Ferret airway epithelial cell cultures support efficient replication of influenza B virus but not mumps virus

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Ferrets have become the model animal of choice for influenza pathology and transmission experiments as they are permissive and susceptible to human influenza A viruses. However, inoculation of ferrets with mumps virus (MuV) did not lead to successful infections. We evaluated the use of highly differentiated ferret tracheal epithelium cell cultures, FTE, for predicting the potential of ferrets to support respiratory viral infections. FTE cultures supported productive replication of human influenza A and B viruses but not of MuV, whereas analogous cells generated from human airways supported replication of all three viruses. We propose that in vitro strategies using these cultures might serve as a method of triaging viruses and potentially reducing the use of ferrets in viral studies.

INTRODUCTION

The ferret is a small animal model that has become increasingly popular in recent years for the analysis of influenza A virus transmission and pathogenesis (Belser et al., 2011; Margine & Krammer, 2014; Thangavel & Bouvier, 2014). Ferrets display clinical signs reminiscent of those seen in infected humans, and express a similar profile of sialic acid (SA) receptors on the surface of the epithelial cells lining the respiratory tract (van Riel et al., 2007; Jia et al., 2014). Ferrets have also been used successfully to study infection and pathogenesis of several other human, animal or zoonotic viruses including CDV, SARS, RSV and Nipah (Bossart et al., 2009; Coates & Chanock, 1962; Martina et al., 2003). However, direct nasal inoculation of ferrets with clinical isolates or with vaccine strains of measles or mumps virus (MuV) did not lead to virus replication or disease (Parker et al., 2013; Pillet et al., 2009; Xu et al., 2013).

Unlike influenza A virus, influenza B virus is thought of as an exquisitely human pathogen. Influenza B viruses isolated from seals and pigs were likely acquired by reverse zoonotic events (Bodewes et al., 2013; Ohishi et al., 2002; Osterhaus et al., 2000; Ran et al., 2015). Although several studies have described infection of ferrets with influenza type B viruses (Huang et al., 2014; Kim et al., 2009), we were interested in understanding at the cellular level how appropriate the ferret model is for the study of human influenza B viruses. In particular, we wanted to assess whether the primary target cells, the ciliated epithelium of the respiratory tract, were as permissive to influenza B virus infection as are the same cells derived from humans. We therefore developed a 3D cell culture system of respiratory airway epithelium derived from ferret trachea. Similar cultures have been described by Zeng et al. (2013) as being permissive to influenza A viruses. Here, we compared replication of influenza A or B viruses and MuV in ferret and human airway cultures.

RESULTS

Establishment of well-differentiated FTE cultures

We established FTE cultures from young adult ferrets (age 16–18 weeks) that were seronegative for influenza A and B viruses. By light microscopy, we observed a mixture of ciliated and non-ciliated cells on a thin basal layer. There were notable differences between ferrets in the levels of differentiation and ciliation, likely due to the outbred nature of ferrets. Nonetheless, the patterns of virus infection described below were recapitulated in FTE cultures derived from several animals.
FTE cells are permissive to infection with a panel of influenza A and B viruses

We infected HAE or FTE cultures with a pH1N1 influenza A virus, A/England/195/2009, or an influenza B virus, B/England/683/2010, each virus at an approximate m.o.i. of 0.001 (Fig. 1a, b). In either culture system, the A/195 virus yielded higher titres than B/683 virus at early time points, but the type B virus eventually reached high titres, in excess of $10^7$ p.f.u. ml$^{-1}$.

A further two influenza A (H3N2) viruses, A/Sydney/05/1997 and A/England/691/2010, and a further two influenza B viruses, B/Beijing/1/1987 and B/Florida/04/2006, also replicated in the FTE cultures (Fig. 1c). The A/691 virus did not achieve peak titres as high as the older H3N2 or the pH1N1 virus had done. The influenza B viruses had slower growth kinetics, but achieved higher peak titres at 72 h than the A/691 virus.

A set of FTE cultures generated from a separate animal were infected with B/683 virus again, and also with three other isolates of influenza B virus representing each of the two antigenically distinct lineages of influenza B virus. Influenza B/England/683/2010, B/England/168/2011 and B/England/171/2011 were of the Victoria-like lineage whereas influenza B/England/169/2011 was of the Yamagata-like lineage. All four clinical influenza B isolates replicated in the FTE cultures (Fig. 1f). There was no segregation depending on lineage.

**Fig. 1.** Influenza A and B viruses replicate efficiently in primary cultures of ferret well-differentiated airway epithelium but MuV does not. (a) Infection of HAE cell cultures at an m.o.i. of 0.001 with A/England/195/2009, or B/England/683/2010. (b) Infection of FTE cell cultures at an m.o.i. of 0.001 with A/England/195/2009, or B/England/683/2010. (c) Infection of FTE cell cultures at an m.o.i. of 0.001 with A/England/691/2010 (H3N2), A/Sydney/05/1997 (H3N2), B/Beijing/1/87 or B/Florida/04/2006. (d) Infection of HAE cell cultures at an m.o.i. of 0.001 with clinical MuV AFZ315/HAE9. (e) Infection of FTE cell cultures at an m.o.i. of with 1 clinical MuV AFZ315/HAE9. Apical surface viral titre was assessed at 24 h intervals, then at 4 and 8 days by washing in 200 μl of serum-free medium and then by plaque assay titration on Vero cells. (f) Infection of FTE cell cultures at an m.o.i. 0.001 with B/England/683/2010, B/England/168/2011, B/England/171/2011 and B/England/169/2011. Viral titre accumulated at the apical surface was assessed at 24 h intervals by washing in 200 μl of serum-free medium and then by plaque assay titration on MDCK. The vertical lines are error bars represent standard deviation from the mean, n=3.
Influenza Beijing/87 virus robustly infects ferrets
We infected two ferrets intranasally with $10^6$ p.f.u. B/Beijing/1/87 virus that had replicated well in the FTE cultures. We monitored virus shedding in daily nasal wash samples and weight loss. Both ferrets were robustly infected and shed virus in nasal wash over the following 6 days in a biphasic manner, reminiscent of influenza A virus (Fig. 2a). The peak titre of virus shed on day 1 post-inoculation was $10^5$ p.f.u. ml$^{-1}$ nasal wash. This value is around one log less than we have reported from animals infected with influenza A H3N2 or pH1N1 virus (Roberts et al., 2012). We did not observe any overt clinical signs: weight loss was minimal (Fig. 2b).

MuV did not replicate in cultured FTE cells
Ferrets were not infected with MuV. Following inoculation with a vaccine strain or the low passage clinical isolate, AFZ315, no virus was detected in nasal wash or respiratory tissue (Parker et al., 2013). We therefore attempted to investigate whether FTE cells were capable of supporting MuV replication. The same clinical MuV isolate as that used in the in vivo ferret studies was propagated in HAE cells (AFZ315/HAE9), and then used to infect HAE or FTE cultures. Infection of HAE cultures at a low multiplicity m.o.i. of 0.001 resulted in measurable MuV titres in apical washes that increased continuously, reaching a modest titre in excess of $10^4$ p.f.u. ml$^{-1}$ by day 8 (Fig. 1d). This increase over titre at 24 h was highly significant ($P<0.0001$, Dunn’s multiple comparisons). No virus was detected in basolateral media. Infection of the FTE cultures with the same virus at an m.o.i. of 0.001 yielded no infectious virus in apical wash or in basolateral medium, and no signal by quantitative reverse transcriptase PCR using primers designed for the SH gene of MuV, in samples collected for the next 14 days. We increased the virus dose used for FTE inoculation, such that the m.o.i. was 1. We then detected extremely low titres of infectious virus released apically from MuV infected FTE at day 8 ($P=0.0389$). Virus at 24 h post-infection may have been residual virus from the inoculum, since no virus was detected at 48 and 72 h (Fig. 1e).

Detection of virus antigen expression by immunofluorescence in infected FTE
Virus inoculated FTE cultures were fixed and stained at 24 h with virus antigen specific antibodies and anti-tubulin antibody to stain cilia. Abundant antigen positive cells were detected following infection with influenza A and influenza B viruses. All three strains of influenza A viruses tested showed a strong correlation between antigen positive and tubulin positive cells (Fig. 3a). Fewer antigen positive cells were stained after infection with A/691 than for E/195 or A/Sydney virus, an observation that corresponded with the lower viral yields measured in the growth curves (Fig. 1a). The two strains of influenza B virus that underwent immunostaining, B/Florida and B/Beijing, also showed a spatial correlation between antigen positive cells and tubulin staining, indicating that influenza B virus also preferentially infected ciliated cells in the FTE cultures (Fig. 3b). No viral antigen staining was detected in MuV inoculated FTE cells (Fig. 3c).

The same FTE cell cultures were also subjected to a second round of staining with SNA lectin (Fig. 4). A Z-stack set of images of these cells displays both tubulin antibody and SNA lectin binding to the same cells, including cells infected with influenza B/Florida (Fig. 4a). Indeed, a higher magnification of the triple stained cells shows clearly that the cells infected by the influenza B virus are both tubulin and SNA positive (Fig. 4b).

DISCUSSION
Our primary aim in developing cultures of ferret airway cells was to be able to triage viruses in vitro to identify those that are not suitable for in vivo study in this animal model. We hope that in the long term, this approach will
reduce numbers of animals used for research and development, and in particular prevent multiple attempts to use ferrets to study viruses that do not replicate productively in them. We focussed our studies on influenza B viruses, because they have a narrow host range, and are considered more restricted to human hosts than influenza A viruses. In total in this study, we inoculated ferret airway cells with six different influenza B viruses isolated between 1987 and 2011, and found that they all replicated efficiently in FTE cultures with little variation between strains (Fig. 1a, b, c, f). Moreover, the replication of influenza B virus was as efficient in ferret as in human airway cultures, and in both ferret and human cell cultures, influenza B virus titres accumulated more slowly than those of influenza A virus (Fig. 1a, b). Several recent reports have described replication of influenza B virus in ferrets (Huang et al., 2011; Kim et al., 2009), and we also show here that ferrets inoculated with an influenza B virus that grew efficiently in the ferret airway cells shed virus from their nose over a similar time course, and with similar kinetics, as seen following inoculation with influenza A virus (Fig. 1a, b). However, the titres of influenza B virus shed from the ferret nose were lower than those we typically detect for influenza A viruses of the H3N2 or pH1N1 sub-types (Roberts et al., 2012). Since the replication of influenza B virus in FTE was slower than that of influenza A virus, it is likely that the lower nasal titres are accounted for by decreased levels of replication in epithelial cells, although it is also possible that influenza B virus amplification in vivo in the ferret is controlled by other cell types that are not present in the respiratory epithelium cultures. Huang et al. (2014) recently compared the replication of four different strains of influenza B virus in ferrets. Although all four viruses clearly accumulated in the nasal wash, only one of the four viruses was isolated from trachea. Our findings here suggest that this was unlikely to be explained by an inability of influenza B viruses to replicate in ferret tracheal cells, since all six of the viruses we tested replicated well in FTE, and one of these viruses was B/Florida/04/2006, which Huang et al. (2014) did not isolate from trachea. It is possible that in the experiments by Huang et al. (2014), B/Florida virus replicated in vivo in trachea at a different time point than was assessed by those authors, or that in the protocol used in their study, replication was limited to the ferret nose.

In contrast to our results with influenza B virus, we found that MuV did not replicate efficiently in the ferret airway cells, whereas it did replicate in equivalent cell cultures derived from human tissue (Fig. 1d, e). This may explain why Parker et al. (2013) struggled to establish the ferret model for MuV, and illustrates how these primary cultures could be used in the future to ascertain the suitability of different species as model hosts for human or zoonotic viruses.

![Fig. 3. Influenza A and B viruses infect ciliated cells in FTE cultures but MuV inoculation does not lead to antigen expression. Influenza A (a) and Influenza B (b) virus-infected FTE cells were fixed at 24 h post-infection and stained for influenza antigens, NP (Influenza A) or M1 (Influenza B) (green), and α-tubulin for ciliated cells (red). DAPI is shown in blue. (c) MuV-infected FTE cells were fixed at 24 h post-infection and stained for MuV F protein (green) and α-tubulin (red) to demarcate ciliated cells. Bar, 100 μm.](http://vir.sgmjournals.org)
In accordance with the lack of detection of infectious virus, we did not find any FTE cells staining for MuV antigen at 24 h after inoculation, whereas multiple FTE cells stained for influenza antigens at this time. Interestingly, the ciliated cell was the primary cell type infected by influenza A and B viruses (Fig. 3a, b; 4a, b). We confirmed the observations of Zeng et al. (2013), that the ciliated cell type in FTE cultures abundantly expressed α2,6 SA, and showed that these cells were the targets of influenza B/Florida/04/2006 (Yamagata lineage) virus infection. Recent reports have described lineage specific SA binding preferences (the Yamagata-like lineage displaying an α2,6 linkage preference and the Victoria-like viruses an α2,6 and α2,3 binding affinity) (Wang et al., 2012). We did not test here whether strains of influenza B virus from different lineages had a different cell tropism, but if they did, it had no effect on the replication of viruses in FTE (Fig. 1f). The rich expression of α2,6 SA on ciliated cells of ferret airway cultures contrasts with observations by us and others that SNA lectin predominantly stained non-ciliated cells in human airway cultures, and in accordance with their preference for α2,6 SA receptor, human adapted influenza viruses tended to infect these non-ciliated cells (Matrosovich et al., 2004; Thompson et al., 2006). The significance of the species difference for the use of the ferret model for influenza pathogenicity and transmission studies is not clear. Other notable differences between the glycosylation patterns on ferret and human tissues are that much of the α2,3 linked NeuAc in the ferret trachea was associated with the Sda tetrasaccharide (Jia et al., 2014), which may block access to the virus, and that LacDiNAc, α2,6 linked SA was particularly abundant in ferret. Like the HA protein of influenza, the haemagglutinin-neuraminidase (HN) glycoprotein of MuV binds to SA as a cellular receptor (Markwell, 1991). However, whereas it is believed that the ability of the influenza virus HA protein to attach to the α2,6 linked form of SA is important for successful replication in the upper respiratory tract of humans and ferrets, the exact specificity of the MuV HN protein for linkages of SA is less well characterized. Whether or not the stringency of the ferret airway for viruses that bind α2,6 SA rather than α2,3 SA receptors underlies the inability of ferrets to support MuV replication, or whether other host range barriers exist, would require further investigation.

![Fig. 4. Ciliated cells in ferret tracheal epithelium cultures express α2,6 SA and are targets for influenza B virus infection.](image-url)
METHODS

Generation of ferret tracheal epithelium cultures. To generate the FTE cultures, the tracheas of 16–18 week old ferrets were removed immediately after a schedule one cull, cut in half along their length and added to 10 ml of ice-cold Hams F12 medium (Gibco; Life Technologies) supplemented with 1 × antimitotic, antibiotic (CnT-ABM10; CellaTec) and Pronase (Roche Diagnostics) to a final concentration of 1.2 mg ml⁻¹. The trachea was then incubated for 18 h at 4°C on a rotating platform.

FBS (1 ml; Labtech.com) was added and supernatant was removed and placed on ice. The trachea was washed twice with 10 ml of Hams F12 medium (by repeated inversions) and all the washes were pooled and centrifuged for 10 min at 600 g to pellet cells. The cells were resuspended in F12 medium containing 0.5 mg ml⁻¹ of DNase1 and incubated for 5 min on ice prior to cell pellet centrifugation at 600 g for 5 min.

The cells were resuspended in CnT-17.5 (CellTec) with components A, B and C, and supplemented with antimitic, antibiotic (CnT-ABM10; CellaTec) and 10 % FBS. Fibroblasts were depleted by a 4 h incubation on fibroblast selective plates. Non-adherent cells were counted and plated onto the apical layer of transwell dishes at 2 × 10⁶ cells per well seated in 12 well plates containing 1 ml of the supplemented CnT-17.5 medium.

The cells were maintained at 37°C in 5 % CO₂ for 5 weeks, with media changes every 3 days. Once the trans-epithelial resistance exceeded 600 Ω, apical medium was removed and the basal medium was changed to CnT-23.5 (CellTec) to encourage differentiation. Basal medium was changed every 3 days. Four weeks later, after the appearance of ciliated cells, the airway cells were infected with virus.

Cell culture. MDCK cells obtained from ATCC, and Vero cells from the National Institute for Biological Standards and Control (NIBSC), were maintained in Dulbecco’s modified Eagle’s Medium (DMEM; Gibco; Life Technologies) supplemented with 10 % FBS and 1 % penicillin and streptomycin (P/S; Gibco; Life Technologies) at 37°C with 5 % CO₂.

Human airway epithelial (HAE) cultures (Mucil Air; Epithelix-Sarl) were purchased differentiated. They were incubated at 37°C at 5 % CO₂ in maintenance media supplied by Epithelix.

Virus source and propagation. A/England/195/2009 (H1N1), B/Florida/04/2006 and B/Beijing/1/87 viruses were derived from reverse genetics systems either by de novo synthesis (GeneArt) from published sequence data [195 (Brookes et al., 2010) and B/Florida – this project] or by cloning from WT virus stocks (B/Beijing/1/87)[Jackson et al., 2002]. A/Sydney/97 (H3N2) virus was obtained from PHE (Herlocher et al., 2001). Influenza viruses, A/England/691/2010 (H3N2), B/England/683/2010, B/England/171/2011, B/England/168/2011 and B/England/169/2011 are clinical strains isolated by Public Health England (PHE) as part of the MOSAIC study (Elderfield et al., 2014).

All influenza viruses were propagated in flasks of confluent MDCK cells in the presence of DMEM (1 % P/S) and 1 μg ml⁻¹ TPCK treated trypsin ( Worthington Biosciences).

Clinical MuV AFZ315/HAE9 was isolated from a patient saliva sample taken 2 days after the onset of parotitis by inoculation onto the apical surface of MucilAir HAE cells. At day 9 post-infection, the propagated virus was harvested from apical cell surface washings and virus titre confirmed by plaque assay on Vero cells (Afzal et al., 2005).

Viral growth kinetics. Prior to infection, the apical surfaces of HAE or FTE cells were washed by a 30 min incubation in 250 μl of DMEM. After removal of medium, virus was added at an m.o.i. of 0.001, or m.o.i. of 1 for MuV, in a volume of 200 μl of DMEM. After 1 h, the inoculum was removed and the cell layer washed with DMEM. The cultures were incubated at 37°C at 5 % CO₂, and at 24, 48 and 72 h post-infection, virus released from the apical surface was collected in 200 μl of DMEM. MuV samples were also harvested at days 4 and 8 post-infection. Infections were performed in triplicate. The supernatant harvested from the apical layer was assayed for viral titre by plaque assay on either MDCK cells (influenza) or Vero cells (MuV).

Animal studies. Animal studies were conducted as previously described (Roberts et al., 2011). Female ferrets (14–16 weeks old) weighing 400–650 g were used. Body weight was measured daily. After acclimatization, the ferrets were lightly anaesthetized with ketamine (32 mg kg⁻¹) and xylazine (0.9 mg kg⁻¹) and inoculated intranasally with 10⁶ p.f.u. of B/Beijing/1/87 virus diluted in PBS (0.1 ml nostril⁻¹).

All animals were nasal washed daily, while conscious, by instilling 2 ml of PBS into the nostrils, and the expectorate was collected in modified 250 ml centrifuge tubes. Infectious virus was titrated by plaque assay of the nasal wash on MDCK cells.

Immunofluorescence. Virus infected FTE cells were fixed at 24 h post-infection and stained for influenza antigens, mouse anti-NP (influenza A) and mouse anti-M1 (influenza B) (1 in 500) with Alexafluor 488 (1 in 500) secondary (Invitrogen), and for a-α-tubulin expression in ciliated cells (rabbit anti-α-tubulin, 1 in 40; Spring Bioscience) with Alexafluor 568 (1 in 500) secondary (Invitrogen). DAPI was used for nuclear staining. Additionally an SNA (Sanbucus nigro) lectin conjugated to biotin (Vector Laboratories) was used with an Alexafluor 647 streptavidin secondary (1 in 500). MuV-infected FTE cells were fixed at 24 h post-infection and stained with rabbit polyclonal MuV anti-F antibody, kindly provided by Dr Steven Rubin, FDA (1 in 10 000), and goat anti-rabbit IgG-FITC (1 in 200, Calbiochem) to detect viral antigen and with mouse monoclonal anti-β-tubulin IV antibody (1 in 200; Sigma T7941) followed with goat anti-mouse IgG TExAS RED conjugate (1 in 50; Merck Millipore 401230) to demarcate ciliated cells. Images were captured using either a Zeiss axiovert 40 CFL with an AxioCam MR3 with AxioVision imaging (images with DAPI) or a Zeiss LSM pascal with AxioPan 2 imaging (images with 647).

Acknowledgements

This work was funded by NC3Rs grants G100033/1 and NC/ K00042X/1. Animal work was performed under UK Home Office License, PPL/70/6643. We are grateful for the expert assistance from the Imperial College London central biological services team, to Aisling Vaughan for assistance with confocal microscopy, to Dr Catherine Thompson at PHE for isolation of the influenza B clinical viruses and to Dr John McCauley and his team at the WHO collaborating centre for antigen typing the clinical influenza B virus isolates, both as part of the MOSAIC study.

References


Bodewes, R., Morick, D., de Mutsert, G., Osinga, N., Bestebroer, T., van der Vliet, S., Smits, S. L., Kuiken, T., Rimmelzwaan, G. F. & other


