Replication kinetics and cellular tropism of emerging reoviruses in sheep and swine respiratory *ex vivo* organ cultures

Giovanni Di Teodoro[a], Alessio Bortolami[a,b], Liana Teodori[a], Alessandra Leone[a], Nicola D'Alterio[a], Daniela Malatesta[a], Alfonso Rosamilia[a], Maria Loredana Colaianni[c], Antonio Petrini[a], Calogero Terregino[b], Giovanni Savini[a], Francesco Bonfante[b], Alessio Lorusso[a,*]

[a] OIE Reference Laboratory for Bluetongue, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM), Teramo, Italy
[b] Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), Italy
[c] Istituto Zooprofilattico Sperimentale di Puglia e Basilicata (IZSPB), Foggia, Italy

ARTICLE INFO

ABSTRACT

Ex vivo organ cultures (EVOCs) are extensively used to study the cellular tropism and infectivity of different pathogens. In this study, we used ovine and porcine respiratory EVOCs to investigate the replication kinetics and cellular tropism of selected emerging reoviruses namely Pteropine orthoreovirus, an emerging bat-borne zoonotic respiratory virus, and atypical Bluetongue virus (BTV) serotypes which, unlike classical serotypes, do not cause Bluetongue, a major OIE-listed disease of ruminants. BTV failed to replicate in ovine EVOCs. Instead, PRV showed slight replication in porcine lower respiratory EVOCs and a more sustained replication in all ovine respiratory tissues. By confocal laser scanning microscopy, PRV was demonstrated to infect bronchiolar and type I pneumocytes of ovine tissues. Overall, respiratory EVOCs from different animal species, eventually obtained at slaughterhouse, are a useful tool for testing and preliminarily characterize novel and emerging viruses addressing the essential *in vivo* animal work. Further experiments are, indeed, warranted in order to characterize the pathogenesis and transmission of these emerging reoviruses.

1. Introduction

The family *Reoviridae* is one of the most complex in all of virology, currently comprising 15 recognized genera within two subfamilies of viruses with genomes composed of multiple (10–12) segments of double-stranded RNA. Individual viruses within the family infect a remarkable variety of hosts, including mammals, birds, reptiles, amphibians, fish, mollusks, crustaceans, insects, plants and fungi. Emerging reoviruses with compelling importance in human medicine comprise also bat-borne fusogenic orthoreoviruses included in the Nelson Bay orthoreovirus species (family *Reoviridae*, subfamily *Spinareovirinae*, genus *Orthoreovirus*) also known as Pteropine orthereovirus (PRV). PRV causes in humans acute respiratory disease or influenza-like illness (Tan et al., 2017). This infection has been described as mild and self-limiting; nonetheless, some patients developed severe illness which may include high fever, followed by cough or sore throat, with some systemic symptoms such as generalized weakness and myalgia (Tan et al., 2017). PRVs have been primarily isolated from fruit bats (Gard and Marshall, 1973) (family *Pteropodidae*) and human infection seems to be related to

the direct exposure with these animals. Human to human transmission has been also hypothesized (Chua et al., 2011). PRV human infections are common in South-East Asia, areas where fruit bats densely live (Chua et al., 2007; Voon et al., 2015; Tan et al., 2017; Uehara et al., 2019; Singh et al., 2015a). To date no information upon the capability of PRV to infect other mammalian hosts in field conditions is available.

Bluetongue virus (BTV, family *Reoviridae*, subfamily *Sedoreovirinae*, genus *Orbivirus*) is the causative agent of bluetongue (BT), one of the major OIE-listed infectious diseases of ruminants. Clinical signs of BT are mainly observed in sheep. Unlike PRV, BTV is transmitted by arthropods belonging to the *Culicoides* genus (MacLachlan et al., 2009). Up to 2008, 24 antigenically distinct serotypes of BTV were officially recognized (Maan et al., 2008). However, in the last years novel serotypes have been described in sheep and goats (Hofmann et al., 2008a; Maan et al., 2011a; Zientara et al., 2014; Schulz et al., 2016; Sun et al., 2016; Bumbarov et al., 2016; Savini et al., 2017; Marcacci et al., 2018; Lorusso et al., 2018). These viruses are also known as atypical BTV serotypes in light of the unique features they do possess. These serotypes are asymptomatic in the infected hosts, some of them are not
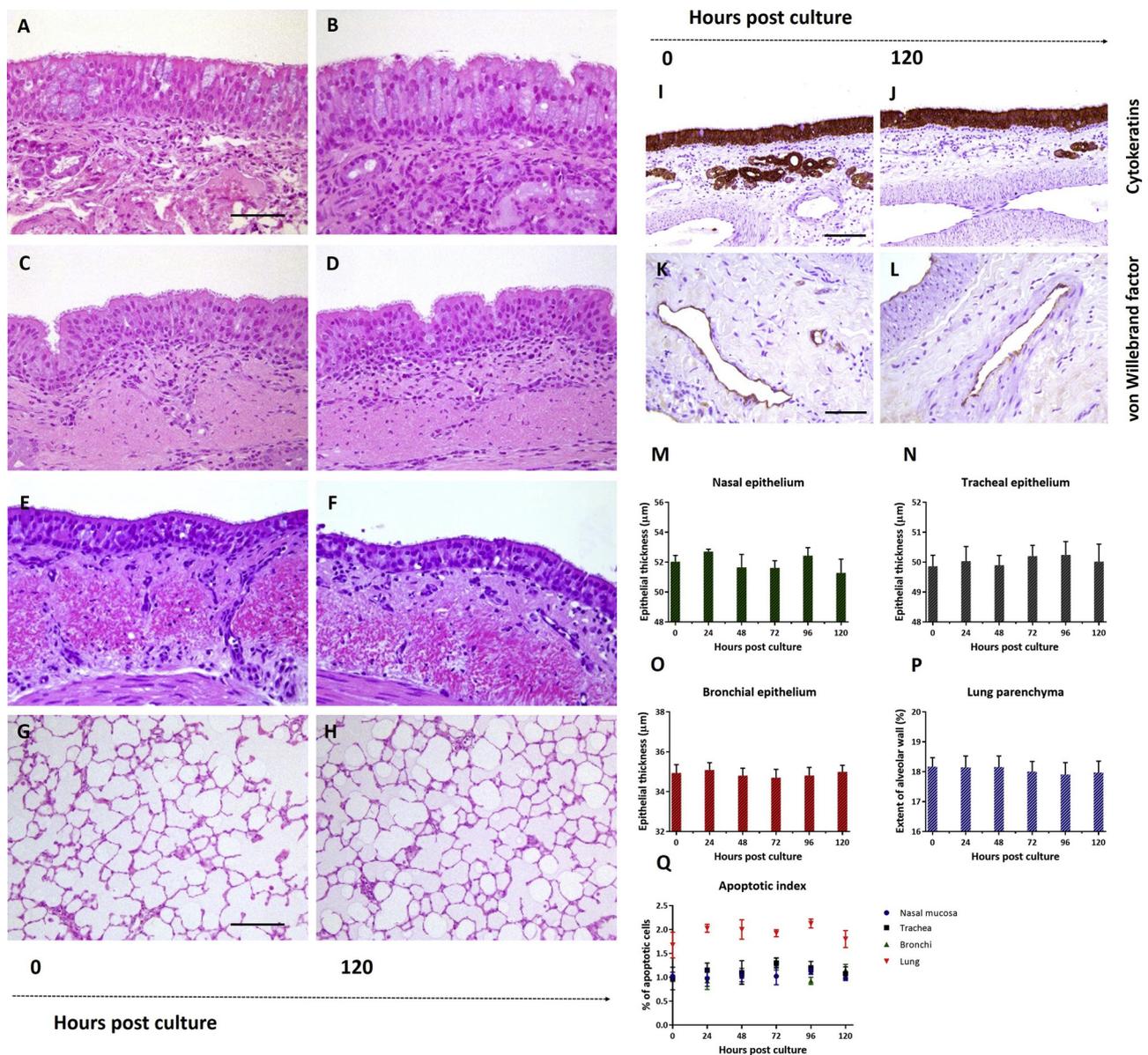
Fig. 1. Morphologic evaluation and analysis of viability of ovine respiratory EVOCs. The morphology of nasal (A–B), tracheal (C–D), bronchial (E–F) epithelium was preserved during the entire time course of the experiment (up to 120 h p.c.). Aspect and density of the cilia of epithelial cells, as well as those of glands and blood vessels of the *lamina propria*, did not show alterations (A–F). The morphology of lung (G–H) was also well preserved. The extent of alveolar sacs and the aspect of bronchiolar and alveolar cells remained unchanged from 0 to 120 h p.c. H&E stain. Scale bar: 50 µm (A–F), 100 µm (G, H). Final magnification: ×400 (A–F), ×200 (G, H).

Immunoreactivity for cytokeratins was evident and specific in correspondence of the tracheal epithelium and glands in control tissue promptly fixed at the slaughterhouse (I) and in a selected tracheal EVOC at 120 h p.c. (J). Similarly, the expression of von Willebrand factor was observed within the endothelial cells of the nasal mucosa immediately fixed at the slaughterhouse (K) and in a selected nasal EVOC at 120 h p.c. (L). Mayer's hematoxylin counterstain. Scale bar: 100 µm (I, J), 50 µm (K, L). Final magnification: ×200 (I, J), ×400 (K, L).

The thickness of nasal (M), tracheal (N) and bronchial (O) epithelium remained constant during the EVOCs cultivation as significant changes were not observed from 0 to 120 h p.c. ($P \geq 0.05$). The extent of the alveolar walls – expressed as percentage (%) of the entire microscopic field of observation – remained also constant (P) from 0 to 120 h p.c. ($P \geq 0.05$).

During cultivation, % of apoptotic cells in all EVOCs (Q), did not increase significantly ($P \geq 0.05$). The experiments were performed in six replicates, from three different animals and data were reported and represented as the mean ± SD.

able to grow onto currently available cell-cultures, and, at least for few of them, vector-free transmission route was demonstrated or strongly suggested (Chaignat et al., 2009; Vögtlin et al., 2013; Batten et al., 2013, 2014; Savini et al., 2017; Bréard et al., 2018).

Ex vivo organ cultures (EVOCs) of the respiratory tract from different animal species have been developed to study the cellular tropism and infectivity of viral respiratory pathogens (Van Poucke et al., 2010; Pena et al., 2012; Chan et al., 2013a; Gonzalez et al., 2014; Cousens

et al., 2015; Chan et al., 2016). In this setting, the three-dimensional structure and cell diversity of the respiratory mucosa and lung parenchyma are preserved together with other important physiological features (*e.g.* normal expression levels of cell receptors, effective mucus production and ciliary activity).

In this study we investigated whether PRV has tropism for the respiratory *ex vivo* organ cultures (EVOCs) of sheep and swine. Moreover, we evaluated the tropism of atypical BTV serotypes for respiratory
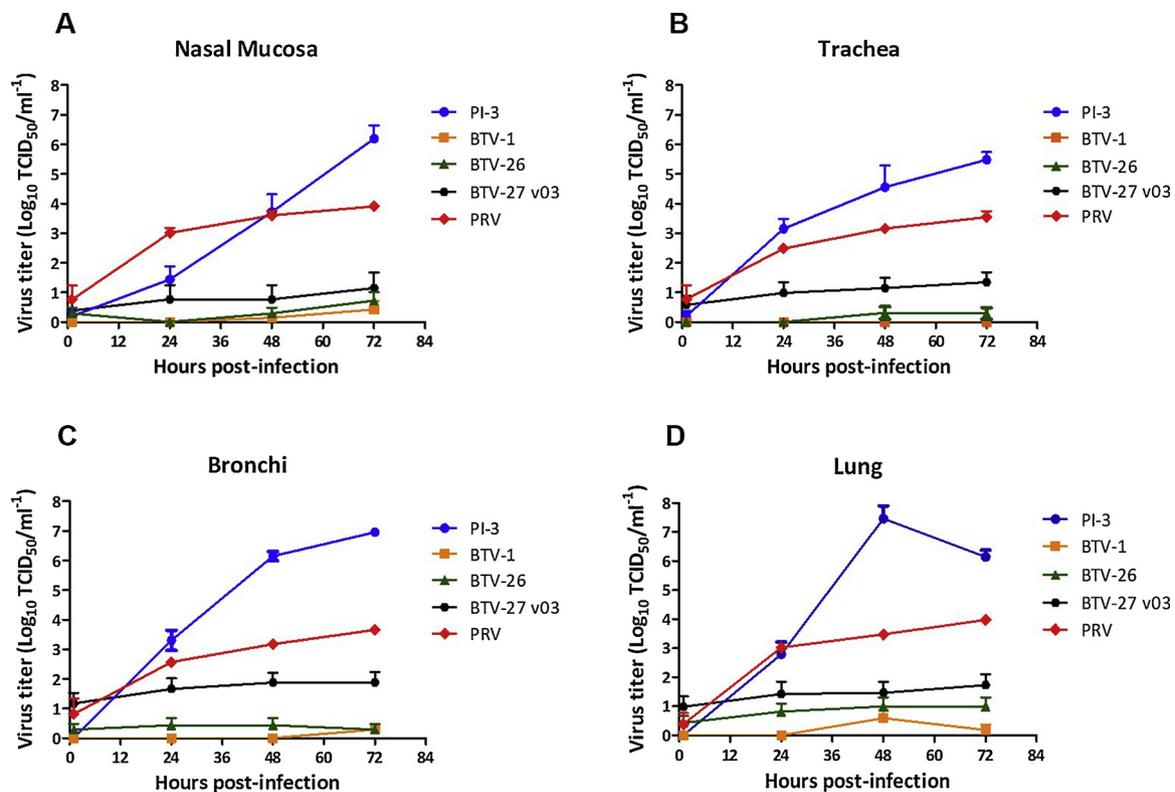
Fig. 2. Viral replication in ovine respiratory EVOCs. Growth kinetics of PI-3, PRV, and BTVs strains in nasal (A), tracheal (B), bronchial (C) and lung EVOCs (D). PI-3 titers significantly increased between 0 and 72 h post infection (hours p.i.) ($P \leq 0.001$) in all respiratory districts. PRV replicated along the entire respiratory tract of sheep between 0 and 72 h p.i. ($P \leq 0.001$), reaching the highest titer in lungs. BTV strains included in this study did not significantly increased in titer from 0 and 72 h p.i. ($P \geq 0.05$) in all EVOCs. Only a mild replication of BTV-27 v03 was observed from 0 and 72 h p.i., however this replication was not significant ($P \geq 0.05$) and less than 1 log. The titer of BTV-27 v03 from 24 to 72 h p.i. was significantly higher ($P \leq 0.05$) in tracheal and bronchial EVOCs than the other BTVs. Titers showed represent the mean of six replicates of three independent experiments and bars indicate the SD.

ovine EVOCs. We also employed two viruses with clear tropism for the respiratory tract of sheep and swine, and used as positive control for the viability of EVOCs, namely Bovine Parainfluenza-3 virus (PI-3, family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Respirovirus*) and H3N2 swine influenza virus (family *Orthomyxoviridae*, genus *Alphainfluenzavirus*), respectively.

2. Materials and methods

2.1. Selection of animals and ethics

Ovine EVOCs were obtained from three 4-months old male lambs originating from herds located in the Abruzzi region (central Italy). Sampling was performed in accordance with internal guidelines of the slaughterhouse (Centro Carni Val Tordino, Mosciano S. Angelo, Teramo-Italy) while animals were officially slaughtered. All procedures including handling and processing of tissues were performed according to the internal guidelines of the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM). Porcine EVOCs were obtained from three 6-week-old male piglets originating from a herd located in the Veneto region (North-East Italy). Sampling of porcine tissues was carried out according to the internal guidelines of the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe). Ethic approval n° 8B654. NDJA was obtained from the Italian Ministry of Health.

2.2. Viruses

Four different viral species were used, including PRV strain Indonesia/2010 (Lorusso et al., 2015, GenBank acc. nos. KM279380-KM279389); BTV-26 strain KUW2010/02 (Maan et al., 2011b, acc. nos.

JN255156-JN255165); BTV-27 v03 (Schulz et al., 2016, kindly donated by Stephan Zientara and Emmanuel Breard, Anses-France, acc. nos. KU760997-KU761006); reference BTV-1 strain from South Africa and available at IZSAM; PI-3 virus (T1 strain, Dawson et al., 1965, Weybridge-UK), and an Eurasian H3N2 swine influenza virus A/swine/Italy/8088/2006 (Patrono et al., 2015) that were used as positive control respiratory viruses for ovine and porcine EVOCs, respectively. In order to generate working stocks, BTV-1, low cell passages of BTV-26 and BTV-27 v03 and the 3rd passage of PRV were propagated onto VERO cells. PI-3 was propagated onto MDBK cells.

For titration, supernatant was collected and titrated by tissue culture infection dose ($TCID_{50}$) onto VERO cells for BTV-1, BTV-26 and PRV, MDBK cells for PI-3 and BSR cells for BTV-27 v03. Titers were calculated following the Reed-Muench method (Reed and Muench, 1938). A working stock of H3N2 was obtained by inoculating 9–11 days old specific pathogen free (SPF) embryonated chicken eggs (Charles River, Italy) *via* the allantoic cavity. For viral titration, 100 µl of 10-fold diluted viral suspension was inoculated in SPF eggs and the median embryo infectious dose (EID_{50}) was calculated according to the Reed and Muench method. All cell lines were cultured in eagle's minimum essential medium (MEM; Biowest) supplemented with 1% of L-glutamine (200 mM; Biowest), 100 U.I./ml of penicillin (Sigma), 100 µg/ml of streptomycin (Sigma), 1.25 µg/ml of gentamycin (Sigma), nystatin 50 U.I./ml (Sigma) and 10% of fetal bovine serum (FBS; Carlo Erba) at 37 °C in 5% CO_2.

2.3. Screening of serum and tissue samples

Serum samples were collected from animals before tissue collection and tested for the presence of BTV (lamb) antibodies by cELISA
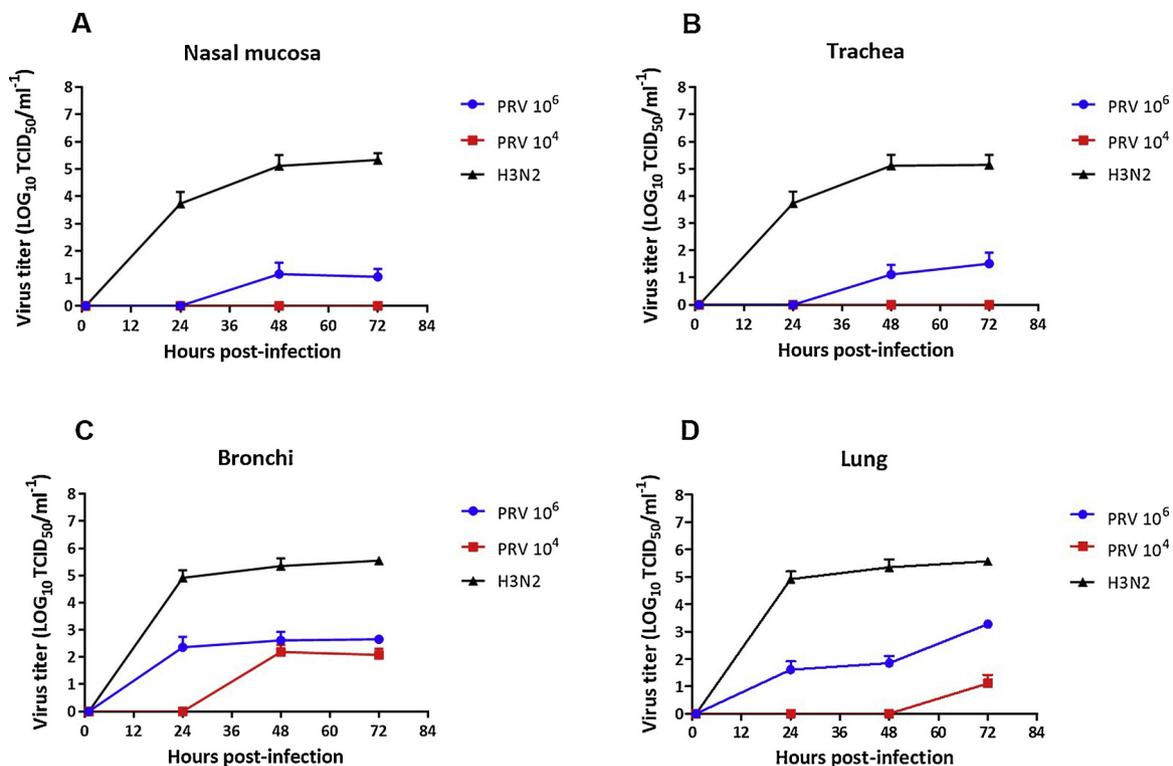
Fig. 3. Viral replication in swine respiratory EVOCs. Growth kinetics of PRV at two different infection doses (10^4 and 10^6 TCID$_{50}$/ml) and H3N2 in nasal mucosa (A), tracheal (B), bronchial (C) and lung (D) EVOCs. PRV replicated in all of the tissues when a dose of 10^6 TCID$_{50}$/ml was administered, while it replicated only in bronchial and lung EVOCs when infected with a dose of 10^4 TCID$_{50}$/ml. H3N2 replicated at significantly higher titers throughout the experiments ($P \leq 0.001$). PRV titers at the highest challenge dose were significantly higher than those observed in EVOCs challenged with the lowest dose, irrespective of the time of infection ($P \leq 0.01$). The values are the mean of six replicates of three independent experiments, bars show the SD.

(Lorusso et al., 2016), Influenza A by haemagglutination inhibition (HI, swine) (Pedersen, 2008), PI-3 and PRV (lamb and swine) by virus neutralization (Campolo et al., 2018). Nucleic acids purified from spleen samples of lambs were also tested for the presence of BTV RNA (Hofmann et al., 2008b) while those purified from lungs and tracheobronchial lymph nodes were tested for bovine respiratory syncytial virus (BRSV) and PI-3 and RNA by means of a commercially available molecular test (VetMAX BRSV & PI3, LSI).

2.4. Culture of sheep and pig respiratory EVOCs

Porcine EVOCs were performed at IZSVe and their viability was analyzed as previously described (Van Poucke et al., 2010; Patrono et al., 2015). EVOCs from lambs were developed at IZSAM according to published protocols for bovine respiratory EVOCs (Di Teodoro et al., 2018). To evaluate the morphological features, sheep EVOCs were collected at 0, 24, 48, 72, 96 and 120 h post culture (p.c.), fixed in 10% neutral buffered formalin and routinely processed for histology and stained by Hematoxylin and Eosin (H&E). Morphologic assessment and analysis of viability was performed following previous protocols (Di Teodoro et al., 2018). In addition, apoptotic index was evaluated by peroxidase *in situ* apoptosis detection kit (ApopTag®, Merck Millipore) according to the manufacturer's instructions. Positive cells were counted in 10 high-power fields (final magnification = 400×) for each time point, per respiratory EVOC and expressed as percentage of total cells present in the microscopic fields. Histological images were analyzed using *ImageJ* software (National Institutes of Health, Bethesda, USA) and data submitted for statistical analysis.

2.5. Infection of ovine and porcine respiratory EVOCs

After 24 h p.c., all sheep EVOCs were transferred in 24-well plates

and infected with a dose of 10^3 TCID$_{50}$/ml of PI-3, PRV, BTV-1, and BTV-27 v03 viruses by submerging the explanted tissues in 1 ml of each virus dilution. Pig EVOCs were infected with the same method, using doses of either 10^4 or 10^6 TCID$_{50}$/ml of PRV and 10^6 PFU/ml of H3N2. After 1 h at 37 °C in 5% CO_2, EVOCs were washed with warm phosphate buffered saline (pH 7.4, PBS) three times to remove non-attached virus particles and placed back into 6-well plates, in 2.5 ml of fresh tissue culture media up to 72 h at 37 °C in 5% CO_2. Virus-free culture medium was used for mock-infected EVOCs. To study viral replication kinetics, 200 µl of supernatant was collected at 1, 24, 48 and 72 h post infection (p.i.) and titrated by TCID$_{50}$.

2.6. Virus immunodetection

To study virus localization and cellular tropism, additional six replicates of virus- and mock-infected ovine and porcine respiratory EVOCs were fixed in 10% neutral buffered formalin at 1, 24, 48 and 72 h p.i. and routinely processed for histology and immunohistochemistry (IHC). For IHC, three µm-thick sections of EVOCs were dried at 37 °C, dewaxed and rehydrated by standard procedures. Antigen retrieval was performed by autoclaving at 121 °C for 10 min in 0.01 M citrate buffer, pH 6. Sections were incubated overnight at 4 °C with a rabbit anti-PRV polyclonal antiserum (Campolo et al., 2018) and a murine anti-BTV VP7 monoclonal antibody (Portanti et al., 2005). Immunoreactions were visualized using a biotin-streptavidin amplification method and 3-3′-diaminobenzidine as chromogen (Dako REAL™ detection system). For the double labelling indirect immunofluorescence (DLIIF) and confocal laser scanning microscopy (CLSM) investigations with ovine tissues, three µm-thick tissue sections of PRV-infected EVOCs were incubated with the anti-PRV polyclonal antiserum (final dilution = 1:1000) and, at the same time, with a murine anti-cytokeratins monoclonal antibody (a specific marker for epithelial cells
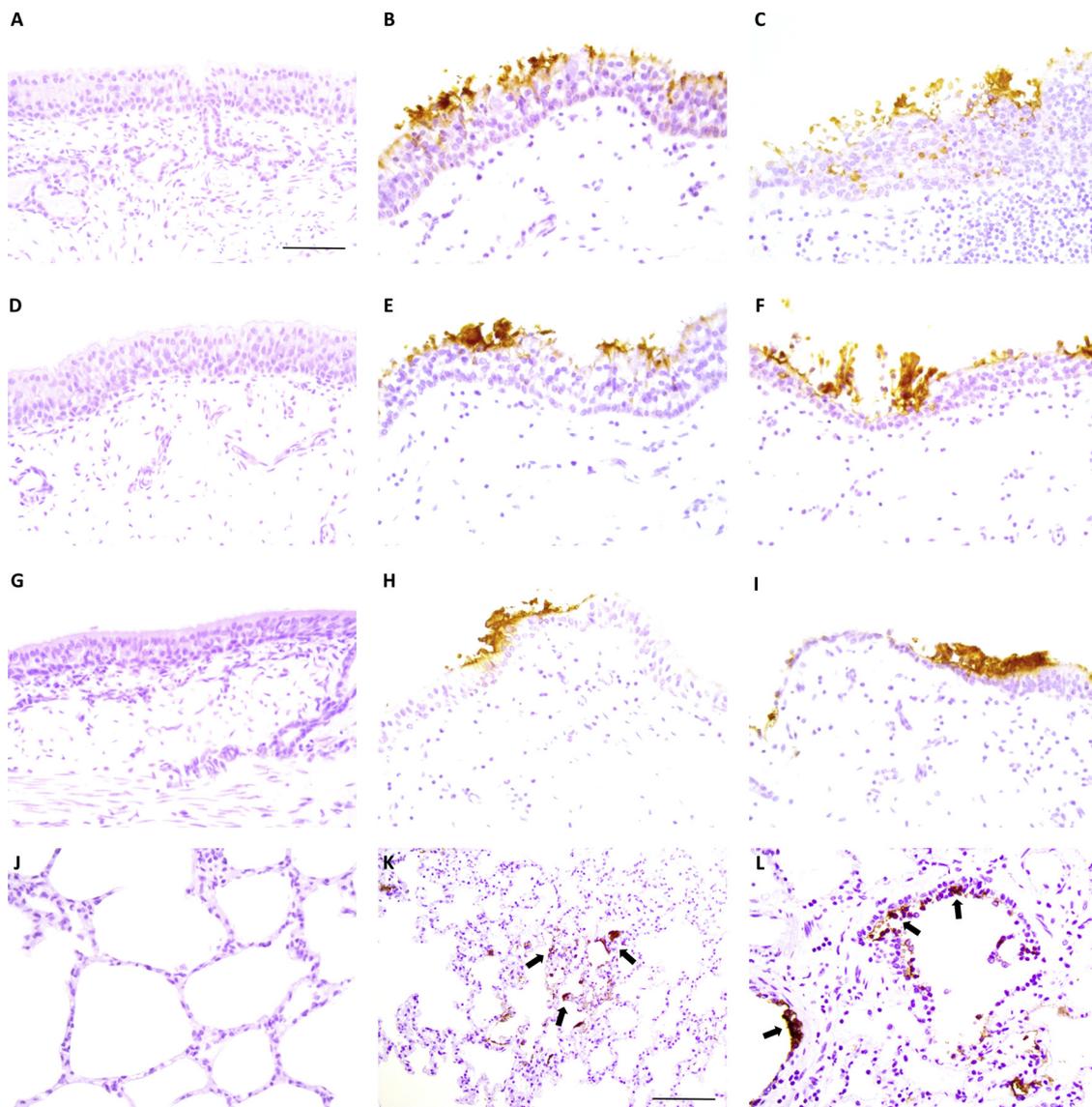
Fig. 4. IHC for PRV in sheep respiratory EVOCs. PRV antigens were detected on the surface and in the epithelial cells of nasal (B, C), tracheal (E, F), bronchial (H, I) epithelium and in alveoli (K, black arrows) and bronchiolar epithelium (L, black arrows) of the lung. Cellular damage, epithelial desquamation and cellular debris in the upper respiratory tract were evident at 24 (B, E, H) and 72 h p.i. (C, F, I) while histological lesions were not observed at 24 h p.i. in mock-infected EVOCs. Furthermore, specific immune reactivity against PRV was not detected in mock-infected tissues (A, D, G, J). Mayer's hematoxylin counterstain. Scale bar: 50 μm (A–J, L), 100 μm (K). Final magnification: ×400 (A–J, L), ×200 (K).
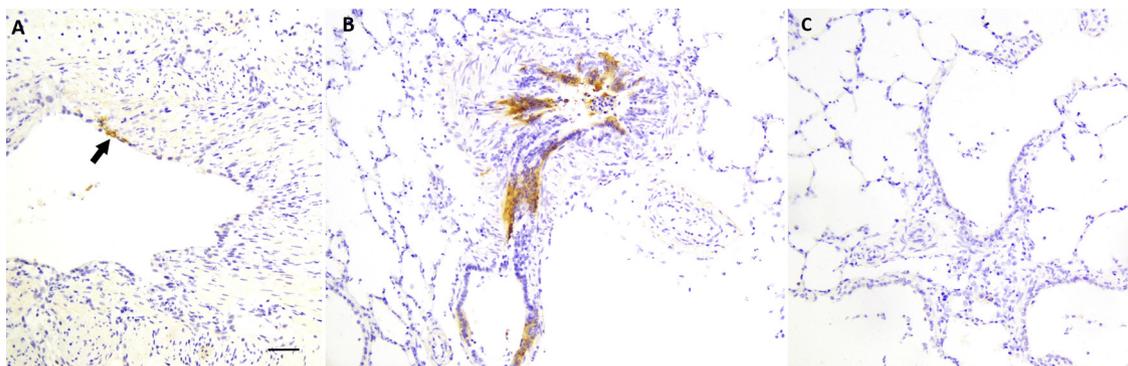
Fig. 5. IHC for PRV in swine respiratory EVOCs. Small amount of PRV antigens were seen on the epithelial layer of a bronchus EVOC (A, black arrow); PRV specific immunoreactivity was also detected in lung EVOCs (B); no PRV immunoreactivity was shown in a mock infected lung EVOC (C). Mayer's hematoxylin counterstain. Scale bar: 50 μm. Final magnification: ×200.
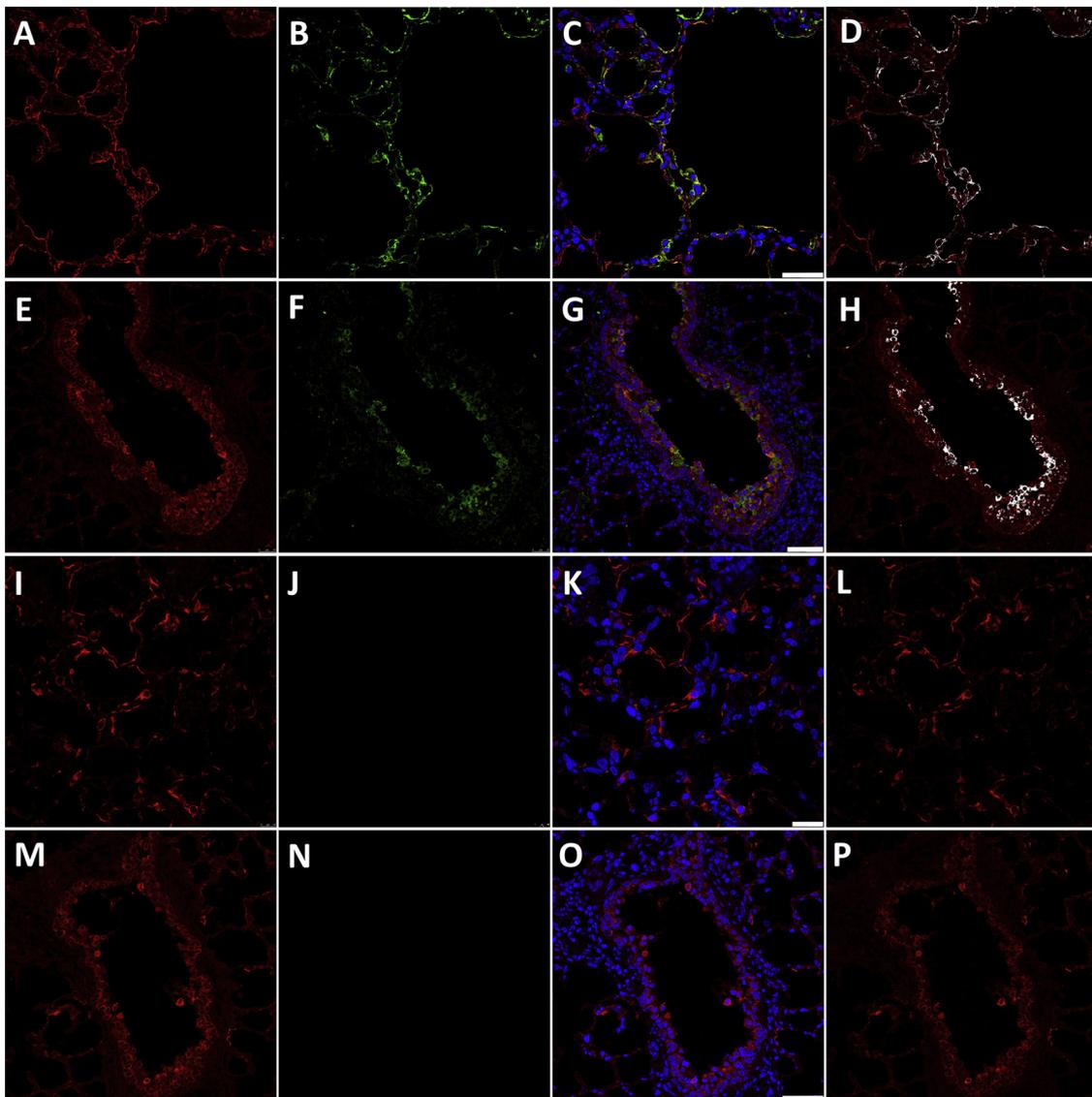
Fig. 6. CLSM investigation on mock and PRV-infected sheep lung EVOC. PRV antigens (green color, B, F) were observed in bronchiolar epithelial cells (red color, E) and type I pneumocytes (red color, A) detected with a monoclonal anti-cytokeratins antibody. Merging of fluorochromes showed the close association between lung (both brochiolar and type I pneumocytes) epithelial cells and PRV (C, G). Co-localization of red and green signals was shown in white color (D, H) and indicated the presence of PRV within the cytoplasm of type I pneumocytes and bronchiolar epithelial cells (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
In mock-infected EVOCs no specific PRV immunofluorescence was observed (J, N), while specific bronchiolar cells and type I pneumocytes immunofluorescence for cytokeratins (red color, M, I) was evident. K and O indicated merging of fluorochromes in mock-infected EVOCs. No co-localization was evident in mock-infected EVOCs (L, P). Cell nuclei were stained in blue with DAPI (C, G, K, O). All pictures derived from PRV-infected (A–H) and mock (I–P) lung EVOC 72 h p.i.. Scale bar: 50 μm (A–H), 20 μm (I–L), 50 μm (M–P).

including bronchiolar cells and type I pneumocytes; Dako, final dilution = 1:400) as primary antibodies. Sections were mounted using an antifade medium with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Inc.), and imaged using a Leica TCS SP5 II confocal laser microscope.

2.7. Statistical analysis

Sample size was calculated by G*Power software (Heinrich-Heine-Universität Düsseldorf, Germany). All statistical analyses were performed using GraphPad Prism software, version 8.0 (GraphPad Software Inc., San Diego, USA). Results were expressed as means ± standard deviation (SD) derived from six replicates of three independent experiments (one animal for each experiment). Multiple Student *t* test was performed to compare sets of data. Differences were

considered statistically significant when *P* value was ≤ 0.05.

3. Results

3.1. Ovine respiratory EVOCs morphology and viability

All selected lambs resulted serologically negative for BTV, PI-3 and PRV. Likewise, collected tissues tested negative for BTV, BRSV and PI-3 RNA. Histological morphology of ovine EVOCs was considered satisfactory during the entire time course of the experiments (Fig. 1A–H). The immunoreactivity of the selected cellular markers was intense, specific and well maintained between 0- and 120 -h p.c. (Fig. 1I–L). No significant changes in epithelial thickness and extent of alveolar septa were observed up to 120 h p.c. (Fig. 1M–P) and cultivation did not influence the apoptotic index (Fig. 1Q).

3.2. PRV and BTVs replicate differently in ovine respiratory EVOCs

BTV strains failed to replicate in ovine respiratory tissues (Fig. 2). Only BTV-27 v03 showed a slight replication in sheep EVOCs reaching titers higher than BTV-1 and BTV-26. Conversely, PRV showed a sustained replication, mostly in the nasal and lung EVOCs (Fig. 2A–D) in which the virus reached 10^4 $TCID_{50}$/ml. On the other hand, PI-3 replicated at several orders of magnitude higher than the other viruses in all EVOCs, mostly in the bronchus and lung parenchyma (Fig. 2C–D).

3.3. Mild replication of PRV in porcine respiratory EVOCs

Porcine respiratory EVOCs challenged with a dose of 10^6 $TCID_{50}$/ml of PRV were poorly receptive to the infection at the level of bronchi and lungs, as titers of up to 10^2-10^3 $TCID_{50}$/ml were recorded (Fig. 3C–D). Nasal and tracheal swine EVOCs recorded a very limited PRV replication reaching titers in the range of 10 $TCID_{50}$/ml (Fig. 3A–B). When EVOCs were infected with a dose of 10^4 $TCID_{50}$/ml, PRV was detected in the bronchi and lung while all of the nasal mucosa and trachea EVOCs were negative. Growth in the bronchi and lung tissues was only detectable from 48 to 72 h p.i. and titers were significantly lower than the ones recorded in EVOCs challenged with 10^6 $TCID_{50}$/ml. H3N2, used as positive control for swine respiratory EVOCs, replicated as expected (Fig. 3; Patrono et al., 2015).

3.4. PRV antigens visualized in ovine and porcine EVOCs

Immunoreactivity for BTV was not revealed in infected sheep EVOCs (data not shown), while small amounts of PRV antigens were observed in the epithelium of nasal, tracheal and bronchial ovine EVOCs. Cellular damage and disepithelialization were observed in PRV-positive tissues (Fig. 4B, C, E, F, H, I, and L). PRV antigens were also observed during the entire duration of the experiment in bronchiolar and alveolar cells (Fig. 4K, L). Immunoreactivity was seen also in PRV-infected porcine EVOCs. More in detail, small foci of PRV antigens were detected in bronchial epithelium of bronchi EVOCs (Fig. 5A) and in correspondence of epithelial cells lining bronchioles of lung EVOCs (Fig. 5B).

3.5. PRV infects type I pneumocytes and bronchiolar epithelial cells of ovine EVOCs

CLSM and DLIIF analyses performed in ovine lung EVOCs identified PRV-immunoreactivity in association to cytokeratin-positive cells demonstrating that the PRV-infected cells were type I pneumocytes and bronchiolar epithelial cells (Fig. 6D, H). No specific fluorescence against PRV was seen in mock-infected lung EVOCs (Fig. 6J, N). CLSM and DLIIF analyses were not performed in porcine tissues as for the lower viral titers which were observed in these EVOCs.

4. Discussion

In recent years, respiratory EVOCs were used to characterize innate immune responses, host range, and tropism of different viruses (Nicholls et al., 2007; Niesalla et al., 2009; Chan et al., 2013a, b; Gonzalez et al., 2014). In this regard, while porcine EVOCs are currently extensively and commonly adopted in several laboratories dealing with respiratory pathogens, as far as we know a limited number of studies describing the employment of ovine respiratory EVOCs is available (Fernandes et al., 2004; Abeynaike et al., 2010; Cousens et al., 2015). Thus, in this experiment, the assessment and standardization of ovine respiratory EVOCs was provided. The viability of ovine respiratory tissues was confirmed by the excellent morphological parameters recorded in mock infected tissues and by the marked replication of a well-known respiratory pathogen as PI-3, used in this study, as positive control respiratory virus.

PRVs are emerging zoonotic respiratory reoviruses and related infections are increasingly reported in humans in South-East Asia (Chua et al., 2007; Singh et al., 2015a; Voon et al., 2015; Tan et al., 2017; Uehara et al., 2019). Except for two murine models of disease, which have been recently described (Egawa et al., 2017; Kanai et al., 2018), evidence of natural infection in other animal species has not been yet reported.

The results of this study demonstrated that PRV is able to replicate in EVOCs of the upper and lower respiratory tracts of sheep, while replication in swine tissues was negligible. Only the higher dose challenge revealed mild replication in porcine tissues. Whether on the one hand, the diverse PRV kinetics observed in porcine EVOCs may be related to the infectious doses which have been used for challenge, on the other, the more efficient replication of PRV in sheep EVOCs may be, with all due caution, potentially associated to a higher susceptibility of this species to virus infection. Reasonably, only a proper *in vivo* animal experiment may better elucidate these preliminary data and confirm the differences which have been observed in this trial.

Both classical (BTV-1) or emerging atypical BTV serotypes (BTV-26 and BTV-27 v03) failed to replicate in the respiratory tract of sheep in our experimental setting. Therefore, proper *in vivo* experiments are also warranted to establish the transmission route of these newly emerged reoviruses.

Respiratory EVOCs were used for the prediction of host range and tissue/cell-tropism of different viral species including avian influenza A (H5N1) (Shinya et al., 2006). In this regard, it was already demonstrated that mammalian orthoreoviruses (genus *Orthoreovirus*) replicate in type I pneumocytes, causing severe pneumonia in mice and rats (Morin et al., 1996; Gauvin et al., 2013). Likewise, it was recently showed that PRV (strain Miyazaki-Bali/2017, PRV-MB) is able to infect type I pneumocytes causing severe pneumonia in BALB/c mice (Egawa et al., 2017). Based upon the results obtained in this study, we provided evidence, by IHC and CLSM analyses, that PRV Indonesia/2010 is able to infect bronchiolar epithelial cells and type I pneumocytes of sheep. In this regard, further experiments are currently ongoing in order to evaluate the early events characterizing the inflammatory response in the infected respiratory EVOCs.

5. Conclusions

The differences in viral replication observed between sheep and swine EVOCs suggest that the sheep might represent a more suitable host for PRV compared to pigs, where higher infectious doses are needed to cause a mild infection. In addition, it is worth to point out that sheep respiratory EVOCs, once infected with Indonesia/2010 PRV strain, supported viral replication at the same magnitude of that observed in mice experimentally infected with PRV-MB (Egawa et al., 2017), a virus strictly related to Indonesia/2010 strain (Lorusso et al., 2015; Singh et al., 2015b). This study confirmed that respiratory EVOCs from different animal species, eventually obtained at slaughterhouse, are a useful tool for testing and preliminarily characterize novel and emerging viruses before the assessment of the essential *in vivo* animal trials.

Competing interests

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.06.001.

References

Abeynaike, L., Meeusen, E.N., Bischof, R.J., 2010. An ovine tracheal explant culture model for allergic airway inflammation. J. Inflamm. (Lond). https://doi.org/10.1186/1476-9255-7-46.

Batten, C.A., Henstock, M.R., Steedman, H.M., Waddington, S., Edwards, L., Oura, C.A., 2013. Bluetongue virus serotype 26: infection kinetics, pathogenesis and possible contact transmission in goats. Vet. Microbiol. 162, 62–67.

Batten, C., Darpel, K., Henstock, M., Fay, P., Veronesi, E., Gubbins, S., Graves, S., Frost, L., Oura, C., 2014. Evidence for transmission of bluetongue virus serotype 26 through direct contact. PLoS One 9, 96049. https://doi.org/10.1371/journal.pone.0096049.

Bréard, E., Schulz, C., Sailleau, C., Bernelin-Cottet, C., Viarouge, C., Vitour, D., Guillaume, B., Caignard, G., Gorlier, A., Attoui, H., Gallois, M., Hoffmann, B., Zientara, S., Beer, M., 2018. Bluetongue virus serotype 27: experimental infection of goats, sheep and cattle with three BTV-27 variants reveal atypical characteristics and likely direct contact transmission BTV-27 between goats. Transbound. Emerg. Dis. 65, 251–263.

Bumbarov, V., Golender, N., Erster, O., Khinich, Y., 2016. Detection and isolation of bluetongue virus from commercial vaccine batches. Vaccine 34, 3317–3323.

Campolo, M., Lorusso, E., Leone, A., Teodori, L., Fogola, T., Ronchi, F.G., Di Sabatino, D., Lorusso, A., 2018. Development of a serum neutralization assay to detect Pteropine Orthoreovirus Indonesia/2010 neutralizing antibodies. Vet. Ital. https://doi.org/10.12834/VetIt.1313.7252.1.

Chaignat, V., Worwa, G., Scherrer, N., Hilbe, M., Ehrensperger, F., Batten, C., Cortyen, M., Hofmann, M., Thuer, B., 2009. Toggenburg Orbivirus, a new bluetongue virus: initial detection, first observations in field and experimental infection of goats and sheep. Vet. Microbiol. 138, 11–19.

Chan, R.W., Chan, M.C., Agnihothram, S., Chan, L.L., Kuok, D.I., Fong, J.H., Guan, Y., Poon, L.L., Baric, R.S., Nicholls, J.M., Peiris, J.S., 2013a. Tropism of and innate immune responses to the novel human betacoronavirus lineage C virus in human ex vivo respiratory organ cultures. J. Virol. 87, 6604–6614.

Chan, M.C., Chan, R.W., Chan, L.L., Mok, C.K., Hui, K.P., Fong, J.H., Tao, K.P., Poon, L.L., Nicholls, J.M., Guan, Y., Peiris, J.S., 2013b. Tropism and innate host responses of a novel avian influenza A H7N9 virus: an analysis of ex-vivo and in-vitro cultures of the human respiratory tract. Lancet Respir. Med. 1, 534–542.

Chan, L.L., Bui, C.T., Mok, C.K., Ng, M.M., Nicholls, J.M., Peiris, J.S., Chan, M.C., Chan, R.W., 2016. Evaluation of the human adaptation of influenza A/H7N9 virus in PB2 protein using human and swine respiratory tract explant cultures. Sci. Rep. 6, 35401. https://doi.org/10.1038/srep35401.

Chua, K.B., Crameri, G., Hyatt, A., Yu, M., Tompang, M.R., Rosli, J., McEachern, J., Crameri, S., Kumarasamy, V., Eaton, B.T., Wang, L.F., 2007. A previously unknown reovirus of bat origin is associated with an acute respiratory disease in humans. Proc. Natl. Acad. Sci. U. S. A. 104, 11424–11429.

Chua, K.B., Voon, K., Yu, M., Keniscope, C., Abdul Rasid, K., Wang, L.F., 2011. Investigation of a potential zoonotic transmission of orthoreovirus associated with acute influenza-like illness in an adult patient. PLoS One 6, e25434. https://doi.org/10.1371/journal.pone.0025434.

Cousens, C., Alleaume, C., Bijsmans, E., Martineau, H.M., Finlayson, J., Dagleish, M.P., Griffiths, D.J., 2015. Jaagsiekte sheep retrovirus infection of lung slice cultures. Retrovirology 12, 31. https://doi.org/10.1186/s12977-015-0157-5.

Dawson, P.S., Darbyshire, J.H., Lamont, P.H., 1965. The inoculation of calves with parainfluenza 3 virus. Res. Vet. Sci. 6, 108–113.

Di Teodoro, G., Marruchella, G., Di Provvido, A., Orsini, G., Ronchi, G.F., D'Angelo, A.R., D'Alterio, N., Sacchini, F., Scacchia, M., 2018. Respiratory explants as a model to investigate early events of contagious bovine pleuropneumonia infection. Vet. Res. 49, 5. https://doi.org/10.1186/s13567-017-0500-z.

Egawa, K., Shimojima, M., Taniguchi, S., Nagata, N., Tani, H., Yoshikawa, T., Kurosu, T., Watanabe, S., Fukushi, S., Saijo, M., 2017. Virulence, pathology, and pathogenesis of Pteropine orthoreovirus (PRV) in BALB/c mice: Development of an animal infection model for PRV. PLoS Negl. Trop. Dis. 11, 6076. https://doi.org/10.1371/journal.pntd.0006076.

Fernandes, L.B., D'Aprile, A.C., Self, G.J., Harnett, G.B., Goldie, R.G., 2004. The impact of respiratory syncytial virus infection on endothelin receptor function and release in sheep bronchial explants. J. Cardiovasc. Pharmacol. 44, 202–206.

Gard, G.P., Marshall, I.D., 1973. Nelson Bay virus. A novel reovirus. Arch. Gesamte Virusforsch. 43, 34–42.

Gauvin, L., Bennett, S., Liu, H., Hakimi, M., Schlossmacher, M., Majithia, J., Brown, E.G., 2013. Respiratory infection of mice with mammalian reoviruses causes systemic infection with age and strain dependent pneumonia and encephalitis. Virol. J. 10, 67. https://doi.org/10.1186/1743-422X-10-67.

Gonzalez, G., Marshall, J.F., Morrell, J., Robb, D., McCauley, J.W., Perez, D.R., Parrish, C.R., Murcia, P.R., 2014. Infection and pathogenesis of canine, equine, and human influenza viruses in canine tracheas. J. Virol. 88, 9208–9219.

Hofmann, M.A., Renzullo, S., Mader, M., Chaignat, V., Worwa, G., Thuer, B., 2008a. Genetic characterization of Toggenburg orbivirus, a new bluetongue virus, from goats. Switzerland. Emerg. Infect. Dis. 14, 1855–1861.

Hofmann, M.A., Griot, C., Chaignat, V., Perler, L., Thür, B., 2008b. Bluetongue disease reaches Switzerland. Schweiz. Arch. Tierheilkd. 150, 49–56. https://doi.org/10.1024/0036-7281.150.2.49.

Kanai, Y., Kawagishi, T., Okamoto, M., Sakai, Y., Matsuura, Y., Kobayashi, T., 2018. Lethal murine infection model for human respiratory disease-associated Pteropine orthoreovirus. Virology 514, 57–65.

Lorusso, A., Teodori, L., Leone, A., Marcacci, M., Mangone, I., Orsini, M., Capobianco-Dondona, A., Camma', C., Monaco, F., Savini, G., 2015. A new member of the Pteropine Orthoreovirus species isolated from fruit bats imported to Italy. Infect. Genet. Evol. 30, 55–58.

Lorusso, A., Baba, D., Spedicato, M., Teodori, L., Bonfini, B., Marcacci, M., Di Provvido, A., Isselmou, K., Marini, V., Carmine, I., Scacchia, M., Di Sabatino, D., Petrini, A., Bezeid, B.A., Savini, G., 2016. Bluetongue virus surveillance in the Islamic Republic of Mauritania: is serotype 26 circulating among cattle and dromedaries? Infect. Genet. Evol. 40, 109–112.

Lorusso, A., Sghaier, S., Di Domenico, M., Barbria, M.E., Zaccaria, G., Megdich, A., Portanti, O., Seliman, I.B., Spedicato, M., Pizzurro, F., Carmine, I., Teodori, L., Mahjoub, M., Mangone, I., Leone, A., Hammami, S., Marcacci, M., Savini, G., 2018. Analysis of bluetongue serotype 3 spread in Tunisia and discovery of a novel strain related to the bluetongue virus isolated from a commercial sheep pox vaccine. Infect. Genet. Evol. 59, 63–71.

Maan, S., Maan, N.S., Ross-smith, N., Batten, C.A., Shaw, A.E., Anthony, S.J., Samuel, A.R., Darpel, K.E., Veronesi, E., Oura, C.A., Singh, K.P., Nomikou, K., Potgieter, A.C., Attoui, H., van Rooij, E., van Rijn, P., De Clercq, K., Vandenbussche, F., Zientara, S., Bréard, E., Sailleau, C., Beer, M., Hoffman, B., Mellor, P.S., Mertens, P.P., 2008. Sequence analysis of bluetongue virus serotype 8 from the Netherlands 2006 and comparison to other European strains. Virology 377, 308–318.

Maan, S., Maan, N.S., Nomikou, K., Batten, C., Antony, F., Belaganahalli, M.N., Samy, A.M., Reda, A.A., Al-Rashid, S.A., El Batel, M., Oura, C.A., Mertens, P.P., 2011a. Novel bluetongue virus serotype from Kuwait. Emerg. Infect. Dis. 17, 886–889.

Maan, S., Maan, N.S., Nomikou, K., Veronesi, E., Bachanek-Bankowska, K., Belaganahalli, M.N., Attoui, H., Mertens, P.P., 2011b. Complete genome characterization of a novel 26th bluetongue virus serotype from Kuwait. PLoS One 6, e26147. https://doi.org/10.1371/journal.pone.0026147.

Maclachlan, N.J., Drew, C.P., Darpel, K.E., Worwa, G., 2009. The pathology and pathogenesis of bluetongue. J. Comp. Pathol. 141, 1–16.

Marcacci, M., Sant, S., Mangone, I., Goria, M., Dondo, A., Zoppi, S., van Gennip, R.G.P., Radaelli, M.C., Cammà, C., van Rijn, P.A., Savini, G., Lorusso, A., 2018. One after the other: a novel Bluetongue virus strain related to Toggenburg virus detected in the Piedmont region (North-western Italy), extends the panel of novel atypical BTV strains. Transbound. Emerg. Dis. 65, 370–374.

Morin, M.J., Warner, A., Fields, B.N., 1996. Reovirus infection in rat lungs as a model to study the pathogenesis of viral pneumonia. J. Virol. 70, 541–548.

Nicholls, J.M., Chan, M.C., Chan, W.Y., Wong, H.K., Cheung, C.Y., Kwong, D.L., Wong, M.P., Chui, W.H., Poon, L.L., Tsao, S.W., Guan, Y., Peiris, J.S., 2007. Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. Nat. Med. 13, 147–149.

Niesalla, H.S., Dale, A., Slater, J.D., Scholes, S.F., Archer, J., Maskell, D.J., Tucker, A.W., 2009. Critical assessment of an in vitro bovine respiratory organ culture system: a model of bovine herpesvirus-1 infection. J. Virol. Methods 158, 123–129.

Patrono, L.V., Bonfante, F., Zanardello, C., Terregino, C., Capua, I., Murcia, P.R., 2015. Phylogenetically distinct equine influenza viruses show different tropism for the swine respiratory tract. J. Gen. Virol. 96, 969–974.

Pedersen, J.C., 2008. Hemagglutination-inhibition test for avian influenza virus subtype identification and the detection and quantification of serum antibodies to the avian influenza virus. Methods Mol. Biol. 436, 53–66.

Pena, L., Vincent, A.L., Loving, C.L., Henningson, J.N., Lager, K.M., Lorusso, A., Perez, D.R., 2012. Restored PB1-F2 in the 2009 pandemic H1N1 influenza virus has minimal effects in swine. J. Virol. 86, 5523–5532.

Portanti, O., Luciani, M., Ronchi, G.F., 2005. Rapid detection of bluetongue virus in blood and organ samples using a capture enzyme-linked immunosorbent assay. Vet. Ital. 41, 15–21.

Reed, L.J., Muench, H.A., 1938. Simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27, 493–497.

Savini, G., Puggioni, G., Meloni, G., Marcacci, M., Di Domenico, M., Rocchigiani, A.M., Spedicato, M., Oggiano, A., Manunta, D., Teodori, L., Leone, A., Portanti, O., Cito, F., Conte, A., Orsini, M., Cammà, C., Calistri, P., Giovannini, A., Lorusso, A., 2017. Novel putative Bluetongue virus in healthy goats from Sardinia. Italy. Infect Genet Evol 51, 108–117.

Schulz, C., Bréard, E., Sailleau, C., Jenckel, M., Viarouge, C., Vitour, D., Palmarini, M., Gallois, M., Höper, D., Hoffmann, B., Beer, M., Zientara, S., 2016. Bluetongue virus serotype 27: detection and characterization of two novel variants in Corsica. France. J. Gen. Virol. 97, 2073–2083.

Shinya, K., Ebina, M., Yamada, S., Ono, M., Kasai, N., Kawaoka, Y., 2006. Avian flu: influenza virus receptors in the human airway. Nature 440, 435–436.

Singh, H., Shimojima, M., Ngoc, T.C., Quoc Huy, N.V., Chuong, T.X., Le Van, A., Saijo, M., Yang, M., Sugamata, M., 2015a. Serological evidence of human infection with Pteropine orthoreovirus in Central Vietnam. J. Med. Virol. 87, 2145–2148.

Singh, H., Yoshikawa, T., Kobayashi, T., Fukushi, S., Tani, H., Taniguchi, S., Fukuma, A., Yang, M., Sugamata, M., Shimojima, M., Saijo, M., 2015b. Rapid whole genome sequencing of Miyazaki-Bali/2007 Pteropine orthoreovirus by modified rolling circular amplification with adaptor ligation - next generation sequencing. Sci. Rep. 12, 16517. https://doi.org/10.1038/srep16517.

Sun, E.C., Huang, L.P., Xu, Q.Y., Wang, H.X., Xue, X.M., Lu, P.3, Li, W.J., Liu, W., Bu, Z.G.,

Wu, D.L., 2016. Emergence of a novel bluetongue virus serotype, China 2014. Transbound. Emerg. Dis. 63, 585–589.

Tan, Y.F., Teng, C.L., Chua, K.B., Voon, K., 2017. Pteropine orthoreovirus: An important emerging virus causing infectious disease in the tropics? J. Infect. Dev. 11, 215–219.

Uehara, A., Tan, C.W., Mani, S., Chua, K.B., Leo, Y.S., Anderson, D.E., Wang, L.F., 2019. Serological evidence of human infection by bat orthoreovirus in Singapore. J. Med. Virol. 91, 707–710.

Van Poucke, S.G., Nicholls, J.M., Nauwynck, H.J., Van Reeth, K., 2010. Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution. Virol. J. 7, 38. https://doi.org/10.1186/1743-422X-7-38.

Vögtlin, A., Hofmann, M.A., Nenniger, C., Renzullo, S., Steinrigl, A., Loitsch, A., Schwermer, H., Kaufmann, C., Thür, B., 2013. Long-term infection of goats with bluetongue virus serotype 25. Vet. Microbiol. 166, 165–173.

Voon, K., Tan, Y.F., Leong, P.P., Teng, C.L., Gunnasekaran, R., Ujang, K., Chua, K.B., Wang, L.F., 2015. Pteropine orthoreovirus infection among out-patients with acute upper respiratory tract infection in Malaysia. J. Med. Virol. 87, 2149–2153.

Zientara, S., Sailleau, C., Viarouge, C., Höper, D., Beer, M., Jenckel, M., Hoffmann, B., Romey, A., Bakkali-Kassimi, L., Fablet, A., Vitour, D., Bréard, E., 2014. Novel bluetongue virus in goats, Corsica, France, 2014. Emerg. Infect. Dis. 20, 2123–2125.