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1 **Experimental reproduction of bluetongue virus serotype 8 clinical disease in calves**

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21 Abstract

22 Cattle are commonly subclinically infected following natural or experimental infection with
23 bluetongue virus (BTV). The introduction of BTV serotype 8 (BTV-8) in Europe has been
24 characterized by the manifestation of clinical signs in infected cattle. In order to study the
25 pathogenesis of BTV-8 in this host, an animal model able to reproduce the clinical
26 manifestations of the disease is required. In this work, two calves were subcutaneously and
27 intravenously injected with a low passage cell-adapted strain of BTV-8. Both calves showed
28 typical bluetongue clinical signs, including pyrexia, ocular discharge, conjunctivitis, oral
29 mucosal congestion, development of ulcers and necrotic lesions on the lips and tongue,
30 submandibular oedema, coronitis and oedema of the coronet and pastern region. A score was
31 assigned depending on the severity of the lesions and a total clinical score was calculated for
32 each animal daily and at the end of the experiment. Both calves became viraemic 24 hours
33 post-infection and seroconversion occurred between 7 and 11 days P.I.

34 In this study we present the development of a protocol of infection in calves able to reproduce
35 the severity of the lesions observed with BTV-8 in field conditions.

36
37 **Keywords:** Bluetongue virus, serotype 8, cattle, experimental infection, clinical signs,
38 pathogenesis.

40 1. Introduction

41 Bluetongue virus (BTV) is an arthropod-borne pathogen infecting most species of domestic
42 and wild ruminants and causing high economical loss in the international movement and
43 animal trade (MacLachlan and Osburn, 2006). BTV belongs to the genus *Orbivirus* within the

44 family *Reoviridae*, and at present 24 serotypes have been identified (Mellor and Wittmann,
45 2002). Clinical signs commonly occur in sheep, while goats and cattle remain mostly
46 subclinically infected (Osburn, 1994). Although the pathogenesis of bluetongue is similar in
47 sheep and cattle (MacLachlan et al., 1990), it is uncertain as to why BTV infection causes
48 extensive microvascular injury in sheep, but less in cattle. Recently the role of species-
49 specific endothelial cell-derived inflammatory and vasoactive mediators has been shown
50 (DeMaula et al., 2002).

51 Since the summer 2006, BTV serotype 8 (BTV-8) appeared in northern and central Europe
52 with striking epidemiological aspects, like the higher proportion of cattle showing clinical
53 signs and the involvement of new *Culicoides* vectors during viral transmission (Saegerman et
54 al., 2008a). During the 2006-2007 outbreaks of BTV-8 in northern and central Europe
55 (Toussaint et al., 2007b), infected adult cattle showed the following clinical signs: ulcerative
56 and necrotic lesions on the muzzle and in the oral cavity, conjunctivitis, lacrimation,
57 periocular dermatitis, photosensitivity-like lesions, drop in milk production, loss of body
58 condition, ulcerative and necrotic lesions on the teats, udder and vulva, oedema of the distal
59 limbs (Thiry et al., 2006; Guyot et al., 2007; Elbers et al., 2008). In Europe BTV-8 appeared
60 to cause transplacental infection, which was normally associated until now to live BTV strains
61 adapted in cell culture (MacLachlan et al., 2000; Vercauteren et al., 2008; Wouda et al.,
62 2008).

63 The understanding of the viral pathogenesis in cattle during BTV-8 infection is still rather
64 poor. There is a real need of a reliable and reproducible experimental model to study the viral-
65 host interactions, including the development of clinical signs associated to the infection.
66 Many experimental inoculation studies have been conducted to characterize BTV
67 pathogenesis in cattle, including a variety of serotypes, animals of different ages and various
68 methods of inoculation. At present, only one study has been published with BTV-8

69 experimental infected sheep and cattle, where the latter did not show obvious clinical
70 manifestations of the disease, although the necropsy revealed a generalized lymphadenopathy
71 and, only in one cattle, petechial haemorrhages on several organs (Darpel et al., 2007).

72 The aim of our study was to reproduce, in experimentally infected calves, the clinical signs of
73 the disease observed in naturally infected cattle with BTV-8. The results obtained infecting
74 two calves with BTV-8 are presented, with particular emphasis to the characterization and
75 quantification of the different clinical signs observed during the experiment.

76

77 **2. Materials and methods**

78 *2.1 Animals*

79 Two 5 months-old female Holstein calves have been used in this study (calf 3179 and 3181).
80 The animals were bought from a French farm located in a BTV-free area and being regularly
81 monitored for BTV. Before the beginning of the experiment, the calves and their dams were
82 tested to confirm their seronegative and non-viremic status for BTV. Furthermore, they have
83 been also tested for bovine viral diarrhoea virus (BVDV) (IDEXX HerdChek, Bovine Viral
84 Diarrhoea Virus Ab; LSI, Kit TaqVet BVD) and infectious bovine rhinotracheitis (IBR) with
85 negative results. A veterinarian was attesting the healthy conditions of the selected calves
86 after a detailed clinical examination.

87

88 *2.2 Virus*

89 Bluetongue virus serotype 8 (BTV-8) strain BEL2006/01 P2, isolated during the 2006
90 epidemic season in Belgium, was used (Toussaint et al., 2007b). First the virus was isolated
91 from the EDTA-blood on embryonated chicken eggs, and subsequently propagated on BHK-

92 21 cell culture. The second passage of the virus on BHK-21 cells had a titer of $10^{5.3}$
93 TCID₅₀/ml and it was used as inoculum in our experimental protocol.

94

95 *2.3 Experimental design*

96 The experimental infection was performed during the winter season, when the vector activity
97 was reduced. The calves were confined in an insect-secure zone at the Experimental
98 Infectiology Platform (PFIE) of the INRA centre of Tours (Nouzilly, France) and insects light
99 traps were installed at the entrance of the experimental area. During the experiments, animals
100 were treated following the International Guiding Principles for Biomedical Research
101 Involving Animals as issued by the Council for the International Organizations of Medical
102 Sciences. Both calves have been infected using BTV-8 strain BEL2006/01 P2. The infection
103 has been performed in the neck using 2 ml of viral inoculum. The dose was administered 1 ml
104 subcutaneously (SC) and 1 ml intravenously (IV). After 14 days post-infection, animals were
105 euthanized following an IV injection of pentobarbital. Spleen, prescapular and mediastinic
106 lymph nodes, tongue were collected from both animals and stored at -80°C for viral RNA
107 detection.

108

109 *2.4 Clinical examination*

110 The animals were monitored daily by a veterinarian for the duration of the experiment (15
111 days). The body temperature and the development of clinical signs were examined daily at the
112 same moment of the day (in the morning) (Jackson and Cockcroft, 2002), and a digital camera
113 was used to record the evolution of the lesions. A standardized clinical form for BTV
114 infection was used during the animal examination, in order to analyse systematically the most
115 affected organs and systems (Saegerman et al., 2008b). In the daily clinical examination, we
116 proceeded in the methodological differentiation of general and specific clinical signs. A major

117 attention was orientated to the skin and the visible mucous membranes, to the locomotor,
118 digestive, respiratory and nervous systems. In addition, for clinical data, a score was
119 attributed for each observed clinical sign in order to quantify the severity of the lesion, and
120 the score of an individual calf was defined as the sum of all clinical signs present. A total
121 clinical score was calculated for each animal daily, at the end of the experiment and also for
122 each group of clinical signs.

123

124 *2.5 Laboratory analysis*

125 *2.5.1 Serological analysis*

126 For serological analysis, whole blood was collected daily. It was centrifuged at 3000 rpm for
127 10 minutes and the serum was stored subsequently at -20°C. The ID Screen® Bluetongue
128 Competition ELISA kit (ID VET, Montpellier, France) was used to detect the appearance of
129 antibodies directed against BTV. The cELISA was performed following manufacture
130 instructions. Besides the kit controls, a two-fold dilution series of an anti-BTV antibody
131 positive reference serum was included and the results were expressed as % negativity (PN) as
132 described by Vandebussche et al. (2008).

133

134 *2.5.2 BTV detection in biological samples*

135 EDTA-blood samples were collected daily and stored at +4°C. These samples were used to
136 detect the levels of viraemia by real-time RT-PCR (RT-qPCR) according to Vandebussche et
137 al. (2008). In order to amplify BTV genome, a RT-qPCR amplifying a region of BTV
138 segment 5 (RT-qPCR_S5) was used (Toussaint et al., 2007a). Bovine beta-actin was
139 contemporarily amplified as internal control (Toussaint et al., 2007a). Cycle threshold values
140 (Ct) for each sample were used to compare the daily blood viral load.

141 BTV detection by RT-qPCR was also performed from spleen, tongue, prescapular and
142 mediastinic lymph nodes collected during necropsy and stored at -80°C. Starting from 100 mg
143 of tissue, the RNA was extracted using Trizol-LS reagent (Gibco, Invitrogen). BTV and beta-
144 actin RT-qPCR were performed as described above.

145

146 *2.6 Statistical analysis*

147 The trends of clinical pattern in time and by group of clinical signs were compared by using
148 Spearman's rank correlation, called r_s in the text (Dagnelie, 1998). A P value < 0.05 was
149 considered as significant.

150

151 **3. Results and discussion**

152 In this study we describe for the first time the occurrence of clinical signs in calves
153 experimentally infected with BTV-8. In the Bluetongue chapter of the OIE Terrestrial Manual
154 2008, the use of wild type virus with no ECE or cell culture passages is recommended during
155 the challenge of vaccinated animals in order to test the efficacy of the vaccine. The two calves
156 included in the study received a subcutaneous and an intravenous injection of a BTV-8 strain
157 with only two passages on BHK-21 cell culture and with a high viral titer. The intravenous
158 injection of a virulent BTV strain is probably at the origin of the successful reproduction of
159 the severe clinical signs that have been observed in the field. Recently severe bluetongue
160 disease has been experimentally induced in sheep using BTV-4 with the intravenous injection
161 of infectious blood (MacLachlan et al., 2008). The possibility to reproduce the clinical
162 manifestations associated to BTV infection seems to be highly increased by the use of a
163 virulent strain inoculated intravenously. The accuracy and reproducibility of the described
164 experimental protocol will be evaluated using a larger number of infected animals and taking
165 into account the kind of inoculum.

166 The clinical consequences observed in the two calves were not dissimilar to the signs induced
167 spontaneously by BTV-8. The day after the infection the calves had pyrexia, a sign which has
168 been already observed in previous experimental infection with BTV (Odeón et al., 1997;
169 Darpel et al., 2007). Probably related to the high levels of viraemia of the following days, we
170 measured a slight increase in body temperature between 7 and 10 days P.I. The daily body
171 temperature, expressed in °C and recorded in the two calves during the experiment, is shown
172 in Figure 1 A. Since the first day P.I, a bilateral aqueous ocular discharge developed in calf
173 3179. In the following days, the ocular discharge progressively became abundant, with a
174 mucus-purulent feature (Figure 2 A), associated to a bilateral conjunctivitis since 3 days P.I
175 (Figure 2 B). An enhanced bilateral mucous ocular discharge was observed starting from 12
176 days P.I. In calf 3181 the appearance of the ocular discharge started since 4 days P.I and it
177 was complicated by conjunctivitis at 10 days P.I. After 4 days P.I and for the entire duration
178 of the observation, calf 3181 had an increased nasal discharge, although it conserved a
179 physiological watery feature (Figure 2 C). No sign of dyspnea was observed in both calves. In
180 calf 3179 a diffuse congestion of the oral mucosa was observed already 2 days P.I, and
181 multiple erosions appeared in the inner part of the upper lip 4 days P.I (Figure 2 D and E).
182 The lesions were associated with a congestion of the gum and the oral mucosa of the inner
183 lips. These erosions healed within some days with the development of a scab 9 days P.I. Four
184 new smaller ulcers appeared 10-11 days P.I. These ulcers were round and localized at the
185 junction of the oral mucosa and the skin at the right and left side of the lower and upper lip. A
186 generalized congestion of the gums and of the oral mucosa was observed 5 days P.I in calf
187 3181. The first oral ulcers in this animal appeared 11 days P.I and their localization and aspect
188 were very similar to the lesions described in calf 3179 at the same time of the infection. Viral
189 replication in the mouth was underlined by BTV RNA detection in the tongue of both calves
190 (data not shown). This finding and the continuous generation of new lesions in the oral cavity

191 underlined the active *in situ* replication of the virus. The importance of the oral cavity as a
192 privileged localization during viral pathogenesis was furthermore underlined by the high
193 scores attributed to the oral lesions developed by both calves (Table 1). The frequent
194 appearance of oral clinical signs during spontaneous bluetongue disease in cattle was already
195 reported (Saegerman et al., 2007; Elbers et al., 2008).

196 Starting from 6 days P.I, a characteristic submandibular oedema appeared in both animals
197 (Figure 2 F). No other oedemas were detected in the different regions of the head or in other
198 body areas. Coronitis was detected in both calves one week after the infection. This lesion
199 started with a reddening around the coronary band at 5 days post-infection (Figure 3 A),
200 followed by an aggravation of the inflammation and the addition of a localized oedema in the
201 coronet and pastern region at 8-9 days post-infection (Figure 3 B, C and D). No one of the
202 calves showed lameness or difficulties in standing up, but the presence of a rubber carpet was
203 probably reducing the pain during the contact of the feet on the ground.

204 A total daily clinical score was calculated for each animal and it allowed appreciating the
205 progression of the disease (Table 1). The calves had few or unapparent clinical signs the first
206 three days P.I, while starting from the 4th day, the infection became clinically manifest. Since
207 day 7 P.I, the severity of the disease enhanced with the development of multiple clinical
208 signs: ocular discharge and conjunctivitis, ulcers in the oral mucosa, lesions in the coronet and
209 pastern region and submandibular oedema (Table 1). In addition, a very good correlation was
210 observed between the two calves for daily total score and total score by group of clinical signs
211 ($r_s = 0.86$ and 1 respectively; $P < 0.0001$) indicating a highly similar clinical pattern in the two
212 calves.

213 The development of viraemia and the seroconversion of the inoculated calves represent
214 evidences of the occurred infection (Figure 1 B and C). BTV RNA was amplified in every
215 EDTA-blood samples starting from 24 hours post-infection. Since 4 day P.I, the Ct values

216 progressively decreased, showing a constant increment of the viral RNA load in the blood of
217 the two calves (Figure 1 B and C). The quantification of the BTV genome in the blood of the
218 two calves, allowed us to follow the dynamic of the viraemia. The early detection of the viral
219 RNA in the blood after 24 hours post-infection was probably due to the intravenous injection
220 of the virus. The temporarily reduction of the viral RNA load in the blood stream until the
221 third day P.I, was probably to refer to the distribution of the virus to the sites of primary and
222 secondary replication, such as lymph nodes, and spleen. Lymphoid organs are important sites
223 of viral replication in sheep and cattle (MacLachlan et al., 1990). The subsequent increase of
224 the viraemia 4 days P.I, was probably to refer to the transit of the virus from the sites of
225 massive replication to the blood stream. In the literature for other BTV serotypes, it is
226 reported that in cattle the release of BTV from the spleen is followed by high titer viraemia
227 which persists for several weeks post-infection (MacLachlan et al., 1990). After 14 days P.I,
228 the virus was detected by RT-qPCR in the spleen and the tongue of both animals, while the
229 tested lymph nodes were negative (data not shown). These results are probably explained by
230 the fugacity of the viral replication in the lymph nodes during the earliest phase of the
231 infection, and by the longer persistence of the virus in the spleen. Similar findings were
232 previously observed in intravenously infected calves (MacLachlan et al., 1990; MacLachlan et
233 al., 2008).

234 The development of clinical signs in cattle infected with BTV-8 is atypical because the
235 disease in this animal host is more commonly undetectable. Before the outbreak of BTV-8 in
236 Europe, some authors have suggested that the rare clinical manifestations in cattle could be
237 explained by a hypersensitivity reaction and to the development of IgE-specific antibody to
238 BTV (Anderson et al., 1985). At present no evidences of the involvement of an
239 immunopathological reaction in the development of the lesions showed by cattle infected with
240 BTV-8, have been documented. The mechanisms explaining the severity of the lesions

241 produced by BTV-8 in cattle have to be investigated. In particular, increased host
242 susceptibility and higher virulence of BTV-8 are the factors to be elucidated. The availability
243 of a model to characterize bluetongue disease in cattle will be crucial for future investigations.
244 The experimental protocol presented in this study successfully reproduced the lesions
245 observed in naturally infected cattle and confirmed the severity of the clinical signs induced
246 by BTV-8 in northern Europe.

247

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256

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327

328 **Figure captions**

329 **Figure 1 :** The variation of the body temperature of calf 3179 and 3181 during the
330 experimental infection with BTV-8 is shown in Figure A. The two black horizontal lines
331 delimit the physiological body temperature range in calves (between 38.5°C and 39.5°C).
332 Figure B and C illustrate the antibody response and the dynamic of viraemia in calf 3179 and
333 3181, respectively. The results of the serological analysis are expressed as % of negativity and
334 were calculated referring to the negative control. BTV detection in EDTA-blood was
335 performed by RT-qPCR and the results were expressed in Ct values.

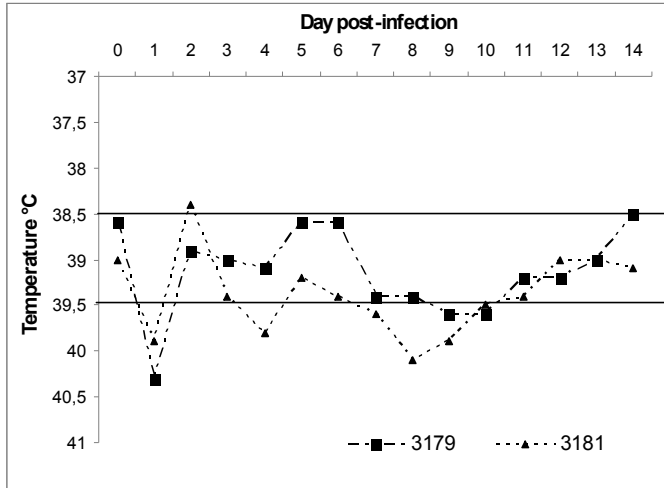
336

337 **Figure 2:** Figure A shows the severity of the ocular discharge observed in calf 3179 after 4
338 days PI. After 3 days P.I a remarkable conjunctivitis was observed in calf 3179 (Figure B).
339 Figure C illustrates the nasal discharge observed in calf 3181. A diffused oral congestion was
340 observed already 2 days P.I in calf 3179 while the first multiple oral erosions appeared in the
341 inner part of the above lip after 4 days P.I (Figure D and E, respectively). Figure F shows
342 submandibular oedema observed 6 days post-infection in calf 3179.

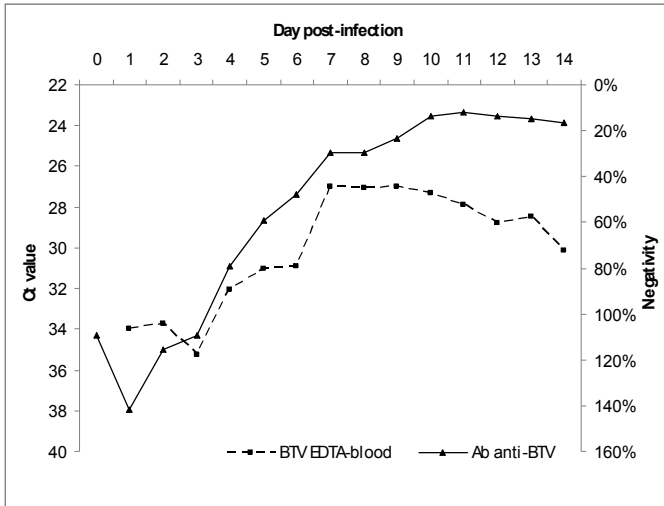
343

344 **Figure 3:** After 5-6 days post-infection, we observed reddening of the coronary band (A),
345 followed by reddening and oedema in the coronet and pastern region at 8-9 days post-
346 infection (B, C and D).

[A]



[B] calf 3179



[C] calf 3181

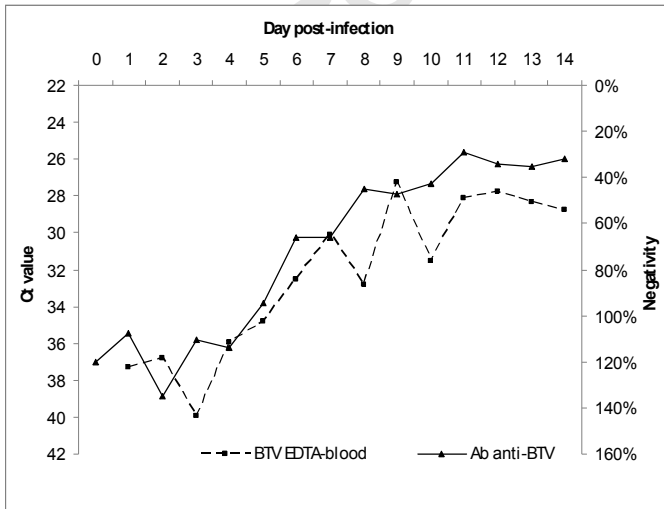
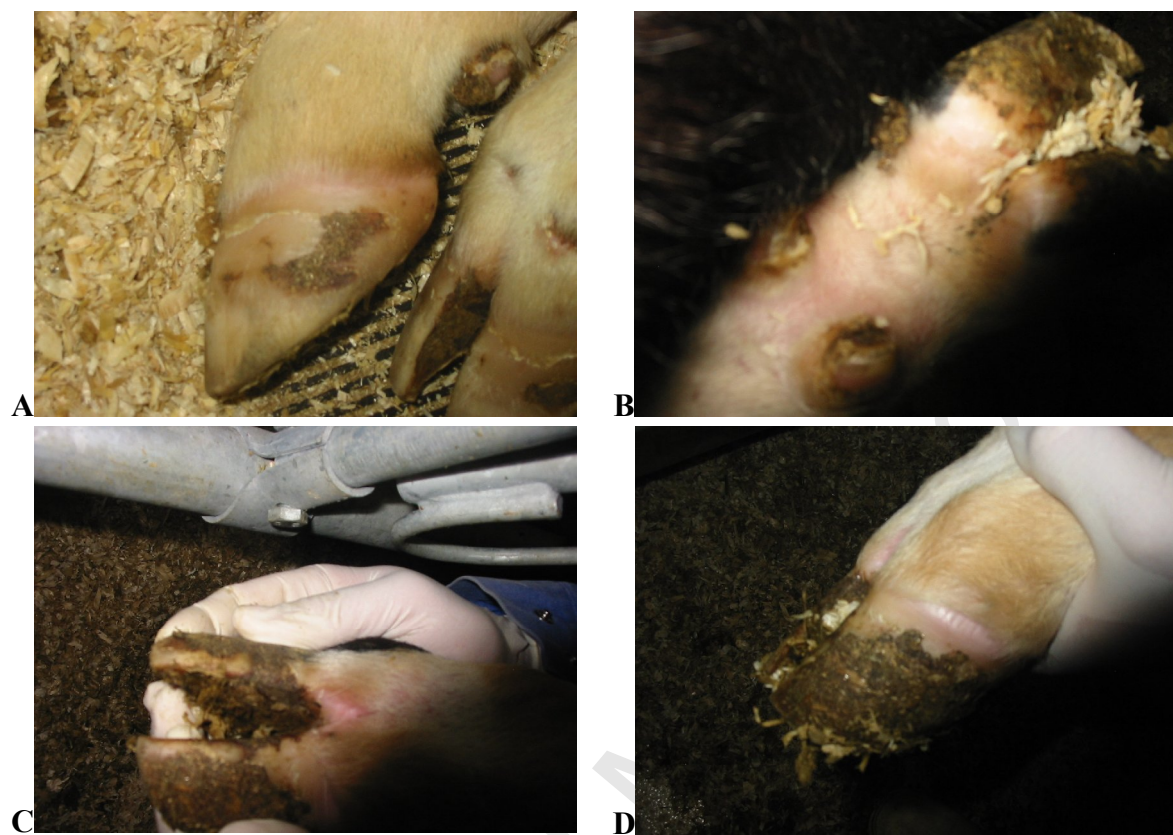


Figure 2



Figure 3



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TABLE 1: Clinical scores applied to BTV-8 induced lesions observed in calf 3179 and 3181.

	DAYS POST-INFECTION														TOTAL SCORE	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13		14
BODY TEMPERATURE ^a																
3179	0	2	0	0	0	0	0	0	0	1	1	0	0	0	0	4
3181	0	1	0	0	1	0	0	1	2	1	0	0	0	0	0	6
OCULAR SIGNS ^b																
3179	0	1	1	2	2	2	1	1	1	0	0	0	1	2	1	15
3181	0	0	0	0	1	1	1	1	1	1	2	2	0	0	0	10
ORAL LESIONS ^c																
3179	0	0	0	0	3	3	3	3	3	1	3	3	3	3	3	31
3181	0	0	0	0	0	1	1	1	1	1	1	3	3	3	3	18
LESIONS TO THE LOCOMOTOR SYSTEM ^d																
3179	0	0	0	0	0	1	1	1	2	2	2	2	2	2	2	17
3181	0	0	0	0	0	0	0	1	1	1	2	2	2	2	2	13
OEDEMA ^e																
3179	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	9
3181	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	9
TOTAL CLINICAL SCORE ^f																
3179	0	3	1	2	5	6	6	6	7	5	7	6	7	8	7	76
3181	0	1	0	0	2	2	3	5	6	5	6	8	6	6	6	56

^a Body temperature score: 0 (body temperature $\leq 39.5^{\circ}\text{C}$), 1 (body temperature $\geq 39.6^{\circ}\text{C} \leq 39.9^{\circ}\text{C}$), 2 (body temperature $\geq 40^{\circ}\text{C} < 41^{\circ}\text{C}$), and 3 (body temperature $> 41^{\circ}\text{C}$).

^b Ocular lesions score: 0 (absence of ocular clinical signs), 1 (ocular discharge), 2 (conjunctivitis was contemporarily observed), and 3 (with complicated ocular dermatitis).

^c Oral lesions score: 0 (absence of clinical signs), 1 (mucosal congestion), 2 (presence of one oral erosion or one ulcer), and 3 (multiple erosions or ulcers). In the presence of a scab as a consequence of the healing process, a score of 1 was attributed.

^d Locomotor system score: 0 (absence of clinical signs), 1 (congestion of the coronary band), 2 (congestion and oedema), and 3 (lameness).

^e Oedema score: 0 (absence of oedemas), 1 (1 oedema), (2 with 2 oedemas), and 3 (multiple oedemas localized in different regions).

^f Total daily clinical score.

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