

Detection of Antibodies against *Pasteurella multocida* Using Immunohistochemical Staining in an Outbreak of Rabbit Pasteurellosis

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(Received 15 June 2000/Accepted 11 October 2000)

ABSTRACT. To detect serum antibody against *Pasteurella multocida* (*P. multocida*) in infected rabbits, a modified immunoperoxidase assay was applied. An outbreak of *P. multocida* infection in rabbits started from sudden death. The infected rabbits had severe fibrinous and purulent pneumonia with hemorrhage, and a large number of *P. multocida* (A:12) was isolated from the trachea and lungs of the animals. Antibodies of IgM and IgG to *P. multocida* were assessed by immunohistochemical staining using the sera of the animals as primary antibodies and applying them to formalin-fixed, paraffin-embedded sections of *P. multocida* attached to calf fibrin. IgM antibodies to *P. multocida* were first detected 7 days after the onset of the disease. IgG antibodies began to rise on the 7th or 14th day. These results suggested that the modified immunoperoxidase assay could detect antibodies against *P. multocida*.

KEY WORDS: antibody, *Pasteurella multocida*, rabbit.

J. Vet. Med. Sci. 63(2): 171–174, 2001

It is well known that *Pasteurella multocida* (*P. multocida*) is an important causal agent of snuffles, pneumonia, otitis media, abscess formation and conjunctivitis in rabbits [4, 8, 9], and these diseases are prevalent in rabbit colonies throughout the world. The clinical and pathological features of the diseases have been reported previously by many researchers [7–9]. However, there is little information on time course change of IgM and IgG antibody responses in rabbits naturally infected with *P. multocida*. The authors have previously reported a useful immunoperoxidase assay for detection of serum antibodies of a few infectious diseases [18, 19]. To determine the relationship between infection and development of class-specific antibody responses, we report an application of immunohistochemical staining to detect IgM and IgG antibodies in rabbits infected with *P. multocida*.

From December 1998 to January 1999, an outbreak of *P. multocida* infection was seen in 40 New Zealand White rabbits kept in a park. Two young rabbits (nos. 1 and 2) died suddenly, and then 3 to 10 days later, three more (nos. 3–5) died. Four weeks after the initial death (nos. 1 and 2), one adult female with poor health (no. 6) was euthanized with an intravenous injection of pentobarbital sodium and necropsied. The initial clinical sign of the disease was a high body temperature (up to 40°C), which was commonly accompanied by depression and anorexia. Rabbits that survived the infection had severe respiratory signs consisting of snuffling, coughing and fever.

For bacterial examination, samples of the liver, spleen, kidneys, heart, lungs and trachea from six rabbits (nos. 1–6) were collected and cultured on plates of 5% sheep blood agar, chocolate agar, desoxycholate hydrogen sulfide agar and mannitol salt agar at 37°C under aerobic conditions for 48–72 hr. In the cultures of the trachea and lungs, 1.5×10^5 colony forming units per gram (cfu/g) and 7.5×10^6 cfu/g of *P. multocida*, respectively, were counted in the six rabbits.

In the cultures of the liver, spleen, kidneys and heart, no bacteria were isolated. Isolation and identification of *P. multocida* were performed as described previously [13]. The isolated *P. multocida* demonstrated capsular A antigen by indirect hemagglutination (IHA) test [1] and somatic antigen that represented major reaction with type 12 antiserum by gel diffusion precipitin (GDP) test [10].

For histopathological examination, organs and tissues from six rabbits (nos. 1–6) were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Sections were cut at 5 µm and stained with hematoxylin and eosin (HE). Microscopically, mild purulent bronchitis, bronchiolitis and alveolitis with hemorrhage, which corresponded to the initial stage of the disease, were observed in nos. 1 and 2. Nos. 5 and 6 showed severe fibrinopurulent pneumonia, where alveoli were filled with neutrophils, macrophages and lymphocytes with necrotic foci surrounded by fibroblasts. Severe tracheitis with hemorrhage and severe lymphadenitis in the bronchial and mediastinal lymph nodes were observed in rabbits nos. 1–3 (Table 1).

For serological examination, serum samples were collected at intervals of 7 days from five rabbits with respiratory signs (nos. 6–10). To assess serum IgM and IgG titers to *P. multocida*, immunohistochemical staining was per-

Table 1. Histological lesions in *P. multocida*-infected rabbits

Tissue	Histological lesion in rabbit no.					
	1	2	3	4	5	6
Trachea	2+	3+	2+	+	+	–
Lung	2+	2+	3+	3+	2+	2+
Lymph nodes	2+	3+	2+	+	+	–

Histological lesion: –, negative; +, slight; 2+, moderate; 3+, severe.

Rabbit nos. 1 and 2 died suddenly without clinical signs.

Rabbit nos. 3, 4 and 5 died within three, six and ten days after the onset of the disease, respectively.

Rabbit no. 6 was euthanized at four weeks after the onset of the disease.

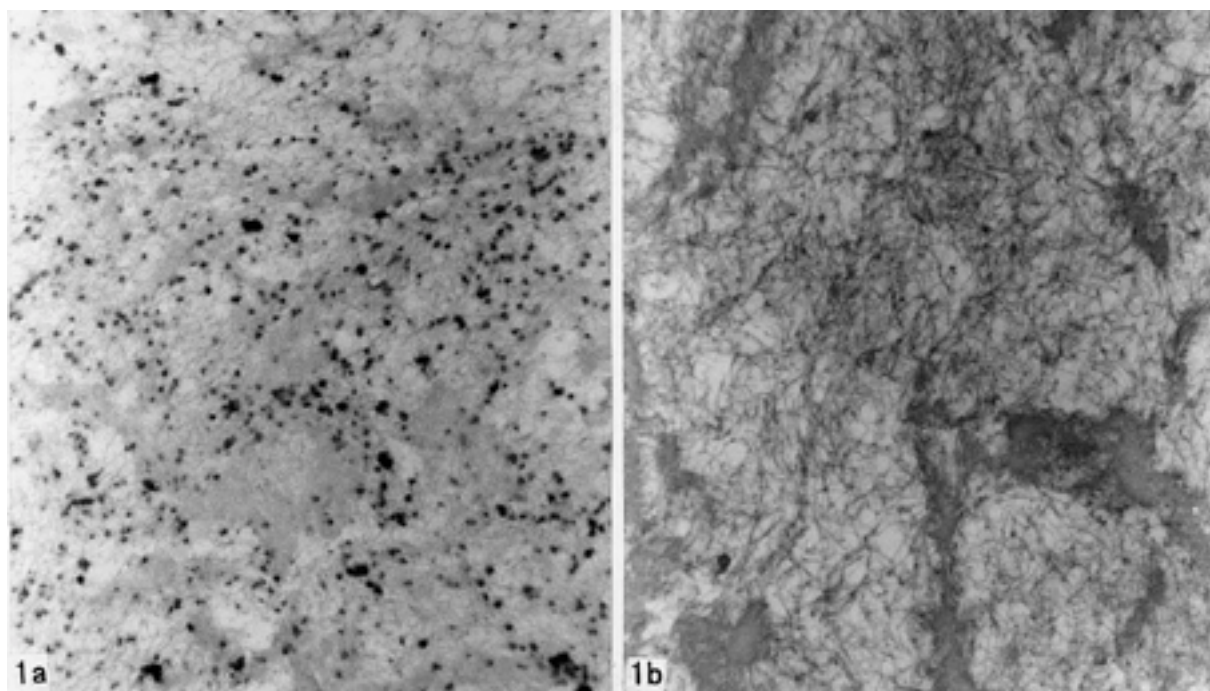


Fig. 1a. Immunohistochemical staining for *P. multocida* attached to calf fibrin. *P. multocida* attached to the fibrin reacted intensely to test serum. A dilution of biotinylated goat anti-rabbit IgM was used as the secondary antibody. The dilution of the test serum from rabbit no. 6 on 14 days after the onset of the disease was 1:800. ABC stain, $\times 720$.

Fig. 1b. Immunohistochemical staining of normal calf fibrin. No positive reaction was observed in the fibrin with test serum. The serum was the same sample as that in Fig. 1a. ABC stain, $\times 720$.

formed as described previously [18]. Briefly, *P. multocida* isolated from rabbit no. 1 was attached to calf fibrin, fixed in 10% neutral-buffered formalin, embedded in paraffin wax and sectioned. The sera from rabbits nos. 6–10 were applied to the sections as primary antibodies and binding to the antigen (*P. multocida*) was detected by ABC method. A dilution of biotinylated goat anti-rabbit IgM or IgG (Southern Biotechnology Associates Inc., U.S.A.) was used as the secondary antibody. The ABC titers were recorded as the highest serum dilutions with specific staining (Figs. 1a, 1b). The results are shown in Fig. 2. All five rabbits examined (nos. 6–10) were determined to be serologically positive using the ABC method. IgM antibody activities were first detected 7 days after the onset of the disease. IgG antibodies started to increase on the 7th or 14th day and remained high until the 28th day.

To compare with the sensitivity of this assay, enzyme-linked immunosorbent assay (ELISA) was performed as described by Klaassen *et al.* [14] using *P. multocida* isolated from rabbit no. 1. A 24-hr growth from a heavily seeded agar plate was suspended in 1.0 ml of 0.85% NaCl solution containing 0.3% saturated solution of formaldehyde. The suspension of the cells was heated in an oil bath at 100°C for 1 hr. The cells were sedimented by centrifugation, and the supernatant was used for the antigen in ELISA. Serum samples from five rabbits (nos. 6–10) were applied as primary

antibodies. A dilution of peroxidase-conjugated goat anti-rabbit IgM or IgG (Sigma Chemical Co., U.S.A.) was used as the secondary antibody. The results are shown in Fig. 3. The data indicated that the sera contained IgM and IgG antibodies to *P. multocida*. The ELISA showed antibodies on 7 to 28 days after the onset of the disease. Kawamoto *et al.* [11] reported that ELISA revealed IgG antibodies against *P. multocida* capsular serotype A in rabbits at one to four weeks post-inoculation. A quantitative analytical study on antibody responses against *P. multocida* in rabbits indicated that the ABC method was as sensitive as ELISA (Figs. 2, 3).

There are many reports of pneumonia in rabbits induced by *P. multocida* [7–9]. In the present study, rabbits naturally infected with *P. multocida* showed severe clinical signs and acute purulent pneumonia with serositis. The histopathological features of *P. multocida* infection in these rabbits correlated well with those reported in the literature [7–9].

This study defined the relationship between duration of natural infection with *P. multocida* and serum antibody responses. There are only a few reports regarding antibody production in rabbit pasteurellosis [11, 14]. ELISA, GDP test, agglutination test and IHA test have been evaluated for the detection of antibodies to *P. multocida* in both naturally- and experimentally infected rabbits [1, 2, 11, 12]. The present results showed that IgM or IgG antibodies to *P. multocida* were detectable by ABC method from 7 to 28 days

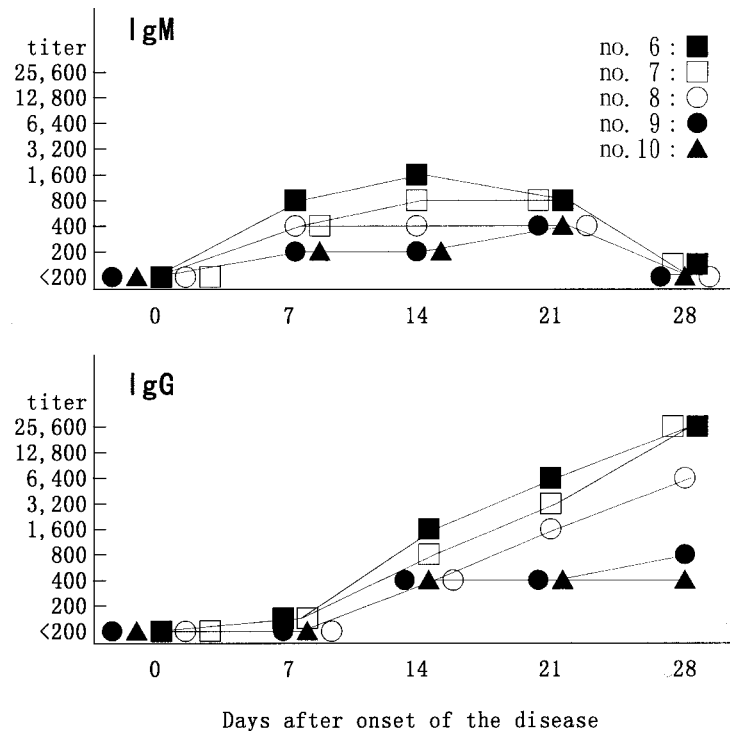


Fig. 2. Detection of antibodies in *P. multocida*-infected rabbits using ABC method. IgM and IgG antibody responses in five rabbits (nos. 6–10) naturally infected with *P. multocida*. The titers represent the highest dilution with specific reaction. Serum samples were obtained at 7-day intervals for four weeks after the onset of the disease. Rabbit no. 6 was euthanized at four weeks after the onset of the disease. Rabbit nos. 7 and 8 showed severe respiratory signs. Rabbit nos. 9 and 10 showed slight clinical signs.

after the onset of the disease. The time course change of IgM and IgG responses demonstrated by ABC method was similar to that detected by ELISA. Deep *et al.* [3] and DiGiacomo *et al.* [6] reported development of the IgM and IgG antibody responses in naturally infected rabbits using ELISA. In the present study using ABC method, the IgM antibody responses increased after the onset of the infection and then declined, whereas the IgG titers continued to increase and remained elevated. These findings support the recommendation of Deep and DiGiacomo *et al.* Cross-reacting antigens have been detected between *P. multocida* and certain other Gram negative organisms [16]. However, it seems unlikely that titers of this magnitude were produced by exposure to other organisms prior to the *P. multocida* infection. Although many serotypes of *P. multocida* have been classified based on combinations of capsular and somatic antigens, predominant serotypes of rabbit strains are limited [1, 2, 12, 17]. Particularly, major somatic antigens are only two to four types. Therefore, serological diagnosis of rabbit pasteurellosis by immunohistochemical staining may be useful if the predominant strains are prepared as antigens. This method is simple and can be per-

formed in 4 hr, similarly to ELISA. Furthermore, formalin-fixed specimens on slide glasses can be preserved for a long time. The only drawback in this method is inability to deal with a great many samples at a time.

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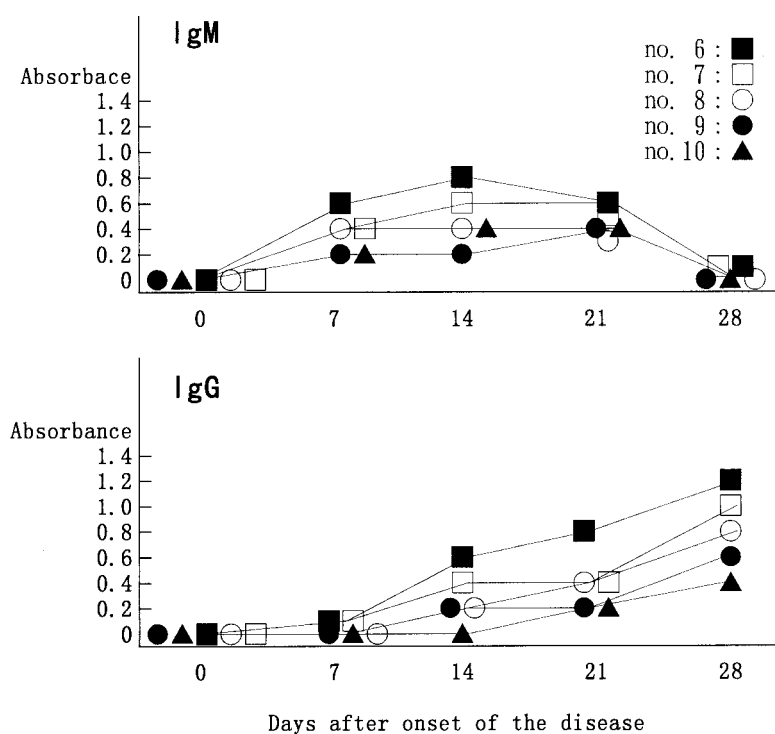


Fig. 3. Detection of antibodies in *P. multocida*-infected rabbits using ELISA. IgM and IgG antibody responses in five rabbits (nos. 6–10) infected with *P. multocida*. Samples were identical to the sera shown in Fig. 2.

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