

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.

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

48 We have investigated alternative options to substitute existing primary cell and embrionated egg based
49 production of poultry vaccines on a reduced cost. The described technologies are based on the
50 continuous cell lines CR and CR.pIX that were obtained by biochemical immortalization of primary retina
51 cells of the domesticated muscovy duck. A detailed summary of the design and development of the two
52 avian cell lines has been published previously [28]. The cells were shown to be free of adventitious
53 agents, and sensitive assays against reverse transcriptase suggest that the anatine cell lines (as opposed
54 to galline cells) do not release active endogenous retroviral particles [29,30]. The cell lines have
55 furthermore been adapted to suspended proliferation in chemically defined medium, and propagation of
56 recombinant and wild type viruses of different families to very high yields has been demonstrated [31–
57 35]. This publication describes the production parameters for additional animal viruses that can be used
58 as vaccines against poultry diseases, namely egg drop syndrome, infectious bursal disease (this is kind of
59 misleading since IBV does not replicate), infectious bronchitis, Marek's disease, Newcastle disease and
60 avian influenza.

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62 Results and discussion

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64 Egg drop syndrome

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66 Egg drop syndrome EDS-76 is caused by a member of the *Atadenovirus* genus in the *Adenoviridae* family
67 [36], viruses with double-stranded genomic DNA enclosed by an icosahedral capsid without lipid
68 membranes. Synonymous names for EDSV are duck adenovirus 1 (DAdV-1) and Duck atadenovirus A (the
69 name proposed by the ICTV). The natural hosts appear to be ducks and geese and the virus has a world-
70 wide distribution. Clinical disease in poultry often is subacute and only evident in the production of
71 fewer eggs (the "egg drop") and disturbances in pigmentation and shell quality. Transmission of DAdV-1
72 occurs vertically and horizontally via orally ingested fomites.

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

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

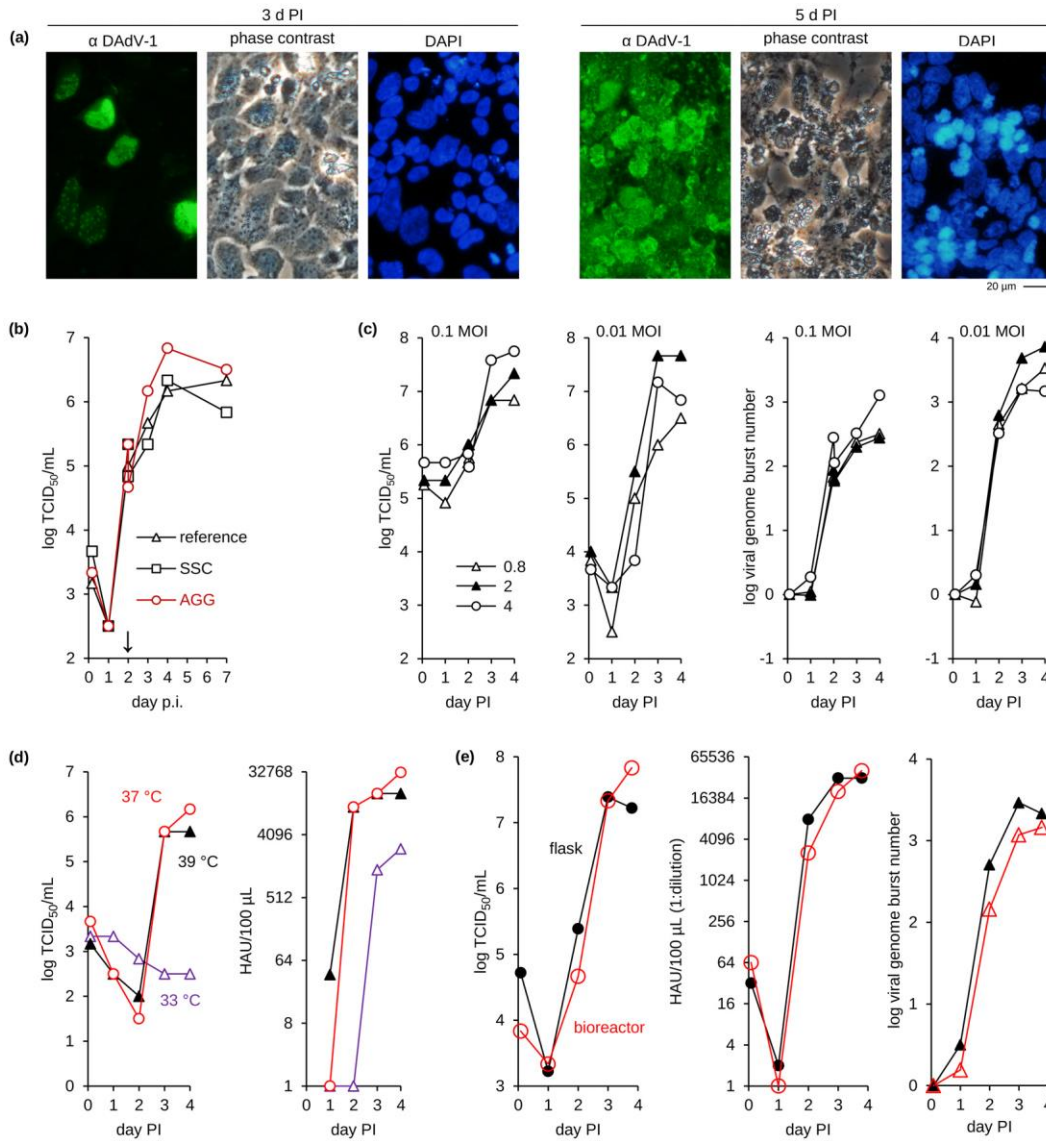
84 phenomenon previously during the development of a production process for poxviruses and have
85 induced suspended cell aggregates to augment transmission of the cell-associated infectious units [32].
86 The aggregates were formed by addition of a chemically defined virus production medium at the time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

102
103 The next set of optimizations focused on MOI and cell density at the time of infection (Figure 1 (c)) and
104 revealed that a MOI of 0.01 (as opposed to 0.1) at intermediate cell densities (2×10^6 /mL) should be
105 optimal for subsequent refinements. Because of the relatively slow replication kinetic we also used qPCR
106 to follow the dynamic of genome replication in parallel to that of the infectious units. Using a calculation
107 described previously for relative quantification of gene expression [37] we confirmed a robust
108 amplification of the viral genome over almost 4 orders of magnitude under the selected conditions
109 (Figure 1 (c)).

110
111 Replication at different temperatures (33, 37 and 39 °C) was studied with the intention to further
112 improve viral titers (Figure 1 (d)). DAdV-1 replicated to high titers at 37 °C and 39 °C (the core
113 temperature of ducks is 41 °C) but appeared not to be able to be propagated efficiently at 33 °C. We
114 noted a surprising discrepancy between infectious and haemagglutinating units (HAU) that suggests that
115 a measurable amount of non-infectious particles with HA activity are formed at 33 °C. Replication may be
116 slightly faster at 39 °C because HA activity indicative of particle formation is higher 24 h PI but this head
117 start appears not to be maintained at later time points. Stability of EDSV appears to be slightly lower at
118 the higher temperature and therefore the optimal temperature for production of DAdV-1 was chosen to
119 be 37 °C.

120
121 An overlay of the optimized process in flasks and a bioreactor (Figure 1 (e)) confirms scalability and good
122 replication of DAdV-1 at MOI of 0.01 with 2×10^6 CR.pIX cells/mL, at 37 °C. Maximum yields were $6.8 \times$
123 10^7 TCID₅₀/mL, $> 1:2^{15}$ HAU/100 µL and 3000-fold amplification of the viral genome (relative to 2 h PI).

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 128 **Figure 1. Propagation of DAdV-1 in CR.pIX cell lines. (a) Infection of adherent cell monolayers is visible only by**
 129 **immunofluorescence staining 3 days PI but virus spreads efficiently until 5 days PI. (b) Efficient replication in suspension**
 130 **cultures requires addition of an aggregation-inducing medium 2 days PI. The reference is a culture without feed, SCS was**
 131 **given cell proliferation medium and AGG virus production medium 2 days PI. (c) Effect of MOI on infectious yields (left panel)**
 132 **and amplification of genomic DNA (right panel). The viral genome burst number was calculated from the ratios of viral**
 133 **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)**
 134 **Propagation at 33 °C does not release infectious units but appears to lead to a measurable amplification of antigen (HAU,**
 135 **right panel). Optimal temperature was determined to be 37 °C. (e) The final process scaled to bioreactors. Comparison of**
 136 **yields of infectious units, hemagglutinating activity and genome amplification in bioreactors and shake flasks. CPE, cytopathic**
 137 **effect; PI, post infection; SCS, single cell suspension; AGG, suspended aggregate culture format; HAU, hemagglutinating units.**
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139 **Newcastle disease**

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

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

148
149 ND caused by velogenic strains is a highly contagious disease associated with a significant economic
150 burden [40]. NDV has a very broad host range and is capable of zoonosis but symptoms caused in
151 humans even by velogenic strains are usually restricted to a mild and transient conjunctivitis or laryngitis
152 [41].

153
154 The vaccine against ND in poultry consists of live, vectored live and killed preparations of lentogenic
155 strains [40]. Live or vectored live vaccines are often preferred because they can be applied directly in the
156 hatchery using automated vaccination equipment. NDV has also the highly promising capacity to
157 replicate in human tumors and is being tested to augment therapies against neoplastic diseases [42].
158 However, because this inherent oncolytic activity correlates with virulence in birds, research on and
159 application of NDV-based virotherapy is being restricted by federal regulation (9 CFR Part 121 i guess) in
160 the USA.

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

166
167 However, infection of CR.pIX suspension cultures yielded NDV at the lower range of titers reported for
168 other continuous cell lines even in the presence of trypsin. Although titers are difficult to compare
169 because infectious units are given in different units it appears that volumetric yields are generally higher
170 in eggs than in cell cultures ($>10^9$ EID₅₀/mL in eggs [46,47], $10^{8.5}$ PFU/mL in LSCC-H32 and secondary
171 chicken embryo cells [48], $10^{6.1}$ to 10^7 TCID₅₀/mL in QT35 [46] and DF-1 cells [47], respectively). Trypsin is
172 not expected to be inhibited because the chemically-defined cell proliferation medium for CR and CR.pIX
173 cells has a negligible low protein content (10 ng/mL of recombinant IGF) and no hydrolysates or other
174 substances that may inhibit proteases.

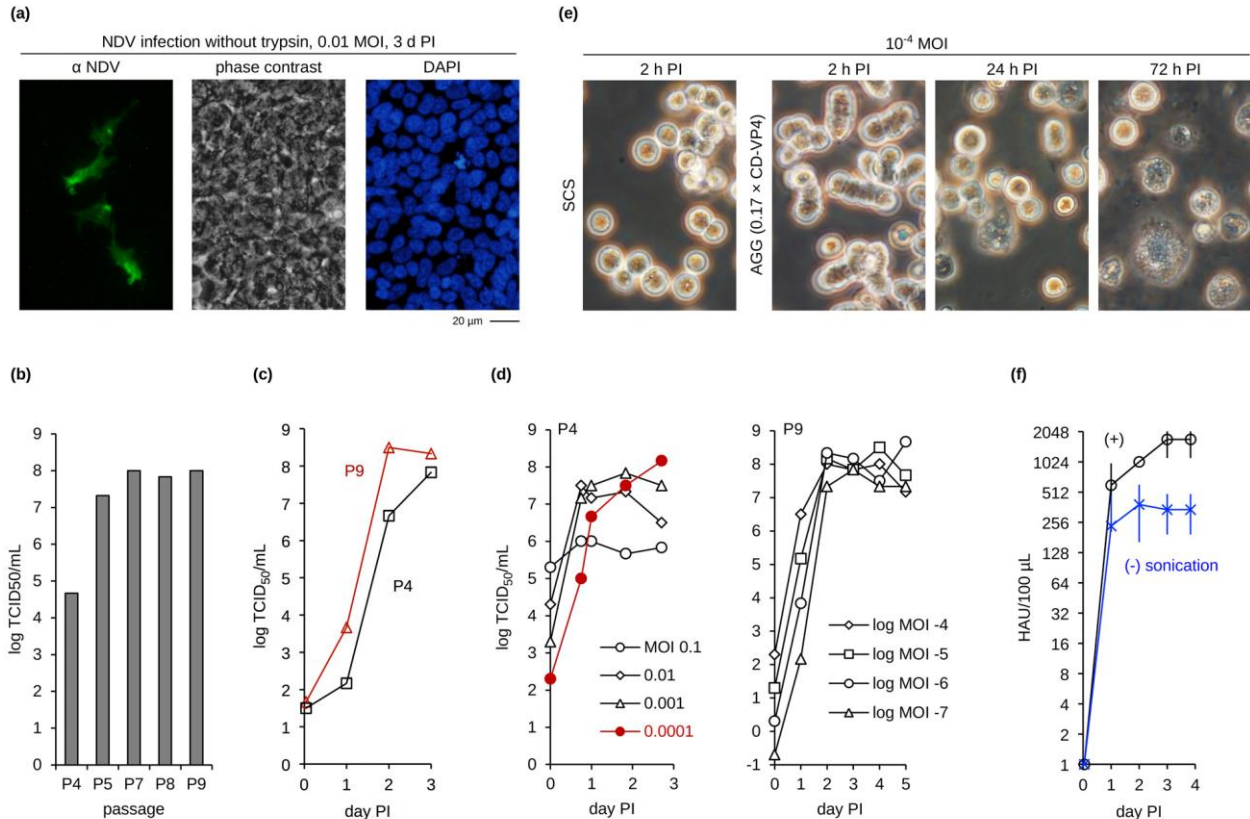
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176 We next suspected that the chemically defined proliferation medium should be supplemented with
177 cholesterol because this compound is not present in the medium but has been reported to be essential
178 for NDV replication [49]. However, biosynthesis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

182
183 Passage of NDV in CR cell suspensions was performed next to investigate whether low titers in cell
184 cultures are due to adaptation of our strain of NDV to eggs. A 1000-fold increase in infectious titers was
185 indeed observed between passages 4 and 7 and appeared to remain stable for at least 2 additional
186 passages (Figure 2 (b)). A direct comparison of virus isolates at passage levels 4 and 9 indicated that

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

193
194 A very low (rather than higher) MOI appears to be the most critical parameter for production of the NDV
195 at passage level 4 (Figure 2 (d)). Titers remained above 10^8 TCID₅₀/mL for virus at passage level 9 at MOI
196 from 10^{-4} down to 10^{-7} . Infectious units with virus at passage level 9 in the improved process are in the
197 upper range of those reported for continuous cell lines and replication at lowest MOI spans almost 9
198 orders of magnitude. However, hemagglutinating (HA) activity was still low with maximum values usually
199 in the range of 1:265 to 1:512/100 µL. To further improve yields we paid more attention to the clumping
200 of the cells that sometimes occurred 24 h post infection. This phenomenon may reflect formation of
201 syncytia and provides an additional mechanism for spread of infectious units [50]. We therefore added
202 virus production medium to further augment the aggregate formation [32], but we also sonicated the
203 final lysate to separate clusters of cell debris and viruses, speculating that the true HA titers are
204 decreased proportionally to the number of particles bound within the aggregates. These additional steps
205 are compatible with scalable processes in bioreactors (Figure 2 (e) and [32]) and did improve HA yields
206 (Figure 2 (f)). The final process consists of supplementation with 1/6th of the culture volume with virus
207 production medium and sonication of the complete lysate for maximum HA levels of 1:1024 to 1:2048 at
208 day 3.

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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

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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.

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IBV is disseminated by aerosol and fomites and mainly causes damage in the respiratory and the urogenital 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].

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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].

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

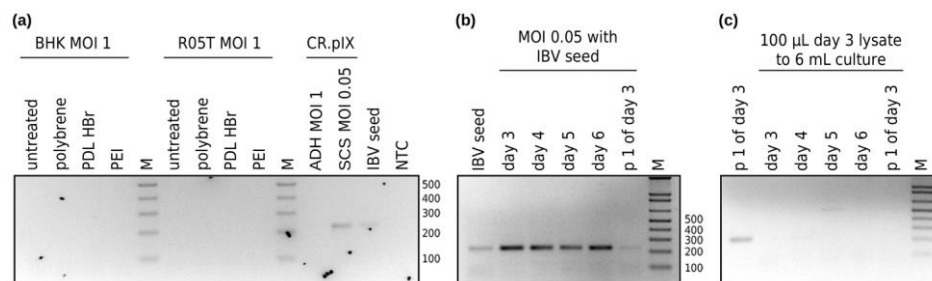
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247 One barrier for permissivity maps to the spike protein (S) of the viral particles [63,64]. This S protein of
248 IBV is processed by cellular proteases into two subunits, S1 and S2 [65]. The S1 subunit mediates
249 attachment to the target cell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

254
255 We attempted adaptation of the M41 and H120 strains to our culture system. Adaptation, however,
256 requires at least low-level replication of the viruses so that suitable mutations can accumulate.
257 Speculating that entry of viruses into the cells is the main block we also tested different mammalian cell
258 lines (hoping for pseudoaffinity [61]) and added polymers (such as polybrene, poly-L-lysine and
259 polyethylenimine) known to augment interaction of viruses (or lipid vesicles) with plasma membranes
260 [68] (Figure 3 (a)). Potential infection was assayed by RT-PCR with primers against the ORF1a
261 polyprotein.

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263 We detected a signal for genomic RNA in infected suspension cultures. This signal was maintained for
264 one week, could be rescued into one subpassage, but was lost with the second subpassage (Figure 3 (b,
265 c)). In adherent cultures we did not observe any signals indicative of entry of any viruses. One important
266 difference in the handling of adherent and suspension cultures was that adherent cultures were washed
267 2 h after inoculation with seed virus. Although interference by bovine serum present in the medium for
268 adherent cultures is a formal possibility, we consider that detection of the input virus is the most
269 parsimonious explanation for transient detection of viral genome and consistent with similar
270 observations and reports on virus stability in the literature [56,69].

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

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

291 292 Marek's disease

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

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

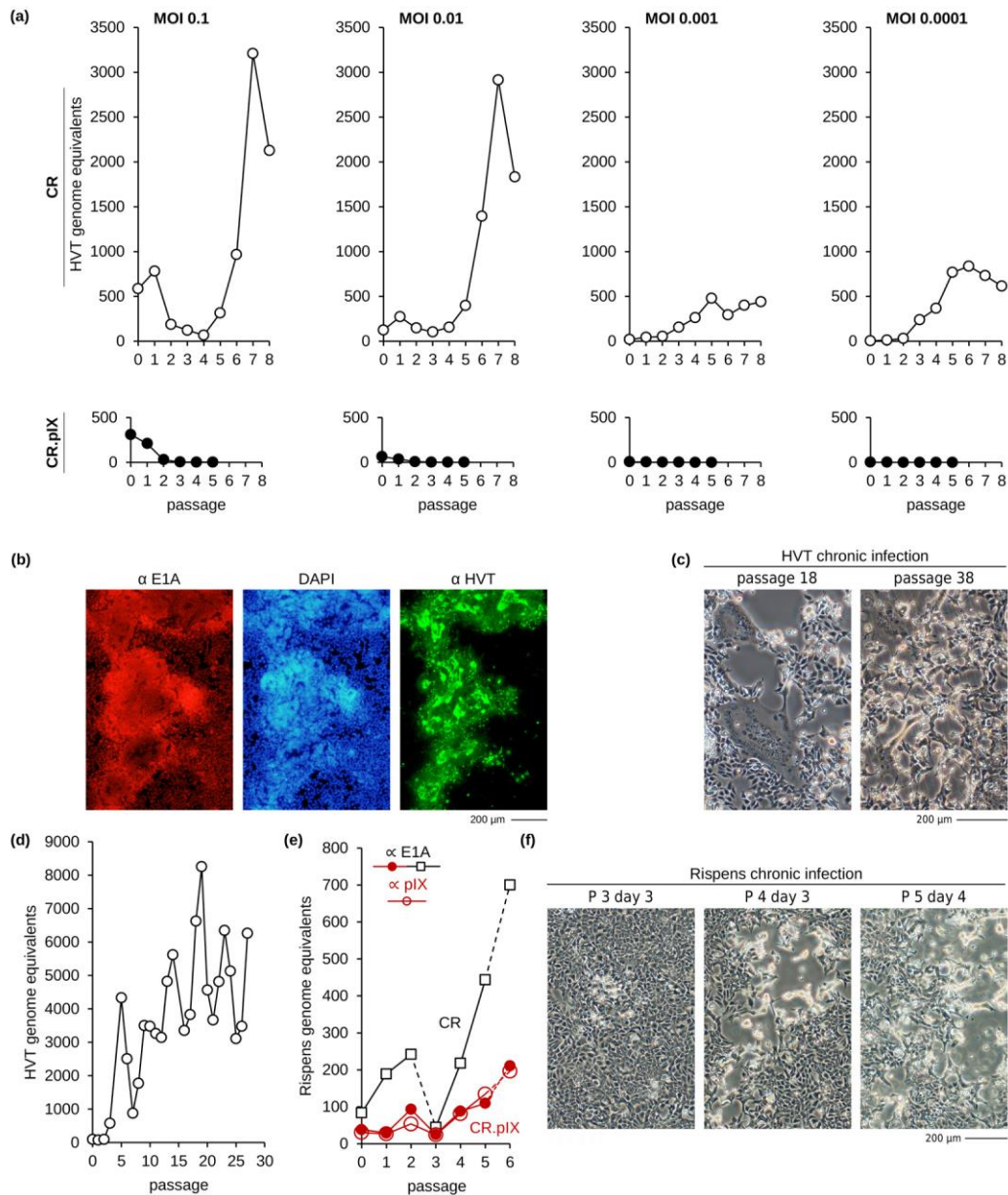
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 304 Vaccines against MD are based on live-attenuated strains of GaHV-2 (for example, Rispens strain), non-
 305 pathogenic GaHV-3, and more importantly on non-pathogenic HVT. HVT is also widely used as vector
 306 virus, and with appropriate insert genes HVT represents the new generation of poultry vaccines being
 307 able to provide good protection against several important avian diseases such as Newcastle disease,
 308 avian influenza, infectious bronchitis or infectious laryngotracheitis. Vaccine production is usually
 309 performed in CEFs and occasionally in duck embryo fibroblasts [72]. Continuous cell lines reported to be
 310 permissive for mardiviruses include the DF-1 chicken cell line [77] and the QM7 quail muscle cell line
 311 [78]. The QT-35 quail fibrosarcoma cell line is also permissive at least for HVT but, surprisingly, appears
 312 to harbour also a previously uncharacterized latent virus possibly related to GaHV-2 [79]. Another cell
 313 line reported to be permissive is the chronically infected lymphoblastoid cell JM-1 that was derived from
 314 chickens infected with MDV and that can transmit this virus to CEFs in cocultivation experiments [80].

315
 316 However, there are also unpredictable restrictions in the permissivity for mardiviruses *in vitro*. For
 317 example, MDV was shown not to replicate in the chicken cell line LSCC-H32 that is permissive for a wide
 318 spectrum of other viruses [48], and only a small fraction of cells, 5 %, were positive for viral antigens in

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

323
 324 We infected adherent CR and CR.pIX cultures with different mardiviruses, including HVT, a vector HVT
 325 that expresses an antigen to protect against NDV [81,82] (Vectormune ND, Ceva Animal Health), and
 326 Rispens. The results shown in Figure 4 (a) and (b) were obtained by inoculation with revitalized CEF cells
 327 carrying Vectormune ND where MOI indicates ratio of infected fibroblasts to CR or CR.pIX. Propagation
 328 of HVT was tracked by qPCR against the viral genome and the cellular DNA.

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334 **Figure 4. Mardivirus propagation in adherent CR and CR.pIX cells.** (a) CR and CR.pIX cells were inoculated with the freshly
335 revitalized Vectormune ND preparation at the indicated ratios (for example, MOI of 0.1 corresponds to 1 CEF of the vaccine
336 for every 10 cells of the anatine continuous cell line). The cultures were subpassaged twice per week by 5-fold dilution. DNA
337 was isolated at each passage and qPCR was performed with primers against mardiviruses and against the E1A gene of the
338 continuous cell line to normalize results to the number of host cells. CR cells are fully permissive for mardiviruses but several
339 attempts failed to establish a productive infection in CR.pIX cells. (b) Immunofluorescence staining against HVT parent virus
340 of Vectormune ND and the cellular E1A gene demonstrates extensive spread of HVT in a culture that has been subpassaged
341 nine times. (c, d) Chronic infections could be maintained for 30 passages. CPE is characterized by extensive cell damage and
342 large syncytia. (e, f) Both, CR and CR.pIX cells were fully permissive for the Rispens strain (GaHV-2). This virus induced strong
343 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
344 cell lines) indicate addition of 1/10th of uninfected cells because cytopathic effect appeared to be too strong to continue
345 subpassaging. The genome equivalents of Rispens were also determined with primers against pIX in the CR.pIX cultures; as
346 expected, pIX could not be detected in CR cultures and the corresponding genome equivalents are therefore not shown.

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349 CR cells are fully permissive for HVT (Figure 4 (a) to (d)) and Rispens ((Figure 4 (e, f)) and infection leads
350 to a progressively expanding CPE that is characterized by frequent plaque formation (centrally dislodged
351 cell layer framed by highly refractile cells) or large syncytia. Appearance of CPE was delayed and is often
352 negligible in the first 3 passages. Chronic infections were readily established and one culture was HVT-
353 positive for at least 30 passages until CPE developed to an extent that required addition of uninfected
354 cells prior subpassage (Figure 4 (c, d)). Because of the delayed CPEs we did not titrate virus by TCID₅₀
355 protocols but quantified virus propagation via qPCR. The qPCR cycle threshold levels for the cellular E1A
356 gene in the full lysates were used to normalize the number of viral genomes to the number of cells in the
357 culture. With this quantitative measurement, that should be independent of cell numbers, we observed
358 what appears to be a periodicity in the kinetic of virus propagation. The maximum yields and kinetic we
359 observe for MDV and Rispens in adherent CR cultures compares well with previous results in chickens
360 that measured a maximum of 10⁸ HVT genomes per 10⁶ feather follicle cells [73].

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362 CR.pIX cells were not permissive for HVT but allowed productive propagation of Rispens with strong
363 cytopathic effect yet lower titers compared to CR cells (Figure 4 (a, e)). Especially the block in HVT
364 replication is very surprising because CR.pIX and CR cells differ only by the expression of the pIX protein
365 [28], a minor structural and presumably also regulatory factor of human adenoviruses [83,84]. We have
366 proposed previously that pIX may also augment binding of Hsp70 and Hsp90 in the avian cell line [28].
367 The resulting activation of the heat shock response can be beneficial for replication of some viruses
368 [85,86] but may also elevate antiviral responses as signaling cascades of the two pathways communicate
369 [87,88]. Mardiviruses have also been reported to interact with Hsp70 [89,90], and may depend on this
370 factor for entry into chicken embryo fibroblasts [90]. By directing Hsp70 into complexes with Hsp90, pIX
371 may interfere with availability of Hsp70 at the cell surface in a way that can be partially compensated by
372 Rispens but not by HVT. Alternatively, pIX may indirectly cause an elevated state of innate immunity.
373 HVT does not code for several virulence factors that are present in Rispens [91,92] and may therefore be
374 unable to block the cellular defenses. (why is this grey???)

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376 **Avian influenza**

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

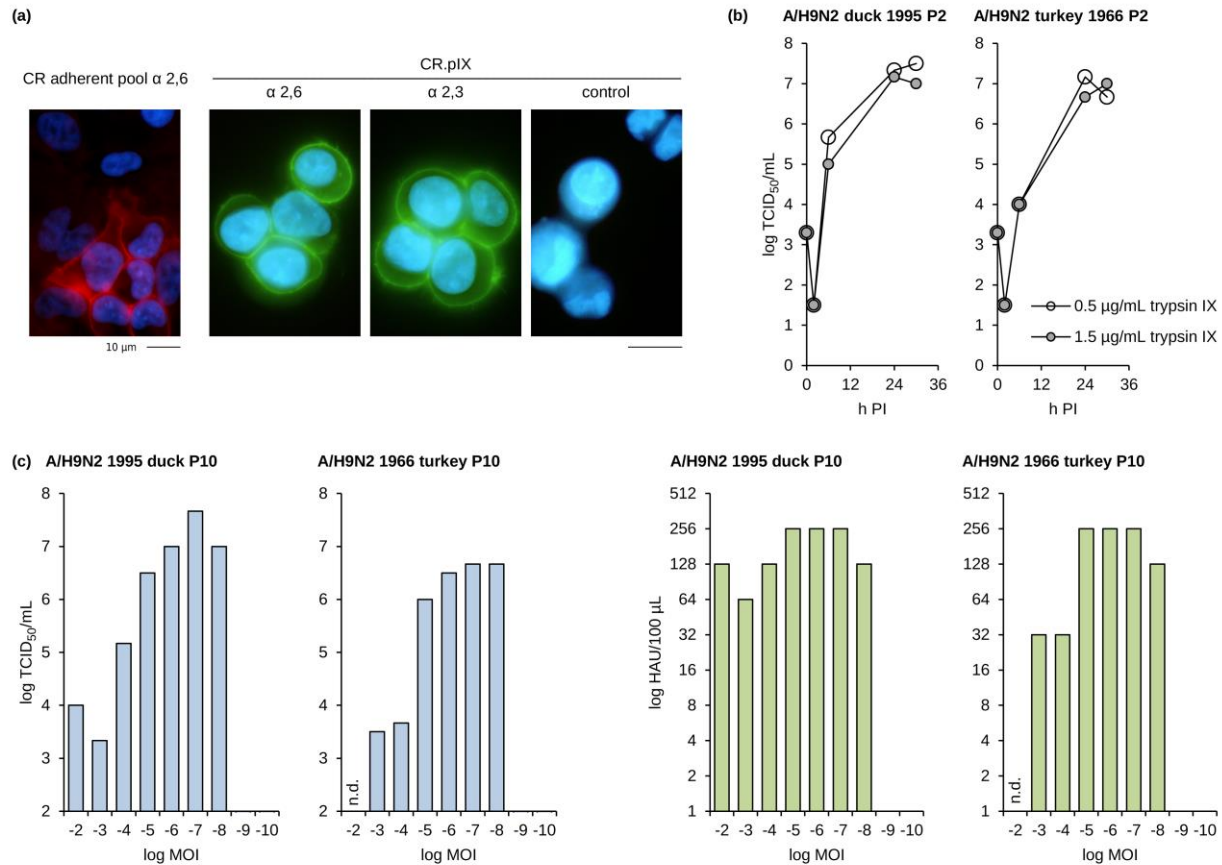
384
385 AIV pathogenicity is determined by a number of factors that range from tropism (for example, viruses
386 that infect the lower respiratory tract tend to cause more severe disease than those that replicate in the
387 upper regions) to interaction with innate immunity (the case fatality rate is higher for strains that induce
388 hypercytokinemia or "cytokine storms") [95,96]. Tropism and host range is mainly determined by the
389 haemagglutinin and neuraminidase proteins (HA and NA, respectively) on the virion surface and the
390 nonstructural protein PB2, a subunit of the viral RNA polymerase. A wide variety of strains have been
391 used to protect against infection with H9N2, H5N2, H5N1, H7N3, H7N7 viruses in various parts of Asia,
392 North America and North Africa [107]. Problems that are sometimes associated with the current
393 production system are low yields for certain strains so that expensive concentration steps are necessary
394 to increase potency, considerable amounts of egg shells that remain as biohazardous solid waste, and
395 dependence on a continuous supply with embryonated eggs free of adventitious agents [107,109,110].
396 Improved control over supply with embryonated eggs is achieved by constructing breeding facilities
397 immediately adjacent to the production site, but this comes at substantial costs [111]. We have
398 therefore tested replication of H9N2 as a highly representative strain for LPAIVs in suspension cultures of
399 CR.pIX cells to provide an additional production substrate that is independent of primary material (FIG
400 5).

401
402 Because the host spectrum of AIVs is partially determined by preference for 2,3 or 2,6 α -glycosidic
403 linkage at the terminal sialic acid [103] we first characterised surface glycosylation of the CR and CR.pIX
404 cell lines. Avian isolates tend to recognize 2,3 α -glycosidic bonds that predominate in the gastrointestinal
405 and respiratory tract of ducks and chickens whereas human isolates tend to recognize the 2,6 α -
406 configuration that predominates in our upper respiratory tract [96,112]. Whereas early passage cell
407 pools are not uniformly positive for the 2,6 α -configuration we observe that lectins specific for either
408 configuration yield signals in the final CR.pIX cell clone (FIG 5 (a)). This result suggests that AIV isolates
409 should be able to replicate in CR.pIX cultures independent of a mammalian or avian origin, a property
410 that has also been described for the MDCK cell line that is considered a gold standard in influenzavirus
411 research [113,114].

412
413 Orthomyxoviruses require proteolytic activation of the viral receptor similar to the paramyxoviruses [43–
414 45]. The proteases contained in embryonated eggs are replaced by exogenous trypsin in cell cultures. We
415 observed good replication of the two H9N2 isolates in the presence of 0.5 and 1.5 $\mu\text{g}/\text{mL}$ of trypsin, well
416 below the 2.5 $\mu\text{g}/\text{mL}$ as maximum amount of trypsin that is tolerated by CR.pIX (FIG 5 (a)). Replication of
417 both isolates (duck/1995 and turkey/1966) at passage level 2 appeared to be very fast with maximum
418 titers in the range of 5×10^7 TCID50/mL and 1:256 HAU/100 μL within 24 h of infection. However,
419 although both isolates are H9N2 strains they differed in maximum yields, a property that was maintained

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

422
 423 AIVs isolated with the help of cell lines have been proposed to be suitable as vaccine seeds [114]. The
 424 hemagglutinating and infectious activities of the LPAIV propagated in CR.pIX cells in true suspensions
 425 cultures in chemically-defined medium without microcarriers and at extremely low MOIs compare
 426 favourably to the results reported with H9N2 in MDCK cells [114,115].
 427



428
 429
 430 **Figure 5. Replication of LPAIV H9N2 isolates in CR.pIX cultures. (a)** Lectin binding indicates that both terminal α -glycosidic
 431 sialic acid configurations utilized by influenza A viruses as receptor are available on CR.pIX suspension cells. The initial pool of
 432 cells appeared not uniformly positive for the 2,6 configuration. **(b)** Very fast replication of the two H9N2 isolates in the CR.pIX
 433 suspension cells to high titers in presence of low and high amounts of trypsin. **(c)** Infectious and hemagglutinating activity of
 434 passage 10 of the two isolates as function of MOI.

435
 436 **Infectious bursal disease**

437
 438 IBDV is a member of the *Birnaviridae*, genus *Avibirnavirus*. The disease caused by IBDV, infectious bursal
 439 disease (IB) or Gumboro disease, is a serious economic burden to the poultry industry [116]. Only
 440 chicken appear to be susceptible to overt disease; turkeys, ducks and other poultry appear not to be
 441 affected by IBDV [117]. Infected chickens develop an acute depression of the humoral immune response

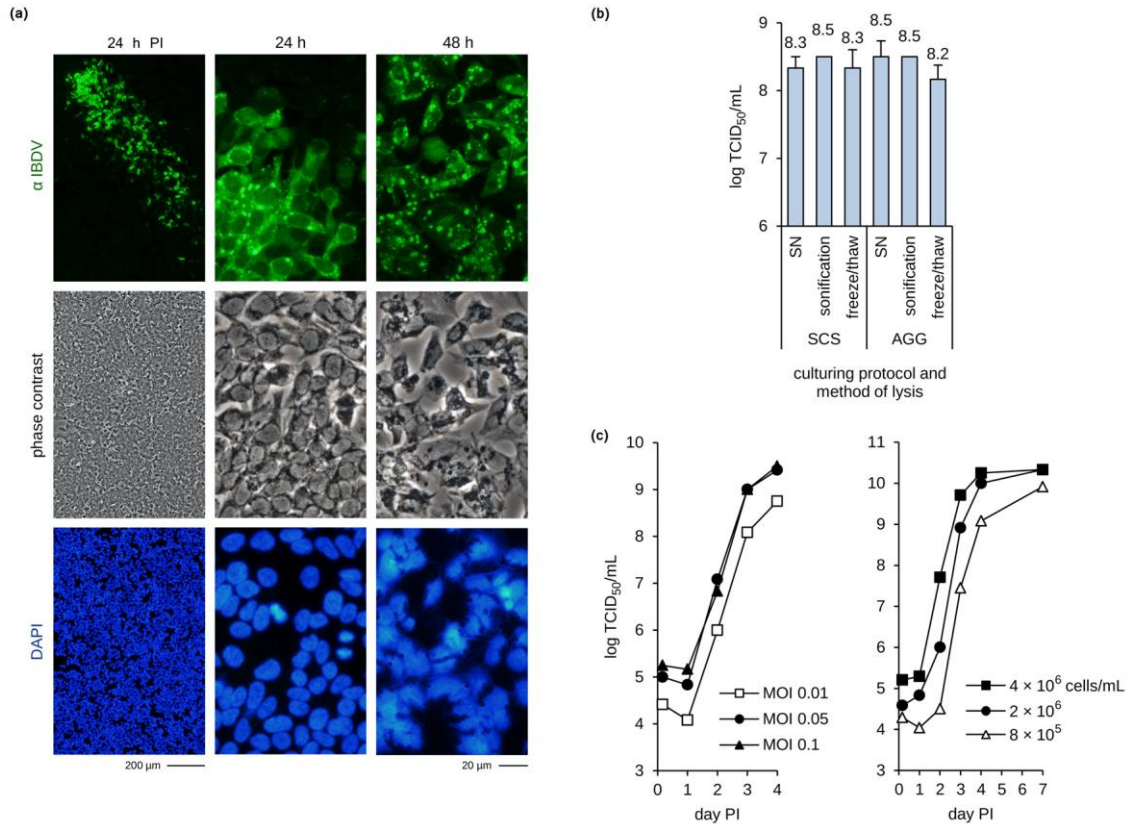
442 due to extensive lesions in the bursa of Fabricius. Such animals are more susceptible to secondary
443 infections and fail to respond adequately to routine vaccination [76].

444
445 The viral capsids are icosahedral with diameters of 60-70 nm. They are not enveloped and contain two
446 segments of double stranded RNA of 2.8 kbp and 3.4 kbp, respectively. The two structural proteins (VP2
447 and VP3) are released by the viral protease (VP4) contained in the autocatalytically active tripartite
448 polyprotein. The infectious virion is very stable in the environment [76]. Control of IBDV is furthermore
449 complicated by the evolution of the pathogen towards greater antigenic diversity and increased
450 virulence [76,117]. The preferred vaccine is live IBDV that has to be monitored for a correct level of
451 attenuation: viral replication should be high enough to induce protective immunity also in the presence
452 of maternal antibodies and against the emerging variant field strains but below levels that may lead to
453 immunosuppression. The challenges are being addressed by immune-complex vaccines, HVT-vectored
454 VP2, and by prime/boost regimes using live and killed vaccines for high value animals such as breeder
455 hens.

456
457 IBDV (strain B87) replicated rapidly in adherent monolayers of CR.pIX cells (Figure 6 (a)). We observed
458 prominent comet formation indicative of efficient release of viruses 24 h PI followed by widely scattered
459 and overt CPE 48 h PI. The results in adherent cultures are confirmed by the high yields of infectious
460 units in various suspension culture formats (Figure 6 (b)): IBDV replicated in single-cell suspensions as
461 efficiently as in cultures with induced aggregates that were designed to support propagation of cell-
462 associated viruses [32]. Titers were also not increased by lysis of the cells in either of the culturing
463 system suggesting that none or negligible infectious units remain within the host cell at the end of the
464 replication cycle.

465
466 Propagation of IBDV in single-cell suspension cultures was robust with high titers at MOIs of 0.1 or 0.05
467 (lower MOIs did not increase yields) and at intermediate to high cell densities ($2 - 4 \times 10^6$ CR.pIX
468 cells/mL). For production processes yields beyond 10^{10} TCID₅₀/mL can be expected 4 days post infection.

469



470
 471
 472 **Figure 6. Replication of IBDV in CR.pIX cells. (a) Immunofluorescence staining of infected adherent cell monolayers. IBDV**
 473 **replicates fast and causes formation of large comets. (b) Similar yields independent of culture format or harvest suggests**
 474 **robust replication. SN, supernatant; SCS, single-cell suspension; AGG, suspended aggregates. (c) Replication kinetic depending**
 475 **on MOI (left panel) and cell density at infection (right panel).**

476
 477

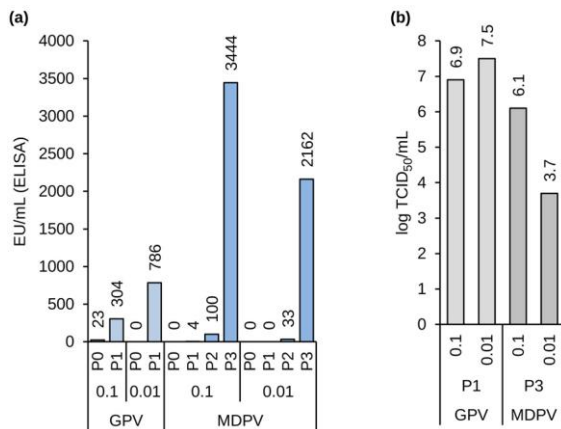
478 **Derzsy's disease**

479
 480 Derzsy's disease appears to affect only geese and muscovy ducks, and has been described in Europe, Asia
 481 and North America ([118] and references therein). It is a highly contagious and economically damaging
 482 disease caused by strains of Goose parvovirus (GPV) and Muscovy duck parvovirus (MDPV), species in
 483 the *Dependovirus* genus in the *Parvoviridae* family. The small non-enveloped viruses are shed in large
 484 amounts by acutely infected birds and remain stable against environmental and thermal stress [119].
 485 Disease can be subclinical in adult animals but is usually fatal in birds in the first month after hatching.
 486 The preferred vaccines are based on live attenuated viruses produced in primary duck embryo cells, and
 487 recently, a transformed (tumorigenic in nude mice) cell line [120–122].

488
 489 We have previously described replication of a goose parvovirus [33] and here briefly confirm this result
 490 with an extension to successful propagation of MDPV (Figure 7). Although they are closely related, the
 491 two viruses had profoundly different properties. GPV was propagated easily and reached a maximum
 492 titer of 7.5 log TCID₅₀/mL within one passage whereas MDPV required three passages of the infected
 493 cells to reach a maximum titer of 6.1 log₁₀ TCID₅₀ (Figure 7 (b)). Concentration of antigen and infectious

494 units also appear not to be congruent in a comparison of the two viruses (Figure 7 (a, b)). For example,
 495 790 EU/mL (ELISA Units/ml How is it defined it should be mentioned in MaM) of antigen yield for GPV
 496 corresponds to a more than ten-fold higher infectious dose relative to MDPV in a sample with 3400
 497 EU/mL. Another difference is that yields are higher if infection with GPV is performed with a low MOI
 498 whereas titers of MDPV benefit from infection with a greater MOI. (Did you use the same sera in the
 499 ELISA and was it validated for the two viruses? The sera may not bind to the two virus with equal affinity.
 500 With other word just because you measure higher ELISA titer at one virus does not mean that you have
 501 higher quantity of that virus. ELISA of Derzsy should be in the MaM)

502
 503 In summary, the yields for both GPV and MDPV both compare favorably to a vaccine dose of 2.6 – 4.8 log
 504 TCID₅₀ or _____ EU (<- WHAT IS TYPICAL FOR VACCINE!?) (I do not have the faintest idea Pénczes might
 505 know about it they have vaccine against Derzsy) so that also for these viruses CR.pIX may provide a
 506 versatile production substrate to replace for the currently predominating primary cells.
 507



508
 509
 510 **Figure 7. Propagation of goose (GPV) and muscovy duck parvoviruses (MDPV) in adherent CR.pIX cultures. (a) Yields of**
 511 **parvovirus antigens after infection with MOI of 0.1 or 0.01. (b) Infectious titers of the parvoviruses in CR.pIX cells. GPV**
 512 **replicates to expected titers within the first passage, MDPV replicates to high titers after three passages. All values shown**
 513 **were obtained from complete lysates 3 days post infection.**

514
 515 **Summary**

516
 517 A delicate balance among costs , economic benefits and biological risk guides the decision on whether a
 518 given vaccine is or is not to be used for agricultural animals with a short life-span. One complication
 519 introduced into these considerations for poultry vaccines is that industrial farmers and those with low
 520 resources both require a diversity of vaccines, but that robust and cost-effective production appears to
 521 be skewed primarily towards industrial use.

522
 523 Most poultry vaccines are manufactured with material derived from embryonated eggs of a high quality.
 524 Dependence on embryonated eggs introduces costs and sometimes fragile logistics into vaccine
 525 production processes that could be circumvented if a continuous cell line permissive for a broad
 526 selection of vaccine strains and suitable for vaccine production were available. CR and CR.pIX are well
 527 characterized established cell lines for research and for manufacturing of vaccines. They are available as

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

538

539 **Material and methods**

540

541 **Cells and viruses**

542

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

547

548 **Determination of infectious units**

549

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

557

558 Detection of NDV or H9N2 replication was facilitated by immunostaining: the cells were fixed in
559 methanol for 10 min, allowed to dry to completion, and rehydrated with PBS containing 0.05 % Tween-
560 20. NDV antibody (CSI) or H9-antiserum (GD Animal Health, VLDIA150) were added to a dilution of
561 1:2000 in PBS containing 1 % fetal calf serum and incubated for 1 h at room temperature. After two
562 washes with PBS, secondary antibody (anti-chicken, Alexa Fluor 488 labelled, host rabbit, _____)
563 was added at a dilution of 1:2000 for 2 h at ambient temperature or overnight at 4 °C. Infected wells
564 were identified by fluorescence after two washes with PBS. Calculation of TCID₅₀ values was performed
565 according to Spearman and Kärber using the implementation of the FAO [123,124].

566

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

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

573
574 IBDV titers were determined on adherent CR.pIX cells. Infection was performed with samples in 10-fold
575 serial dilutions, one day after the indicator cells were seeded at 4×10^6 per 96-well plate. Wells with CPE
576 were identified microscopically (without immunostaining) 3 days post infection. As for EDSV, a change of
577 medium or addition of trypsin was not performed to titer this virus.

578
579 **Haemagglutination assay**

580
581 Haemagglutination units (HAU) as correlates for number of viral particles were determined with chicken
582 erythrocytes and serial dilutions of infected cell suspensions in PBS in steps of 2. The erythrocytes were
583 isolated from complete blood of what?. Briefly, erythrocytes in 7 mL of a complete blood suspension (25
584 % in alsever buffer, E200, Labor Dr. Merk & Kollegen GmbH, Germany) were washed thrice in PBS.
585 Centrifugation for the washing steps was performed at room temperature with $500 \times g$, brake set to low,
586 for 5 min each. The final erythrocyte suspension was diluted in PBS to a concentration that yields an OD
587 of 2.9-3.1 at 576 nm. This suspension was stored for up to 2 weeks at 4 °C.

588
589 The assay was performed in round-bottom 96-well microtiter plaes. The first row of the plate was filled
590 with 200 μ L of the infected cell suspension (wa it clarified or it contained cells too?) and all other rows
591 received 100 μ L of PBS. Next, 100 μ L of virus sample was added to the second row, the pipetting tips
592 were replaced and the suspension mixed with fresh tips. This two-fold dilution was continued serially
593 into the next row, and, for samples with high potency, continued into a second plate. Each well thus
594 contained 100 μ L of diluted virus sample and was subsequently supplemented with 100 μ L of the
595 erythrocyte suspension. This volume was mixed by pipetting and the turbid solution allowed to stand
596 undisturbed for 30 min at ambient temperature (22 °C). HAU/100 μ L are given by the highest dilution
597 where formation of a dot was visible.

598
599 Some samples were further homogenized by sonication with a sonotrode H3 (Hielscher, Germany) of 3
600 mm diameter powered by an UP400S device at 50 % pulse and 50 % amplitude for 5 min (100 mL). This
601 step increased HA titers of NDV by 2 to 4 dilution steps but was not performed in all experiments.

602
603 **Detection and titration by qPCR**

604
605 DNA was isolated with the QIAamp DNA Blood Mini kit (Qiagen). Purified DNA was eluted into 100 μ L,
606 and 5 μ L thereof were added to PCR reactions. Alternatively, DNA was isolated from 20 μ L of infected
607 cell culture suspension by addition of 5 μ L of QuickExtract DNA Extraction Solution 1.0 (101094, Biozym).
608 This sample was heated to 65 °C for 15 min and to 98 °C for 5 min. After dilution with 50 μ L of water, 8
609 μ L therefrom???????? was added to a PCR reaction with a final volume of 20 μ L.

610
611 Quantitative PCR of EDSV was performed using the Power SYBR Green PCR Master Mix (Applied
612 Biosystems) in an ABI 7000 unit. Primers were added to 70 nM each per reaction and real-time PCR was

613 launched with a single incubation for 10 min at 95 °C, followed by 40 cycles of 94 °C for 15 seconds and
614 60 °C for 1 min. The primer sequences were TgA CTC Cgg TCC TTC TAA CAC A and TCA Cgg CAA CTg gTT
615 TAA Tgg for detection of the E1A gene used to immortalize the avian host cell as internal reference [28],
616 and gAg CgT gAT CAA gTC gAT gg and CAC TTA CCg CgA gCA TAT CC against the polymerase of EDSV
617 (designed using GenBank sequence with accession number Y09598). Amplification of the genomic DNA
618 was calculated via an adaptation described previously for relative quantification of gene expression [37].
619 We first determined the differences in the ct values obtained with primers against viral genome and
620 against cellular DNA relative to the first data point, usually 2 h post infection. The differences were raised
621 to the power of 2, assuming a uniform efficiency of 2.0 for each cycle of amplification, and the ratios of
622 changes of viral to cellular DNA, each relative to the first data point, is the burst number.

623
624 HVT titers were calculated similarly, but here, because of the seeding with HVT infected CEFs that do not
625 contain a cellular reference, we did not normalize to an arbitrary initial time of infection, but rather
626 depicted the ratio of viral to cellular DNA (as "genome equivalents") for each passage. The primers used
627 to detect E1A were the same that were used in the EDSV study and primers against the cellular pIX gene
628 were ACC TAC gAg ACC gTg TCT g and gAg CCg TCA ACT TgT CAT C, but primers were added to 90 nM
629 each. The primer sequences were TCg gCg CCC TTT gAT ATA CT and TAg Agg AAg TgT Tgg gCA gg (against
630 the HVT012 gene of MeHV-1, Genbank AF291866), and TCCgTCgTgAATTTgTACgC and CgA CAA TTC CAC
631 CTT Agg Cg (against the MDV011 gene of GaHV-2, Genbank DQ530348).

632
633 RNA-extraktion was performed with the Innu PREP RNA Mini kit (Analytik Jena) and cDNA transcribed
634 with the Cloned AMV First-Strand cDNA-Synthesis kit (Invitrogen) using random hexamers. PCR was
635 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
636 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
637 reactions with the thermocycler programmed to 94 °C for 180 seconds, followed by 30 cycles at 94 °C for
638 30 s, 55 °C for 30 s and 72 °C for 60s. A fragment of IBDV genomic RNA segment B was amplified with
639 primers gAC gAg TTC CTA gCC gAg Tg and CTg gCT gTg gCT AgA Agg AC. The primers bind to what appear
640 to be conserved islands determined by alignment of GenBank sequences JQ411013 (isolate Hungary
641 903/78), GQ451331 (strain HLJ-0504), JX682710 (isolate QL), FJ695139 (Chinese strain YS07), and
642 JX682712 (strain ZZ-11). H9N2 genome was quantified with AAg CCg AAA TCC Tgg gAA Tg and CTC CCT
643 CTC AAA gTC gTA TC against segment 5 encoding NP (Genbank DQ067440), and IBV was detected with
644 TgT TgT gCC AgT CCT CTC AT and gTT TCA CAg TAg CAg AgC gg against the ORF1a gene of strain H120
645 (Genbank FJ888351).

646
647

648 **Lectin staining**

649
650 Cells were collected with 200 × g, fixed with 1 % formaldehyde in PBS and incubated with 30 µg/mL of
651 biotinylated agglutinin of *Sambucus nigra* (Vector Laboratories, USA, B-1305) to detect α 2,6 bonds, or of
652 *Maackia amurensis* (B-1265) to detect α 2,3 bonds on the surface of the plasma membrane (the cells
653 were not permeabilized). Binding was allowed for 45 min at ambient temperature and protected from
654 light. The cells were washed once with PBS and the bound lectins were visualized with streptavidin
655 conjugated to Texas Red (016-070-084) or fluorescein (DTAF, both from Jackson Immuno Research,

656 #016-010-084). DAPI (4',6-diamidino-2-phenylindol) in methanol was added to 1 µg/mL as a nuclear
657 counterstain. The cells were washed once more with PBS prior to fluorescence microscopy.

658

659 **Bioreactor**

660

661 The DASBox (DASGip, Eppendorf) bioreactor units were equipped with a Marine impeller with 3 blades
662 and 60-250 mL working-volume vessels. Gas mixing was performed with N₂, air, CO₂ and O₂, pH was
663 adjusted with CO₂ and 1 M Na₂CO₃. Inoculation was usually performed to 1 × 10⁶ cells/mL in CD-U3
664 medium and the culture was allowed to proliferate for 3 days to approximately 4 × 10⁶ cells/mL. The
665 parameters for the cell proliferation phase were 37 °C culture temperature, 60 % DO (dissolved
666 Oxygen????) saturation in the medium, 150 rpm for the impeller, and a pH gradient that decreased from
667 7.25 to 7.00 units in the cell culture during cell proliferation. The pH was usually kept at 7.0 units during
668 infection and impeller speed was increased to 180 rpm.

669

670 Variations were introduced by addition of one volume of virus production medium (100 mL, fed at a rate
671 of 40 mL/h) 2 days after infection to cultures infected with EDSV, and 1/6th volume at the time of
672 infection with NDV. Propagation of NDV and H9N2 virus was furthermore supported by feeding trypsin
673 (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
674 per day for a final concentration of 0.5 µg/mL of reactor volume per day. Recombinant trypsin (rTrypsin,
675 Novozym 6395020) was fed into the infected culture from a solution kept at 4 °C with an activity
676 adjusted such that a feeding rate of 0.17 mL/h (4 mL per day) resulted in a final concentration of 8 U/mL
677 of culture volume each day. Recombinant or porcine trypsin were equivalent in activity. The incubation
678 temperature was set to 35 °C for propagation of NDV. None of the mardiviruses replicated in suspension
679 cultures, also not if the cultures were supplemented with virus production medium or bovine serum.

680

681 **Acknowledgements & conflicts of interest**

682

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684

685 Some authors (IJ, VS, ZP ...) are employed by companies involved in vaccine production.

686

687

688 **References**

689

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