

逆受身赤血球凝集反応によるウシの血中と尿中のミオグロビンの微量測定

誌名	Japanese journal of veterinary science
ISSN	00215295
著者	納, 敏 一条, 茂
巻/号	49巻6号
掲載ページ	p. 995-1001
発行年月	1987年12月

Microassay of Bovine Serum and Urinary Myoglobin by Reverse Passive Hemagglutination

Satoshi OSAME, and Shigeru ICHIJO

Department of Veterinary Internal Medicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080, Japan.

(Received 16 February 1987/Accepted 16 July 1987)

ABSTRACT. A sensitive microassay by reverse passive hemagglutination (RPHA) was carried out for the measurement of bovine myoglobin (Mb). Sheep erythrocytes fixed with glutaraldehyde and treated with tannic acid were coated with anti-bovine Mb antibodies purified by affinity chromatography. The RPHA test was done by a microtiter method and minimal detectable Mb levels were 10 ng/ml in serum and 2 ng/ml in urine. Mb levels of 15 normal calves and 15 normal cows ranged from <10 to 80 ng/ml in serum and from <2 to 16 ng/ml in urine. Sixteen calves with nutritional myopathy showed markedly increased Mb levels in serum, 640 to 51,200 ng/ml, and in urine, 640 to 256,000 ng/ml. Serum Mb levels in calves recovered from nutritional myopathy decreased to nearly normal ranges within 3 to 5 days. Although each of the 51 cows at the onset of parturient paresis showed high levels of serum Mb ranging from 320 to 20,480 ng/ml, while about 30 per cent of the cows showed normal serum CPK activities. Serum Mb levels in cows recovered from parturient paresis rapidly decreased to the normal levels after 2 to 4 days. Serum Mb in cases slaughtered continued at high levels until the last treatment of parturient paresis. It would be concluded that RPHA method is useful for Mb determination in evaluating the myolytic state of bovine myopathy and for early detection of the disease.—**KEY WORDS:** bovine myoglobin, nutritional myopathy, parturient paresis, reverse hemagglutination.

Jpn. J. Vet. Sci. 49(6): 995–1001, 1987

Clinical diagnostic tests for bovine myopathies have been principally performed with examination of serum enzyme activities, such as creatine phosphokinase (CPK), glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT) and lactate dehydrogenase (LDH) [2, 9, 10, 11, 20]. Since these serum enzymes are widely distributed in various tissues [5], these enzymes are not suited for a specific diagnosis for early detection of muscular damage. To establish sensitive and specific tests on myopathy, detection of serum and urinary myoglobin (Mb) by immunodiffusion method was reported [14, 15]. This method facilitated detection of Mb in serum and urine of cattle with myopathy [15], but the method was not sensitive enough for detection of serum and urinary Mb of normal or mildly increased levels. In

the present study, therefore, a microassay of Mb by reverse passive hemagglutination (RPHA) method and its clinical application for early diagnosis of bovine myopathies were carried out.

MATERIALS AND METHODS

Purification of Mb: The methods for purification of bovine Mb were described previously [14]. Mb was purified from bovine skeletal muscle by fractionation with ammonium sulfate and two cycles of crystallization in phosphate buffer. Then pure crystals of bovine metmyoglobin were obtained. The purified Mb was freeze-dried and stored at 4°C.

Preparation of anti-bovine Mb serum: Purified Mb (10 mg) was dissolved in physiological saline, emulsified in an equal

volume of Freund's complete adjuvant and injected into rabbit's foot pads. Three weeks later, additional immunizations were performed 3 times by injecting 5 mg of Mb to the animals at weekly intervals. The rabbits were bled at 7 days after the last booster injection to check the antibody titer, which reached the peak at 5 to 6 weeks after immunization.

Extraction of antibody to bovine Mb: Specific antibodies to the bovine Mb were purified by affinity chromatography from a rabbit antiserum using CNBr-activated Sepharose 4B coupled to the purified bovine Mb [7]. Two grams of Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) activated by CNBr were mixed with 8 ml of diluent (0.5 M NaCl, 0.1 M NaHCO₃, pH8.3) containing 2 mg of purified Mb. The mixture was rotated at room temperature for 2 hr and the remaining active Sepharose unconjugated with Mb were blocked with 10 ml of 1 M glycine, pH8.0 for 2 hr. The gel was washed in 3 cycles with 0.5 M NaCl-0.1 M acetate buffer at pH4.0 followed by a solution containing 0.5 M NaCl and 0.1 M NaHCO₃ at pH8.3. This material was packed into an 8-mm diameter column. Twenty ml of anti-Mb serum was applied to the column, and then washed with a solution containing 0.5 M NaCl and 0.1 M NaHCO₃ at pH8.3. Specific antibody to bovine Mb was eluted with 0.2 M glycine buffer, pH2.5 in 0.5 ml fractions. Fractions were pooled and dialyzed against PBS (0.15 M NaCl, 0.01 M phosphate buffer, pH7.2) at 4°C for 24 hr.

Coating of the erythrocytes with purified antibodies: Sheep blood was collected in Alsever's solution. After 5 washes with PBS the erythrocytes were fixed by mixing equal volumes of a 10% suspension and 1% glutaraldehyde in PBS, then the mixture was incubated at 37°C for 20 hr [3]. The fixed cells were washed 4 times with distilled water and suspended in PBS at the concen-

tration of 10%. The suspension was mixed with an equal volume of 50 µg/ml tannic acid (Wako Pure Chemical Co., Ltd. Japan) and incubated at 37°C for 15 min [6]. After incubation the cells were washed 5 times with PBS, suspended in PBS at the concentration of 10% and mixed with an equal volume of PBS containing specific antibodies to bovine Mb described above. After incubation at room temperature for 1 hr, the cells were washed 2 times with PBS and 1% suspension with PBS containing 1% bovine serum albumin (Sigma Chemical Co., St Louis, Mo) was prepared.

Treatment of sera and urine: Sera were inactivated by heating at 56°C for 30 min before use and subsequently absorbed at room temperature for 30 min with 9 volumes of 0.2% suspension of glutaraldehyde-fixed sheep erythrocytes. Urine samples were absorbed at room temperature for 30 min with 1 volume of glutaraldehyde-fixed sheep erythrocyte suspension. The supernatant of samples absorbed was used for RPHA test.

RPHA test: RPHA test was carried out by a microtiter method with microplate of U type. Twofold dilutions of the test samples with PBS were made in 50 µl volumes and mixed with 50 µl of 1% suspension of sheep erythrocytes coated with purified antibodies to bovine Mb. The plate was incubated at room temperature for 1 hr and the results were read. Control serial dilutions were prepared by using glutaraldehyde-fixed erythrocytes treated with tannic acid only. To determine for sensitivity of erythrocytes coated with purified antibodies, serial dilutions of standard Mb solution were made in each test.

Samples: Serum and urine samples were prepared from 15 normal Holstein calves of one week to 3 months of age, 15 normal cows, 51 cows with parturient paresis, and 16 Japanese Black calves of 20 to 130 days of age suffering from nutritional myopathy.

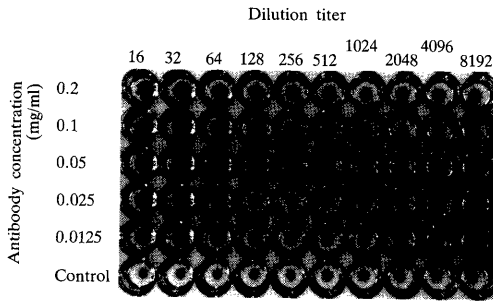


Fig. 1 Results of RPHA test of various amounts of purified antibodies (from 0.0125 to 0.2 mg/ml) used for coating of sheep erythrocytes. Standard Mb solution (1 $\mu\text{g/ml}$) was prepared by twofold dilutions.

Samples from 51 cows suffering from parturient paresis within 3 days after parturition were collected during the period of September to December 1985, in Tokachi district, Hokkaido. Of these, low concentrations of serum calcium and phosphorus were found in 44 cows, but no such change was seen in 7 cows. Serum enzyme activities at the onset of parturient paresis showed elevations of CPK activities over 100 international unit (IU) in 36 cases out of 51 cows (70.6%) and of GOT activities over 200 karmen unit (KU) in 3 cases. Six cases out of 51 cows were slaughtered during 4 to 9 days after the onset because of no response to therapy. The calves with nutritional myopathy were studied during the period from January 1984 to June 1985 in the same district. They were diagnosed by clinical symptoms of recumbency or stiffness and marked elevations of serum enzyme activities *i. e.*, over 2,900 IU in CPK and over 1,360 KU in GOT respectively.

RESULTS

Results of RPHA test of the various amount of purified antibody used for coating of sheep erythrocytes is shown in Fig. 1. Erythrocytes coated with antibody concentrations from 0.0125 to 0.2 mg/ml were given to serial twofold dilutions of Mb

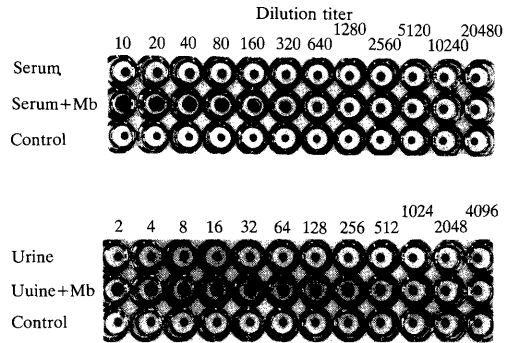


Fig. 2 Affection to RPHA test with serum and urine. Purified Mb (1 $\mu\text{g/ml}$) was added to normal serum and urine.

solution of 1 $\mu\text{g/ml}$ in PBS. Coated erythrocytes agglutinated in every serial dilutions of Mb: the distinguishable amount of antibodies for hemagglutination was 0.05 mg/ml. Based on this finding, erythrocytes coated with 0.05 mg/ml of purified antibodies were used in the following examination. Since positive hemagglutination was observed to maximum dilution titer of 1:512 (Fig. 1), RPHA test showed detectable titer for Mb of 1:512 to 1:1024, *i. e.*, the lowest detectable level of as little as 1 ng/ml of Mb by the technique. The Mb level was expressed by multiplying 1 ng/ml by the highest dilution titer of hemagglutination. RPHA tests were carried out by using 10-fold dilutions of sera and twofold dilutions of urine for absorption of nonspecific reaction. Therefore, the detectable sensitivity of Mb in samples was 10 ng/ml in sera and 2 ng/ml in urine. Affection to RPHA test with serum and urine was examined, to which purified Mb was added at the concentration of 1 $\mu\text{g/ml}$. Hemagglutination was observed to dilution titer of 1:640 in normal serum and 1:512 in normal urine (Fig. 2). Additional Mb concentration in serum was from 640 to 1,280 ng/ml and in urine from 512 to 1,024 ng/ml. No adverse influence on hemagglutination by serum and urine was seen.

Quantitative determination of normal

serum and urinary Mb in 15 calves and 15 cows was performed by RPHA method (Table 1). In calves, serum Mb levels ranged from <10 to 80 ng/ml and the urinary Mb ranged from <2 to 4 ng/ml. Serum and urinary Mb levels of cows were from <10 to 40 ng/ml and from <2 to 16 ng/ml, respectively. There were no appreciable differences in Mb levels between calves and cows.

In 16 calves with nutritional myopathy serum Mb levels ranged from 640 to 51,200 ng/ml and urinary Mb levels from 640 to 256,000 ng/ml (Fig. 3). In 6 calves recovered from nutritional myopathy, changes of serum Mb levels are shown in Fig. 4. Serum Mb levels showed high concentrations at the onset and rapidly decreased to normal values 3 to 5 days after administration of vitamin E or vitamin E-selenium.

In 51 cows with parturient paresis, Mb

levels at the onset ranged from 320 to 20,480 ng/ml in serum and from <2 to 81,920 ng/ml in urine (Fig. 5). High levels of serum Mb were seen in all cows, but urinary Mb levels were lower than 20 ng/ml in 9 cows. Changes of serum Mb concentrations of 4 cows recovered from parturient paresis are shown in Fig. 6. Two cows (case No. 16, 18)

Table 1. Serum and Urinary Mb concentrations (ng/ml) in normal calves and cows

Specimen	Calf		Cow	
	Serum	Urine	Serum	Urine
1	<10	2	<10	4
2	40	2	<10	4
3	<10	4	10	< 2
4	<10	2	<10	< 2
5	<10	<2	<10	< 2
6	<10	<2	20	< 2
7	<10	4	10	< 2
8	<10	<2	<10	2
9	<10	2	<10	2
10	<10	2	<10	2
11	<10	<2	<10	< 2
12	<10	<2	<10	8
13	<10	<2	<10	4
14	10	4	40	< 2
15	80	2	10	16

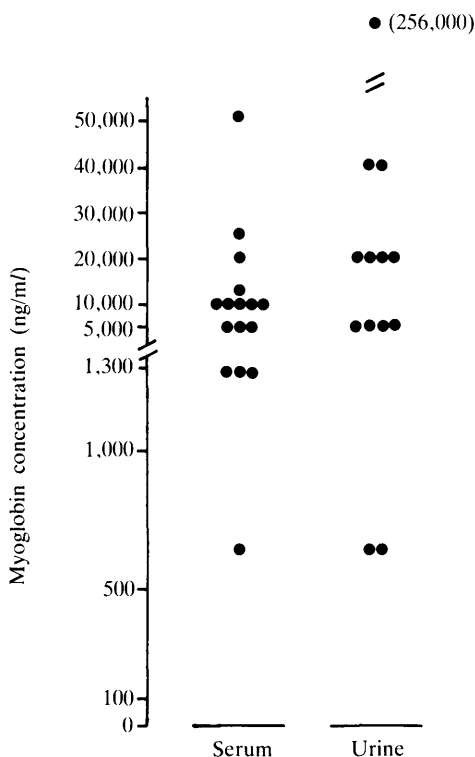


Fig. 3 Serum and urinary Mb levels at the onset of nutritional myopathy in calves.

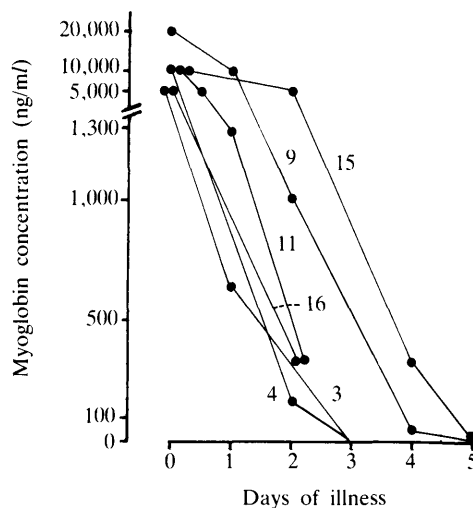


Fig. 4 Changes of serum Mb levels in 6 calves (case No. 3, 4, 9, 11, 15, 16) recovered from nutritional myopathy.

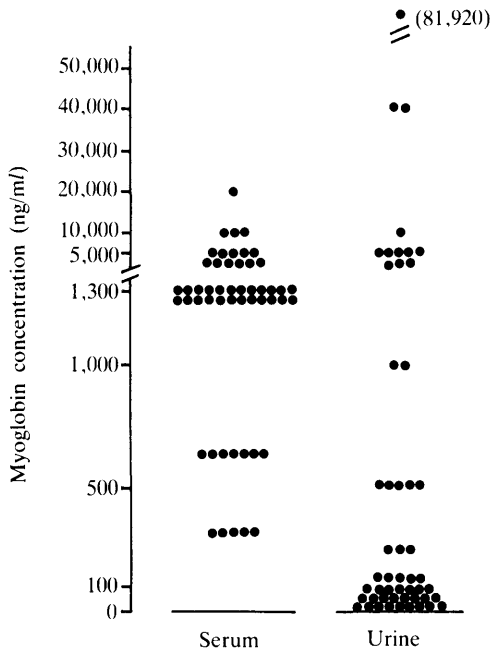


Fig. 5 Serum and urinary Mb levels at the onset of parturient paresis in dairy cows.

recovered in 1 hr after the first Ca injection, and other 2 cows (case No. 51, 27) recovered at 6 hr and 2 days after the onset respectively. The serum Mb levels in 4 cows recovered, which ranged from 1,280 to 10,240 ng/ml at the onset, rapidly decreased to normal levels 2 to 4 days after recovery. Of 6 cows slaughtered, which were suffering from parturient paresis, serum Mb levels at the onset ranged from 320 to 10,240 ng/ml, and high Mb levels were detected continuously until the last treatment of parturient paresis (Fig. 7).

DISCUSSION

In measuring of serum and urinary Mb levels, radioimmunoassay (sensitivity 1 ng/ml) was reported in human Mb [12, 13, 17] and immunodiffusion method was done in horse Mb [18, 19]. Immunodiffusion method for bovine Mb determination was reported, and this method was successful in

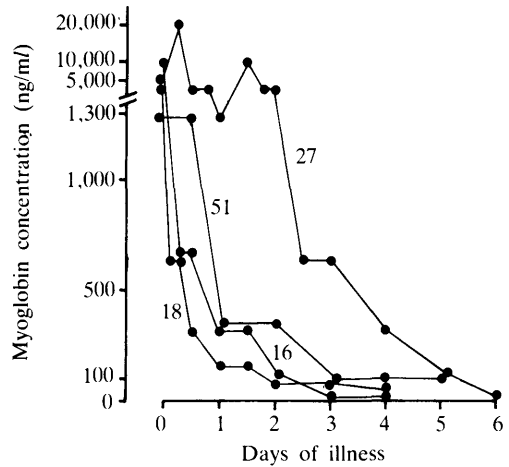


Fig. 6 Changes of serum Mb levels in 4 cows (case No. 16, 18, 27, 51) recovered from parturient paresis.

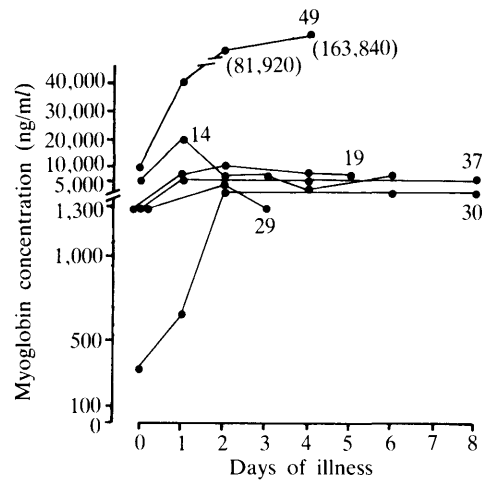


Fig. 7 Changes of serum Mb levels in 6 cows (case No. 14, 19, 29, 30, 37, 49) slaughtered with parturient paresis. Six cows remained recumbency to the last treatment of paresis.

the measurement of serum and urinary Mb in calves with nutritional Myopathy [14, 15].

The present paper describes the sensitive microassay of bovine Mb by RPHA method, using sheep erythrocytes fixed with glutaraldehyde, treated with tannic acid and coated with purified anti-Mb antibody by affinity chromatography. The lowest level of detection was 1 ng/ml of Mb; the sensitivity

is comparable to radioimmunoassay. However, since samples were diluted for absorption, the method enabled detection of Mb at 10 ng/ml in sera and 2 ng/ml in urine. The RPHA method has the disadvantage in expressing Mb value by multiplying dilution titer, but it can be calculated within a short time.

Normal serum and urinary Mb levels, of which there was no difference between calves and cows, were in the range of <10 to 80 ng/ml and from <2 to 16 ng/ml, respectively. It has been reported by Stone *et al.* [17] that the normal serum levels of human Mb was within the range of 6 to 85 ng/ml (28.9 ± 17.3 ng/ml) and below 30 ng/ml (13.1 ± 6.1 ng/ml) by Miyoshi *et al.* [13]. No study on normal Mb values in serum and urine of animals was reported. The present results indicate that high levels in bovine Mb are over 100 ng/ml in serum and over 20 ng/ml in urine.

In human Mb, Miyoshi *et al.* [12, 13] reported elevations of serum Mb levels in patients with various diseases. Particularly, in case of acute myocardial infarction, determination of serum Mb was important. Hällgren *et al.* [8] and Stone *et al.* [17] emphasized importance of serum Mb in the disease. The authors described the detection of serum and urinary Mb in calves with nutritional myopathy by immunodiffusion [15]. However, myoglobinemia and myoglobinuria were not detected in all calves of nutritional myopathy, possibly due to the low sensitivity of the method. RPHA method was sensitive enough to evaluate serum and urinary Mb levels in calves with nutritional myopathy and in cows with parturient paresis.

The present study clearly confirms elevations of serum and urinary Mb in all calves with nutritional myopathy. Mb levels of calves with nutritional myopathy ranged from 640 to 51,200 ng/ml in serum and from 640 to 256,000 ng/ml in urine. Serum Mb

levels of calves recovered rapidly decreased to normal levels.

The present paper also describes changes of serum and urinary Mb levels in dairy cows with parturient paresis. All cows showed elevations of serum Mb at the onset over 320 ng/ml. In contrast, elevations in serum CPK activities were found in 70.6% of the cows with parturient paresis. These data suggested that Mb was released into circulation from damaged muscle cells within a short time after the onset. It became also clear that muscular damage in dairy cows with parturient paresis started at the early stage of the disease. Serum Mb levels of cows recovered from parturient paresis decreased rapidly to normal levels after 2 to 4 days, while slaughtered cows showed continuous high levels from the onset to the last treatment of parturient paresis indicating the progressive changes of muscular damage.

Studies on muscular degeneration in various bovine diseases with recumbency have dealt mainly with serum enzyme activities [2, 4, 9, 10, 11, 20] and pathological changes [11, 16]. However, determination of serum Mb levels in bovine myopathy is more useful for early diagnosis of muscular damage than measurement of serum enzyme activity.

In conclusion, microassay of serum and urinary Mb by RPHA method can be utilized as an important clinical diagnostic tool for bovine myopathy and will be an aid in elucidating the status of the disease.

REFERENCES

1. Allen, W. M., Bradley, R., Berrett, S., Parr, W. H., Swannack, K., Barton, C. R. Q., and Macphee, A. 1975. Degenerative myopathy with myoglobinuria in yearling cattle. *Br. Vet. J.* 131: 292-308.
2. Anderson, P. H., Berrett, S., and Patterson, D. S. P. 1976. The significance of elevated plasma creatine phosphokinase activity in muscle disease of cattle. *J. Comp Path.* 86: 531-538.
3. Avrameas, S., Taudou, B., and Chuilon, S. 1969.

- Glutaraldehyde, cyanuric chloride and tetraazotized 0-dianisidine as coupling reagents in the passive hemagglutination test. *Immunochemistry*. 6: 67-76.
4. Björnsell, K. A., Holtenius, P., and Jacobsson, S. O. 1969. Studies on parturient paresis with special reference to the downer cow syndrome. *Acta. Vet. Scand.* 10: 36-43.
 5. Boyd, J. W. 1962. The comparative activity of some enzymes in sheep, cattle and rats—normal serum and tissue levels and changes during experimental liver necrosis. *Res. Vet. Sci.* 3: 256-268.
 6. Boyden, S. V. 1951. The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera. *J. Exp. Med.* 93: 107-120.
 7. Cuatrecasas, P., Wilcheck, M., and Anfinsen, C. B. 1968. Selective enzyme purification by affinity chromatography. *Proc. Natl. Acad. Sci. U. S. A.* 61: 638-643.
 8. Hällgren, R., Karlsson, F. A., Roxin, L. E., and Venge, P. 1978. Myoglobin turnover—influence of renal and extrarenal factors. *J. Lab. Clin. Med.* 91: 246-254.
 9. Ichijo, S., Hasegawa, M., Kim, D. H., Kimata, T., Ogawa, S., Konishi, T., Ono, T., Morishita, T., and Tanaka, I. 1981. Clinical and Clinicopathological findings on leukomyoma in calves. *J. Jpn. Vet. Med. Assoc.* 34: 573-579 (in Japanese with English summary).
 10. Johnston, W. S., and Murray, I. S. 1975. Myopathy in young cattle associated with possible myoglobinuria. *Vet. Rec.* 97: 176-177.
 11. Jönsson, G., and Pehrson, B. 1969. Studies on the downer syndrome in dairy cows. *Zentbl. Vet. Med.* 16A: 757-784.
 12. Miyoshi, K., Kawai, H., Hiasa, M., and Iwasa, M. 1977. Myoglobin and skeletal muscle or myocardial disorders. pp. 581-594. *In: The Function and Structure of Muscle* (Sakai, T., Endo, M., and Sugita, H. eds.), Igaku Shoin Ltd., Tokyo (in Japanese).
 13. Miyoshi, K., Saito, S., Kawai, H., Kondo, A., Iwasa, M., Hayashi, T., and Yagita, M. 1978. Radioimmunoassay for human myoglobin: methods and results in patients with skeletal muscle or myocardial disorders. *J. Lab. Clin. Med.* 92: 341-352.
 14. Osame, S., and Ichijo, S. 1986. Bovine Myoglobin: Its purification and detection by the anti-serum. *Jpn. J. Vet. Sci.* 48: 441-444.
 15. Osame, S., and Ichijo, S. 1986. Bovine myoglobin: Detection of myoglobin in sera and urine of calves with nutritional myopathy by immunodiffusion method. *Jpn. J. Vet. Sci.* 48: 829-832.
 16. Smith, D. L., Palmer, S. J., Hulland, T. J., Mcsherry, B. J., and Blackwell, T. E. 1985. A nutritional myopathy enzootic in a group of yearling beef cattle. *Can. Vet. J.* 26: 385-390.
 17. Stone, M. J., Willerson, J. T., Gomez-Sanchez, C. E., and Waterman, M. R. 1975. Radioimmunoassay of myoglobin in human serum. Results in patients with acute myocardial infarction. *J. Clin. Invest.* 56: 1334-1339.
 18. Watanabe, H., Ikeda, S., Yamaoka, S., Kameya, T., Nagasawa, Y., Takizawa, I., and Hasegawa, M. 1978. Evaluation of myoglobin determination for the diagnosis of tying-up syndrome in racehorses in Japan. *Exp. Rep. Equine Hlth Lab.* No. 15: 79-90.
 19. Watanabe, H., Yamaoka, S., Ikeda, S., and Kameya, T. 1975. Detection of horse myoglobin by the immunodiffusion method. *Exp. Rep. Equine Hlth Lab.* No. 12: 64-73.
 20. Yano, Y., Hamana, K., Kuroda, H., and Honda, K. 1985. Clinical and hematological findings and treatment of parturient paresis in dairy cows. *J. Jpn. Vet. Med. Assoc.* 38: 356-361 (in Japanese with English Summary).

要 約

逆受身赤血球凝集反応によるウシの血中と尿中のミオグロビンの微量測定：納 敏・一条 茂（帯広畜産大学家畜内科学教室）——アフィニティクロマトグラフィーにより精製した抗ウシ Mb 抗体で感作したグルタルアルデヒド固定タンニン酸処理羊赤血球を用い、マイクロタイター法によって逆受身赤血球凝集反応（RPHA）によるウシミオグロビン（Mb）の微量測定法を実施した。血中では 10ng/ml、尿中では 2ng/ml の Mb の検出が可能であった。健康牛の血中 Mb は <10-80ng/ml、尿中 Mb は <2-16ng/ml であった。子牛の栄養性ミオパチー 16 例の Mb は高値を示し、血中で 640-51,200ng/ml、尿中で 640-256,000ng/ml であったが、治療開始後 3-5 日には血中 Mb は正常値に復帰した。乳牛の産後起立不能症 51 例の血中 Mb は高値を示し、発症時に 320-20,480ng/ml であり、約 30% の例では血清 CPK 活性値は正常値を示した。起立後、血中 Mb 濃度は急速に低下して 2-4 日後には正常値となったが、廃用例では終診時まで高値が持続した。以上から、RPHA 法により、骨格筋障害の早期かつ的確な診断が可能であると考えられた。