 samples were then not treated with trypsin. Viral titers were determined with a plaque assay. C1: 10 µl virus activated with trypsin. C2: 10 µl of the virus preparation not treated with trypsin. The mean of three different incubations including standard deviation is shown.

B. Aliquots of the samples from A were subjected to SDS-PAGE and western blot using antisera against the HA_2 subunit. Controls: virus particles with uncleaved (C2) and cleaved HA (C1) as size marker for the SDS-PAGE mobility of HA_0 and HA_2, respectively. Numbers indicate the size (kDa) of molecular weight markers.

C. A preparation of avian virus A/duck/77 (10 µl) was incubated for 20 min with 60 µl gizzard fluid serially diluted with buffer adjusted to pH 6.5. Viral titers were determined with a plaque assay. C1: 10 µl virus activated with trypsin. C2: 10 µl of the preparation not treated with trypsin. The mean of three different incubations including standard deviation is shown.

(Fig. 3. left part). In the absence of trypsin, neither hemagglutinating nor infectious viruses were detected in the supernatant, but in the presence of trypsin FPV M1 grows to high titers (~10^7 pfu/ml), very similar to their growth in MDCK II cells, a standard cell line to produce virus particles. A similar growth curve was obtained for A/duck/Bavaria/1977 (data not shown).

Human influenza viruses are (often) not able to replicate in the intestine of ducks if infected orally (Kida et al., 1980; Webster et al., 1978), a genetic trait which has been linked to the neuraminidase gene (Fujimoto et al., 2016; Hinshaw et al., 1983; Kobasa et al., 2001). However, the 8E11 cells support replication of the human lab strain WSN (H1N1) to similar titers as FPV M1 and as in MDCK II cells (although somewhat slower), but only if trypsin is present in the medium (Fig. 3, right part).

The dependence on trypsin suggests that 8E11 cells are not able to properly process HA with a monobasic cleavage site. To confirm this, we infected 8E11 cells with FPV M1 at an MOI of 1, added trypsin to the cellular supernatant and analyzed cell lysates by western blotting with an HA_2 specific antiserum. With exogenous trypsin HA is almost completely cleaved into the HA_2 subunit, whereas in its absence HA remains predominantly uncleaved (Fig. 4A). The small amount of an HA_2 band that runs below the major HA_2 band generated by trypsin is probably functional, since virus grown in the absence of trypsin exhibits low infectivity (2×10^4), which is activated ~100-fold by trypsin treatment (2×10^6). Thus, similar to most other cell lines, 8E11 cells do not express a protease that completely activates influenza viruses having an HA with a monobasic cleavage site.

To investigate whether 8E11 cells process HA with a polybasic cleavage site, HA from the authentic FPV strain (cleavage site PSKKRKKR) and from the mutant 1 (cleavage site PSKGR) were expressed and analyzed by western blotting. As expected, HA with a monobasic cleavage site also remains almost completely uncleaved, whereas HA with a polybasic site is properly processed in transfected 8E11 cells (Fig. 4B).

HA of HPAIV is usually cleaved by the ubiquitous transmembrane protease furin, which is located in the trans-Golgi network and at the cell surface and cleaves HA during its intracellular transport (Schafer et al., 1995; Stieneke-Grober et al., 1992). To investigate whether furin is also responsible in 8E11 cells for cleavage of HA with polybasic cleavage site, we used the peptidomimetic furin inhibitor MI-701, which blocks processing of H5 and H7 subtype HAs and replication of the corresponding viruses in MDCK cells (Lu et al., 2015). Presence of the drug in 8E11 cells transfected with polybasic HA clearly inhibits processing at concentrations between 25 µM and 150 µM (Fig. 4C), which are only slightly higher than reported for MDCK cells (Lu et al., 2015).

3.3. Virus incubation with intestinal fluid

Having shown that HA from HPAIV, but not from LPAIV is activated in 8E11 cells, we asked whether this could be accomplished by the digestive fluid of the intestine, which contains trypsin and chymotrypsin as main digestive enzymes. We first tested the effect of a wide concentration range of trypsin on titers of a FPV M1 preparation grown in 8E11 cells in the absence of trypsin. Every tested concentration of trypsin activates the virus ~100-fold, i. e. virus titers increased from 2×10^4 to 2×10^6 (Fig. 5A). Western blotting with an aliquot of the incubation showed that the two lowest concentrations of trypsin (2 and 20 µg/ml, which are usually used in plaque assays and multiple step growth experiments) cleaved ~50% and 100%, respectively, of HA into its subunits. At higher concentrations an additional band with a molecular weight clearly lower than genuine HA_2 appeared, indicating that the protease is beginning to cleave other residues in HA, but this apparently does not affect the infectivity of the virus preparation as a whole.

We then tested the effect of serial dilutions of gut fluid on virus

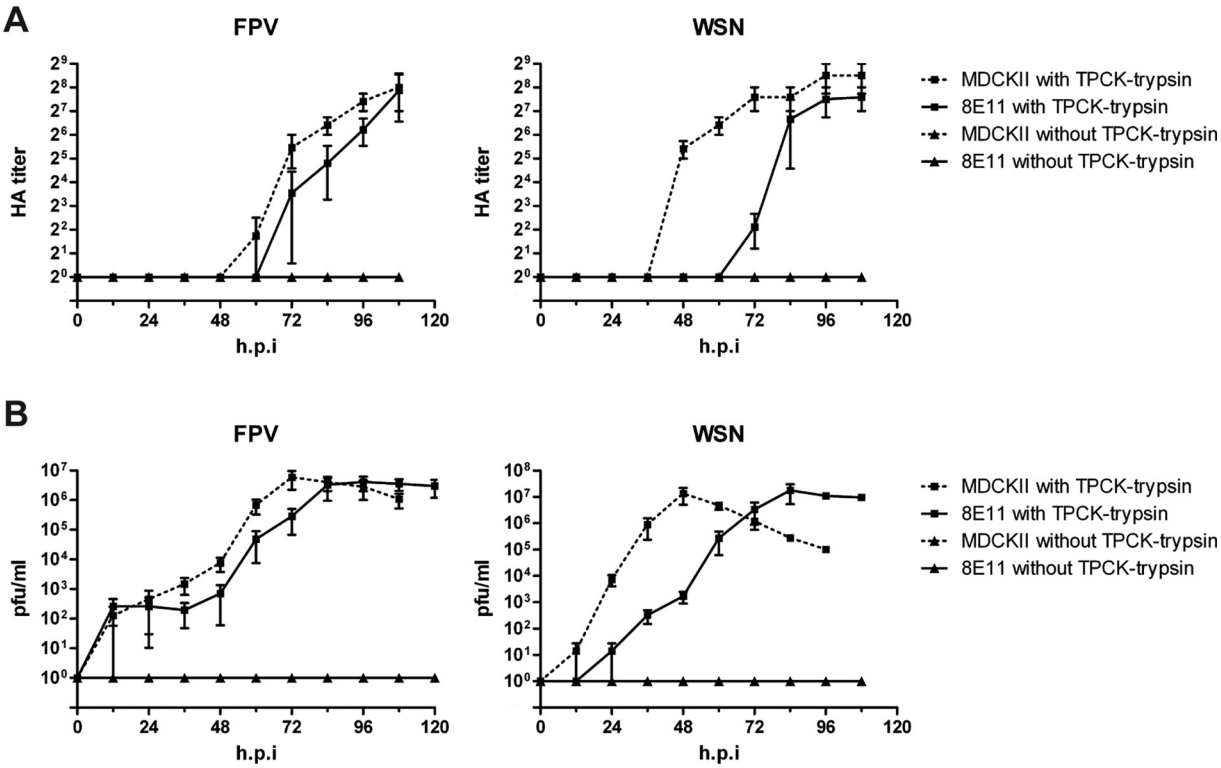


Fig. 3. Avian FPV M1 and human WSN virus grow to high titers in the chicken epithelial gut cell line 8E11, but only in the presence of trypsin.
8E11 and MDCK II cells were infected with avian FPV M1 or human WSN virus at an MOI of 0.00005 and incubated in the absence or presence of trypsin. At the indicated time points aliquots of the supernatant were removed and HA- (**A**) and plaque-titers (**B**) were determined. Results are shown as the mean including standard deviation of three experiments.

activation and integrity of HA of FPV mutant M1. Only at a dilution of 1:1000 the virus preparation with an uncleaved HA was activated ~10-fold, i.e. an increase in virus titer from 2.8×10^4 to 2.3×10^5 was observed (Fig. 4B). Since trypsin treatment (2 µg/ml) caused an increase in viral titers to 5.1×10^6 it can be calculated that ~5% of potentially infective virus particles were activated by the gut fluid. Western blotting showed that only a small fraction of HA_0 was processed into functional HA_2 and most bands detected by the antiserum had a higher molecular weight suggesting HA cleavage at amino acids other than the authentic cleavage site. The same two HA degradation products prevail in less diluted gut fluid whereas undiluted fluid almost completely degraded HA (Fig. 5B, left). Since the pattern of HA bands is different from that obtained with viruses incubated with high concentrations of trypsin it indicates that other enzymes, such as chymotrypsin, also digest the virus particles. This increasing degradation of HA is reflected by a decrease of the virus titers. At a dilution of 1:100 no virus particles were activated, i.e. the virus titer is slightly lower (1.4×10^4) compared to samples not treated with trypsin (2.8×10^4). At lower dilutions of gut fluid the residual viral infectivity is further diminished; after incubation with undiluted gut fluid 95% of virus particles are inactivated. However, in contrast to experiments with the gizzard fluid about 2000 virus particles remained infectious after

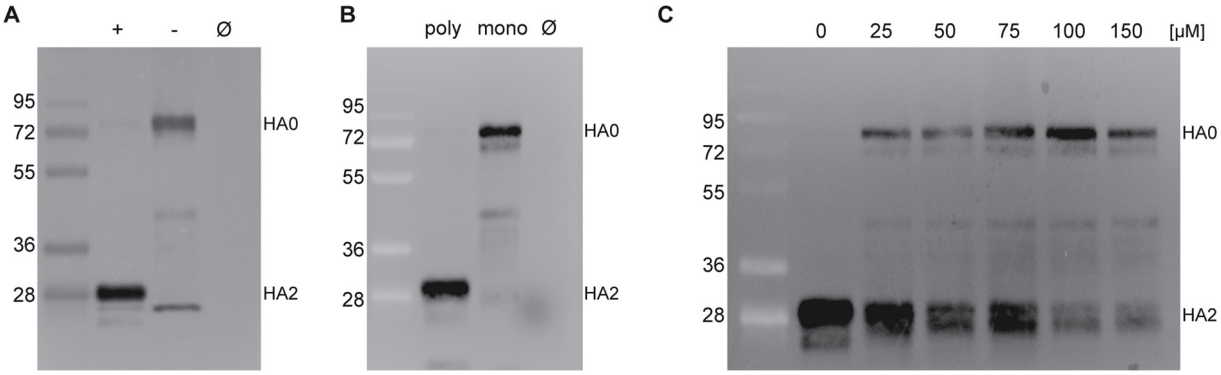
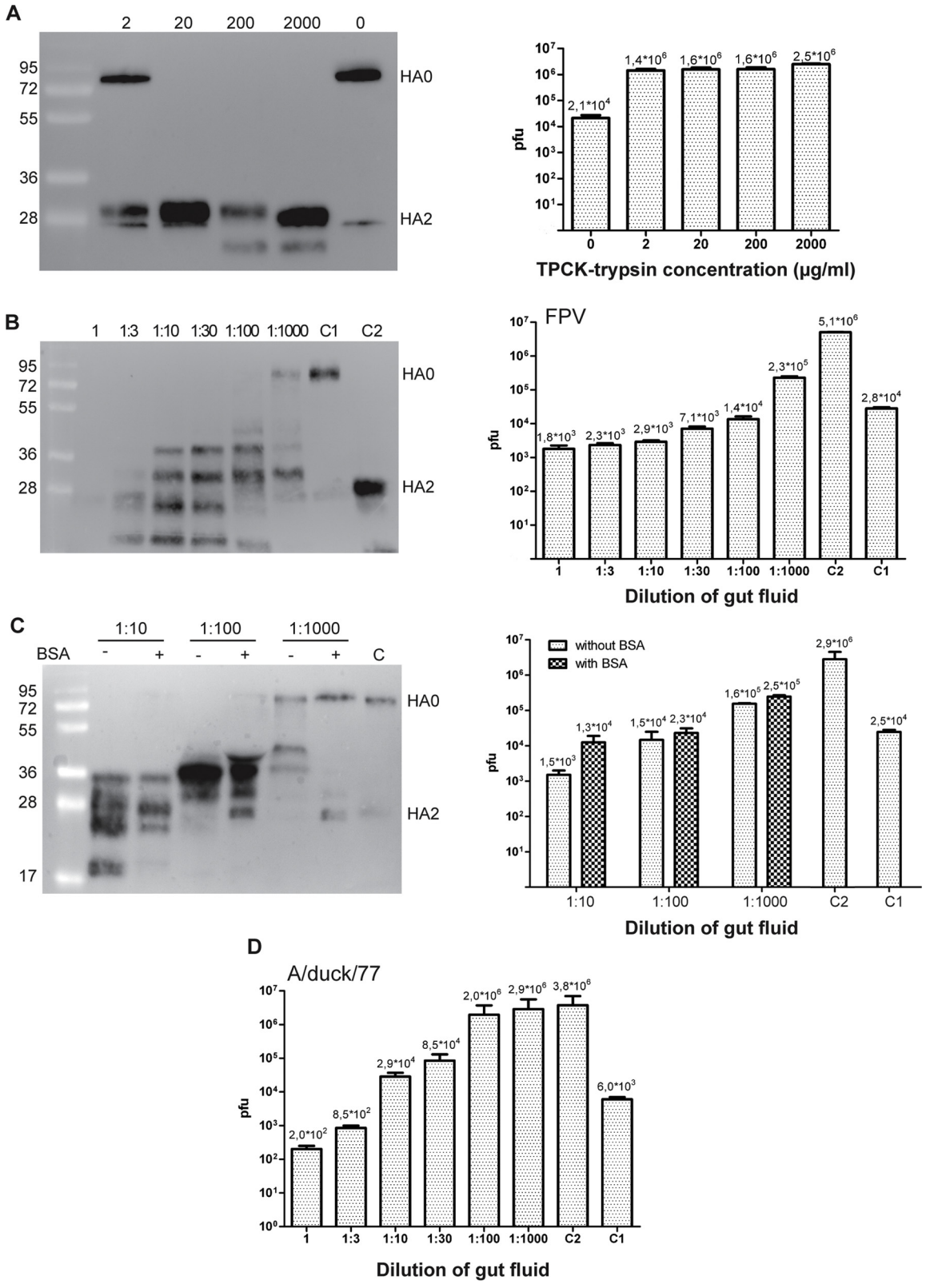


Fig. 4. 8E11 cells cleave HA with polybasic, but not with monobasic cleavage site.
A. 8E11 cells were infected at an MOI of 1 with FPV M1 containing HA with a monobasic cleavage site and incubated in the absence (-) or presence (+) of trypsin (2 µg/ml) for 12 h. B. HA from FPV containing a monobasic (mono) or a polybasic (poly) cleavage site was expressed in 8E11 cells. Cell lysates were then subjected to western blot using antiserum against the HA_2 subunit. Ø: uninfected or untransfected cells.
C. HA from FPV containing a polybasic cleavage site was expressed in 8E11 cells. 1 h after transfection the furin inhibitor MI-701 was added to the indicated final concentrations (µM). Cell lysates were subjected to western blot using antiserum against the HA_2 subunit. Note that at the highest MI-701 concentrations an additional HA_2 band becomes apparent which might be due to cleavage of HA at another site.



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incubation with gut fluid.

We repeated this experiment with combined gut fluids from two additional chickens and added the protein albumin at a high final concentration (1 μg/μl) to better mimic digestion of food in the duodenum. Nevertheless, essentially the same result was obtained. The presence of albumin had a slightly beneficial effect on virus titers, especially at the dilution of gut fluid of 1:10, but HA was still degraded to similar extent compared to samples not supplemented with albumin (Fig. 5C).

We then performed this experiment with the low-pathogenic avian

Fig. 5. Incubation of FPV and A/duck/77 with intestinal fluid.
A. FPV M1 was incubated with the indicated concentration of trypsin (2 μg/ml) for 20 min. Aliquots of samples were then subjected to SDS-PAGE and western blot using antisera against the HA_2 subunit (left part) or to plaque assays (right part).
B. FPV M1 was incubated with serial dilutions of gut fluid for 20 min. Aliquots of samples were then subjected to SDS-PAGE and western blot using antisera against the HA_2 subunit or to plaque assay. Controls: virus particles with uncleaved (C1) and cleaved HA (C2).
C. FPV M1 was incubated with serial dilutions of gut fluid for 20 min without (-) or with (+) additional BSA (1 μg/μl). Aliquots of samples were then subjected to SDS-PAGE and western blot using antisera against the HA_2 subunit or to plaque assay. Controls: virus particles with partially cleaved HA (C), uncleaved (C1) and cleaved HA (C2). Results of plaque assays are shown as the mean including standard deviation of three experiments.
D. A/duck/77 was incubated with serial dilutions of gut fluid for 20 min. Samples were then subjected to plaque assay. C1: 10 μl of the preparation not treated with trypsin. C2: 10 μl virus activated with trypsin. The mean of three different incubations including standard deviation is shown for all plaque assays.

virus that was isolated from cloacal swabs of a duck. Plaque assays revealed that this virus is already activated at the low dilution of 1:10; at a dilution of 1:100 and 1:1000 (almost) to the same extent as trypsin treatment (Fig. 5D). However, undiluted gut fluid and gut fluid diluted 1:3 reduced virus titers, but some infectivity persists. Thus, the duck virus is apparently better adapted to grow in the intestine of birds compared to FPV M1.

4. Discussion

We analyzed whether two avian influenza viruses might survive the passage through the gastrointestinal tract of birds by incubating virus particles with fluids obtained from the gizzard and the first part of the intestine of chickens. Gizzard fluid completely inactivates 5×10^6 infectious particles of a mutant of fowl plague virus (FPV) harboring a HA with a monobasic cleavage site, even if the gizzard fluid is highly diluted at acidic pH. The same was observed for a virus originally isolated from cloacal swabs of a duck. This is due to the low pH of the gizzard (pH 3.5), since at pH 4 the same amount of virus is also (almost) completely inactivated. In addition, HA (and probably other viral proteins) are most likely degraded by the protease pepsin even if samples were supplemented with additional protein (Fig. 1)

These inactivation experiments are compatible with recent studies about survival of two human enveloped viruses in juices from the gastrointestinal tract. Hantavirus (titer 5×10^5) is completely inactivated after incubation for 15 min with human gastric juice adjusted to pH 1 or pH 3. Juice adjusted to pH 4 or pH 5 inactivated 99% and 90%, respectively of infectious particles (Witkowski et al., 2017). Likewise, MERS-coronavirus (titer 7.5×10^6) is completely inactivated after an incubation for 30 min in fasted state simulated gastric fluid (pH 1.6, containing pepsin), but stable in a solution simulating the gastric fluid after food uptake (pH 5, but no pepsin) (Zhou et al., 2017). Taken together, enveloped viruses are apparently rapidly and quantitatively inactivated at a pH below 5. In contrast, non-enveloped viruses known to be transmitted by the fecal-oral route, such as rotaviruses are completely stable at pH 4, partly and slowly inactivated at pH 3 and only at pH 2 rapidly and completely lose their infectivity (Weiss and Clark, 1985).

We only had access to intestinal fluids from chickens, but the natural host and the reservoir of avian influenza viruses are water birds and one might argue that less acidic conditions might prevail in their stomachs. However, a recent investigation points out that chickens possess the highest pH in the stomach of all *Aves* (~3.5). The pH of the gizzard in water birds susceptible to influenza infection is more acidic; pH 2.2 in the mallard duck (*Anseriformes*), 1.5 in black headed gull and 1.2 in common pied oystercatchers (*Charadriiformes*) (Beasley et al., 2015).

However, if the gizzard fluid is diluted with neutral buffer, a different picture emerges (Fig. 2). Even at a low dilution of 1:10 viruses having an uncleaved HA are activated almost to the same extent as cleavage with trypsin. Thus, it is tempting to speculate that if water contaminated with viruses is swallowed by a water bird, it might neutralize the otherwise destructive acidic pH of the gizzard and therefore virus particles are neither denatured nor digested by the acidic protease pepsin. Even more, a trypsin-like protease present in the gizzard fluid now becomes active that cleaves HA at the proper site and thus activates virus particles. More research is required to characterize this proteolytic activity further.

Whether Influenza viruses can infect epithelial cells of the gizzard has not been investigated. Histologically, the gizzard consists of a dense and thick inner coat (depending on the diet), a protein-carbohydrate complex termed koilin, which covers a mucous membrane (Akester, 1986). Both koilin and the mucus probably represent a mechanical and biochemical barrier for infection of underlying epithelial cells. However, gizzard pathology has been observed in infections mainly with bacteria and fungi, but also with (non-enveloped and thus more resistant) adenoviruses (Gjevre et al., 2013). As for influenza infections, a recent report describes that a co-infection with H9N2 virus potentially exacerbates gizzard ulceration induced by feed-borne *Bacilus cereus* contamination, but the virus alone does not cause gizzard damage (Zhang et al., 2019). Thus, it seems unlikely that Influenza viruses infect cells of the gizzard on a regular basis.

In the present study, we also show that both an avian and human influenza virus can productively infect a new cell line derived from the chicken intestine and both viruses are able to replicate to high titers (Fig. 3). Several studies have shown that an avian virus, but not a human virus can replicate in the intestine of ducks and is shed into the feces (Hinshaw et al., 1983; Kida et al., 1980; Webster et al., 1978). Assuming that the properties of the 8E11 cell line faithfully reflects native cells in the bird's intestine, one can conclude that the reason for a replication defect of human influenza viruses in birds cannot be found at the cellular level. Indeed, more recent studies have shown that avian and human viruses differ in the pH optimum of their neuraminidase, which is involved in release of virus particles from infected cells (Fujimoto et al., 2016; Kobasa et al., 2001).

8E11 cells processes HA with polybasic cleavage site, probably by furin, but FPV M1 with a monobasic cleavage site requires trypsin for growth under multiple cycle conditions (Fig. 3 and 4). Thus, the results indicate that only highly pathogenic influenza viruses are released by chicken gut cells as infectious particles while low pathogenic influenza viruses require cleavage of HA. In principle, this can be achieved by the digestive enzyme trypsin present in the gut fluid, which activates virus particles with uncleaved HA even at concentrations 100-fold higher (2000 μg/ml) than usually used in multiple cycle growth experiments, although some degradation of the HA_2 band is observed under those conditions (Fig. 5a). Indeed, viruses are activated by the gut fluid, but a remarkable difference exists between the viruses isolated from a chicken (FPV) and from cloacal swabs of a duck (A/duck/77). Both are slightly inactivated by undiluted gut fluid, but the duck virus is already activated at a dilution of 1:10, whereas FPV M1 requires a dilution of 1:1000 for the same effect (Fig. 5). Thus, the duck virus is apparently better adapted to grow in the intestine of birds compared to FPV M1.

However, it remains to be shown whether such a conclusion can be generalized and more viruses isolated from ducks or other water birds needs to be analyzed and compared with viruses isolated from chicken. This might allow identifying molecular markers on viral proteins that confer better adaption to grow in the avian intestine. These observations raise the question whether (and how) viruses survive the passage through the gastrointestinal tract, if they are transmitted between birds via the fecal-oral route, as it is generally assumed (Webster et al.,

1992). We found only a few studies were chickens or ducks have been experimentally infected only via the oral route (Fujimoto et al., 2016; Hatta et al., 2002; Hinshaw et al., 1983; Kida et al., 1980; Kobasa et al., 2001; Webster et al., 1978). Usually, high doses of virus are required, indicating that this mode of infection is not very effective. Most experimental studies inoculated viruses via the upper respiratory tract, by aerosol, intravenously or by a combination of inoculation routes such that virus passage through the gizzard is not required to reach the intestine. On the other hand, it has been shown that avian viruses can replicate in cells of the intestine since viral antigen has been detected in the epithelium of the cloacal bursa and the small intestine of mallards and ducks experimentally infected with LPAIV (Kida et al., 1980; Kuiken, 2013; Slemons and Easterday, 1977). In addition, viral RNA and infectious particles are routinely recovered from cloacal swabs of both chicken and ducks and virus is present in feces where it is stable for days or weeks, depending on the temperature (Hinshaw et al., 1979; Webster et al., 1978).

Several recent reports pointed out that LPAIV may spread systemically in chicken. After intranasal or intratracheal infection with various LPAIV of subtypes H5, H7 and H9 viral RNA was found by RT-PCR in a broad range of tissues beyond the respiratory and gastrointestinal tract and H7N7 virus could be isolated from several tissues (Post et al., 2012, 2013). Thus, one might speculate that systemic, but asymptomatic infection of LPAIV might occur more often than previously anticipated. This would allow viruses to bypass the stomach and duodenum after oral ingestion, using the blood stream and enter epithelial cells in distal parts of the digestive tract via the basolateral membrane. Here they replicate and are released from the apical membrane into the lumen of the intestine. In distal parts of the intestine, the concentration of digestive enzymes is lower and hence virus particles are not degraded. Instead, viruses with uncleaved HA are processed by trypsin and infectious virus particles are then released via the cloaca. Alternatively, viruses might enter the body from the seawater via the cloaca, replicate in the distal parts of the intestine and again are excreted by feces, as previously suggested (Scholtissek, 1985). Since methods to label influenza virus proteins with fluorophores are now available (Fukuyama et al., 2015; Vahey and Fletcher, 2019) the hypothesis is now amenable to experimental verification by following expression of viral proteins in various tissues and cells after oral infection of birds.

5. Conclusion

We conclude that under certain conditions influenza viruses can withstand the destructive fluids of the avian digestive tract. One important condition is that the acidic pH of the gizzard needs to be neutralized, which happens if viruses are swallowed together with water. This also inactivates pepsin; its enzymatic activity would otherwise degrade virus particles. Moreover, neutralization uncovers a trypsin-like protease that activates influenza viruses with an uncleaved HA. Chicken epithelial cells in the intestine are not able to process HA with a monobasic cleavage site, but the intestinal fluid is able to activate the virus, at least if diluted. A remarkable difference exists in this regard between the chicken virus FPV M1 and the duck virus A/duck/Bavaria/77. While FPV M1 requires a high dilution of 1:1000 to be activated, lower dilutions inactivate the virus and degrade HA In contrast; the duck derived virus is already activated at a dilution of 1:10.

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Declaration of Competing Interest

All authors declare that they have no conflict of interest regarding the publication of this article.

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