Continuous cell lines from the muscovy duck as replacement for primary cells in the production of avian vaccines to increase supply security

Abstract

Veterinary vaccines contribute to food security, interrupt zoonotic transmissions, and help to maintain overall health in livestock. Although vaccines are usually cost-effective, their adoption depends on a multitude of factors. Because poultry vaccines are usually given to birds with a short life span, very low production cost per dose is one important challenge. Other hurdles are to ensure a consistent and reliable supply of very large number of doses, and to have flexible production processes to accommodate a range of different pathogens and dosage requirements. Most poultry vaccines are currently being produced on primary avian cells derived from chicken or waterfowl embryos. This production system is associated with high costs, logistic complexities, rigid intervals between harvest and production, and supply limitations. We investigated whether the continuous cell lines Cairina retina and CR.pIX may provide a substrate independent of primary cell cultures or embryonated eggs. Viruses examined for replication in these cell lines are strains associated with, or contained in vaccines against egg drop syndrome, Marek's disease, Newcastle disease, avian influenza, infectious bursal disease and Derzsy's disease. Each of the tested viruses required the development of unique conditions for replication that are described here and can be used to generate material for in vivo efficacy studies and to accelerate transfer of the processes to larger production volumes.

Keywords: Veterinary vaccines, continuous cell line, vaccine production, CR, CR.pIX, muscovy duck, One Health

Introduction

Animal infectious diseases are a cause for losses to biodiversity [1] and a risk to human health due to zoonosis [2–4]. Infectious diseases that threaten livestock can also lead to economic damages and contribute to persistent poverty [5–7]. Vulnerability is enhanced by an epidemiological environment that consists of a high and dynamic annual population of 40 billion chickens (+turkey duck), high density of animals in intensive farming, contact of free-range and wild animals of different species, potential for coinfection with different pathogens and different strains of the same pathogen, and legal and illegal trade with live birds and poultry products across continents [14–16].

Vaccines for humans [17,18] and animals [19,20] are an efficacious and economic intervention for the control of infectious diseases. Veterinary vaccines are also perceived as the ethically and epidemiologically preferred approach to avoid mass slaughter and culling of animals [21,22].
However the utilization of an animal vaccine depends on its zoonotic capacity, virulence and transmissibility of the pathogen in animal populations, potential effects on livestock productivity, and not at least marketability.

Such complex considerations also apply for poultry diseases with one consequence that the supply of vaccines is optimized for industrial, but less so for non-commercial and semi-intensive livestock poultry keepers. Cost of vaccines and risk to supply is furthermore increased if the production depends on embryonated chicken eggs. For certain avian virus production, the preparation of the primary chicken cells is reported to account for approximately 30 % of the total production costs [26], and such issues do affect poultry farmers with low resources [27].

We have investigated alternative options to substitute existing primary cell and embrionated egg based production of poultry vaccines on a reduced cost. The described technologies are based on the continuous cell lines CR and CR.pIX that were obtained by biochemical immortalization of primary retina cells of the domesticated muscovy duck. A detailed summary of the design and development of the two avian cell lines has been published previously [28]. The cells were shown to be free of adventitious agents, and sensitive assays against reverse transcriptase suggest that the anatine cell lines (as opposed to galline cells) do not release active endogenous retroviral particles [29,30]. The cell lines have furthermore been adapted to suspended proliferation in chemically defined medium, and propagation of recombinant and wild type viruses of different families to very high yields has been demonstrated [31–35]. This publication describes the production parameters for additional animal viruses that can be used as vaccines against poultry diseases, namely egg drop syndrome, infectious bursal disease (this is kind of misleading since IBV does not replicate), infectious bronchitis, Marek's disease, Newcastle disease and avian influenza.

Results and discussion

Egg drop syndrome

Egg drop syndrome EDS-76 is caused by a member of the *Atadenovirus* genus in the *Adenoviridae* family [36], viruses with double-stranded genomic DNA enclosed by an icosahedral capsid without lipid membranes. Synonymous names for EDSV are duck adenovirus 1 (DAdV-1) and Duck atadenovirus A (the name proposed by the ICTV). The natural hosts appear to be ducks and geese and the virus has a world-wide distribution. Clinical disease in poultry often is subacute and only evident in the production of fewer eggs (the "egg drop") and disturbances in pigmentation and shell quality. Transmission of DAdV-1 occurs vertically and horizontally via orally ingested fomites.

Adherent CR.pIX cells are permissive for DAdV-1 but overt CPE develops only 5 days post infection and sometimes it is too weak for clear identification of infection (Figure 1 (a)). A subpassage of the infected cells usually elevates CPE and titers significantly (data not shown). This observation suggests that DAdV-1 may require an active cell cycle and may have difficulties to spread in cultures that have settled into the plateau phase. Augmentation of virus replication by subcultivation may also indicate that mixing and reseeding of the cell monolayer disperses infectious units that are not easily shed into the supernatant.

DAdV-1 replication in suspension cultures was investigated because such production processes can be scaled more easily to large volumes and are therefore preferred to adherent cultures. However, yields in single-cell suspensions were below those obtained in the adherent cultures. We have observed such a

phenomenon previously during the development of a production process for poxviruses and have induced suspended cell aggregates to augment transmission of the cell-associated infectious units [32]. The aggregates were formed by addition of a chemically defined virus production medium at the time of infection [32]. DAdV-1 production may have also benefited from this culture format if spread of cell-associated infectious units contributes to yields. To test this model we compared yields after feed with virus production medium (aggregation inducer) or with cell proliferation medium (that maintains single-cell culture formats). As virus titers still remained below expectation in both regimens we next investigated effects of addition of either medium 2 days or 3 days PI, respectively. This time there was a significant (I see only 2-3 times increase) effect that suggested that virus production medium should be added 2 days PI (Figure 1 (b)), neither earlier nor later. We currently have no satisfying explanation for this observation and speculate that inoculation and production phases may have different requirements for DAdV-1 in our cell and medium combination. Virus attachment to host cells may be more efficient in single cell suspensions in the initial phase of production as few viruses (at low MOIs) are inoculated into the culture. Subsequent virus generations are released to much higher MOIs relative to the uninfected cells. In this later phase it may be beneficial to induce aggregates that include a single or very few infected cells and a majority of yet uninfected, potential host cells. If the ratio of infected (still viable) and uninfected cells is important for productive aggregate induction then such a scenario would also explain the importance of timing.

The next set of optimizations focused on MOI and cell density at the time of infection (Figure 1 (c)) and revealed that a MOI of 0.01 (as opposed to 0.1) at intermediate cell densities (2×10^6/mL) should be optimal for subsequent refinements. Because of the relatively slow replication kinetic we also used qPCR to follow the dynamic of genome replication in parallel to that of the infectious units. Using a calculation described previously for relative quantification of gene expression [37] we confirmed a robust amplification of the viral genome over almost 4 orders of magnitude under the selected conditions (Figure 1 (c)).

Replication at different temperatures (33, 37 and 39 °C) was studied with the intention to further improve viral titers (Figure 1 (d)). DAdV-1 replicated to high titers at 37 °C and 39 °C (the core temperature of ducks is 41 °C) but appeared not to be able to be propagated efficiently at 33 °C. We noted a surprising discrepancy between infectious and haemagglutinating units (HAU) that suggests that a measurable amount of non-infectious particles with HA activity are formed at 33 °C. Replication may be slightly faster at 39 °C because HA activity indicative of particle formation is higher 24 h PI but this head start appears not to be maintained at later time points. Stability of EDSV appears to be slightly lower at the higher temperature and therefore the optimal temperature for production of DAdV-1 was chosen to be 37 °C.

An overlay of the optimized process in flasks and a bioreactor (Figure 1 (e)) confirms scalability and good replication of DAdV-1 at MOI of 0.01 with 2×10^6 CR.pIX cells/mL, at 37 °C. Maximum yields were 6.8×10^7 $TCID_{50}$/mL, > $1:2^{15}$ HAU/100 µL and 3000-fold amplification of the viral genome (relative to 2 h PI).

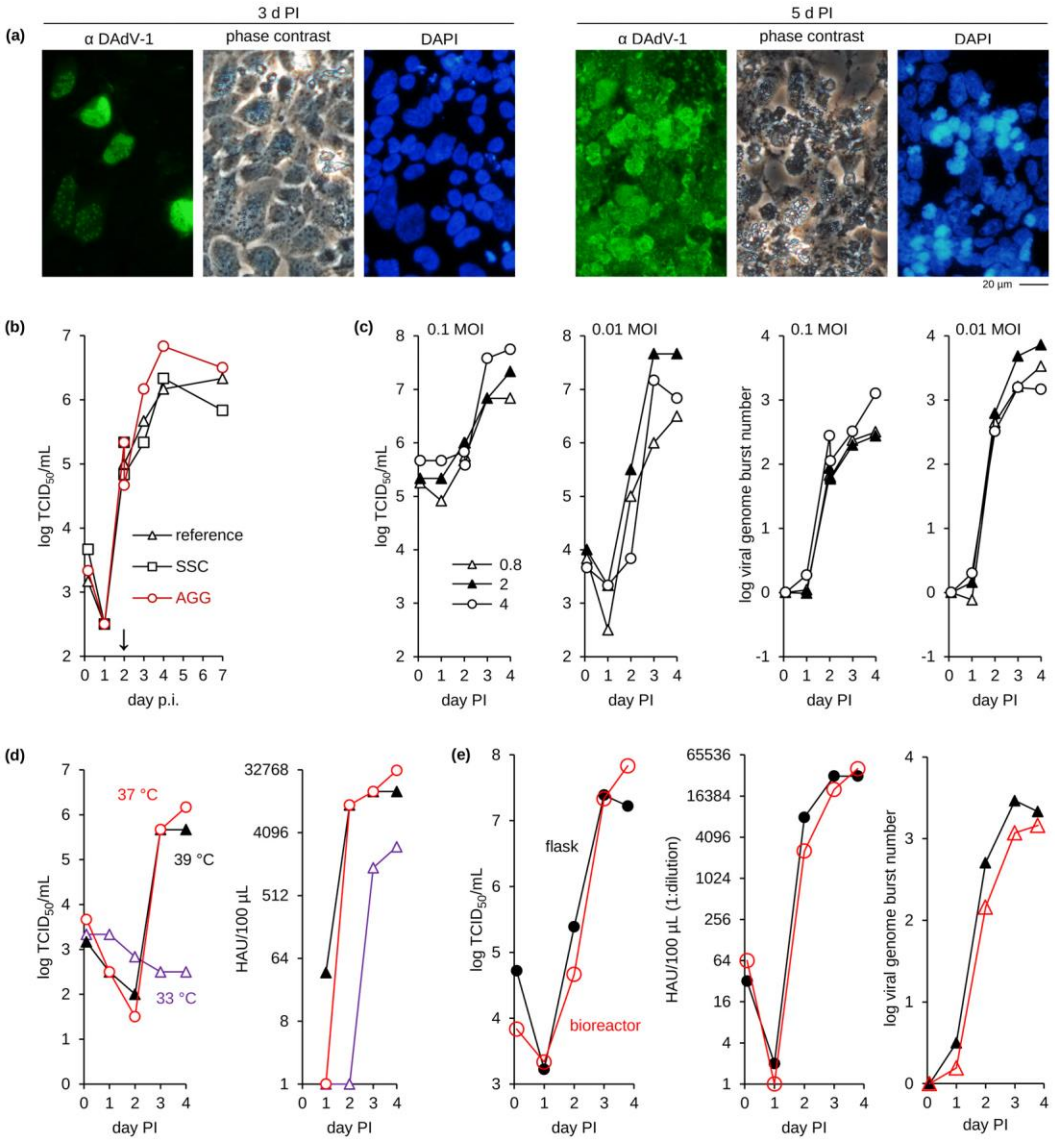

Figure 1. Propagation of DAdV-1 in CR.pIX cell lines. (a) Infection of adherent cell monolayers is visible only by immunofluorescence staining 3 days PI but virus spreads efficiently until 5 days PI. (b) Efficient replication in suspension cultures requires addition of an aggregation-inducing medium 2 days PI. The reference is a culture without feed, SCS was given cell proliferation medium and AGG virus production medium 2 days PI. (c) Effect of MOI on infectious yields (left panel) and amplification of genomic DNA (right panel). The viral genome burst number was calculated from the ratios of viral genome to cellular E1A gene for each time point relative to the ratios at day 0.(what is 0.8, 2 and4??? cell density) (d) Propagation at 33 °C does not release infectious units but appears to lead to a measurable amplification of antigen (HAU, right panel). Optimal temperature was determined to be 37 °C. (e) The final process scaled to bioreactors. Comparison of yields of infectious units, hemagglutinating activity and genome amplification in bioreactors and shake flasks. CPE, cytopathic effect; PI, post infection; SCS, single cell suspension; AGG, suspended aggregate culture format; HAU, hemagglutinating units.

Newcastle disease

Newcastle disease (ND) is caused by a negative-stranded RNA virus of the *Avulavirus* genus in the *Paramyxoviridae* family. NDV is also referred to as serotype 1 in the group of avian paramyxoviruses, APMV-1. Infectivity of the viruses is augmented by proteolytic processing of the F0 precursor protein in

the viral envelope. This cleavage is catalysed by host proteases and is strongly influenced by the number of basic amino acids at the cleavage site [38]. Glycoproteins of highly virulent (or velogenic) strains are cleaved by a greater diversity of proteases, a property that broadens the tropism and allows viruses to spread more easily within an infected animal and to induce greater damage to organs and tissues [39].

ND caused by velogenic strains is a highly contagious disease associated with a significant economic burden [40]. NDV has a very broad host range and is capable of zoonosis but symptoms caused in humans even by velogenic strains are usually restricted to a mild and transient conjunctivitis or laryngitis [41].

The vaccine against ND in poultry consists of live, vectored live and killed preparations of lentogenic strains [40]. Live or vectored live vaccines are often preferred because they can be applied directly in the hatchery using automated vaccination equipment. NDV has also the highly promising capacity to replicate in human tumors and is being tested to augment therapies against neoplastic diseases [42]. However, because this inherent oncolytic activity correlates with virulence in birds, research on and application of NDV-based virotherapy is being restricted by federal regulation (9 CFR Part 121 i guess) in the USA.

Most NDV vaccines are produced in embryonated eggs that naturally contain the proteases required for activation of lentogenic viruses [43–45]. As expected, exogenous trypsin had to be added to CR.pIX cultures for replication of the lentogenic NDV LaSota (Figure 2 (a)). Because adherent CR and CR.pIX cells dislodge easily if they are cultivated without supplementation with bovine serum (that would interfere with the activity of trypsin) we did not further investigate replication in this culture format.

However, infection of CR.pIX suspension cultures yielded NDV at the lower range of titers reported for other continuous cell lines even in the presence of trypsin. Although titers are difficult to compare because infectious units are given in different units it appears that volumetric yields are generally higher in eggs than in cell cultures (>10^9 EID_{50}/mL in eggs [46,47], $10^{8.5}$ PFU/mL in LSCC-H32 and secondary chicken embryo cells [48], $10^{6.1}$ to 10^7 $TCID_{50}$/mL in QT35 [46] and DF-1 cells [47], respectively). Trypsin is not expected to be inhibited because the chemically-defined cell proliferation medium for CR and CR.pIX cells has a negligible low protein content (10 ng/mL of recombinant IGF) and no hydrolysates or other substances that may inhibit proteases.

We next suspected that the chemically defined proliferation medium should be supplemented with cholesterol because this compound is not present in the medium but has been reported to be essential for NDV replication [49]. However, biosynthesis of cholesterol by CR.pIX cells appears to be either sufficient or not the main limitation as no increases in yields were observed by supplementation. Cholesterol supplementation may add further complexity to a final production process and was therefore not included in subsequent experiments.

Passage of NDV in CR cell suspensions was performed next to investigate whether low titers in cell cultures are due to adaptation of our strain of NDV to eggs. A 1000-fold increase in infectious titers was indeed observed between passages 4 and 7 and appeared to remain stable for at least 2 additional passages (Figure 2 (b)). A direct comparison of virus isolates at passage levels 4 and 9 indicated that

replication kinetics has also increased (Figure 2 (c)). We observed no decrease in titers even at 9×10^6 cells/mL indicating that NDV production may also be possible also at very high densities (there is no cell-density effect, [35] and references therein) although this may not further increase absolute yields. Further optimization resulted in a process with incubation temperature of 33 - 35 °C, pH of 7.1 units, and cell density of 2×10^6 /mL. The optimal trypsin concentration is 0.5 µg/mL and should be maintained by daily or continuous supplementation.

A very low (rather than higher) MOI appears to be the most critical parameter for production of the NDV at passage level 4 (Figure 2 (d)). Titers remained above 10^8 $TCID_{50}$/mL for virus at passage level 9 at MOI from 10^{-4} down to 10^{-7}. Infectious units with virus at passage level 9 in the improved process are in the upper range of those reported for continuous cell lines and replication at lowest MOI spans almost 9 orders of magnitude. However, hemagglutinating (HA) activity was still low with maximum values usually in the range of 1:265 to 1:512/100 µL. To further improve yields we paid more attention to the clumping of the cells that sometimes occurred 24 h post infection. This phenomenon may reflect formation of syncytia and provides an additional mechanism for spread of infectious units [50]. We therefore added virus production medium to further augment the aggregate formation [32], but we also sonicated the final lysate to separate clusters of cell debris and viruses, speculating that the true HA titers are decreased proportionally to the number of particles bound within the aggregates. These additional steps are compatible with scalable processes in bioreactors (Figure 2 (e) and [32]) and did improve HA yields (Figure 2 (f)). The final process consists of supplementation with 1/6th of the culture volume with virus production medium and sonication of the complete lysate for maximum HA levels of 1:1024 to 1:2048 at day 3.

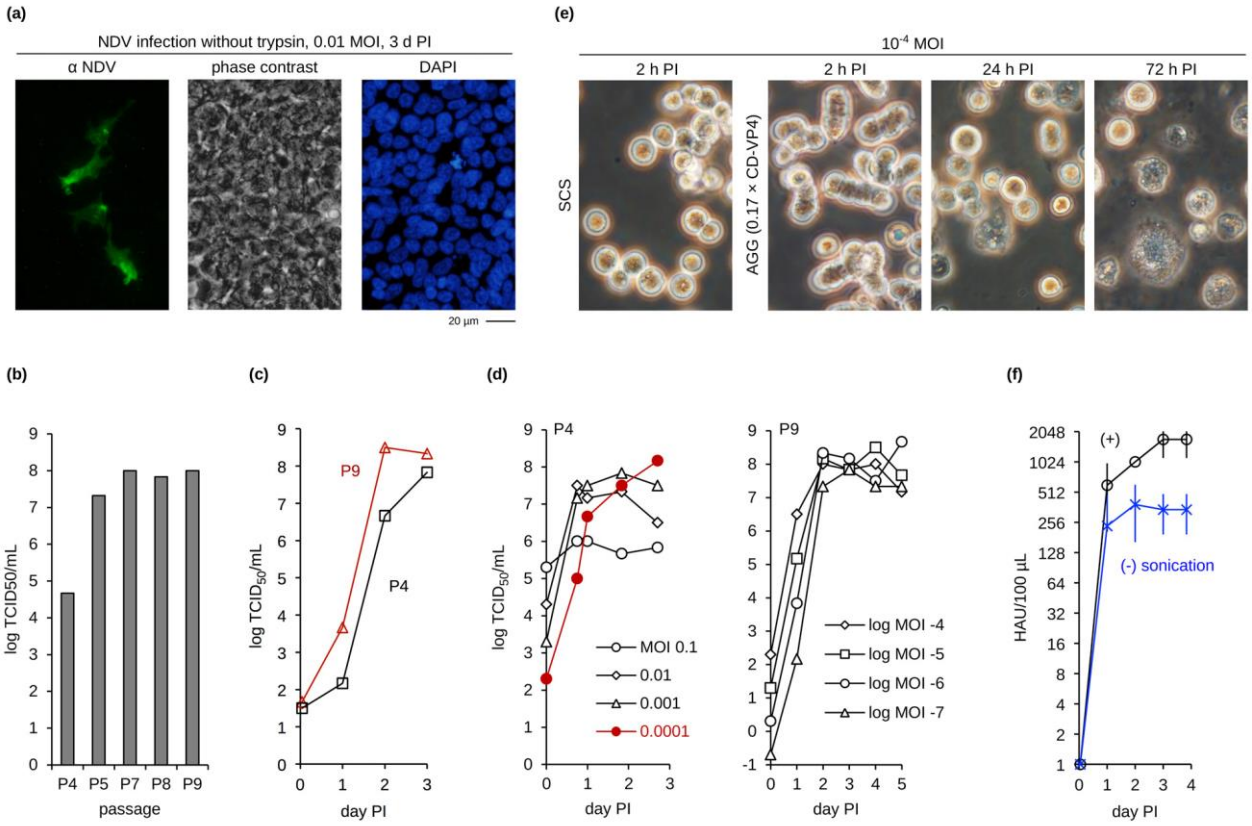

Figure 2. Production of NDV in CR.pIX cultures. (a) Lentogenic NDV enters CR.pIX cells but cannot spread without an exogenous supply with protease. (b) Adaptation of NDV by passaging on CR.pIX suspension cells. Note steep increase of infectious units between passage 4 and 5. (c) Differences in the kinetic of replication of the isolates at different passage levels, P4 and P9. (d) Influence of MOI on replication and yields. (e) Suspension cultures in a bioreactor infected with NDV. Addition of virus production medium CD-VP4 to 1/6-th of the culture volume induces small aggregates. CPE is evident 24 h PI. (f) Sonication further increases HA yields. PI, post infection.

Avian infectious bronchitis

Avian infectious bronchitis virus (IBV) belongs to the *Coronaviridae*, and therein to the *Gammacoronavirus* genus in the *Coronavirinae* subfamily. Coronaviruses are a family of large enveloped viruses with prominent surface proteins or spikes.

IBV is disseminated by aerosol and fomites and mainly causes damage in the respiratory and the uro-genital tract [14]. Overt direct or secondary bacterial disease has been observed only in galliforms [55,56] but waterfowl appears to be susceptible and may serve as asymptomatic reservoir for IBV if chicken and ducks are farmed together [57].

Vaccinations regimes typically include live and killed vaccines but infectious bronchitis appears to be a disease that is not easily controlled. The attenuated strains are generated by serial passages in eggs, and the final passage level of a vaccine virus is selected by balancing the safety and efficacy of the vaccine candidate virus [55].

We were disappointed but not surprised that inoculation of CR or CR.pIX cell monolayers with IBV strains M41, H120 or 11518/2010 originated from 13-day-old broiler with severe lympho-histiocytic interstitial nephritis did not result in any signs of CPE (data not shown). IBV is known to replicate efficiently in embryonated eggs but not in continuous cell lines ([61] and references therein). The attenuated strain Beaudette, that was obtained by serial passaging in embryonated chicken eggs [62], is an exception with a broad cell tropism but reduced value as vaccine strain.

One barrier for permissivity maps to the spike protein (S) of the viral particles [63,64]. This S protein of IBV is processed by cellular proteases into two subunits, S1 and S2 [65]. The S1 subunit mediates attachment to the target cell [58] and appears to contain the dominant positions for neutralizing epitopes and escape mutants [66]. The S2 subunit cooperates with the cognate S1 in receptor recognition and is responsible for fusion of viral and plasma membranes [14,67]. The receptors of IBV are not yet known but glycosylated structures, especially α-2,3-linked sialic acids, appear to be required for infection [14,65]. Both, α-2,3 and α-2,6 configurations are available on CR and CR.pIX cells (Figure 5 (a)).

We attempted adaptation of the M41 and H120 strains to our culture system. Adaptation, however, requires at least low-level replication of the viruses so that suitable mutations can accumulate. Speculating that entry of viruses into the cells is the main block we also tested different mammalian cell lines (hoping for pseudoaffinity [61]) and added polymers (such as polybrene, poly-L-lysine and polyethylenimine) known to augment interaction of viruses (or lipid vesicles) with plasma membranes [68] (Figure 3 (a)). Potential infection was assayed by RT-PCR with primers against the ORF1a polyprotein.

We detected a signal for genomic RNA in infected suspension cultures. This signal was maintained for one week, could be rescued into one subpassage, but was lost with the second subpassage (Figure 3 (b, c)). In adherent cultures we did not observe any signals indicative of entry of any viruses. One important difference in the handling of adherent and suspension cultures was that adherent cultures were washed 2 h after inoculation with seed virus. Although interference by bovine serum present in the medium for adherent cultures is a formal possibility, we consider that detection of the input virus is the most parsimonious explanation for transient detection of viral genome and consistent with similar observations and reports on virus stability in the literature [56,69].

Combined, CR and CR.pIX cell lines appear not to be permissive for and probably not even susceptible to infection with IBV strains M41 or H120. Adaptation may be successful starting with a strain such as Beaudette but this may not be generally desirable. Adaption of IBV to replication *ex vivo* appears to change properties of the virus that may reduce cross-protection as a vaccine. S1-vectored vaccines may provide alternative vaccines against infectious bronchitits [14], so the muscovy duck cell lines may still find a way for IBV vaccines as producers of recombinant viral vectors[32].

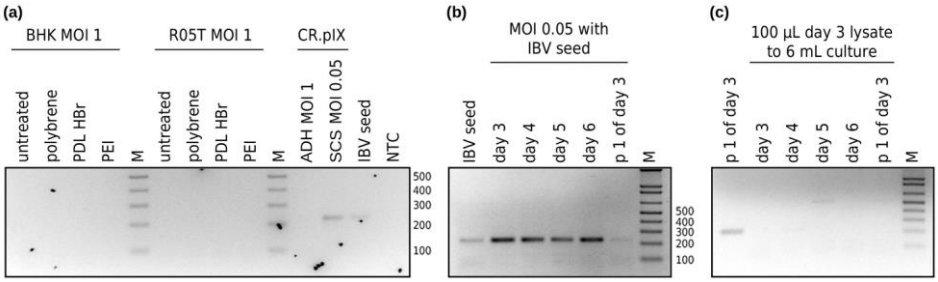

Figure 3. No infection with IBV strains M41 and H120. (a) Adherent cultures of BHK (from the Syrian hamster), R05T (from the Egyptian fruit bat [70]) and CR.pIX cells were inoculated with AIBV at MOI of 1 also in presence of charged polymers with the intention to improve infection [68]. Potential entry of virus was analysed by RT-PCR. A comparison of infection of adherent (a) and suspension (s) cultures initially suggested that suspension cultures may be susceptible. (b) Strong signal in the suspension culture for 6 days but reduced signal of the first passage (performed with the culture 3 days post inoculation). (c) Complete loss of signal in inoculations with lysate from the previously inoculated CR.pIX culture. Passage 1 culture shown in (b) was lysed and used to inoculate fresh CR.pIX cells. No signal indicative of replication-competent virus could be detected suggesting that all earlier signals were caused by the virus from the initial inoculation.

Marek's disease

Marek's disease (MD) is a neoplastic disease caused by some herpesviruses of the *Mardivirus* genus. The type species is referred to as Gallid herpesvirus 2 (GaHV-2) or Marek's disease virus (MDV). Other species in this genus include Gallid herpersvirus 3 (GaHV-3) and Meleagrid herpesvirus 1 (MeHV-1), also referred to as herpesvirus of turkey (HVT). Infections with MDV lead to substantial economic losses each year, while GaHV-3 and HVT are non-pathogenic.

MDV replicates in the skin and feather follicles and is disseminated horizontally via inhalation of fomites. Infiltration of neurons can cause progressive paralysis of legs and wings whereas diffuse lymphomatosis of visceral organs often presents as an acute disease associated with high mortality [71,72].

Vaccines against MD are based on live-attenuated strains of GaHV-2 (for example, Rispens strain), non-pathogenic GaHV-3, and more importantly on non-pathogenic HVT. HVT is also widely used as vector virus, and with appropriate insert genes HVT represents the new generation of poultry vaccines being able to provide good protection against several important avian diseases such as Newcastle disease, avian influenza, infectious bronchitis or infectious laryngotracheitis. Vaccine roduction is usually performed in CEFs and occasionally in duck embryo fibroblasts [72]. Continuous cell lines reported to be permissive for mardiviruses include the DF-1 chicken cell line [77] and the QM7 quail muscle cell line [78]. The QT-35 quail fibrosarcoma cell line is also permissive at least for HVT but, surprisingly, appears to harbour also a previously uncharacterized latent virus possibly related to GaHV-2 [79]. Another cell line reported to be permissive is the chronically infected lymphoblastoid cell JM-1 that was derived from chickens infected with MDV and that can transmit this virus to CEFs in cocultivation experiments [80].

However, there are also unpredictable restrictions in the permissivity for mardiviruses *in vitro*. For example, MDV was shown not to replicate in the chicken cell line LSCC-H32 that is permissive for a wide spectrum of other viruses [48], and only a small fraction of cells, 5 %, were positive for viral antigens in

the chronically infected JM-1 cultures [80]. Gene expression of latent MDV-like virus in QT-35 cells could be reactivated by superinfection with HVT but recovery of infectious units was not possible by cocultivation with CEFs or primary chicken kidney cultures [79]. Isolation was only successful *in vivo* by inoculation of QT-35 cells into chicken embryos [79].

We infected adherent CR and CR.pIX cultures with different mardiviruses, including HVT, a vector HVT that expresses an antigen to protect against NDV [81,82] (Vectormune ND, Ceva Animal Health), and Rispens. The results shown in Figure 4 (a) and (b) were obtained by inoculation with revitalized CEF cells carrying Vectormune ND where MOI indicates ratio of infected fibroblasts to CR or CR.pIX. Propagation of HVT was tracked by qPCR against the viral genome and the cellular DNA.

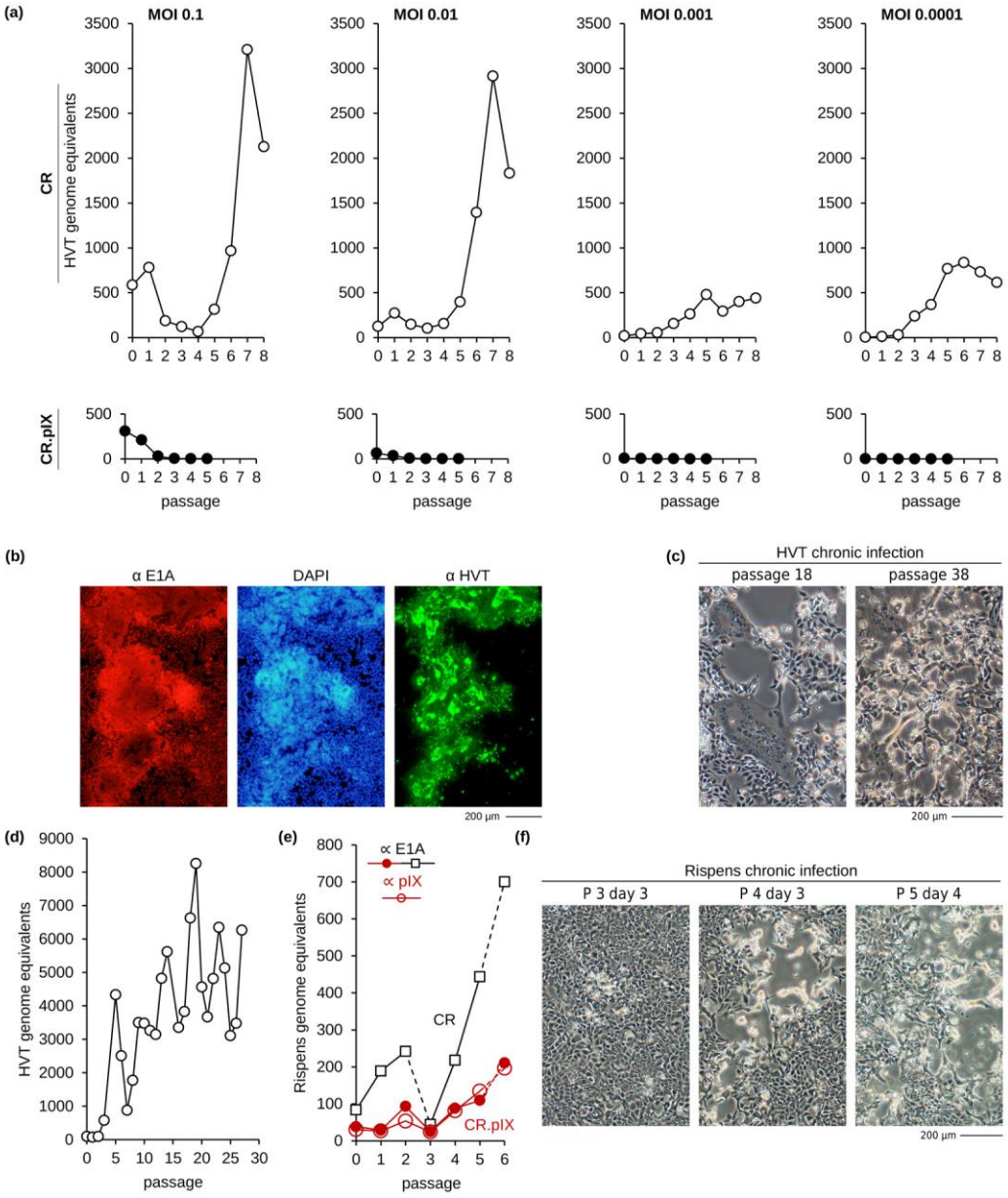

Figure 4. Mardivirus propagation in adherent CR and CR.pIX cells. (a) CR and CR.pIX cells were inoculated with the freshly revitalized Vectormune ND preparation at the indicated ratios (for example, MOI of 0.1 corresponds to 1 CEF of the vaccine for every 10 cells of the anatine continuous cell line). The cultures were subpassaged twice per week by 5-fold dilution. DNA was isolated at each passage and qPCR was performed with primers against mardiviruses and against the E1A gene of the continuous cell line to normalize results to the number of host cells. CR cells are fully permissive for mardiviruses but several attempts failed to establish a productive infection in CR.pIX cells. (b) Immunofluorescence staining against HVT parent virus of Vectormue ND and the cellular E1A gene demonstrates extensive spread of HVT in a culture that has been subpassaged nine times. (c, d) Chronic infections could be maintained for 30 passages. CPE is characterized by extensive cell damage and large syncytia. (e, f) Both, CR and CR.pIX cells were fully permissive for the Rispens strain (GaHV-2). This virus induced strong CPE also in CR.pIX cells but replicated to lower levels. The dashed lines between passage 2 and 3 (CR only) and 5 and 6 (both cell lines) indicate addition of 1/10th of uninfected cells because cytopathic effect appeared to be too strong to continue subpassaging. The genome equivalents of Rispens were also determined with primers against pIX in the CR.pIX cultures; as expected, pIX could not be detected in CR cultures and the corresponding genome equivalents are therefore not shown.

CR cells are fully permissive for HVT (Figure 4 (a) to (d)) and Rispens ((Figure 4 (e, f)) and infection leads to a progressively expanding CPE that is characterized by frequent plaque formation (centrally dislodged cell layer framed by highly refractile cells) or large syncitia. Appearance of CPE was delayed and is often negligible in the first 3 passages. Chronic infections were readily established and one culture was HVT-positive for at least 30 passages until CPE developed to an extent that required addition of uninfected cells prior subpassage (Figure 4 (c, d)). Because of the delayed CPEs we did not titrate virus by $TCID_{50}$ protocols but quantified virus propagation via qPCR. The qPCR cycle threshold levels for the cellular E1A gene in the full lysates were used to normalize the number of viral genomes to the number of cells in the culture. With this quantitative measurement, that should be independent of cell numbers, we observed what appears to be a periodicity in the kinetic of virus propagation. The maximum yields and kinetic we observe for MDV and Rispens in adherent CR cultures compares well with previous results in chickens that measured a maximum of 10^8 HVT genomes per 10^6 feather follicle cells [73].

CR.pIX cells were not permissive for HVT but allowed productive propagation of Rispens with strong cytopathic effect yet lower titers compared to CR cells (Figure 4 (a, e)). Especially the block in HVT replication is very surprising because CR.pIX and CR cells differ only by the expression of the pIX protein [28], a minor structural and presumably also regulatory factor of human adenoviruses [83,84]. We have proposed previously that pIX may also augment binding of Hsp70 and Hsp90 in the avian cell line [28]. The resulting activation of the heat shock response can be beneficial for replication of some viruses [85,86] but may also elevate antiviral responses as signaling cascades of the two pathways communicate [87,88]. Mardiviruses have also been reported to interact with Hsp70 [89,90], and may depend on this factor for entry into chicken embryo fibroblasts [90]. By directing Hsp70 into complexes with Hsp90, pIX may interfere with availability of Hsp70 at the cell surface in a way that can be partially compensated by Rispens but not by HVT. Alternatively, pIX may indirectly cause an elevated state of innate immunity. HVT does not code for several virulence factors that are present in Rispens [91,92] and may therefore be unable to block the cellular defenses. (why is this grey????)

Avian influenza

Members of the *Influenzavirus A* genus in the *Orthomyxoviridae* family, the avian influenzaviruses (AIVs), are among those pathogens with a very high capacity to cause dangerous zoonosis and pandemics [93]. They can infect birds and mammals, and are transmitted by the respiratory route without dependence on hematophagous vectors. The diversity of viral strains is extremely broad and properties span low to high pathogenicity (with case fatality rates from 0.1 % to at least 15 % [94]), low to high transmissibility, and narrow to broad host range.

AIV pathogenicity is determined by a number of factors that range from tropism (for example, viruses that infect the lower respiratory tract tend to cause more severe disease than those that replicate in the upper regions) to interaction with innate immunity (the case fatality rate is higher for strains that induce hypercytokinemia or "cytokine storms") [95,96]. Tropism and host range is mainly determined by the haemagglutinin and neuraminidase proteins (HA and NA, respectively) on the virion surface and the nonstructural protein PB2, a subunit of the viral RNA polymerase. A wide variety of strains have been used to protect against infection with H9N2, H5N2, H5N1, H7N3, H7N7 viruses in various parts of Asia, North America and North Africa [107]. Problems that are sometimes associated with the current production system are low yields for certain strains so that expensive concentration steps are necessary to increase potency, considerable amounts of egg shells that remain as biohazardous solid waste, and dependence on a continuous supply with embryonated eggs free of adventitious agents [107,109,110]. Improved control over supply with embryonated eggs is achieved by constructing breeding facilities immediately adjacent to the production site, but this comes at substantial costs [111]. We have therefore tested replication of H9N2 as a highly representative strain for LPAIVs in suspension cultures of CR.pIX cells to provide an additional production substrate that is independent of primary material (FIG 5).

Because the host spectrum of AIVs is partially determined by preference for 2,3 or 2,6 α-glycosidic linkage at the terminal sialic acid [103] we first characterised surface glycosylation of the CR and CR.pIX cell lines. Avian isolates tend to recognize 2,3 α-glycosidic bonds that predominate in the gastrointestinal and respiratory tract of ducks and chickens whereas human isolates tend to recognize the 2,6 α-configuration that predominates in our upper respiratory tract [96,112]. Whereas early passage cell pools are not uniformly positive for the 2,6 α-configuration we observe that lectins specific for either confirguration yield signals in the final CR.pIX cell clone (FIG 5 (a)). This result suggests that AIV isolates should be able to replicate in CR.pIX cultures independent of a mammalian or avian origin, a property that has also been described for the MDCK cell line that is considered a gold standard in influenzavirus research [113,114].

Orthomyxoviruses require proteolytic activation of the viral receptor similar to the paramyxoviruses [43–45]. The proteases contained in embryonated eggs are replaced by exogenous trypsin in cell cultures. We observed good replication of the two H9N2 isolates in the presence of 0.5 and 1.5 µg/mL of trypsin, well below the 2.5 µg/mL as maximum amount of trypsin that is tolerated by CR.pIX (FIG 5 (a)). Replication of both isolates (duck/1995 and turkey/1966) at passage level 2 appeared to be very fast with maximum titers in the range of 5×10^7 TCID50/mL and 1:256 HAU/100 µL within 24 h of infection. However, although both isolates are H9N2 strains they differed in maximum yields, a property that was maintained

even after 10 passages in the CR.pIX cells (FIG 5(c)). Permissivity of CR.pIX cells appears to be very high as both isolates established productive infections to maximum titers at MOIs as low as 10^{-7} (FIG 5(c)).

AIVs isolated with the help of cell lines have been proposed to be suitable as vaccine seeds [114]. The hemagglutinating and infectious activities of the LPAIV propagated in CR.pIX cells in true suspensions cultures in chemically-defined medium without microcarriers and at extremely low MOIs compare favourably to the results reported with H9N2 in MDCK cells [114,115].

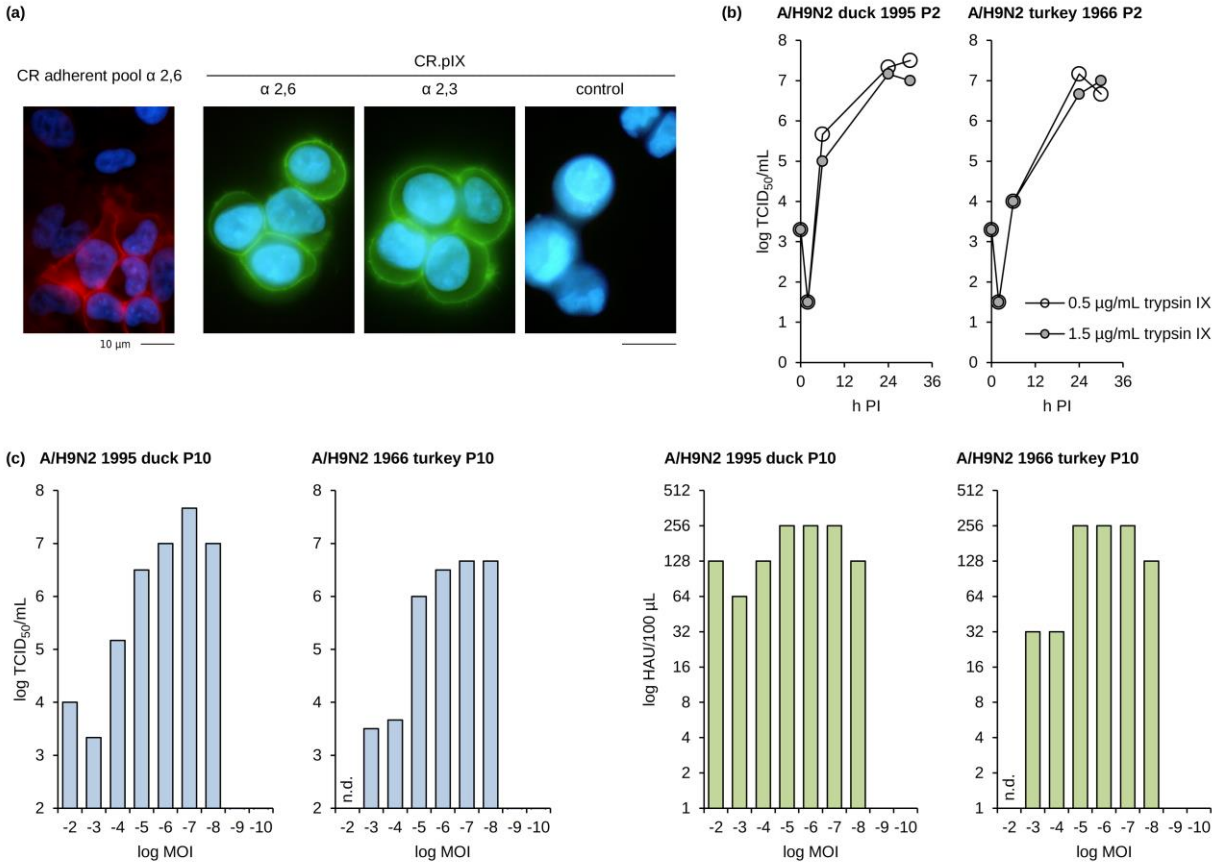

Figure 5. Replication of LPAIV H9N2 isolates in CR.pIX cultures. (a) Lectin binding indicates that both terminal α-glycosidic sialic acid configurations utilized by influenza A viruses as receptor are available on CR.pIX suspension cells. The initial pool of cells appeared not uniformly positive for the 2,6 configuration. (b) Very fast replication of the two H9N2 isolates in the CR.pIX suspension cells to high titers in presence of low and high amounts of trypsin. (c) Infectious and hemagglutinating activity of passage 10 of the two isolates as function of MOI.

Infectious bursal disease

IBDV is a member of the *Birnaviridae*, genus *Avibirnavirus*. The disease caused by IBDV, infectious bursal disease (IB) or Gumboro disease, is a serious economic burden to the poultry industry [116]. Only chicken appear to be susceptible to overt disease; turkeys, ducks and other poultry appear not to be affected by IBDV [117]. Infected chickens develop an acute depression of the humoral immune response

due to extensive lesions in the bursa of Fabricius. Such animals are more susceptible to secondary infections and fail to respond adequately to routine vaccination [76].

The viral capsids are icosahedral with diameters of 60-70 nm. They are not enveloped and contain two segments of double stranded RNA of 2.8 kbp and 3.4 kbp, respectively. The two structural proteins (VP2 and VP3) are released by the viral protease (VP4) contained in the autocatalytically active tripartite polyprotein. The infectious virion is very stable in the environment [76]. Control of IBDV is furthermore complicated by the evolution of the pathogen towards greater antigenic diversity and increased virulence [76,117]. The preferred vaccine is live IBDV that has to be monitored for a correct level of attenuation: viral replication should be high enough to induce protective immunity also in the presence of maternal antibodies and against the emerging variant field strains but below levels that may lead to immunosupression. The challenges are being addressed by immune-complex vaccines, HVT-vectored VP2, and by prime/boost regimes using live and killed vaccines for high value animals such as breeder hens.

IBDV (strain B87) replicated rapidly in adherent monolayers of CR.pIX cells (Figure 6 (a)). We observed prominent comet formation indicative of efficient release of viruses 24 h PI followed by widely scattered and overt CPE 48 h PI. The results in adherent cultures are confirmed by the high yields of infectious units in various suspension culture formats (Figure 6 (b)): IBDV replicated in single-cell suspensions as efficiently as in cultures with induced aggregates that were designed to support propagation of cell-associated viruses [32]. Titers were also not increased by lysis of the cells in either of the culturing system suggesting that none or negligible infectious units remain within the host cell at the end of the replication cycle.

Propagation of IBDV in single-cell suspension cultures was robust with high titers at MOIs of 0.1 or 0.05 (lower MOIs did not increase yields) and at intermediate to high cell densities (2 - 4 $\times 10^6$ CR.pIX cells/mL). For production processes yields beyond 10^{10} $TCID_{50}$/mL can be expected 4 days post infection.

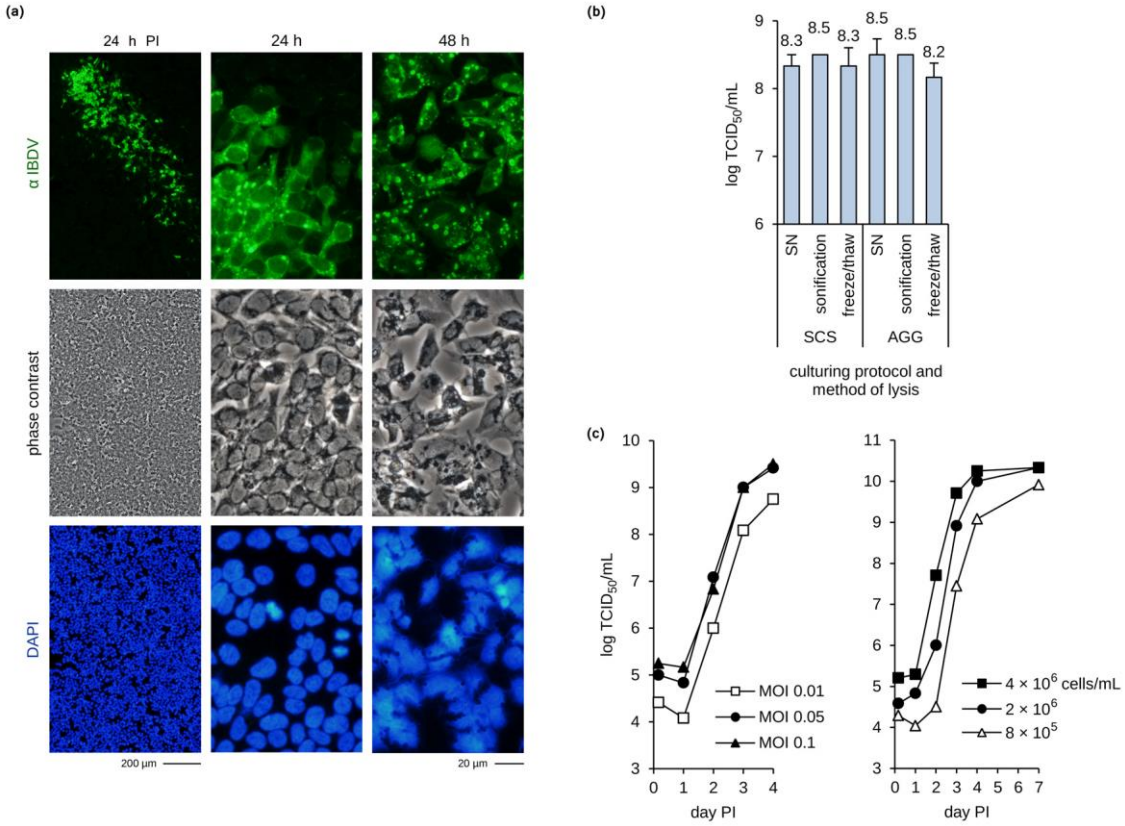

Figure 6. Replication of IBDV in CR.pIX cells. (a) Immunofluorescence staining of infected adherent cell monolayers. IBDV replicates fast and causes formation of large comets. (b) Similar yields independent of culture format or harvest suggests robust replication. SN, supernatant; SCS, single-cell suspension; AGG, suspended aggregates. (c) Replication kinetic depending on MOI (left panel) and cell density at infection (right panel).

Derzsy's disease

Derzsy's disease appears to affect only geese and muscovy ducks, and has been described in Europe, Asia and North America ([118] and references therein). It is a highly contagious and economically damaging disease caused by strains of Goose parvovirus (GPV) and Muscovy duck parvovirus (MDPV), species in the *Dependovirus* genus in the *Parvoviridae* family. The small non-enveloped viruses are shed in large amounts by acutely infected birds and remain stable against environmental and thermal stress [119]. Disease can be subclinical in adult animals but is usually fatal in birds in the first month after hatching. The preferred vaccines are based on live attenuated viruses produced in primary duck embryo cells, and recently, a transformed (tumorigenic in nude mice) cell line [120–122].

We have previously described replication of a goose parvovirus [33] and here briefly confirm this result with an extension to successful propagation of MDPV (Figure 7). Although they are closely related, the two viruses had profoundly different properties. GPV was propagated easily and reached a maximum titer of 7.5 log $TCID_{50}$/mL within one passage whereas MDPV required three passages of the infected cells to reach a maximum titer of 6.1 $\log_{10}$ $TCID_{50}$ (Figure 7 (b)). Concentration of antigen and infectious

units also appear not to be congruent in a comparision of the two viruses (Figure 7 (a, b)). For example, 790 EU/mL (ElISA Units/ml How is it defined it should be mentioned in MaM) of antigen yield for GPV corresponds to a more than ten-fold higher infectious dose relative to MDPV in a sample with 3400 EU/mL. Another difference is that yields are higher if infection with GPV is performed with a low MOI whereas titers of MDPV benefit from infection with a greater MOI. (Did you use the same sera in the ELISA and was it validated for the two viruses? The sera may not bind to the two virus with equal affinity. With other word just because you measure higher ELISA titer at one virus does not mean that you have higher quantity of that virus. ELISA of Derzsy should be in the MaM)

In summary, the yields for both GPV and MDPV both compare favorably to a vaccine dose of 2.6 – 4.8 log $TCID_{50}$ or _____ EU (<- WHAT IS TYPICAL FOR VACCINE!?) (I do not have the faintest idea Pénzes might know about it they have vaccine against Derzsy) so that also for these viruses CR.pIX may provide a versatile production substrate to replace for the currently predominating primary cells.

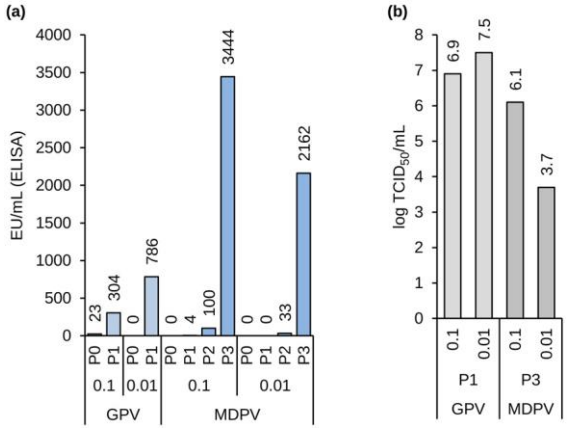

Figure 7. Propagation of goose (GPV) and muscovy duck parvoviruses (MDPV) in adherent CR.pIX cultures. (a) Yields of parvovirus antigens after infection with MOI of 0.1 or 0.01. (b) Infectious titers of the parvoviruses in CR.pIX cells. GPV replicates to expected titers within the first passage, MDPV replicates to high titers after three passages. All values shown were obtained from complete lysates 3 days post infection.

Summary

A delicate balance among costs , economic benefits and biological risk guides the decision on whether a given vaccine is or is not to be used for agricultural animals with a short life-span. One complication introduced into these considerations for poultry vaccines is that industrial farmers and those with low resources both require a diversity of vaccines, but that robust and cost-effective production appears to be skewed primarily towards industrial use.

Most poultry vaccines are manufactured with material derived from embryonated eggs of a high quality. Dependence on embryonated eggs introduces costs and sometimes fragile logistics into vaccine production processes that could be circumvented if a continuous cell line permissive for a broad selection of vaccine strains and suitable for vaccine production were available. CR and CR.pIX are well characterized established cell lines for research and for manufacturing of vaccines. They are available as

adherent cultures and in suspension format in chemically defined media without dependence on bovine sera or other animal-derived components. We now extend earlier studies on permissivity for various pathogens to include several poultry vaccine candidates against egg drop syndrome, Marek's disease, Newcastle disease, infectious bronchitis, avian influenza, infectious bursal disease, and Derzsy's disease. Some of the viruses presented unique requirements for the production process but all, with exception of the two isolates of IBV, could be propagated to high titers in the anatine continuous cell lines. Future studies will focus on two major aspects, one being scale-up to larger bioreactors or transfer to different bioreactor types such as cultivation at high cell-density or with help of perfusion. The other important topics are characterization of safety and immunogenicity of vaccines obtained after the change of the active substance and the production protocol.

Material and methods

Cells and viruses

The CR and CR.pIX cell lines and the development of the chemically defined media has been described previously [28,32]. The CD-U3 and CD-VP4 media used in this study were manufactured by Biochrom (catalog numbers F9185 and F9127, respectively). Viruses were obtained from Ceva Phylaxia (Hungary) and the Friedrich-Löffler-Institut (Germany).

Determination of infectious units

Infectious titers of NDV and H9N2 were determined on Vero cells. 2×10^5 cells in DMEM:F12 medium containing 2 mM GlutaMAX I (both Gibco) and 5 % fetal calf serum (Biochrom) were seeded into a 96-well plate at 100 µL of cell suspension. The medium was replaced on the following day against DMEM:F12 containing 2 mM GlutaMAX I and 1.5 µg/mL trypsin (type IX-S, Sigma T0303), but no fetal calf serum. Serial dilutions in steps of 10 of NDV or H9N2-containing virus samples were prepared in DMEM:F12 medium free of serum, and 10 µL each of the dilutions were added to the Vero cultures. Virus replication was allowed at 37 °C for 72 h.

Detection of NDV or H9N2 replication was facilitated by immunostaining: the cells were fixed in methanol for 10 min, allowed to dry to completion, and rehydrated with PBS containing 0.05 % Tween-20. NDV antibody (CSI) or H9-antiserum (GD Animal Health, VLDIA150) were added to a dilution of 1:2000 in PBS containing 1 % fetal calf serum and incubated for 1 h at room temperature. After two washes with PBS, secondary antibody (anti-chicken, Alexa Fluor 488 labelled, host rabbit, _____) was added at a dilution of 1:2000 for 2 h at ambient temperature or overnight at 4 °C. Infected wells were identified by fluorescence after two washes with PBS. Calculation of $TCID_{50}$ values was performed according to Spearman and Kärber using the implementation of the FAO [123,124].

Infectious titers of EDSV were determined on CS cells, a fibroblastoid and strictly adherent relative of the CR cell line [28]. Similar to titration of NDV, indicator cells were seeded (at 1×10^6 cells/plate) one day prior to infection. Virus samples were applied in 10-fold serial dilutions without change of medium or

addition of trypsin, and replication was allowed for 3 days at 37 °C. Identification of infected wells was facilitated by immunostaining with primary antibody (GD Animal Health, VLDIA050) at 1:2000 and secondary antibody conjugated to Alexa Fluor 488 at 1:2000 after fixation with methanol.

IBDV titers were determined on adherent CR.pIX cells. Infection was performed with samples in 10-fold serial dilutions, one day after the indicator cells were seeded at 4×10^6 per 96-well plate. Wells with CPE were identified microscopically (without immunostaining) 3 days post infection. As for EDSV, a change of medium or addition of trypsin was not performed to titer this virus.

Haemagglutination assay

Haemagglutination units (HAU) as correlates for number of viral particles were determined with chicken erythrocytes and serial dilutions of infected cell suspensions in PBS in steps of 2. The erythrocytes were isolated from complete blood of what?. Briefly, erythrocytes in 7 mL of a complete blood suspension (25 % in alsever buffer, E200, Labor Dr. Merk & Kollegen GmbH, Germany) were washed thrice in PBS. Centrifugation for the washing steps was performed at room temperature with 500 × g, brake set to low, for 5 min each. The final erythrocyte suspension was diluted in PBS to a concentration that yields an OD of 2.9-3.1 at 576 nm. This suspension was stored for up to 2 weeks at 4 °C.

The assay was performed in round-bottom 96-well microtiter plaes. The first row of the plate was filled with 200 µL of the infected cell suspension (wa it claridfied or it contained cells too?) and all other rows received 100 µL of PBS. Next, 100 µL of virus sample was added to the second row, the pipetting tips were replaced and the suspension mixed with fresh tips. This two-fold dilution was continued serially into the next row, and, for samples with high potency, continued into a second plate. Each well thus contained 100 µL of diluted virus sample and was subsequently supplemented with 100 µL of the erythrocyte suspension. This volume was mixed by pipetting and the turbid solution allowed to stand undisturbed for 30 min at ambient temperature (22 °C). HAU/100 µL are given by the highest dilution where formation of a dot was visible.

Some samples were further homogenized by sonication with a sonotrode H3 (Hielscher, Germany) of 3 mm diameter powered by an UP400S device at 50 % pulse and 50 % amplitude for 5 min (100 mL). This step increased HA titers of NDV by 2 to 4 dilution steps but was not performed in all experiments.

Detection and titration by qPCR

DNA was isolated with the QIAamp DNA Blood Mini kit (Qiagen). Purified DNA was eluted into 100 µL, and 5 µL thereof were added to PCR reactions. Alternatively, DNA was isolated from 20 µL of infected cell culture suspension by addition of 5 µL of QuickExtract DNA Extraction Solution 1.0 (101094, Biozym). This sample was heated to 65 °C for 15 min and to 98 °C for 5 min. After dilution with 50 µL of water, 8 µL therefrom???????? was added to a PCR reaction with a final volume of 20 µL.

Quantitative PCR of EDSV was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) in an ABI 7000 unit. Primers were added to 70 nM each per reaction and real-time PCR was

launched with a single incubation for 10 min at 95 °C, followed by 40 cycles of 94 °C for 15 seconds and 60 °C for 1 min. The primer sequences were TgA CTC Cgg TCC TTC TAA CAC A and TCA Cgg CAA CTg gTT TAA Tgg for detection of the E1A gene used to immortalize the avian host cell as internal reference [28], and gAg CgT gAT CAA gTC gAT gg and CAC TTA CCg CgA gCA TAT CC against the polymerase of EDSV (designed using GenBank sequence with accession number Y09598). Amplification of the genomic DNA was calculated via an adaptation described previously for relative quantification of gene expression [37]. We first determined the differences in the ct values obtained with primers against viral genome and against cellular DNA relative to the first data point, usually 2 h post infection. The differences were raised to the power of 2, assuming a uniform efficiency of 2.0 for each cycle of amplification, and the ratios of changes of viral to cellular DNA, each relative to the first data point, is the burst number.

HVT titers were calculated similarly, but here, because of the seeding with HVT infected CEFs that do not contain a cellular reference, we did not normalize to an arbitrary initial time of infection, but rather depicted the ratio of viral to cellular DNA (as "genome equivalents") for each passage. The primers used to detect E1A were the same that were used in the EDSV study and primers against the cellular pIX gene were ACC TAC gAg ACC gTg TCT g and gAg CCg TCA ACT TgT CAT C, but primers were added to 90 nM each. The primer sequences were TCg gCg CCC TTT gAT ATA CT and TAg Agg AAg TgT Tgg gCA gg (against the HVT012 gene of MeHV-1, Genbank AF291866), and TCCgTCgTgAATTTgTACgC and CgA CAA TTC CAC CTT Agg Cg (against the MDV011 gene of GaHV-2, Genbank DQ530348).

RNA-extraktion was performed with the Innu PREP RNA Mini kit (Analytik Jena) and cDNA transcribed with the Cloned AMV First-Strand cDNA-Synthesis kit (Invitrogen) using random hexamers. PCR was performed with 2.5 µL of the cDNA in 25 µL reactions with 94 °C for 180 s, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 60 s. Conventional PCR was performed with 2.5 µL sample in 25 µL reactions with the thermocycler programmed to 94 °C for 180 seconds, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60s. A fragment of IBDV genomic RNA segment B was amplified with primers gAC gAg TTC CTA gCC gAg Tg and CTg gCT gTg gCT AgA Agg AC. The primers bind to what appear to be conserved islands determined by alignment of GenBank sequences JQ411013 (isolate Hungary 903/78), GQ451331 (strain HLJ-0504), JX682710 (isolate QL), FJ695139 (Chinese strain YS07), and JX682712 (strain ZZ-11). H9N2 genome was quantified with AAg CCg AAA TCC Tgg gAA Tg and CTC CCT CTC AAA gTC gTA TC against segment 5 encoding NP (Genbank DQ067440), and IBV was detected with TgT TgT gCC AgT CCT CTC AT and gTT TCA CAg TAg CAg AgC gg against the ORF1a gene of strain H120 (Genbank FJ888351).

Lectin staining

Cells were collected with 200 × g, fixed with 1 % formaldehyde in PBS and incubated with 30 µg/mL of biotinylated agglutinin of *Sambucus nigra* (Vector Laboratories, USA, B-1305) to detect α 2,6 bonds, or of *Maackia amurensis* (B-1265) to detect α 2,3 bonds on the surface of the plasma membrane (the cells were not permeabilized). Binding was allowed for 45 min at ambient temperature and protected from light. The cells were washed once with PBS and the bound lectins were visualized with streptavidin conjugated to Texas Red (016-070-084) or fluorescein (DTAF, both from Jackson Immuno Research,

#016-010-084). DAPI (4',6-diamidino-2-phenylindol) in methanol was added to 1 µg/mL as a nuclear counterstain. The cells were washed once more with PBS prior to fluorescence microscopy.

Bioreactor

The DASBox (DASGip, Eppendorf) bioreactor units were equipped with a Marine impeller with 3 blades and 60-250 mL working-volume vessels. Gas mixing was performed with N_2, air, CO_2 and O_2, pH was adjusted with CO_2 and 1 M Na_2CO_3. Inoculation was usually performed to 1×10^6 cells/mL in CD-U3 medium and the culture was allowed to proliferate for 3 days to approximately 4×10^6 cells/mL. The parameters for the cell proliferation phase were 37 °C culture temperature, 60 % DO (dissolved Oxigen????) saturation in the medium, 150 rpm for the impeller, and a pH gradient that decreased from 7.25 to 7.00 units in the cell culture during cell proliferation. The pH was usually kept at 7.0 units during infection and impeller speed was increased to 180 rpm.

Variations were introduced by addition of one volume of virus production medium (100 mL, fed at a rate of 40 mL/h) 2 days after infection to cultures infected with EDSV, and 1/6th volume at the time of infection with NDV. Propagation of NDV and H9N2 virus was furthermore supported by feeding trypsin (type IX-S, Sigma T0303) into the infected culture from a 1 mg/mL, 16 U/µL solution at 0.17 mL/h, 4 mL per day for a final concentration of 0.5 µg/mL of reactor volume per day. Recombinant trypsin (rTrypsin, Novozym 6395020) was fed into the infected culture from a solution kept at 4 °C with an activity adjusted such that a feeding rate of 0.17 mL/h (4 mL per day) resulted in a final concentration of 8 U/mL of culture volume each day. Recombinant or porcine trypsin were equivalent in activity. The incubation temperature was set to 35 °C for propagation of NDV. None of the mardiviruses replicated in suspension cultures, also not if the cultures were supplemented with virus production medium or bovine serum.

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