Immunohistological Staining for Detection of *Theileria sergenti* Schizonts

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Bovine theileriosis in Japan is a tick-borne disease of cattle caused by the protozoan parasite, *Theileria sergenti* [7]. The disease has been recognized to be one of the major constraints to the development of livestock industry. The life cycles of *T. parva* and *T. annulata*, other two *Theileria* species pathogenic for cattle, are characterized by two stages, a schizont stage within the lymphoid organs and a piroplasm stage within erythrocytes [2, 6]. Recently, the exoerythrocytic forms of *T. sergenti* were also demonstrated in lymph node aspirates of experimentally infected calves [8]. However, it is very difficult to distinguish the schizonts in specimens from infected lymph nodes by Giemsa's stain, because only a few organisms are present in the node and their extracellular location further complicates their detection from the smear preparations used for a routine examination [8]. In the present study, therefore, the suitability of two immunohistological staining methods to demonstrate the schizont stage of *T. sergenti* in infected lymph nodes was examined and compared.

The Ikeda strain of *T. sergenti* was used. It was isolated by the authors from naturally infected cattle in Tochigi prefecture in 1983 [1]. It has been maintained in our laboratory by transmission through splenectomized calves and a *Haemaphysalis* tick colony. The parthenogenetic Okayama strain of *Haemaphysalis longicornis* was used. *H. longicornis* has been recognized as a vector of *T. sergenti* in Japan [1]. The procedure of breeding and feeding of *H. longicornis* for infection with *T. sergenti* was described previously [1]. Three healthy Holstein calves aged 3–5 months were used for this study. For preparation of antiserum, one calf was splenectomized and infected by the feeding of 200 infected ticks with *T. sergenti*. Other two intact calves were used for the detection of schizonts. A *T. sergenti* infection was induced in one of these calves by the feeding of 243 infected *H. longicornis* ticks on it, and the other calf was infested with 176 uninfected ticks as a control. Infected or uninfected *H. longicornis* ticks were applied to the ears of calves according to the method reported previously [3]. After tick infestation, blood smears were taken daily from the calves, stained with Giemsa and examined for *T. sergenti* intraerythrocytic piroplasms.

The two calves infested by either infected or uninfected ticks were killed 10 days after tick infestation. The parotid lymph nodes proximal to the tick infested ear were taken from each calf, and smears were prepared for detection of schizonts. Some of the smears were stained with Giemsa.

Other smears were fixed in chilled acetone for 10 minutes and then stored at -20°C until used for the immunohistological stainings.

The serum used for preparation of labelled antibodies was collected from the experimentally infected splenectomized calf on the 47th day of infection, when the second peak of parasitaemia was recorded. The indirect immunofluorescent antibody titer of the serum was 1:25,000. The crude immunoglobulin (Ig) fraction was obtained by precipitation of globulins from the serum by adding ammonium sulfate up to one third saturation. IgG was then purified from the crude Ig fraction by ion-exchange chromatography on DEAE-cellulose (Whatman DE-52; Springfield Mill, Maidstone, Kent, England) equilibrated with 0.04 M phosphate buffer, pH 6.3. Isolated IgG was divided into two parts. One part was labelled with fluorescein isothiocyanate (FITC; Fluka Co., Switzerland) and the other part with horseradish peroxidase (HRP; type VI, Sigma Chemical Co., St. Louis, Mo, U.S.A.). The FITC labelling was performed as described by Kawamura [4]. The conjugation of HRP with IgG was done in the same method as described by Nakane and Kawaoi [9]. Briefly, 10 mg of periodate-oxidized peroxidase was conjugated with an equal weight of IgG and the conjugate was stabilized by reduction with sodium borohydride.

The acetone fixed smears were subjected to direct immunofluorescent antibody (DFA) staining, essentially as described by Kawamura [5]. Briefly, FITC conjugated infected calf IgG was diluted to 1:50 in phosphate buffered saline (PBS), pH 7.2, containing 0.4% bovine serum albumin, and was permitted to react for 1 hr with the smears before washing in PBS. After washing, smears were mounted in glycerol phosphate buffer, pH 8.0, and examined with a fluorescence microscope (Fluophot, Microphot V Series, Nikon Co., Ltd., Japan). The dilution of the HRP-conjugate and the staining procedure used for direct immunoperoxidase (DPO) staining were almost the same as used for DFA staining except for some steps. Namely, the smears were permitted to react for 5 min with the protein blocking agent (Lipshaw Co., Ltd., U.S.A.) before conjugate application. And for the last step, the antigen-conjugate complex was allowed to react for 20 min to the substrate, and then counterstained with 1.0% methyl green solution. The substrate used for DPO staining was 0.1% hydrogen peroxide with the addition of 0.05% diaminobenzidine (Wako Pure Chemical Industries, Ltd., Japan) in Tris buffer, pH 7.6.

It consumed much of time and required skill to detect schizonts of *T. sergenti* in Giemsa stained smears, because

of their extracellular location and apparent resemblance to circumferential host tissues. Schizonts of *T. sergenti* detected in a routine examination of Giemsa stained smears were, therefore, few in number. In contrast, detection of schizonts by both of the immunohistological staining methods was easier as compared with that by Giemsa staining, and schizonts were detected as specific reactions in many smears examined with those methods. Despite the strong DFA or DPO reactions to schizonts in infected specimens, control smears from the uninfected calf, stained to confirm the specificities of both conjugates,

showed complete absence of specific reactions.

Figure 1-A shows the appearance of a schizont under low magnification ($\times 500$) in a smear from the parotid lymph node of the infected calf after staining by the DFA method. The schizont exhibited a brilliant green fluorescence which could be readily differentiated from the weakly fluorescing lymphocytes. It was not possible, however, to see details of the internal structures of the schizont using this method of staining. Figure 1-B shows the appearance under low magnification ($\times 500$) of a schizont in a smear from the infected parotid lymph node

Fig. 1. *T. sergenti* schizonts and micromerozoites stained by DFA and DPO staining methods.
 A: Schizont stained by the DFA method was clearly seen adjacent to the weakly fluorescing lymphoid tissue ($\times 500$).
 B and C: Schizonts stained by DPO method were clearly differentiated as brown masses and retained the structural characteristic (B: $\times 500$, C: $\times 1,250$).
 D: Micromerozoites stained by the DPO method were seen in cluster. The parasite cell wall and nucleus retained the fine structure ($\times 1,250$).

stained with the DPO method, and another schizont under high magnification ($\times 1,250$) is shown in Fig. 1-C. The schizonts, which were seen as brown masses with many diffuse nuclei, were situated extracellularly.

The structural characteristics of the schizonts revealed by the DPO method were the same as those recognized in Giemsa stained smears. Furthermore, these schizonts were also similar to those described in *T. orientalis* [12] and in *T. buffeli* [11]. Figure 1-D shows the appearance under high magnification ($\times 1,250$) of micromerozoites in a smear from the infected parotid lymph node stained by the DPO method. The parasite cell wall and nucleus were generally distinctly stained, and retained their fine structures.

In contrast to the results obtained using Giemsa staining, schizonts and micromerozoites were readily detected using both DFA and DPO staining methods. In both immunohistological staining methods, schizonts exhibited specific reactions which could be readily differentiated from bovine lymphoid tissue. However, the intensity of fluorescence sometimes makes it difficult to evaluate the morphology of microorganisms [10]. In the present experiment, the internal structures of schizonts were not revealed by DFA staining. The intensity of the fluorescence using this method allowed only a less distinct demonstration of the fine structure.

The sensitivity for both FITC and HRP conjugates used in this study was the same. The demonstration of the internal structures of schizonts by the DPO method, however, seems to be clearer than by the DFA method. The DPO staining method should be an excellent aid in further studies on the mammalian schizogonic stage of *T. sergenti*.

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