



NOTE

Internal Medicine

Acute myelomonocytic leukemia negative for alpha-naphthyl acetate esterase stain in a Holstein cow

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ABSTRACT. A 4-year, 7-month-old Holstein cow presented with anorexia. Physical examination revealed masses in the interscapular region and vagina. Blast cells were detected in the masses and peripheral blood by fine needle aspiration cytology and hematological examination. By bone marrow aspiration, blast cells constituted up to 24.2% of all nucleated cells, and 22% and 2% of non-erythroid cells stained positive for myeloperoxidase and alpha-naphthyl acetate esterase (ANAE), respectively. Pathological examination revealed the mass lesions consisted of a proliferation of tumor cells, which were positive for monocytic markers (HLA-DR and Iba-1). The cow was diagnosed with acute myelomonocytic leukemia (AMML). Even when tumor cells are ANAE-negative, AMML cannot be completely ruled out and should be considered when diagnosing cattle with leukemia/lymphoma.

KEY WORDS: acute myelomonocytic leukemia, alpha-naphthyl acetate esterase, cow, cytochemical staining, diagnosis

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Leukemia/lymphoma is a tumor of hematopoietic tissue that can be divided into two types based on the affected blood cell: myeloid leukemia and lymphoid leukemia [1]. Acute myelomonocytic leukemia (AMML) is a subgroup of myeloid leukemia and, to our knowledge, only two cases have been reported in cattle [6, 12]. Monocytes are usually strongly positive for alpha-naphthyl acetate esterase (ANAE), and ANAE staining is commonly used to diagnose monocytic malignancy [1, 6, 12]. In the present report, a case of ANAE-negative AMML in a Holstein cow was diagnosed by bone marrow aspiration and immunohistochemical staining using lymphocytic markers (CD3 and BLA-36) and monocytic markers (HLA-DR and Iba-1).

A 4-year, 7-month-old Holstein dairy cow presented with anorexia and decreased milk production 2 months after normal delivery. At initial examination by a local veterinarian (day 1), the cow had a body temperature of 39.8°C (reference interval (RI): 38.0–39.17°C), heart rate of 100 beats/min (bpm) (RI: 60–84 bpm), and respiratory rate of 50 breaths/min (RI: 18–28 breaths/min) [2]. Wheezing, rough vesicular breathing, and edema of the dewlap were noted. The cow was tentatively diagnosed with pneumonia and treated with 20 mg/kg ampicillin (Kyoritsu Seiyaku Inc., Tokyo, Japan) and 0.2 mg/kg dexamethasone (Kyoritsu Seiyaku Inc.). However, the general condition of the cow did not improve. On day 8, the cow was transferred to the Animal Teaching Hospital at the Obihiro University of Agriculture and Veterinary Medicine for further examination.

On initial physical examination at the hospital, the cow had a high rectal temperature (39.6°C), normal heart rate (84 bpm), and polypnea (32 breaths/min). Heart sounds were faint on auscultation. Emaciation, wheezing, rough vesicular breathing, edema of the dewlap, and a mass in the interscapular region were noted (Fig. 1). Several masses in the vagina were detected by speculum examination. Rectal palpation revealed several masses (5–10 cm) in the pelvic cavity. Enlargement of superficial lymph nodes was not observed. Fine needle aspiration (FNA) cytology of the masses in the interscapular region and vagina revealed medium to large undifferentiated blast cells with a small amount of cytoplasm undergoing mitosis (Supplementary Fig. 1). Cytoplasmic vacuoles were present in several blast cells. Echocardiography revealed pericardial fluid and adhesion of fibrin-like structures to the pericardium (Supplementary Fig. 2). Blood-like liquid was recovered from the pericardium by thoracentesis. Analysis of the pericardial fluid revealed a red blood cell (RBC) count of 1.00×10^6 / μ l, white blood cell (WBC) count of 43,000/ μ l, total protein (TP) of 4.2 g/dl, and specific gravity (SG) of 1.033. A sediment smear of the blood-like pericardial fluid showed that almost all

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(Supplementary material: refer to PMC <https://www.ncbi.nlm.nih.gov/pmc/journals/2350/>)

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Fig. 1. Emaciation, edema of the dewlap (black arrow), and a mass (black arrowhead) were apparent on Day 8.

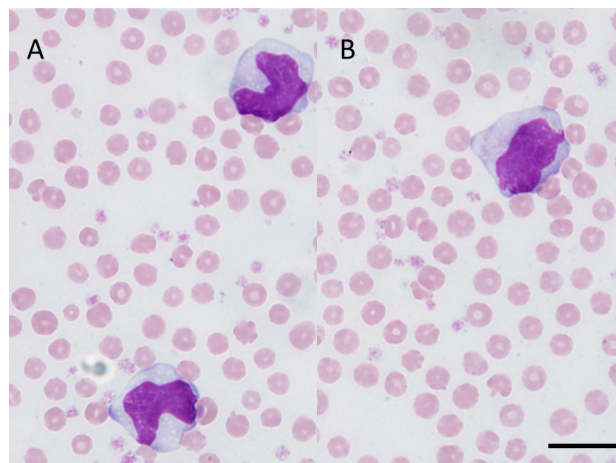


Fig. 2. Medium to large immature mononuclear cells with horse-shoe (A) or round (B) shaped nuclei in a peripheral blood smear. Wright-Giemsa stain. Bar=10 μ m.

WBCs were undifferentiated blast cells, similar to those observed in FNA cytology of the masses. Based on these findings, the cow was tentatively diagnosed with lymphoma.

Hematological examination indicated mild anemia (RBC, $4.56 \times 10^6/\mu\text{l}$; RI: $5.10\text{--}7.60 \times 10^6/\mu\text{l}$; hemoglobin concentration, 8.3 g/dl; RI: 8.5–12.2 g/dl) and moderate thrombocytosis ($99 \times 10^4/\mu\text{l}$; RI: $19.3\text{--}63.7 \times 10^4/\mu\text{l}$) [2]. WBC count ($11,200/\mu\text{l}$; RI: $4,900\text{--}12,000/\mu\text{l}$) was in the normal range [2]. Smear examination of peripheral blood showed that 76% ($8,512/\mu\text{l}$) of WBCs were medium to large immature mononuclear cells with round or horse-shoe-shaped nuclei and basophilic cytoplasm (Fig. 2); cytoplasmic vacuoles were present in several of these cells. In addition, mild lymphopenia (11%; $1,232/\mu\text{l}$; RI: $1,600\text{--}5,600/\mu\text{l}$) and mild neutropenia (13%; $1,456/\mu\text{l}$; RI: $1,800\text{--}6,300/\mu\text{l}$) were noted [2].

Serum biochemical analysis revealed increased total lactate dehydrogenase (LDH) (2,895 U/l; RI: $697\text{--}1,450$ U/l) and thymidine kinase (TK) (30.8 U/l; RI: <5.4 U/l) activities [4, 9]. LDH isozyme analysis showed elevated activities of LDH-2 (955 U/l; RI: $137\text{--}503$ U/l) and LDH-3 (579 U/l; RI: $82\text{--}262$ U/l) [4]. Antibodies against bovine leukemia virus (BLV) were detected with the BLV Antibody Test ELISA kit (JNC, Tokyo, Japan). The BLV proviral load of DNA extracted from the peripheral blood sample was quantified using the CoCoMo[®]-BLV Primer/Probe (Riken Genesis, Tokyo, Japan) and TaqMan[™] Gene Expression Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. BLV copy number in peripheral blood was 19 copies/10 ng DNA. There was no evidence of monoclonal proliferation of B-cells by the B-cell clonality test using a DNA sample of peripheral blood [7].

Bone marrow aspiration was performed to examine the etiology of the hematological abnormalities. Bone marrow smears were stained with Wright-Giemsa, and more than 500 cells were counted. The myeloid to erythroid (M:E) ratio was 1.16, and the composition of bone marrow cells without abnormalities in cell morphology did not differ from those of a reference control (Table 1) [6]. However, blast cells were observed, constituting up to 24.2% of all nucleated cells (ANCs) and 44.8% of all non-erythroid cells (NECs) (Fig. 3A). The majority of blast cells had a round to oval nucleus and a basophilic cytoplasm without azurophilic granules (Fig. 3A). These cells were further examined by myeloperoxidase (MPO) stain using a 3,3'-diaminobenzidine (DAB) staining kit (Muto Pure Chemicals, Tokyo, Japan) and ANAE stain using an α -NA esterase staining kit (Muto Pure Chemicals). Of the NECs, 22% and 2% were positive for MPO and ANAE, respectively (Fig. 3B and 3C).

The cow was euthanized under anesthesia on day 14 for autopsy according to the ethical and animal welfare requirements under the guidelines of the Care and Use of Agriculture Animals of Obihiro University (Approval 18-32). At the gross level, several masses (2–20 cm) were disseminated in the cervical and precordial muscles, heart, diaphragm, abomasum, intestine, and vagina. Mesenteric, gastric, and medial iliac lymph nodes were swollen. The cut surface of masses and affected lymph nodes was firm, homogenous, and white to grey. The sternal bone marrow was pale red.

Histopathologically, neoplastic cells diffusely proliferated in the affected lymph nodes and replaced the normal structure of the lymph nodes. The mass lesions consisted of a proliferation of tumor cells in the cervical and precordial muscles, heart, diaphragm, abomasum, intestine and vagina. Neoplastic cells were about 2–3 times the size of surrounding red blood cells. These cells had atypical round nuclei with a granular pattern of chromatin, as well as scant cytoplasm (Fig. 4). Immunohistochemical analysis revealed that the neoplastic cells were negative for CD3 (Biogenex Laboratories, Fremont, CA, USA) and BLA-36 (Biogenex Laboratories), and positive for HLA-DR (Dako Cytomation, Carpinteria, CA, USA) and Iba-1 (Fujifilm Wako Pure Chemical co., Osaka, Japan) (Fig. 5).

In humans, acute myeloid leukemia (AML) is classified using the World Health Organization (WHO) classification based on a combination of morphology, immunophenotype, genetics, and clinical features [1]. According to the WHO classification, the

Table 1. Myelogram of the case

	Results	Reference ^{a)}	
		Range	Median
Myeloid cells			
Myeloblast	0.4%	0.2–5.6%	3.2%
Promyelocyte	2.4%	1.6–5.0%	2.8%
Myelocyte	5.0%	4.0–20.8%	6.4%
Metamyelocyte	8.0%	6.0–19.6%	10.0%
Band neutrophil	10.4%	10.2–24.0%	16.8%
Segment neutrophil	2.4%	1.4–19.0%	11.6%
Eosinophil	0.4%	5.8–10.6%	7.2%
Basophil	0.0%	0.0–0.4%	0.0%
Monocyte	0.8%	0.0–0.8%	0.4%
Erythroid cells			
Rubriblast	5.8%	3.4–6.4%	4.2%
Prorubricyte	1.2%	0.0–2.6%	0.2%
Rubricyte	20.4%	12.6–30.8%	17.4%
Metarubricyte	18.6%	8.8–14.6%	13.8%
Others			
Lymphocyte	0.0%	0.0–1.6%	0.4%
Blast cell	24.2%	N/A	N/A
M/E ratio	1.16	0.97–2.67	1.76

a) Cited from [6]. N/A, not applicable.

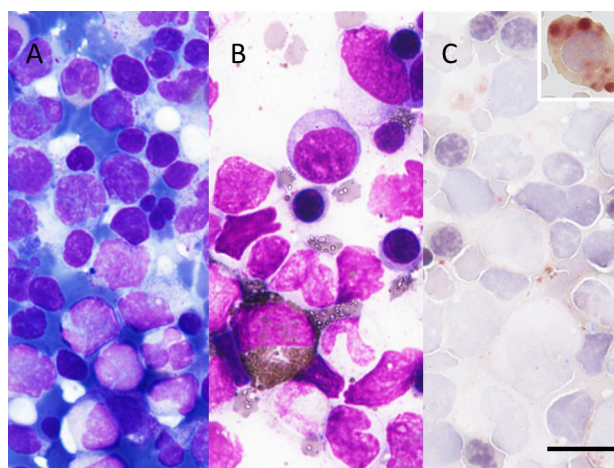


Fig. 3. Morphology and cytochemical staining of blast cells in bone marrow. (A) The majority of blast cells had a round to oval nucleus with distinct nucleoli, a basophilic cytoplasm, and a high nuclear/cytoplasmic ratio. Wright-Giemsa stain. (B) Of the non-erythroid cells, 22% were positive for myeloperoxidase. (C) Most cells in bone marrow were negative for α -naphthyl acetate esterase (ANAE). The upper right inset in Fig. 3C shows an ANAE-positive cell. Bar=10 μ m.

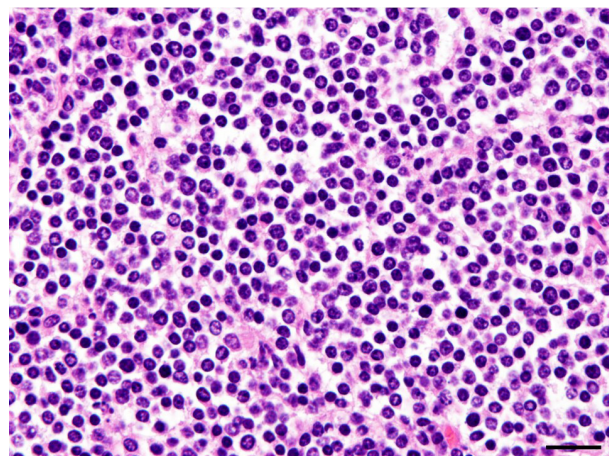


Fig. 4. Histopathology of medial iliac lymph node. Neoplastic cells had a round nucleus with nuclear atypia, granular-patterned chromatin, and scanty cytoplasm. Hematoxylin and eosin stain. Bar=20 μ m.

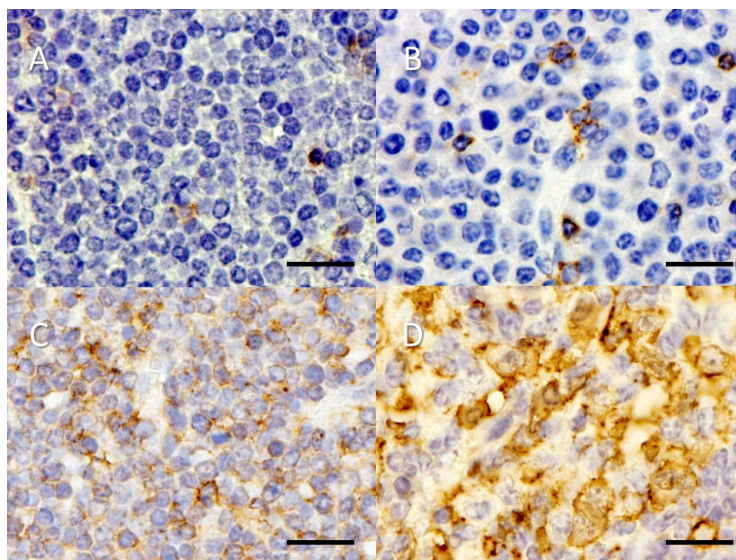


Fig. 5. Immunohistochemistry of mass around ileum. Neoplastic cells were negative for CD3 (A) and BLA-36 (B), and positive for HLA-DR (C) and Iba-1 (D). Mayer's hematoxylin counterstain. Bar=20 μ m.

diagnosis of acute leukemia is established by demonstrating the involvement of $\geq 20\%$ leukemic myeloblasts in bone marrow. AMML and acute monoblastic/monocytic leukemia are subtype of AML in which neoplastic cells show monocytic differentiation. AMML is diagnosed when the percentages of monocytic lineage cells and granulocytic lineage cells in NECs are $\geq 20\%$, and monocytic lineage cells are $\geq 5,000/\mu\text{l}$ in peripheral blood. In the present case, the blast cell count was $8,512/\mu\text{l}$ in peripheral blood and the proportion of blast cells was 44.8% of NECs in bone marrow. In cytochemical staining, 22% and 2% of NECs were positive for MPO and ANAE, respectively. By immunohistochemical analysis, although a portion of cells in tumor tissues were positive for CD3, most cells were negative for lymphocytic markers (CD3 and BLA-36) and positive for monocytic markers (HLA-DR and Iba-1). Therefore, the cow was diagnosed with AMML, despite the ANAE-negative tumor cells. To the best of our

knowledge, this is the third report of bovine AMML and provides additional information regarding the clinicopathological aspects of bovine AMML [6, 12]. Incidence of AMML in cattle has never been investigated, and additional studies are needed to clarify the incidence of AMML in cattle.

Almost all hematopoietic neoplasms reported in cattle are lymphoid malignancies. Enzootic bovine leukosis (EBL) is a lymphoma caused by BLV infection in adult cattle. The present case was initially suspected to be EBL based on physical examination, FNA cytology of masses, hematological examination, serum biochemical analysis, and detection of antibodies against BLV. However, the BLV provirus load was low [11], suggesting that the disease was likely a leukemia/lymphoma other than EBL. In bone marrow aspiration cytology and cytochemical staining, the proportion of blast cells was <90% of NECs, $\geq 10\%$ of NECs were positive for MOP, and <20% of NECs were positive for ANAE. These results suggest that the type of leukemia/lymphoma in the present case was AML with maturation [1]. However, tumor cells with clear granules were not observed, preventing a definitive diagnosis of the type of leukemia/lymphoma. Although tumor cells in bone marrow were ANAE-negative, histopathological examination showed that the ANAE-negative cells were positive for monocytic markers (HLA-DR and Iba-1). In humans, some leukemic monocytes are weakly positive or negative for ANAE [5, 10]. In cases of human AML with a negative ANAE stain, monocytic differentiation of neoplastic cells is evaluated by alpha-naphthyl butyrate esterase (ANBE) stain, flow cytometry, measurement of serum and urinary lysozyme levels, and evaluating the phagocytic ability of the tumor cells [3, 5, 8, 15]. However, these methods have not been validated for use in diagnosing AMML in cattle. Further investigation is required to clarify the usefulness of these methods in diagnosis of bovine AMML.

BLV preferentially infects B-cells, but also infects T-cells, monocytes, and granulocytes [13]. Although the present case was positive for BLV infection, the BLV copy number was low in peripheral blood, suggesting that BLV was not present in tumor cells and was unlikely to be associated with tumorigenesis.

CD14, CD68, and CD163 are commonly used as monocyte/macrophage markers in human. However, both specific and sensitive of these markers were not enough to confidently identify monocytic cells, and frequently a broad panel was warranted [14]. Iba-1 is a marker for identifying neoplasms of monocytic origin with higher sensitivity and specificity than CD14, CD68, and CD163 [14]. In present case and previous bovine AMML case, Iba-1 was used as monocytic marker [6]. Therefore, although usefulness of those markers had not been evaluated in bovine monocytic leukemia, Iba-1 should be used as a monocytic marker in cattle with suspected monocytic leukemia.

Higher activities of LDH and TK suggest aggressive proliferation of hematological tumor cells and these activities have been used as markers of bovine lymphoma [4, 9]. The present case and previous bovine AMML case showed higher activities of these enzymes [6]. Those results suggested that measurement of activities serum LDH and TK can be used as markers of bovine AMML as well as bovine lymphoma though origin of tumor cells cannot be identified by both activities.

In conclusion, our case was diagnosed with AMML, despite having ANAE-negative tumor cells. Even when tumor cells are ANAE-negative, AMML cannot be completely ruled out and should be considered when diagnosing cattle with leukemia/lymphoma.

POTENTIAL CONFLICTS OF INTEREST. The authors declare no conflicts of interest.

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