

Bacillary Hemoglobinuria in Japanese Black Cattle in Hiroshima, Japan: A Case Study

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(Received 30 May 2010/Accepted 25 September 2010/Published online in J-STAGE 8 October 2010)

ABSTRACT. Three Japanese Black cows housed with 6 other cows exhibited main clinical symptoms of severe hemoglobinuria. Hematological analyses conducted after antibiotic therapy demonstrated severe anemia, and biochemical analyses indicated both severe hemolysis and disruption of hepatic function. One of the three cows died. Based on the above analyses and observation of typical clinical symptoms, a speculative diagnosis of bacillary hemoglobinuria was made, and immediate high-dose antibiotic treatment improved the general conditions of the surviving animals. Blood samples from the other 2 cows were collected sequentially after antibacterial therapy. *Clostridium haemolyticum* was detected by a nested polymerase chain reaction analysis of the blood samples. The cows were diagnosed with the second recorded occurrence of bacillary hemoglobinuria in Japan.

KEY WORDS: bacillary hemoglobinuria, cattle, *Clostridium haemolyticum*.

J. Vet. Med. Sci. 73(2): 255–258, 2011

Bacillary hemoglobinuria (BHU; red water disease) is an acute, toxemic and highly fatal disease in cattle caused mainly by phospholipase C (beta-toxin) produced by *Clostridium haemolyticum*; its clinical symptoms are fever, jaundice and hemoglobinuria [5, 6]. This beta-toxin causes hemolysis, necrosis of hepatocytes and damage to the capillary endothelium, all of which lead to hemoglobinuria and loss of vascular fluids in tissues and serous cavities [3]. Although the disease was initially reported in California in 1916 [4] and subsequently in other parts of the world [3], neither the clinical symptoms nor clinicopathologic changes caused by BHU are well known because the course of the illness is short and, in most cases, the cattle are found dead in the pasture without prior manifestation of clinical signs [6]. The routine method for diagnosis of BHU from live or dead cattle involves microbiological analysis for detection of *C. haemolyticum* in blood or tissue samples. However, it has been reported that isolation of *C. haemolyticum* is both time-consuming and very difficult, since it requires strict anaerobic conditions, and clinical specimens are often contaminated with other anaerobic bacteria, including environmental clostridia from the soil, which grow faster than pathogenic bacteria in culture media [8, 10]. Because the clinical course of BHU is rapid and the outcome is almost invariably fatal, early treatment of the disease by administration of a large dose of specific antibiotics before diagnosis has been recommended in response to appearance of typical clinical symptoms [1, 5, 6]. These practical limitations of sampling make it difficult to establish an accurate diagnosis for suspected cases of BHU once the animals have

completely recovered after antibacterial therapy. Recently, Takagi *et al.* [9] reported the first case of BHU in a Japanese Black cow, which recovered completely, and the diagnosis was confirmed by polymerase chain reaction (PCR) analysis using a whole blood sample collected before antibacterial therapy. However, to our knowledge, there is no documented report on the possibility or limitations of using molecular diagnosis to identify *C. haemolyticum* in blood samples collected from infected cattle either during or after antibiotic therapy. In the present study, we report the second occurrence of BHU in Japan, in which two of the three cows recovered completely after antibacterial therapy. The diagnosis was later established by nested PCR analysis using whole blood samples collected sequentially after antibacterial therapy.

In August 2009, a non-pregnant 8-year-old Japanese Black cow (case 1) that was housed with 8 other cows on a farm in Hiroshima, Japan, suddenly developed anorexia and diarrhea and excreted red urine. On the following day, its body temperature was 39.6°C, and a detailed clinical examination demonstrated pyrexia, absence of rumen movement, jaundiced vulval mucous membranes and red-wine-like urine. Urinary analysis was conducted on-site using Uropaper (Eiken Chemical Co., Ltd., Tokyo, Japan), which demonstrated the presence of urobilinogen (+), occult blood (+++) and ketone bodies (+++) in urine samples. Based on the clinical symptoms and urinary examination, a tentative diagnosis of bacillary pyelonephritis was made, and the animal was administered the recommended dose of penicillin and prescribed supportive therapies from days 2 to 4 after onset of the symptoms. During this treatment period, the cow's appetite seemed to increase. However, on day 5, the cow again became anorexic and hypothermic (37.2°C) and exhibited paleness of the mucous membranes; it died on day

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Table 1. Characteristics of primers used in the current study

Name	PCR	Sequence 5'-3' (mers)	Nucleotide positions*	Tm (°C)	Concentration (nM)
FlaF2	1st	AGAATAAACAGAGCTGGAGATG (22)	126-147	54.8	625
FlaF3	2nd	AAGAGGGCTTAATCAAGCTTCAA (23)	191-213	55.1	625
FlahaR3	2nd	TGAATATTATTTCCTTCTCC (23)	666-688	51.5	625
FlahaR2	1st	CTGCTGTACCTTCTATGAACC (21)	799-819	56.5	625

* Nucleotide positions on the flagellin gene sequence (DDBJ accession no. AB058939).

6. Hematological analyses demonstrated anemia [red blood cell count (RBC), $1.74 \times 10^6/\mu\text{l}$; white blood cell count (WBC), $12.8 \times 10^3/\mu\text{l}$; packed cell volume (PCV), 8.8%; and hemoglobin concentration (Hb), 3.2 g/dl]. Hemotrophic parasites were excluded by morphological examination of the erythrocytes. Biochemical analyses indicated slight jaundice [total bilirubin concentration (T-Bil), 0.95 mg/dl] and disruption of hepatic function [aspartate aminotransferase activity (AST), 1,103 U/l; γ -glutamyl transferase activity (GGT), 55 U/l].

In September 2009, 2 Japanese Black cows (case 2, non-pregnant 7-year-old; case 3, nonpregnant 8-year-old) housed within the same herd exhibited clinical signs similar to those of the first cow (case 1). These animals presented with symptoms of anorexia, diarrhea and excretion of red urine. Detailed clinical examination demonstrated pyrexia and absence of rumen motility. In both cases, urine analysis with Uropaper demonstrated a urinary pH of 9 and confirmed the presence of urobilinogen (-), occult blood (+++), ketone bodies (++), glucose (-) and protein (+++) in urine samples. Based on clinical and urinary examinations made on the farm, it was strongly suspected that the cows had a severe urinary tract infections, and a tentative diagnosis of bacillary pyelonephritis or cystitis was made. From day 1, both cows were administered the recommended dose of penicillin and prescribed supportive therapies. A urine sample (case 2) was collected under sterile conditions for bacterial examination and cultured with CHROMagar™ Orientation (Kanto Chemical Co., Inc., Tokyo, Japan) under aerobic conditions at 37°C for 24 hr. As a result, gram-positive cocci were isolated from the urine and the treatments were continued for 3 days. Despite the continuation of both the antibiotic and supportive therapies, the general clinical conditions of the animals did not improve; their clinical symptoms included anorexia, lethargy and hemoglobinuria. On day 4, hematological analyses demonstrated anemia (RBC, WBC, PCV and Hb of $3.45 \times 10^6/\mu\text{l}$, $6.0 \times 10^3/\mu\text{l}$, 17% and 5.7 g/dl for case 2 and $4.59 \times 10^6/\mu\text{l}$, $3.30 \times 10^3/\mu\text{l}$, 25.6% and 8.2 g/dl for case 3, respectively). Biochemical analyses indicated slight jaundice (T-Bil of 0.56 mg/dl and 0.46 mg/dl for cases 2 and 3, respectively) and disruption of hepatic function (AST and GGT of 143 U/l and 20 U/l for case 2 and 101 U/l and 29 U/l for cases 2 and 3, respectively). Based on these findings and the clinical symptoms of the 2 cows, a speculative diagnosis of BHU was made. The animals were treated with a high dose of cefazolin [10 mg/kg intravenously] every 24 hr and ampicillin [10 mg/kg intramuscularly] every 12 hr for a period of 4 days, as recommended

[9]. In addition, fecal examination of 1 cow (case 3) was conducted on day 4, and severe fascioliasis infection was detected. After treatment, appetite and rumen motility recovered by day 6, and a normal urine color was observed on day 8.

For molecular diagnosis of *C. haemolyticum* infection, blood was collected from case 2 on days 4, 6, 18 and 25 and from case 3 on days 3, 5, 17 and 24. A diagnostic assay was conducted using a modified version of the nested PCR method previously reported [9]. As shown in Table 1, the primers used in this method were designated on the basis of the flagellin gene (*fliC*) sequence (DDBJ accession no. AB058939) reported by Sasaki *et al.* [7]. Each blood specimen was transported to Kagoshima University for molecular diagnosis and spotted onto Filter Technology Associates filter paper (FTA cards, Whatman International Ltd., Piscataway, NJ, U.S.A.), allowed to dry and stored at about 4°C. A 1.2-mm diameter disc was punched out of the FTA card using a hole punch (a Harris Micro Punch, Whatman International Ltd.). The disc was placed into a separate 0.2-ml tube for PCR, washed 3 times for 5 min with 100 μl of a washing solution (FTA Purification Reagent, Whatman International Ltd.), rinsed twice for 5 min with 200 μl of Tris-EDTA buffer (pH 8.0) and dried at 60°C for 10 min. The first PCR of the nested PCR was carried out using this treated disc as a template. The second PCR was carried out using 1 μl of the PCR product from the first PCR in 20 μl of reaction mixture containing 10 μl of 2 \times GoTaq Hot Start Green Master Mix (Promega Corp, Madison, WI, U.S.A.), 12.5 pmol of each primer and a template. After the initial denaturation at 94°C for 5 min, 35 cycles of amplification were carried out at a denaturing temperature of 94°C for 30 sec, an annealing temperature of 54°C for 30 sec, and an extension temperature of 72°C for 45 sec. Extension during the last cycle was carried out at 72°C for 5 min. Amplification of the predicted band of the *C. haemolyticum fliC* gene was confirmed using DNA from strain ATCC 9650^T of *C. haemolyticum* in a previous report [7]. In addition, the DNA fragments in the second PCR product were sequenced using a general protocol (Hokkaido System Science, Sapporo, Japan) to identify *C. haemolyticum* in the blood specimens from cases 2 and 3.

The results of the nested PCR are shown in Fig. 1. Predicted bands were amplified in case 2 on days 4, 6 and 18 and in case 3 on days 3, 5, 17 and 24 after the onset of illness. There were no amplified bands in blood from healthy cows as negative controls (data not shown). Additionally, the nucleotide sequence of these bands detected in the 2 ani-

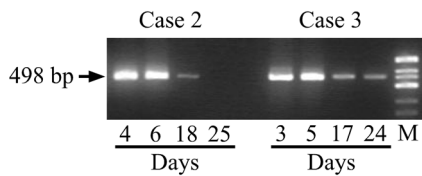


Fig. 1. PCR amplification of DNA from whole blood samples obtained from cases 2 and 3 using the nested PCR methods. The blood samples were collected on days 4, 6, 18 and 25 (case 2) and days 3, 5, 17 and 24 (case 3) after the onset of clinical symptoms. Lane M shows the molecular size markers. bp: base pair.

imals suspected of having BHU corresponded to the sequence previously reported (DDBJ accession no. AB058939) [7]. These results demonstrated that cases 2 and 3 were infected with *C. haemolyticum*, and the molecular diagnosis of BHU was established.

Recently, Takagi *et al.* [9] reported the first occurrence of BHU caused by *C. haemolyticum* infection in Japanese Black cows in Kagoshima, Japan. To our knowledge, the present paper reports the second occurrence of BHU in Japan, which suggests that BHU could occur anywhere in Japan where cattle feed on pasture that has been contaminated by *C. haemolyticum*.

Although previous reports suggested that the clinical course of BHU is rapid and the outcome is almost invariably fatal [2, 12], in both of the present cases as well as previous cases [9] subsequently diagnosed as BHU, the animals successfully recovered with the help of antibacterial therapy. For recovery from BHU, it is critical for the dose and route of antibiotic administration at the first treatment to achieve an effective blood concentration of the antibiotic against *C. haemolyticum* [1, 5, 6, 9]. In the present series, the initial tentative diagnosis was bacillary pyelonephritis or cystitis; thus, the cows were administered normal doses of antibiotics for the treatment of severe urinary tract infections, and then high doses of antibiotics, both intravenously and intramuscularly, were administered from day 4 onward. The results achieved in the present cases strongly suggest that the key to successful treatment of BHU is not only the initial dose of antibiotics, but also the timing of the initial administration of antibiotics after the onset of clinical symptoms of BHU, especially the appearance of wine-like urine.

As indicated above, the illness is of a short duration; prompt treatment with antibiotics should be undertaken on the farm without waiting for microbiological examinations. Accurate diagnosis of BHU by detection of *C. haemolyticum* in samples from suspected animals, especially isolation of *C. haemolyticum* under the strict anaerobic conditions required for culture agar, is quite difficult. Recently, Sasaki *et al.* [7] reported the possibility of the rapid identification of pathogenic clostridia with the help of an established multiplex PCR system. Using the method reported, Takagi *et al.* [9] recently demonstrated the possibility of diagnosing

animals with BHU by PCR analysis using samples collected before administration of antibiotic therapy. The results of the present study using our established nested-PCR analysis of whole blood samples clearly confirmed the presence of the *fliC* gene not only during antibiotic therapy, but also at the clinically complete recovery stage approximately 20 days postantibiotic therapy.

The reason why PCR analysis detected the *fliC* gene of *C. haemolyticum* for about 3 weeks after initiation of antibiotic therapy remains to be elucidated. However, some possible explanations can be proposed. First, there is a possibility that the antibiotic therapy employed in this report did not kill all bacteria in circulation within 3 weeks. Second, some of the bacteria may persist in the liver during this period. Alternatively, components of killed bacteria may continue to circulate for about 3 weeks. Whatever the case, antibiotic therapy should be maintained for more than 3 weeks on the basis that some of these bacteria may remain viable somewhere in the bovine body even during the administration of antibiotics.

It has been reported that under natural conditions in endemic areas, bacterial invasion originates in the alimentary tract after ingestion of contaminated material and fascioliasis is one of the triggering factors that creates local anaerobic conditions for germination of dormant spores [5, 6, 11]. Takagi *et al.* [9] also indicated the efficacy of anti-fascioliasis therapy in preventing BHU in fascioliasis-contaminated pastures. The pasture of the farm in the present study was muddy, and the water drainage was inefficient; thus, it is conceivable that the pasture was contaminated with bacterial spores. Furthermore, the fact that numerous eggs of *Fasciola* sp. were detected by a fecal survey of case 3 is thought to have been a critical factor for the development of BHU on this particular farm. In fact, we advised the farmer not to put other cows out to pasture and to administer anti-fascioliasis medicine to all cows. Since then, no further cases of BHU have been reported in the herd.

In conclusion, our established nested-PCR technique may be useful for rapid and accurate detection of *C. haemolyticum* derived from whole blood samples of suspected cases of BHU collected after antibiotic therapy. Additionally, the past and present cases of BHU suggest the possibility that BHU is not a rare illness among cattle in Japan, especially in a fascioliasis-contaminated cattle herd fed on natural pasture. It will be necessary to add BHU to the list of possibilities when considering a diagnosis in animals showing clinical findings of hemoglobinuria.

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