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

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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 TCID50 of BTV-8, the second group of four animals with 107 TCID50 and the third group, which also included four animals, was infected with 106 TCID50. 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.

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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 (Hoffmann 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, BT 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 BT free Country imports animals upon the respect of certain conditions which...
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 TCID₅₀/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 10³ TCID₅₀ of the BTV-8 virus, while those of group B and Group C with 1 ml containing 10⁴ and 10⁵ TCID₅₀, 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 10⁴ 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% CO₂. The plates were examined after 6 days and the TCID₅₀ 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 10⁻¹⁵ TCID₅₀/ml of BTV-8 and shows a very strong linear correlation between the virus concentration and the Ct value (R² = 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 10⁻³ and 10⁻⁴ TCID₅₀/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 10³ TCID₅₀ dose, the real time RT-PCR resulted positive until day 157 p.i. whereas in those infected with the 10⁴ TCID₅₀ dose, the BTV RNA was
detected up to 151 p.i. Both groups showed similar viraemic trends with threshold cycle (Ct) values ranging from 22 to 38 (Figs. 2 and 3). No correlation was demonstrated between the Ct values and the presence of infectious virus.

4. Discussion

The major problem related to the BTV infection is the ban of animal trade from BTV-infected to BTV-free areas or countries. When BTV-8 affected Northern European Countries where cattle export represents a significant source of income, the trade restriction was difficult to sustain. Vaccination was proposed as a practical solution for eluding the trade limitation. In Europe, most of the BTV vaccination campaigns were implemented to allow safe movement of animals. Vaccine elicits cellular and/or humoral immunity which should prevent animals from viraemia in case of infection. Not all vaccinated animals, however, are protected from viraemia following infection and therefore animals should be checked serologically and virologically for BTV before moving. Many techniques are now available to detect the presence of virus in the blood of the

Fig. 1. Average viraemic and neutralising titers in cattle infected with different doses of European BTV-8.

Fig. 2. RT-PCR Ct values in cattle infected with $10^3$ TCID$_{50}$ of BTV-8 European strain.
animals. Amongst these, the real time RT-PCR is undoubtedly one of the most sensitive and commonly used diagnostic test. It is characterised by having a very high sensitivity and specificity. It could then represent the optimal solution to prevent trading of viraemic animals which is likely the major source of BTV spreading. However, even if the RT-PCR technique detects viral RNA with a very high level of sensitivity, it is not capable of determining whether the sample contains infectious virus or not. The classical virus isolation conversely detects only infectious virus. In this study real time RT-PCR and the classical virus isolation were used to assess the length of BTV-8 viraemia in cattle following infection with various doses of BTV-8. Similarly to what found with other BTV serotypes (Bonneau et al., 2002; MacLachlan et al., 1994; Singer et al., 2001), positivity by real time RT-PCR in blood lasted longer (>150 dpi) than the period over which infectious virus could be isolated (39 dpi). Concerning the maximal predicted duration of viraemia in BTV infected cattle, quite a few data are available in the literature. It has been observed that the length of viraemia depends on the BTV serotype involved, the natural or the experimental infection protocol, the animal's age and the method of isolation (Dal Pozzo et al., 2009). According to Bonneau and MacLachlan (2004), only ruminants whose blood contains BTV as determined by virus isolation were able to infect the vector. Therefore, calves infected with BTV-8 in this study would have been able to infect Culicoides until 39 dpi. After that period they should have been considered epidemiologically safe although still positive to RT-PCR. Any evidence proving a correlation between Ct-values and the animal infectious status could represent a possible solution to the real time RT-PCR limitations. However, in this study no correlation was found between the real time RT-PCR Ct values and the presence of infectious virus. At the beginning of infection, BTV was isolated from samples with Ct values above 30 whereas, after 39 dpi, it was not possible to isolate virus despite the Ct values found in the samples were below 30. In the early phase of infection larger part of RNA virus detected in the blood is likely to derive from live virions as opposed to later phases where most of the RNA was probably deriving from degraded virions, no more capable of infecting vectors. The impact of virus dose on the outcome of infection is generally poorly understood. A strong correlation has been described between dose and length of incubation period, severity of clinical disease and efficiency of transmission. It is generally believed that the higher the dose, the shorter the incubation period and the time from inoculation to onset of viraemia and clinical signs (Alexandersen et al., 2003; Howey et al., 2009; Quan et al., 2004). In this study, the size of the inoculum did not influence the kinetics of viraemia as shown for hepatitis B virus (Asabe et al., 2009), and thus probably also having no effect on the capability of transmission and diffusion of BTV in the environment. The only difference was in animals infected with the 10^6 dose, in which viraemia started earlier, suggesting that they were able to transmit the virus earlier than the other two groups. However, the length and the intensity of viraemia were similar in all experimental groups. As in other viral infections the clearance of Bluetongue virus and a reduction in viral titres occur at the time of seroconversion confirming that neutralising antibodies prevent viraemia and further replication of viruses (Stott and Osborn, 1992). In the present experiment, the immune system of cattle responded more quickly to a low antigen dose than to the higher doses, suggesting that higher BTV infection doses affected the immune response. Although we do not have specific data to explain this unexpected findings, other authors have observed a transient immune-suppression and lympho-cellular depletion in infected lymph nodes during BTV infection (Barratt-Boyes et al., 1992; Odeon et al., 1997; Quist et al., 1997a,b; Lacetera and Ronchi, 2004; Owens et al., 2004). Nonetheless, apart from an earlier presence of the virus in the blood of animals infected with the highest dose, no other outcomes resulted from this different response. In all experimental groups there was a quite long period during which high neutralising antibodies and viraemic titres were present at the same time. This is in line with other studies and further confirms the role played by the red blood cell membranes in protecting bluetongue virus from the attack of the neutralising antibodies (Brewer and MacLachlan, 1994).
It could be concluded that real time RT-PCR is still the technique of choice for detecting infection because it is a very sensitive test and therefore useful as screening to detect BTV. Virus isolation, however, is needed to assess if the sample contains infectious virus or not. The duration of BTV positivity by RT-PCR in blood lasts longer than the period over which infectious virus can be isolated. Consequently, to avoid blocking trading of safe animals, care should be taken in interpreting positive results. The OIE terrestrial code (2009) states that the infectivity period for bluetongue virus shall be 60 days. The infectious period of all infected animals included in this trial was much less then 60 days and this occurred despite the challenge dose used. Regarding the host-virus interaction, apart from either a delay in the immune response observed in the two highest dose groups or an earlier presence of circulating BTV in animals infected with 10^6 dose, no other effects were found in the viraemia and neutralising response when varying the infectious dose.

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