The identification of transmission routes for bluetongue virus (BTV) is essential to improve the control of the disease. Although BTV is primarily transmitted by several species of *Culicoides* biting midges, there has been evidence of transplacental and oral transmission. We now report that IFNAR(−/−) mice are susceptible to oral infection by BTV-8. Viraemia, clinical manifestations and tissue lesions are similar to those in intravenously infected mice. In addition, we show that the oral cavity and oesophagus are susceptible to BTV infection and replication, suggesting that these organs are possible entry routes during BTV oral infection.

In this study, we demonstrate the capacity of BTV-8 to infect IFNAR(−/−) mice by the oral route. We show that the oesophagus and the oral cavity are susceptible to BTV infection and replication. Furthermore, we also show that in IFNAR(−/−) mice, oral infection produces similar levels of viraemia, clinical signs and tissue lesions to those observed after intravenous infection. All these data show that, in principle, BTV could infect animals using the oral route.

In order to determine whether IFNAR(−/−) mice, on a 129 background (B&K Universal Ltd), were susceptible to oral infection with BTV-8, groups of 12 mice (males, 8 weeks old) were fed with DMEM containing BTV-8 (Belgium/2006). To resemble the natural conditions of food intake, mice were allowed to drink the medium without virus in individual cages equipped with a liquid delivery system (consisting of a microcentrifuge tube pierced with a 3 mm hole) before being infected. Consumption of the infectious preparations was carefully monitored. Control mice were fed with 100 µl DMEM and infected mice with the same amount of DMEM containing 10⁶ p.f.u. of BTV-8. Mice were examined for clinical signs daily. Infected mice showed the same clinical signs of BTV infection as mice that were infected intravenously (Calvo-Pinilla et al., 2009a), such as apathy, lethargy and ocular discharges typical of conjunctivitis. Death typically occurred 48 h after the onset of clinical signs. All orally infected animals died between 4 and 8 days post-infection (p.i.) (Fig. 1a). These results confirmed that IFNAR(−/−) mice were infected by ingestion of BTV-8, leading to animal death.
To study virus dissemination after oral infection, viral titres in blood and tissues were analysed. EDTA blood was collected from all animals at regular intervals after inoculation. Whole blood was subjected to three cycles of freeze/thawing and the amount of infectious virus was measured by plaque assay on Vero cells (Calvo-Pinilla et al., 2009a). Viraemia was observed in BTV-8-infected IFNAR\(^{-/-}\) mice with peak titres of about \(5 \times 10^4\) p.f.u. ml\(^{-1}\) at day 7 p.i. (Fig. 1b). Similar virus loads were found in IFNAR\(^{-/-}\) mice infected intravenously with BTV-8 (Calvo-Pinilla et al., 2009a). In addition, virus dissemination was determined in the infected mice. IFNAR\(^{-/-}\) mice were orally infected with BTV-8 and sacrificed 5 days p.i. followed by perfusion with PBS. Several organs (spleen, lung, thymus, liver, brain, heart, tongue, skin and testicles) and lymph nodes (inguinal, mediastinal and mesenteric) were collected. The tissues were homogenized in PBS using a Tissue Lyser homogenizer (Qiagen) and the amount of infectious virus was measured by plaque assay on Vero cells. Significant titres of infectious virus were detected in spleen, thymus, lung and lymph nodes. Titres of up to \(4 \times 10^4\) p.f.u. g\(^{-1}\) were recovered from the thymus, \(2 \times 10^5\) p.f.u. g\(^{-1}\) from the spleen, \(10^5\) p.f.u. g\(^{-1}\) from the lungs, and between \(10^2\) and \(5 \times 10^5\) p.f.u. g\(^{-1}\) from the lymph nodes (Fig. 1c). No infectious virus was detected in the other tissues analysed. The same viral tropism has been observed in IFNAR\(^{-/-}\) mice infected intravenously with BTV (Calvo-Pinilla et al., 2009a) and in naturally BTV-infected calves and sheep where high titres of BTV are present in lung, precapsular and mesenteric lymph nodes, thymus and spleen, which are secondary sites of BTV replication (Barratt-Boyes & MacLachlan, 1994, 1995; Hemati et al., 2009; MacLachlan et al., 1990; Sanchez-Cordon et al., 2010; Worwa et al., 2010).

To study whether the oral BTV infection of IFNAR\(^{-/-}\) mice causes the same pathological effects described for the intravenous infection, histological analyses were performed. Samples from different organs from BTV-8-infected IFNAR\(^{-/-}\) mice were taken at 5 days p.i. and fixed in 10% buffered formalin (pH 7.2), embedded in paraffin and stained with haematoxylin-eosin for histopathological examination.

Macroscopic lesions in the infected animals were characterized by enlarged spleens and lymph nodes, oedema and petechial haemorrhages in the lungs and the spleen. Histological examination of BTV-infected mice showed lymphoid depletion in the spleen (Fig. 2a), with infiltration of neutrophils in the white pulp. Additionally, the lymph nodes (Fig. 2c) showed lymphoid depletion, which was also observed in the thymus. Furthermore, the thymus showed loss of its typical architecture with the medulla and the cortex becoming indistinguishable (Fig. 2e). The lungs of BTV-infected mice (Fig. 2g) showed hyperaemia and increased septum size, with infiltration of lymphocytes, macrophages and neutrophils. Moreover, oedema in the alveolar cavity was observed. These histopathological findings were consistent with interstitial pneumonia. The results showed that oral and intravenous infection of BTV-8 produce the same tissue lesions in IFNAR\(^{-/-}\) mice (Calvo-Pinilla et al., 2009a), regardless of the mode of infection. In addition, these microscopic lesions are similar.
To identify the organs of the digestive tract that are susceptible to BTV-8 after oral infection and taking into account the normal low pH conditions of the stomach, we analysed the susceptibility of BTV-8 at different pH values. BTV-8 (5 × 10^5 p.f.u.) was incubated at different pH values (ranging from 2.5 to 7.5) for 30 min and subsequently titrated (Fig. 3a). BTV-8 was stable from pH 7.5 to 6.5. Incubation at pH 5.5 produced a decrease of 100-fold in virus infectivity and use of lower pH values completely inactivated BTV-8 infectivity. These results suggest that the lower pH of the stomach would inactivate the virus infectivity and that the infection probably occurs before the virus reaches the stomach. Experimental BTV infection of

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![Graph showing pH vs. virus titre](image)

Fig. 3. BTV-8 infects oesophagus *ex vivo* and *in vivo*. (a) Effects of pH on BTV-8 survival. Titrations of BTV-8 infectious virus after incubation of 5 × 10^5 p.f.u. for 30 min at the indicated pH are shown. Mean values from four experiments are represented and SD shown as error bars. (b) Autoradiograph of gel after SDS-PAGE of radio-immunoprecipitation assay conducted with mouse antiserum specific for BTV-8. Oesophageal explants infected (BTV-8) or non-infected (control) with BTV-8 were labelled metabolically with 35S-methionine for 24 h. (c) Immunolabelling by bovine polyclonal serum against BTV-8 in soft palate (×40) and oesophagus (×40) at 6 and 16 h p.i., respectively. BTV-8 labelled cells are indicated by arrows.

**Fig. 2.** Tissue sections of several organs from BTV-8 orally infected IFNAR(-/-) mice. Six mice were fed with 10^6 p.f.u. of BTV-8 and 5 days p.i. several organs were harvested to perform histopathological analysis. Haematoxylin and eosin staining of (a) spleen, (c) lymph node (×40), (e) thymus and (g) lung (×100) are shown. The main lesions were lymphoid depletion in spleen, lymph nodes and thymus, as well as severe pneumonia. Immunolabelling by bovine polyclonal serum against BTV-8 in (b) spleen (×40), (d) lymph node (×40), (f) thymus (×100) and (h) lung (×100) fixed in 10% neutral buffered formalin and subjected to microwave treatment (30 min) in citrate buffer (pH 9.0) for antigen retrieval.

To those observed in the natural host (Maclachlan et al., 2009; Worwa et al., 2010). To confirm that BTV-8 replicates in these tissues, immunohistochemistry with a hyperimmune serum from a BTV-8-infected cow was performed as described by Sanchez-Cordon et al. (2010). BTV-8 labelled cells, mostly macrophages and lymphocytes, were detected in the spleen, lymph nodes, lungs and thymus (Fig. 2b, d, f, h), confirming that the target tissues for BTV-8 are the same in oral and in intravenous infection of IFNAR(-/-) mice. Furthermore, these tissues have been described as the main target tissues for BTV infection in sheep and goats (Sanchez-Cordon et al., 2010).
sheep described the presence of BTV antigens in the oesophagus (Mahrt & Osburn, 1986). This observation suggests that the oesophagus is a potential port of entry for BTV-8 during oral infection.

To analyse this possibility, the infection of the oesophagus with BTV-8 was analysed *ex vivo*. The oesophagus, stomach and intestine were dissected from IFNAR<sup>(−/−)</sup> mice, washed with PBS, and suspended in serum-free and methionine-free minimal essential medium. The tissues were infected in culture with 10° p.f.u. of BTV-8 or used as a control. After 90 min of viral adsorption, [<sup>35</sup>S]methionine was added to the culture and incubated for 24 h at 37 °C. The tissues were then homogenized in RIPA buffer (0.15 M NaCl; 1 % sodium deoxycholate; 1 % Triton X-100; 0.1 % SDS; 0.01 M Tris/HCl pH 7.8; 1 mM phenyl-sulphonyl fluoride). For immunoprecipitation, a mouse antiserum against BTV-8 diluted 1:100 was incubated with the homogenized tissues for 16 h at 4 °C. Immune complexes were precipitated after incubation with protein G agarose (Sigma) for 90 min on ice and washed five times in RIPA buffer. The immunoprecipitates were denatured at 95 °C with 1 volume of SDS buffer containing 2-β-mercaptoethanol, and fractionated by 10 % SDS-PAGE. The results showed the presence of single immunoprecipitated bands of 111, 103 and 38 kDa corresponding to VP2, VP3 and VP7 BTV proteins, respectively, and a double band of 24 and 22 kDa corresponding to BTV NS3 and NS3α proteins in the BTV-8-infected oesophagus (Fig. 3b). These proteins were not immunoprecipitated in the oesophagus of control mice. In contrast, BTV proteins were not observed in stomach and intestine infected with BTV-8 (data not shown). The presence of BTV proteins in the infected oesophagus demonstrates that BTV-8 infects this organ *ex vivo* and that the virus also replicates in it, suggesting the oesophagus is an organ susceptible to BTV-8 infection when the virus is administered orally.

To verify that the oesophagus is the most likely organ of virus entry into the organism, immunohistochemistry of tissues was performed in mice at early time points after oral infection. IFNAR<sup>(−/−)</sup> mice were orally infected with BTV-8 and sacrificed at 6 and 16 h p.i. The soft palate, oesophagus, stomach, intestine, spleen, lungs and thymus were collected for examination of viral antigens by immunohistochemistry. BTV-8-labelled cells, mostly macrophages, were detected in the lamina propria of the mucosa in the soft palate and oesophagus at 6 and 16 h p.i., respectively (Fig. 3c). BTV-8-infected cells were not found in the other tissues analysed. These data confirm that the mucosa in the oral cavity and the oesophagus are tissues of BTV-8 entry into the organism during oral infection of IFNAR<sup>(−/−)</sup> mice. In addition, the soft palate and oesophagus were collected from BTV-8-infected IFNAR<sup>(−/−)</sup> mice at 5 days p.i. BTV-8-infected cells were not found in these tissues (data not shown). These data suggest that, after oral infection, the infected cells migrate to the lymphoid tissues that have been described as the secondary sites of BTV replication (Barratt-Boyes & MacLachlan, 1994, 1995; Hemati et al., 2009; MacLachlan et al., 1990; Sanchez-Cordon et al., 2010; Worwa et al., 2010).

In conclusion, we have confirmed experimentally that BTV-8 can infect IFNAR<sup>(−/−)</sup> mice via the oral route inducing levels of viraemia, clinical signs and pathology similar to those occurring after intravenous infection. In addition, we show that the oral cavity and the oesophagus are susceptible to BTV infection and replication, suggesting that these organs are points of viral entry during BTV oral infection. Our results are consistent with observations made in natural host species and we suggest that the oral route must be considered as a possible route of BTV transmission in the field via ingestion of blood, placenta or other organs from BTV-infected animals. Further research will be needed to identify and characterize the different transmission routes of BTV, especially the oral route in order to improve the control of BT disease.

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