"Ormilo disease" a disorder of zebu cattle in Tanzania: bovine cerebral theileriosis or new protozoan disease?

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Abstract

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33 "Ormilo" disease is a neurological disorder of cattle described by Maasai herders in Tanzania. It is attributed to

34 infection by *Theileria* species, although no detailed data are available in the literature.

35 The authors describe the macroscopical and histological changes observed in 30 brains of indigenous short-horn zebu

36 cattle from Northern Tanzania, aged 2-9 years, with the characteristic neurological signs of "Ormilo". Moreover the

37 ultrastructural details observed in 14 selected brains samples were reported. Areas of congestion and hemorrhages,

38 associated with the obstruction of the cerebral vessels with large numbers of parasitized lymphoid cells, were observed.

39 Electron microscopy showed the presence of intralymphocytic parasites morphologically comparable to flagellated

40 protozoa, not previously described in the lymphoid cells of cattle, but only reported during the sexual stages within the

41 vector. *Theileria taurotragi* was detected by Polymerase Chain Reaction and Reverse Line Blot in 9 samples. The

42 authors hypothesize that the parasite detected by electron microscopy could be a strain of a *Theileria* endemic to this

43 region till now not investigated, having an intralymphocytic phase and being associated with other *Theileria* spp.

44 infestation. Further studies are needed to better understand the etiology of "Ormilo" disease and to characterize the

45 morphology of the observed parasite, clarifying its role in the disease in Tanzania.

46

47 **Key words**: Bovine Cerebral Theileriosis, Ormilo, pathology, short-horn zebu cattle, Tanzania, Theileria.

48

49 **Introduction**

50 Theileriae are tick-transmitted intracellular apicomplexan parasites, which infect a wide range of mammals worldwide

51 (Norval et al. 1992; Dobbelaere and McKeever 2002). After the tick bite, *Theileria* sporozoites invade lymphoid cells of

52 the vertebrate host and induce them to proliferate in an unregulated manner. The cells develop into a multinucleate

53 syncytial schizont (macroschizont), later in microschizonts that differentiate into uninucleate merozoites, that are

54 liberated into the blood where they invade the erythrocytes and become piroplasms (Fig. 1).

55 Several species of *Theileria* are known. *Theileria parva* and *Theileria annulata* are considered the most important

56 pathogens of domestic livestock in the Old World tropical and subtropical regions. *Theileria taurotragi* was first

57 described in 1960 as a benign parasite of the eland (Martin and Brocklesby 1960). Subsequently, in cattle it was

58 demonstrated that this parasite causes a benign parasitosis or fatal infections (De Vos et al. 1981), frequently associated

59 with concurrent presence of *Theileria mutans* and/or *T. parva* (Young et al. 1977; Uilenberg et al. 1982).

60 *T. parva, T. taurotragi,* and more rarely *T. annulata* are considered causing agents of the Bovine Cerebral Theileriosis

61 (BCT) (De Vos et al.1981; Saville 2002; Sudan et al. 2012). BCT is described in tropical and subtropical African

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regions (Mettam et al.1936; Flanagan et al.1957), but more recently two cases caused by *T. annulata* were reported in Turkey (Dabak et al. 2004) and in India (Sudan et al. 2012).

BCT, or Turning sickness, is a fatal disease of indigenous African short-horn cattle of cattle. It usually occurs in young animals and results in a mild febrile reaction of 1-14 days duration, after an incubation period of 10-21 days. Slight enlargement of the superficial lymph nodes is present, but no other clinical signs are reported. The nervous form of BCT is characterized by uncontrollable movements, sometimes unilateral-bilateral blindness (corneal opacity), circling, head pressing, ataxia, opisthotonous and paralysis. Usually the animals collapse after 2-21 days, but occasionally the cases become chronic and they may live for up to 6 months (Van Amstel 1982; Lawrence et al. 2004). The neuropathological changes include congestion and hemorrhages in the meninges and in the brain, and subacute-chronic areas of malacia. Microscopically, the obstruction of the arteries with a large numbers of parasitized lymphoblasts is the most prominent finding (Bader et al. 1986; Lawrence et al. 2004).

Cases of disease in cattle similar to BCT have been reported from Maasai herders in northern Tanzania (Arusha, Manyara and Tanga regions) since the mid-1980's (Field et al. 1988; Nsengwa 1993). Although its incidence has been increasing, especially in northern Maasai land, detailed pathological studies have never been documented. Local Maasai livestock keepers call the disease "Ormilo" and, from information gathered during a rapid rural in 2001, they consider it as the highest disease priority and, thus, as a severe constraint to livestock production (Lynen, unpublished data).

This paper reports the macroscopical and histological changes observed in 30 brains of indigenous short-horn zebu cattle, ranging from to 2 to 9 years of age, living in Northen Tanzania (Arusha Region, Ngorongoro District, Endulen ward), that presented neurological signs characteristic of "Ormilo". Ultrastructural features and Polymerase Chain Reaction (PCR) investigations are also described.

Materials and methods

The brains of 30 East-African short-horn Zebu cattle aging from 2 to 9 years old, were collected between 2001 and 2003 in Endulen, Ngorongoro Conservation Area, Arusha region, north-eastern Tanzania. In this area, mixed herds of small ruminants and cattle are kept under a traditional pastoralist livestock system.

These animals were selected according to the clinical description by Maasai herders of Endulen ward confirmed by the veterinarians of the Integrated Tick and Tick-borne Disease Control Project. The most frequently reported clinical signs was circling, associated with para-paresis and paralysis, generally followed by recumbency. In one animal, difficulties in chewing and walking were also present. Except in one case, found dead, all the other animals were slaughtered due to the worsening of their clinical signs.

92 Immediately after death/slaughter, the brains were fixed in 10% neutral buffered formalin and sent to the Department of

93 Veterinary Science of Torino University to perform pathological investigations. Not all the collected samples were

94 suitable for ultrastructural investigations (TEM) due to conservation problems. Therefore, meningeal blood vessels,

95 choroid plexuses and thrombosed brain vessels of 14 animals were fixed in 2.5% glutaraldehyde for TEM.

96 Tissues were postfixed in 1% osmium tetroxide, deydrated in acetone and embedded in Spurr epoxy resin. Semithin

97 sections (0.90 μm) were cut and stained with toluidine blue. Ultrathin sections of 70 nm were then collected onto 200

98 mesh copper grids and contrasted by uranyl acetate and lead citrate. The grids were evaluated by Electron Microscopy

99 109 JD.

100 Brain tissues from the same 14 animals were also subjected to DNA extraction with the DNeasy tissue kit (QIAGEN,

101 Hilden, Germany). PCR was carried out to amplify the V4 hyper variable region of the 18S rRNA gene of *Theileria* and

102 *Babesia* spp. Species identification was carried out by reverse line blot (RLB) hybridization of the amplified products

103 on a membrane containing oligonucleotides specific for six bovine *Theileria* species and two *Babesia* spp. and a catch-

104 all *Theileria/Babesia* control probe, according to the protocol previously described (Nijhof et al. 2003).

105

106 **Results**

107 Macroscopically, moderate to severe congestion of the leptomeningeal blood vessels, disseminated sub-meningeal

108 hemorrhages and/or multifocal to diffuse hemorrhages into the brain tissue (Fig. 2a, b, c), disseminated or focal areas of

109 sub-acute/chronic malacia were the most important observed features (Fig. 2d). Histological investigations revealed

110 congestions/hemorrhages of the meningeal vessels, frequently thrombosed and necrotic, hemorrhages and

111 plasmorrhages around the ventricles and especially in the choroid plexuses (Fig. 2e), and focal to multifocal necrotic

112 areas. Severe accumulation of mononuclear cells in the cerebral and meningeal blood vessels was the most significant

113 lesion observed (Fig. 2f). Morphologically large lymphoblastic cells predominated, including sometimes small and

114 medium sized lymphocytes, rare plasma cells and macrophages. Most lymphocytic cells showed a variable number of

115 the schizonts in the cytoplasm. The main involved areas were the cortical meninges and the rostral brainstem, while the

116 pons and medulla were rarely affected by meningeal and parenchymal hemorrhages only.

117 Ultrastructurally, all the analyzed blood vessels revealed the presence of cytoplasmatic irregular structures,

118 morphologically similar to parasitic forms, in lymphoid cells. The structures observed were considered to be free

119 schizonts in the cytoplasm of the host cells whose nuclei were arranged at their periphery. Moreover, numerous free

120 merozoites were present in the cytoplasm of lymphoid cells and in the lumen of the blood vessel. In all cases, such

121 merozoites showed a flagellum and one cytoplasmatic vacuole containing a small spherical electrondense structure.

122 These morphological data allowed the authors to propose a developing cycle of the parasite in the lymphoid cells of the

123 cattle as represented in the Fig. 3 (a - f). The first stage observed was a young schizont - spherical in shape, electron

124 dense, with diameters of about 7-9μm characterized by spherical dividing nuclei, numerous mitochondria and many

125 spherical organelles such as vacuoles (Fig 3a). Later the schizont appears as a spherical vesicle with 2-3 nuclei located

126 at the periphery of the cytoplasm, numerous vacuoles and some merozoites. The merozoites are spherical or pear-

127 shaped, measure about 1.6μm in diameter and show some micronemes, vacuoles and numerous microtubules (Fig. 3b).

128 In some cases the microtubules are observed in cross section in both poles of the merozoites, in other cases they are

129 longitudinally sectioned and organized to form a flagellum which is located at the apical pole or sometimes crossing the

130 whole merozoite from one pole to the other. In the following stage the schizont appears as an electron dense vesicle

131 containing only few mithocondria and vacuoles, whereas the merozoites, measuring about 2.1-2.2 μm in diameter,

132 invade the cytoplasm of the host cell (Fig. 3c). Subsequently the schizont seems to degenerate and the merozoites

133 escape from the host cell and are observed free into the lumen of the blood vessel. The merozoites free in the blood

134 measure from about 1.8x2.5 to 3.5x3.7μm and show numerous microtubules and a clear flagellum-like structure. The

135 medium length of these merozoites is of about 4 μm (Fig. 3d, e, f).

136 Nine DNA extracts from brain tissues were positive to the PCR and specifically hybridised with the *T. taurotragi*

137 oligonucleotide on the RLB membrane (Fig. 4, samples 10 to 14, 34 and 37 to 39). The remaining five samples were

138 negative (samples 9,15,16,35,36).

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140 **Discussion**

141 Despite the limited information available, the clinical signs reported by Maasai herders in the cattle of the present study

142 are similar to those described in the literature in cases of BCT (Van Amstel 1982; Lawrence et al. 2004). Contrary to

143 what has generally been observed in BCT, several cases of the present study (n=13 animals) were older than 5 years,

144 reaching 8 and 9 years of age in 6 cases.

145 Macroscopical and histological lesions resemble those reported by other authors in BCT caused by *Theileria* species

146 (Giles et al.1978; Bader et al. 1986; Lawrence et al. 2004), and particularly the accumulation of parasitized lymphoid

147 cells in the cerebral and meningeal blood vessels represents the pathognomonic feature of these neurological cases as

148 reported in the cerebral form of theileriosis (Giles et al.1978; Bader et al. 1986; Lawrence et al. 2004).

149 Ultrastructurally, most of the knowledge on developmental stages in the tick and mammalian host cells of theileriae

150 species comes from studies on *T. parva* and, to a lesser extent, *T. annulata*. In the authors' opinion, the morphology of

151 developmental stages of *T. taurotragi* in lymphoid cells is still unknown. Shein and colleagues accurately described by

152 means of electron microscopy, the morphology of the four most important *Theileriae* spp. affecting bovine species (*T.*

153 *parva, T. annulata, T. mutans,* and *T. lawrencei*) during the schizogony (Schein et al. 1978). These authors evaluated

154 the development of a young schizont, spherical in shape with diameters of about 2μm converting into a multinucleate

155 mature schizont (20-50 nuclei) that produces merozoites. The latter, pear-shaped, show a length of about 1 μm and a

156 diameter of 0.6 μm; they show an apical pole where is present a polar ring system at which microtubules are anchored,

157 3-4 rhoptries, some micronemes and numerous mitochondria. This development destroys the host cell, the merozoites

158 become free and in a position to infect the next host cell, the erythrocyte. Inside the erythrocyte the piroplasms are

159 spherical and variable in size; they may measure from 1.0 to 1.5 μm in *T. parva*, they can reach up to 2.5 μm in *T.*

160 *mutans* and *T. annulata,* and are even larger in *T. taurotragi* and *T. orientalis* (Schein et al. 1978). When infected

161 erythrocytes are ingested by the tick, lysis of the erythrocytes occurs and many free piroplasms can be seen in gut

162 smears of the ticks. A variable proportion of piroplasms undergo development into sexual stages. In the tick gut, ray

163 bodies are formed by development of ovoid or spherical intra-erythrocytic stages (Mehlhorn and Schein 1984). The ray

164 bodies are spindle-shaped, measuring 8-12 μm in length with a diameter of 0.8 μm. These parasites, in cross section,

165 could show up to four nuclei and up to four flagella-like protrusions with up to 14 tubules within such protrusions.

166 The samples described in the present paper show mature schizonts in the host cells comparable to those described in the

167 literature in the *Theileria* species cycle. However, they measure 7-9 μm in diameter compared to 2-10 μm reported by

168 Schein et al. (1978). The merozoites observed in the schizont are relatively bigger (about 1.6μm in diameter) compared

169 to the same described by Shein and coll (Schein et al. 1978) (1 x 0.6 μm). The dimensions of the merozoites free in the

170 cytoplasm of the lymphoid cells and in the blood vessels are not reported in the literature and are thus not comparable.

171 The final structure of the merozoites observed in the present paper is very similar to that described in the literature.

172 They are pear-shaped and limited by a cell membrane. At the apical pole they show two inner membranes which form a

173 polar ring system at which subpellicular microtubules are anchored. Several rhoptries and few micronemes and

174 vacuoles are present. Particularly one cytoplasmatic vacuole is always present containing a spherical structure strongly

175 electrondense (with a diameter of about 0.40μm). Moreover, it is interesting to point out that the merozoites showed in

176 the schizonts or free in the vessels, always show an evident flagellum-like protrusion so far not described in the

177 literature in these phase of the life cycle. This flagellum seems to start from the pole opposite to the apical one;

178 sometimes the microtubules forming the flagellum seem to cross the whole merozoite. Some merozoites show clear

179 microtubules in cross sections in both the poles. A comparable morphology, characterized by the presence of numerous

180 microtubules and flagella-like structures was reported in details only during the sexual stages within the tick gut (Young

181 et al. 1980; Norval et al. 1992), but nobody described it in the final host such as the lymphoid cells of the cattle. These

182 flagellated protozoa were observed in all samples.

183 Out of 14 tissue samples tested by molecular assays, nine samples were positive and *T. taurotragi* was detected as the

184 unique infectious agent by RLB. Our samples belong to a larger sample of 78 tissues (brain, lymph nodes, spleen) from

185 suspected Ormilo cases from Arusha region tested at the Division of Parasitology and Tropical Veterinary Medicine,

186 Utrecht University, by PCR/RLB. Overall, 59% of the processed samples hybridized exclusively with the *T. taurotragi*

187 oligonucleotide. None hybridized with the *T. parva* oligonucleotide, thereby convincingly excluding *T. parva* as causal

188 agent of the cerebral theileriosis in the study area (Lynen et al., unpublished data).

189 The neurological signs observed and the neuropathological lesions characteristic of cerebral theileriosis, led the authors

190 to speculate that *Theileria* spp. could be considered the possible etiologic agent in these "Ormilo" disease cases.

191 Based on the electron microscopy observations, the morphology of the schizonts and the merozoites observed during

192 schizogony is partially different from the morphology of the *Theileria* species described in the literature. Moreover the

193 absence of intra-erithrocitic parasites, not even when numerous merozoites are demonstrated free in the blood, leads the

194 authors to assume that the whole cycle of this parasite is different from the one of the *Theileria* species reported in the

195 literature (*T. parva, T. annulata, T. mutans* and *T. lawrencei*) (Schein et al., 1978). On the basis of the PCR/RLB results

196 *T. taurotragi* could be hypothesized the etiological agent of "Ormilo" cases.

197   It is also possible that the observed parasites belong to a different strain of *Theileria* genus endemic to this region till

198 now not investigated, with a development cycle different from what is supposed for other species characterized by a

199 distinct morphology. In this case, it is to be assumed that additional factors could determine the simultaneous

200 involvement of the usually benign *T. taurotragi* (Martin and Brocklesby 1960; Young et al. 1977; Grootenhuis 1979;

201 Uilenberg et al. 1982*)* in the cerebral theileriosis pathogenesis, such as the impairment of the immunity by viruses or

202 other concomitant tick-borne infections or heavy tick infestations. Simultaneous occurrence of different tick-borne

203 pathogens within the same host is in fact a frequent feature, but little is known about their possible interactions. Other

204 contributing factors considered for BCT are parasitoses like trypanosomiasis in Kenya (Moll et al. 1985), or

205 *Amblyomma variegatum* tick infestation (Lloyd and Walker 1993).

206 As the role of the observed parasite in the "Ormilo" syndrome is at the moment unknown, further studies are needed to

207 better understand its morphology and its potential pathogenic importance.

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Conflict of interest statement

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Figure captions

311 **Fig. 1** Generalized representation of the typical life cycle of the genus Theileria. Sporozoites are inoculated into a

312 mammalian host when an infected tick takes a meal. The sporozoites invade lymphoid cells, and develop into a

313 multinucleate syncytial schizont or macroschizont (**a**). At the same time, the parasite induces host cell transformation

314 and proliferation (**b**). Then the schizonts differentiates into merozoites and invade erythrocytes (**c**). Ticks become

315 infected by ingesting infected erythrocytes, and gametogenesis and fertilization takes place in the gut lumen (**d**). The

316 resulting zygote invades a gut epithelial cell where it remains during the tick moult cycle and develops into a single

317 motile kinete (**e**). The motile kinete egresses the gut cell and invades the salivary glands (**f**). Tick initiates rapid

318 sporozoite development in the salivary glands, and infective sporozoites that survive in the gut epithelium are

319 transmitted to another mammalian host when the resulting post-moult nymphs or adults feed (**g**) Modified from

320 http://www.theileria.org/ahdw/background.htm [accessed 27-02-2015]

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322 **Fig. 2 a** Multifocal congestion of the meningeal blood vessels covering cerebral hemispheres and cerebellum.

323 Hemorrhages surrounding the brainstem and the cervical spinal cord. **b** Severe hemorrhages involving the

324 leptomeninges, the IV ventricle, the central canal and the surrounding areas. **c** Punctiform hemorrhages in the cortical

325 white matter. **d** Focus of chronic malacia in the capsula interna area. **e** Cerebellum: hemorrhages involving the

326 leptomeninges and severe congestion of the meningeal vessels. Multifocal hemorrhages and plasmorrhages in the

327 nervous tissue around the IV ventricle (H&E). **f** Severe accumulation of mononuclear cells in the meningeal blood

328 vessels many of them showing irregular round to ovoidal bodies in the cytoplasms corresponding to parasitic schizonts

329 (H&E). (e bar=100 μm, f bar=50 μm)

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331 **Fig. 3** Electron microscopy of bovine lymphoid cells with endocellular parasites (**a – f**). **a** Lymphoid cells containing a

332 young schizont (arrow) spherical in shape, electrondense, characterized by spherical dividing nuclei, numerous

333 mitochondria and many spherical organelles such as vacuoles. **b** Lymphoid cells with the cytoplasm occupied by a

334 voluminous schizont (arrow) with two evident nuclei located at the periphery (#), numerous vacuoles and some

335 spherical or pear-shaped merozoites (asterisks). **c** In this following stage the schizont (arrow) appears as an electron

336 dense vesicle containing only some mitochondria and few vacuoles whereas the merozoites (asterisks) invade the

337 cytoplasm of the host cell. **d** Merozoites free into the lumen of the blood vessel. **e, f** High magnification of the

338 merozoites free into the vessels. They measure from about 1.8x2.5 to 3.5x3.7μm. At the apical pole the merozoites

339 show a polar ring system at which subpellicular microtubules are anchored to form a clear flagellum-like structure

340 (asterisk). Few rhoptries (long arrows) and micronemes (short arrows) and vacuoles are present. Scale bar 1µm

341 (**a,b,c,d**); 0.2µm (**e,f**)

342

343 **Fig. 4** Reverse Line Blot results showing species-specific oligonucleotides of the 18S rRNA gene of *Theileria* spp. in

344 the horizontal lanes and PCR products in the vertical lanes. Fourteen brain tissue samples from Ormilo cases from

345 Endulen were analyzed (vertical lanes: 9 to 16 and 34 to 39). Positive *Theileria* spp. and *Babesia spp.* controls (line 1:

346 *T. parva*, 2: *T. taurotragi*, 3: *T. buffeli*, 4: *T. mutans*, 5: *B. bovis*, 6: *B. bigemina*, 7: *T. annulata*) as well as a negative

347 control (water, line 8) are included. Other vertical lanes (17 to 33 and 40) refer to amplified DNA of tissue samples

348 collected by the authors in other study areas in Arusha region.

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