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The length of BTV-8 viraemia in cattle according to infection doses and diagnostic techniques

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ABSTRACT

Four groups of BTV free Frisian and cross bred calves were used to determine the length of viraemia following infection with different doses of BTV-8 Italian isolate. The first group of five animals was infected with 10 $TCID_{50}$ of BTV-8, the second group of four animals with 10^3 $TCID_{50}$ and the third group, which also included four animals, was infected with 10^6 $TCID_{50}$. A placebo containing uninfected tissue culture medium was given to the four animals of the fourth group. The viraemia was evaluated by real time RT-PCR and virus isolation. In all infected groups, virus isolation was able to detect infectious virus up to 39 days post infection (dpi) while RT-PCR was positive up to 151–157 dpi. Infectious dose did influence neither the length nor the pattern of BTV-8 viraemia and confirmed that real time RT-PCR remains positive although no circulating virus is detectable in the peripheral circulation.

1. Introduction

Bluetongue is a non-contagious, infectious, vector-borne viral disease of domestic and wild ruminants caused by Bluetongue virus (BTV), a member of the *Reoviridae* family, *Orbivirus* genus (Mertens et al., 2004). The virus is transmitted by haematophagous midges of the genus *Culicoides* and their presence is essential to determine the occurrence of the disease and/or the virus circulation. BTV has been reported from latitudes 35°S to 53°N (Gibbs and Greiner, 1994; OIE, 2009). Several species of *Culicoides* may act as BTV vectors (Mellor, 1992). *C. imicola* is the main vector in southern Europe (Mellor and Wittmann, 2002), while species of the Obsoletus and Pulicaris complexes, and *C. dewulfi* are responsible for the BTV diffusion in the central and northern parts of the continent (Wilson and Mellor, 2009). BTV is a double strand RNA virus with a segmented genome consisting of 10 segments which encode for seven structural (VP1–VP7) and four non-structural proteins (NS1–NS3 and NS3A). Twenty-four serotypes have been formally identified (Erasmus, 1990; Gibbs and Greiner, 1994) and a likely 25th has been recently suggested (Hofmann et al., 2008).

The disease is characterised by various clinical forms, ranging from acute to chronic. Acute clinical signs include fever, ulceration of the mucosa, facial oedema and limp, while chronic forms are in general characterised by weakness, emaciation, muscle stiffness, fragile fleece with consequent alopecia (Verwoerd and Erasmus, 2004).

Not long ago BT was included in the World Organization for Animal Health (OIE) list A of diseases. Although now BT is among the notifiable diseases, affected countries or provinces are still banned to move ruminant livestock. This restriction has severe economical repercussions on national and international trade. It is well established that, at least within the European scenario, most of the economical importance of BT is related to animal movement restrictions rather than to the direct costs caused by animal disease and production losses.

In 1998, BTV entered in Europe and, between 1998 and 2006, caused an unprecedented epidemic with the incursions of six different serotypes into several European Countries around the Mediterranean basin. In August 2006, BTV-8 occurred for the first time in Northern Europe affecting the most important European exporting Countries. Outbreaks were reported in Holland, Belgium, Germany, Luxembourg and France (Mellor et al., 2009; Saegerman et al., 2008). European BTV-8 is a particular virulent strain which, unlike other field isolates, is capable of passing the placental barrier and infecting foetal tissues (Dal Pozzo et al., 2009). An animal to be safely moved from an infected area must not be viraemic. Viraemia and particularly its duration are crucial for BTV transmission. Amongst susceptible species, cattle can harbour infectious virus in the blood for up to 2 months. They play an important epidemiological role by acting as BTV *reservoirs* for *Culicoides* (MacLachlan, 1994). The OIE recommends that a BTV free Country imports animals upon the respect of certain conditions which

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basically ensure the absence of the virus in the blood of the moving animal. This should result negative when tested for the presence of BTV antibodies or for the presence of virus in the blood. Today most of the labs use molecular technology to detect virus in blood or tissue samples. RT-PCR and, more recently, real time RT-PCR assays provide a versatile system able to give information on virus serogroup and serotype within a few hours. They are also highly sensitive and capable of detecting very low concentration of viral RNA. In addition the real time assay is able to quantify the viral genome. Because of all these advantages, in most labs these new techniques are preferred to the classical viral detection techniques which requires 3–4 weeks to be completed. However, RT-PCRs are actually not able to distinguish whether the RNA detected in the animal is part of an infectious virus or just RNA of degraded virus not any longer able to infect the vector. The classical virus isolation is still the only method able to reveal the presence of infectious virus in an animal. This information becomes crucial when an animal from an infected area has to be moved. Several studies have confirmed that, at least for some BTV serotypes, the RNA could be detected in the blood of susceptible species longer than the infectious virus (Bonneau et al., 2002; MacLachlan et al., 1994; Singer et al., 2001). For BTV-8, the duration of viraemia as detected by RT-PCR or viral isolation still needs to be elucidated (Dal Pozzo et al., 2009). Also, it is critical to know how long an animal can maintain an infectious BTV-8 in the blood and if this period is affected by the infection dose.

This study was aimed to evaluate the relationship between BTV-8 infection dose and viraemia pattern in cattle by using classical virus isolation method (OIE, 2008) and real time RT-PCR (Polci et al., 2007).

2. Materials and methods

2.1. Virus

The isolate of BTV serotype 8 used in this trial is a field strain isolated at the Virology Department of the Istituto G. Caporale Teramo from an Italian cow belonging to a herd in which numerous animals were imported yearly from France. Before use, the virus was propagated on Vero cells (African green monkey kidney) cultivated in minimum essential medium (MEM) (Eurobio, France) containing L-glutamine, antibiotics (penicillin 100 IU/ml (Sigma, Munich), streptomycin 100 μg/ml (Sigma), gentamicin 5 μg/ml (Sigma), nystatin 50 IU/ml (Sigma)) and 3% foetal calf serum (FCS) (Sigma). Virus titration was carried out on Vero cells using standard microtitration assay (Savini et al., 2004). The virus titre was calculated using the Reed and Muench formula (Reed and Muench, 1938) and expressed as $TCID_{50}$/ml.

2.2. Animals and infection

Frisian and crossbred calves of 3 months of age, free of respiratory, digestive, umbilical and osteo-articular disease were used in the trial. The animals originated from BTV-free herd located in a BTV-free area and resulted negative for BTV-antibodies, when tested by competitive ELISA.

The animals were divided into four groups, one of 5 animals (Group A), and 3 of 4 animals each (Groups B, C and D). Animals of group A were infected subcutaneously with 1 ml suspension containing 10 $TCID_{50}$ of the BTV-8 virus, while those of group B and Group C with 1 ml containing 10^3 $TCID_{50}$, and 10^6 $TCID_{50}$, respectively. One milliliter suspension containing uninfected tissue culture medium was inoculated to the animals of the group D as placebo.

2.3. Sampling and laboratory tests

From each animal, ethylenediaminetetraacetic acid (EDTA) blood and serum samples were taken before the infection and three times a week for 10 weeks. Serum samples were tested for the presence of BTV-8 antibodies by serum neutralisation (Savini et al., 2004). Blood samples were tested for the presence of BTV by using both the classic virus isolation method (Savini et al., 2004, 2005; OIE, 2008) and real time RT-PCR (Polci et al., 2007). In group C the viraemia was assessed using only the classic virus isolation. Intravenous egg inoculation followed by two blind passages in VERO cells was used to isolate BTV-8 from EDTA blood samples, according to the method described by Savini et al. (2005). Virus titres were determined in the blood of viraemic animals as follows: the blood cells were washed three times in phosphate buffer saline (PBS) containing antibiotics. After the last washing, the sample was re-suspended in MEM with antibiotics (1/10 v/v) and sonicated. Four 10-fold dilutions of each sample suspension (from 1:10 to 1:10,000) were inoculated into a 96 flat bottomed microtitre plate wells, following the method described by the OIE (2008). Four replicates were made for each dilution. Approximately 10^4 cells, in a volume of 100 μl of MEM plus antibiotics and 3% FCS, were added per well and the plates incubated in an incubator at 37 °C under 5% CO_2. The plates were examined after 6 days and the $TCID_{50}$ calculated by using the Reed and Muench formula (Reed and Muench, 1938).

The real time RT-PCR used was that described by Polci et al. (2007) and adapted for BTV-8 (unpublished data). The method is able to detect $10^{1.55}TCID_{50}$/ml of BTV-8 and shows a very strong linear correlation between the virus concentration and the Ct value (R^2 = 0.994). The efficiency of the method is also high (91.43%).

To avoid the possible presence of inhibitors and to prevent false negative results, an Internal Control (IC), was included in the real time RT-PCR assay used in this trial. The Internal Control is a non-infectious synthetic RNA representing a fragment of the NS5-2 region of West Nile Virus (HNY1999) acquired from Ambion diagnostics. For the IC amplification specific primers and a probe labelled with VIC at the 5′-end and TAMRA at the 3′-end were used. Samples with a threshold cycle (Ct) value of less than 39 cycles were considered as positive.

3. Results

Fig. 1 shows the viraemia and neutralising antibody titre patterns obtained when the blood and serum samples of cattle infected with different doses of BTV-8 were tested by using the classic virus isolation and the serum neutralisation assay, respectively. Except for one animal of the group A which resulted negative to both the real time RT-PCR and the virus isolation for the entire experimental period, all infected animals had viraemic titres after infection. Conversely, no viraemia was detected in the animals of the control group.

In groups A and B, BTV was detected from day 5 post infection (p.i.) to day 39 p.i. In group C, BTV was firstly detected on day 2 p.i. but the last day of viraemia was the 39th day p.i. as the other groups. In all infected groups the highest viraemic titre (between $10^{5.3}$ and $10^{5.5}$ $TCID_{50}$/ml) was reported on day 12 p.i. Animals of group A showed first neutralising titres on day 5 p.i. whereas those of the other two groups on day 12 p.i. (Fig. 1).

The same animals which resulted viraemic by classical virology were reactive to the real time RT-PCR. As in the virus isolation, either in animals of group A or in those of group B the viraemia started on day 5 p.i. In calves infected with the 10 $TCID_{50}$ dose, the real time RT-PCR resulted positive until day 157 p.i. whereas in those infected with the 10^3 $TCID_{50}$ dose, the BTV RNA was