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Occurrence and potential causative factors of immune-mediated hemolytic anemia in cattle and river buffaloes

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Abstract

The main objectives of this study were to determine the occurrence and potential causative factors of Immune-mediated hemolytic anemia (IMHA) in native cattle and water buffaloes from southwest of Iran. Fifty-three anemic animals (37 cattle and 16 buffaloes) were studied. A full clinical history and physical examinations were undertaken for all animals. Four clinically healthy cattle and four healthy buffaloes were also used as control animals. Blood samples were subjected to a complete blood count, Coombs' test, erythrocyte osmotic fragility test and serum biochemical analysis. IMHA was diagnosed in 12 (32.43%) cattle and 6 (37.50%) buffaloes based on the Coombs' test. Underlying or concurrent diseases, including theileriosis, anaplasmosis, vaccination, and pneumonia were detected in 11 cattle and four buffaloes. Primary or idiopathic IMHA was identified in one cattle and two buffaloes that their Coombs' test was positive. Hematologic and biochemical findings in the cattle with IMHA included a nonregenerative anemia, leukopenia, thrombocytopenia, increased osmotic fragility, hyperbilirubinemia and elevated serum alkaline phosphatase, aspartate aminotransferase and lactate dehydrogenase activities. It can be concluded that IMHA occurs in a significant proportion of anemic cattle and river buffaloes in southwest of Iran. The occurrence of IMHA in both cattle and buffaloes is mostly secondary to infectious diseases especially theileriosis and anaplasmosis. Clarification of the mechanisms of primary or idiopathic and secondary IMHA in cattle and buffaloes require further studies.

رخداد و علل بالقوه آنمی همولیتیک با واسطه ایمنی در گاو و گاومیش رودخانه‌ای

چکیده

هدف اصلی این مطالعه بررسی وقوع و علل احتمالی آنمی همولیتیک با واسطه ایمنی (IMHA) در گاوها و گاومیش‌های بومی جنوب غربی ایران می‌باشد. پنجاه و سه رأس دام (۳۷ رأس گاو و ۱۶ رأس گاومیش) دچار کم خونی مورد مطالعه قرار گرفتند. اخذ تاریخچه کامل و معاینات بالینی برای هر دام انجام گرفت. چهار گاو سالم و چهار گاومیش سالم نیز به‌عنوان گروه کنترل مورد استفاده قرار گرفتند. شمارش خون کامل، تست کومبس، آزمایش شکنندگی اسمزی اریتروسیت‌ها و آنالیز بیوشیمیایی سرم بر روی نمونه‌های خون جمع‌آوری شده انجام گرفت. IMHA در ۱۲ رأس گاو (۳۲/۴۳ درصد) و شش رأس گاومیش (۳۷/۵۰ درصد) براساس تست کومبس تشخیص داده شد. بیماری‌های اولیه یا همزمان شامل تیلریوز، آناپلاسموز، واکسیناسیون و پنومونی در ۱۱ رأس گاو و چهار رأس گاومیش مشاهده گردید. IMHA از نوع ایدیوپاتیک یا اولیه در یک رأس گاو و دو رأس گاومیش دارای تست کومبس مثبت، تشخیص داده شد. یافته‌های هماتولوژیک و بیوشیمیایی در گاوهای دچار IMHA شامل کم خونی جبران ناپذیر، لکوپنی، ترومبوسایتوپنی، افزایش شکنندگی اسمزی، هایپربیلیروبینمی و افزایش فعالیت سرمی آنزیم‌های آلکالاین فسفاتاز، آسپارتات آمینوترانسفراز و لاکتات دهیدروژناز بود. در مجموع می‌توان چنین نتیجه‌گرفت که IMHA در بخش زیادی از گاوها و گاومیش‌های دچار آنمی در جنوب غربی ایران ایجاد می‌شود. رخداد IMHA هم در گاو و هم در گاومیش اغلب ثانویه و به دنبال بیماری‌های عفونی به ویژه تیلریوز و آناپلاسموز می‌باشد. جهت شناسایی مکانیسم ایجاد IMHA اولیه یا ایدیوپاتیک و ثانویه در گاو و گاومیش مطالعات بیشتری مورد نیاز است.

واژه های کلیدی: آنمی همولیتیک با واسطه ایمنی، تست کومبس، گاو، گاومیش

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Introduction

Immune-mediated hemolytic anemia (IMHA) is an increased destruction of red blood cells (RBCs) due to antibodies bound to erythrocytes (type II hypersensitivity). It is one of the most commonly recognized autoimmune diseases reported in dog, cat, horse, and cattle.[1-4]

The removal of the antibody-coated RBCs occurs by macrophages through their fragment crystallizable (FC) receptors, primarily in the spleen and liver (extravascular hemolysis) or less frequently mediated by complement (intravascular or extravascular hemolysis).[5] The IMHA may develop primarily with the formation of antibodies against the normal RBC surface antigens which is a rare condition in large animals. This kind of anemia may be due to the incompatible blood transfusions, neonatal isoerythrolysis, or idiopathic autoimmune hemolytic anemia.[6,7] When antibodies are formed against abnormal substances covering the RBC, secondary IMHA occurs. It is usually associated with infection, neoplasia, and exposure to drugs or toxins.[8]

The IMHA is diagnosed based on demonstration of one or more of the following laboratory findings: auto-agglutination, spherocytosis and positive direct anti-globulin test (Coombs' test).[1,5] The Coombs' test detects immunoglobulin associated with the surface of RBCs using species-specific antisera.

There are few published reports of IMHA in cattle and, to the best of authors' knowledge, it has not been reported in river buffaloes. This study was performed to investigate the occurrence of immune mediated hemolytic anemia in native cattle and water buffaloes in southwest Iran. In addition, a clinical, hematologic and serum biochemical assessment was performed to find potential causative factors and complications involved in IMHA.

Materials and Methods

Animals. This study was performed in Ahvaz city, a subtropical area located in the southwest of Iran. A total of 37 anemic cattle (23 female and 14 male), and 16 anemic buffaloes (11 females and 5 males), referred to the Veterinary Hospital, Shahid Chamran University of Ahvaz, Iran from June 2013 through February 2014, were included in this study. Cattle and buffaloes with the hematocrit (HCT) lower than 27.00% and 30.00% respectively, were considered anemic.[9,10] The age of cattle ranged between 9 day and 12 years and buffaloes were between one month and seven years old. The animals used in this study were crossbred cattle and indigenous water buffaloes. For each animal a full clinical history was obtained and physical examinations were undertaken. In addition, blood samples from four clinically healthy cattle (three females and one males), and four healthy buffaloes (two females and two males), based on physical examination and laboratory testing were collected and used as control samples for the analysis of hematologic and biochemical results. All studies were conducted in compliance with institutional animal care and use policies.

Preparation of anti-cattle and anti-buffalo immunoglobulin. To produce rabbit anti-cattle and anti-buffalo immunoglobulin, gamma globulins from five bovine and five buffalo bulk sera were precipitated with 45.00% saturated ammonium sulfate and dialyzed against phosphate buffered solution (PBS) at 4 ˚C for 48 hr. The protein concentrations of the dialyzed solutions were measured using the Bradford method,[11] and the dialyzed solutions were then diluted with PBS until a concentration of 200 µg per 500 µL was achieved. The later diluted solutions were mixed with 500 µL of the Ferund's complete adjuvant (Razi Vaccine and Serum Research Institute, Karaj, Iran) and were intramuscularly injected into two groups of rabbits. Immunization was repeated two weeks later with the same amount of immuno-globulins but with Ferund's incomplete adjuvant. Blood serum was collected from the rabbits after two weeks. The rabbit anti-cattle and anti-buffalo immunoglobulins were detected via agglutination of cattle/buffalo immuno-globulin coated sheep RBCs.[11]

Direct Coombs' test. The EDTA-anticoagulated cattle or buffalo blood samples were washed three times with PBS and after the final washing, a 2.00% suspension of red blood cells in PBS was prepared. The Coombs' test was performed in a 96-well microplate. Rabbit anti-cattle or anti-buffalo antisera (50 µL) was added to 50 µL of PBS in the first well of the row, and then increasing dilutions of 1:2 antisera in PBS were prepared through 1:4096. Afterwards, 50 µL of washed RBCs suspension was added to each well and incubated at 37 ˚C for 30 min and then for 30 min at room temperature (25 ˚C). The additional 30 min incubation at room temperature was conducted to permit RBCs to settle and agglutination patterns to form. Wells were recorded as negative if they contained a button of RBCs that would disperse when the microtiter plate was slanted; positive wells exhibited mat formation that did not disperse when slanted, and the titer of positivity was recorded. Coombs' test was considered positive if agglutination occurred at 1:8 antibody dilutions.

Hematology and clinical chemistry assessments. Blood samples collected from the jugular vein into tubes containing EDTA were used to perform a CBC, Coombs' test, and erythrocyte osmotic fragility test. The concentration of NaCl causing 50.00% hemolysis was expressed as median corpuscular fragility (MCF). Blood samples which were collected into tubes lacking anticoagulant were used for serum separation. Following serum separation, serum biochemical analysis was performed using an automated analyzer (BT 1500; Biotecnica Instruments, Rome, Italy). Glucose concentration, total protein, albumin, total and direct bilirubin and activities of aspartate aminotransferase

(AST), γ-glutamyl transferase (GGT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were measured using commercial kits (Pars Azmoon, Tehran, Iran). All blood samples were examined for theileriosis and anaplasmosis agents using blood films. Parasitemia ratio was assessed by counting the number of infected red blood cells by screening at least 200 microscopic fields.

Statistical analysis. The normal distribution of data was assessed by Shapiro-Wilk's test. Analysis of variance (ANOVA) and Tukey's post hoc test were employed to compare the groups using SPSS (version 16.0; SPSS Inc., Chicago, USA). A p value less than 0.05 was considered as statistically significant.

Results

Direct Coombs' test. Based on the Coombs' test, IMHA was diagnosed in 12 cattle and six buffaloes. Titers of 1:8 to 1:512 were observed in different examined cases.

Clinical findings. The most frequent clinical signs at the time of admission were anorexia (12 cases) and diarrhea (six cases) in cattle with IMHA (Table 1). Pale mucosal membranes, icterus, and fever (> 39.50 ˚C) were observed in two, three and five cattle cases, respectively. Underlying or concurrent diseases were detected in 11 Coombs'-positive cases, including: theileriosis (9), anaplasmosis (3), FMD vaccination (FMD) (1) and infectious diseases including pneumonia (4). In one Coombs' positive cattle underlying cause could not be identified, and a diagnosis of primary or idiopathic IMHA was made (case 5). Concurrent infections were noticed in all buffaloes with IMHA except two cases (1 and 5) in which no underlying disease was detected (Table 1).

Hematologic analysis. Hematologic assessment revealed that mean HCT, hemoglobin concentration and RBC count of Coombs'-positive cattle were significantly lower than Coombs'-negative and nonanemic control animals ($p < 0.05$). The minimum MCV value was also observed in cattle with IMHA. Mean red cell distribution width (RDW) was increased in both anemic groups (Coombs'-positive and Coombs'-negative) compared to the control group. Neither marked spherocytosis nor auto-agglutination were observed in anemic cattle blood smears. Although no statistically significant difference was observed in leukocyte total count; neutrophil count was significantly lower ($p < 0.05$) and lymphocytes were higher in cattle with IMHA compared to the control animals. Despite the fact that IMHA did not affect mean platelet count statistically, thrombocytopenia (platelet counts < 160×10^3 per μL) was recorded in four Coombs' positive anemic cattle. Anemia was associated with an increase in MCF in Coombs' positive as well as Coombs' negative animals. However, these changes were not statistically significant ($p > 0.05$). Hematologic data showed significant reductions in HCT ($p < 0.05$) along with a decrease in RBC count, hemoglobin concentration and MCV ($p > 0.05$) among anemic buffaloes with and without IMHA, while RDW was increased in the mentioned animals ($p > 0.05$). In examination of blood smears, spherocytes or autoagglutination were not observed. A significant drop in total leukocyte and neutrophil counts was also observed in Coombs'-positive anemic buffaloes compared to the control group ($p < 0.05$). Thrombocytopenia was noted in three buffaloes with IMHA. However, mean platelet count was not statistically different among buffaloes with IMHA and control group (Table 2).

Table 1. History and clinical findings in anemic cattle and buffaloes with immune-mediated hemolytic anemia (IMHA).

Case	Sex	Age	Titer of Coombs' test	History and clinical signs	BT (˚C)	HR (bpm)
Cattle						
1	M	1 m	1:8	Severe theileriosis, Anorexia, pneumonia	41.00	120
2	F	9 d	1:8	Severe theileriosis, Lethargy, anorexia, coughing, diarrhea (yellow watery stool), icterus	40.00	56
3	M	7 m	1:8	Severe theileriosis, anorexia, dyspnea, diarrhea, limbs tremor,	39.30	54
4	M	1 m	1:8	Theileriosis and anaplasmosis, anorexia, coughing, chronic diarrhea, hair loss, arthritis,	40.70	70
5	F	12 y	1:16	Pregnancy, anorexia, decreased milk production, bloat, stranguria, gastrointestinal impaction	38.50	90
6	F	3 y	1:16	Theileriosis and anaplasmosis, lethargy, anorexia, recumbency, decreased milk production,	40.50	90
7	F	5 y	1:32	Anaplasmosis, pregnancy, anorexia, icterus, bruxism, , pale mucous membranes	38.50	84
8	F	8 y	1:32	Theileriosis, anorexia, diarrhea, dysuria, coughing,	38.50	76
9	M	2 m	1:32	Theileriosis, anorexia, recumbency, polydipsia, diarrhea, , pale mucous membranes	39.00	108
10	M	8 m	1:32	Theileriosis, anorexia, weight loss, icterus,	40.30	100
11	M	6 m	1:128	FMD vaccination 15 days ago, Anorexia, hair loss, arthritis	39.00	145
12	F	2 m	1:256	Anorexia, hyperpnea, diarrhea, lymph adenomegaly, skin lesions	39.40	86
Buffaloes						
1	M	1 y	1:32	Arthritis	41.00	90
2	F	2 y	1:32	Anorexia, coughing, purulent ophthalmitis, bruxism	39.50	60
3	F	7 y	1:64	Anorexia, severe weight loss, watery diarrhea	38.00	90
4	F	4 y	1:64	Anorexia, enteritis, watery diarrhea	39.50	70
5	F	1 m	1:128	Recumbency, acidosis	37.50	56
6	F	2 y	1:512	Infectious bovine rhinotracheitis (IBR), Anorexia, nasal and ocular discharge	37.30	45

M: Male, F: Female, d: Day, m: Month, y: Year, BT: Body temperature, HR: Heart rate, FMD: Foot and mouth disease.

Table 2. Hematologic results in Coombs'-negative, -positive, and control cattle and buffaloes. Data are expressed as mean ± SE.

Parameters	Cattle			Buffaloes		
	Control (n = 4)	Coombs' – (n = 25)	Coombs' + (n = 12)	Control (n = 4)	Coombs' – (n = 10)	Coombs' + (n = 6)
Hematocrit (%)	33.65 ± 1.70[a]	20.83 ± 1.98[b]	14.19 ± 1.53[c]	35.32 ± 2.41[a]	25.8 ± 0.87[b]	25.51 ± 1.03[b]
Red blood cells (10^6 µL^{-1})	6.76 ± 0.65[a]	4.39 ± 0.55[b]	3.21 ± 0.42[b]	6.61± 1.17	4.8 ± 0.52	5.29 ± 0.27
Hemoglobin (g dL^{-1})	9.17 ± 0.69[a]	6.93 ± 0.76[a]	4.04 ± 0.50[b]	10.27 ± 1.36	8.26 ± 1.17	8.13 ± 0.46
MCH (pg)	13.65 ± 0.96	14.96 ± 0.70	12.55 ± 0.94	16.00 ± 1.60	16.8 ± 2.03	15.45 ± 0.96
MCHC (%)	27.25 ± 1.84[a]	32.88 ± 1.05[b]	28.20 ± 0.92[a]	28.67 ± 1.91	31.3 ± 1.62	31.75 ± 1.01
MCV (fL)	50.57 ± 3.23	48.86 ± 3.98	47.93 ± 3.79	56.45 ± 5.93	53.9 ± 4.51	48.65 ± 2.22
RDW (%)	15.03 ± 0.83	18.30 ± 1.13	18.07 ± 0.97	15.00 ± 1.01	16.7 ± 1.48	16.16 ± 1.33
White blood cells (10^3 µL^{-1})	9.46 ± 2.14	7.13 ± 1.29	6.79 ± 1.07	9.10 ± 1.66[a]	8.34 ± 1.70[ab]	4.85 ± 0.93[b]
Neutrophils (10^3 µL^{-1})	8.15 ± 1.22[a]	2.88 ± 0.88[ab]	2.71 ± 0.80[b]	4.84 ± 0.63[a]	4.05 ± 0.96[a]	1.81 ± 0.36[b]
Lymphocytes (10^3 µL^{-1})	2.97 ± 0.90	4.42 ± 1.35	4.04 ± 0.51	2.75 ± 1.64	2.13 ± 0.88	1.90 ± 0.36
Eosinophils (10^3 µL^{-1})	0.06 ± 0.06	0.07 ± 0.03	0.11 ± 0.08	0.41 ± 0.41	0.18 ± 0.07	0.04 ± 0.02
Monocytes (10^3 µL^{-1})	0.00 ± 0.00	0.03 ± 0.03	0.01 ± 0.01	0.00 ± 0.00	0.05 ± 0.05	0.01 ± 0.01
Platelet (10^3 µL^{-1})	210.00 ± 33.19	302.00 ± 49.59	193.90 ± 27.78	256.25 ± 71.64	278.31 ± 67.24	178.00 ± 18.31
MCF (%)	0.64 ± 0.00	0.68 ± 0.005	0.67 ± 0.00	ND	ND	ND

MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, MCV: Mean corpuscular volume, RDW: Red blood cell distribution width, MCF: Median corpuscular fragility, and ND: Not determined.
[abc] Different letters in each row indicate significant difference between groups for each species ($p < 0.05$).

Biochemical analysis. Despite no significant differences in biochemical profile among various groups, the amount of total and direct bilirubin was considerably increased in Coombs'-positive anemic cattle compared to control and Coombs'-negative groups. A remarkable rise in serum ALP, AST and LDH activities was observed in Coombs'-positive anemic buffaloes. However, these alterations were statistically insignificant ($p > 0.05$, Table 3).

Discussion

The IMHA was found in 32.43% of anemic cattle and 37.50% of anemic buffaloes examined in this study based on positive Coombs' tests. There are a few reports of IMHA in cattle,[4,12,13] while, it has not been described in buffaloes previously. It is less prevalent in ruminants compared with dogs and cats. The IMHA does not seem to be affected by breed or gender. However, it is still an important cause of anemia in these species.[5]

Immune mediated hemolysis is associated with un-specific signs such as lethargy, anorexia, fever, weakness and icterus.[12,14,15] In the present study, anorexia was observed most frequently whereas only three cattle with IMHA displayed icterus. The majority of these symptoms are attributable to anemia and hypoxia in different vital organs including liver, lung, heart, kidney, and intestines. Other signs vary depending on the underlying diseases.

The main underlying causes of IMHA in both cattle and buffaloes in this study were infectious diseases including blood parasites (*Theileria* spp. and *Anaplasma* spp.), bacterial and viral infections.

Most reported cases of IMHA in cattle were secondary to infectious, neoplastic, and other immune-mediated diseases or to drug administration.[5,12,16] An association between infection and autoimmunity has been described based on the hypothesis of molecular mimicry. This model assumes that antigenic cross-reaction exists between epitopes expressed by infectious agents and self-molecules or autoantigens.[4]

Theileriosis and anaplasmosis are demonstrated to induce IMHA,[17] but the pathophysiologic mechanism of this anemia is currently unknown. Alteration of the RBC surface, which induces acceleration in clearance of

Table 3. Serum biochemical test results in Coombs'-negative, -positive, and control cattle and buffaloes. Data are expressed as mean ± SE.

Parameters	Cattle			Buffaloes		
	Control (n = 4)	Coombs' – (n = 25)	Coombs' + (n = 12)	Control (n = 4)	Coombs' – (n = 10)	Coombs' + (n = 6)
Glucose (mg dL^{-1})	109.67 ± 36.02	97.66 ± 22.48	85.00 ± 12.48	123.50 ± 57.50	108.34 ± 45.61	140.00 ± 28.00
Protein (g dL^{-1})	6.53 ± 1.46	7.40 ± 1.38	6.84 ± 0.50	6.50 ± 0.40	8.27 ± 2.58	4.97 ± 1.04
Albumin (g dL^{-1})	3.33 ± 0.61	3.43 ± 0.73	3.20 ± 0.52	3.35 ± 0.55	3.16 ± 0.60	3.00 ± 0.36
Total bilirubin (mg dL^{-1})	0.90 ± 0.47	0.64 ± 0.02	1.84 ± 0.66	0.69 ± 0.22	0.51 ± 0.27	0.57 ± 0.13
Direct bilirubin (mg dL^{-1})	0.28 ± 0.14	0.25 ± 0.00	0.60 ± 0.29	0.34 ± 0.18	0.17 ± 0.05	0.19 ± 0.02
ALP (U L^{-1})	101.00 ± 14.00	174.67 ± 66.13	112.14 ± 17.82	229.00 ± 83.00	137.72 ± 45.33	446.57 ± 106.20
GGT (U L^{-1})	23.66 ± 0.88	27.00 ± 4.04	36.71 ± 6.37	43.50 ± 2.50	37.69 ± 6.16	39.00 ± 8.51
AST (U L^{-1})	91.00 ± 31.17	88.33 ± 39.82	94.16 ± 28.73	97.50 ± 78.50	134.23 ± 51.65	142.50 ± 48.50
LDH (U L^{-1})	2758.00 ± 833.26	1936.30 ± 146.51	2744.60 ± 394.62	1861.00 ± 339.00	1381.76 ± 159.20	2399.70 ± 363.38

ALP: alkaline phosphatase, GGT: γ-glutamyl transferase, AST: aspartate aminotransferase, LDH: lactate dehydrogenase.

RBCs from peripheral blood, may occur in infected cattle. Phosphatidylserine molecules, which normally are localized on the inner leaflets of cell membranes, have been shown to be translocated to the external surface of RBC in *Theileria*-infected cattle. Exposure of phosphatidyl-serine on the cell surface can induce an antibody response and function as a marker for phagocytic clearance of RBCs by macrophages.[18]

The anemia in anaplasmosis results largely from extra vascular destruction of parasitised erythrocytes.[19] However, the degree of anemia is often out of proportion to the prevailing parasitemia.[20] This situation is attributable to immune-mediated destruction of non parasitized erythro-cytes in addition to that of parasitised erythrocytes. Immunological studies indicate that during anaplasmosis, the host produces antibodies directed against the organism as well as against its own red cells.[20,21]

Recent vaccination along with arthritis was recorded in a Coombs' positive anemic cattle in the present study. Vaccination can initiate immune mediated diseases, such as IMHA,[22] polyarthritis,[23] and bovine neonatal pancytopenia.[24] The mechanism of autoimmune reactions following immunization has not yet been clarified. One of the possibilities is molecular mimicry; when a structural similarity exists between some viral antigen (or other component of the vaccine) and a self-antigen. This similarity may be the trigger to the autoimmune reaction.[25]

A diagnosis of primary or idiopathic IMHA was made in one cattle and two buffaloes on the basis of positive Coombs' test and exclusion of other possible causes of hemolytic anemia including infectious agents and effects of drug administration. The term "idiopathic" is usually used, as primary IMHA should only represent the cases in which an autologous antibody has been produced against a normal constituent antigen and this is often difficult to prove.[12,13]

In this study, a nonregenerative anemia was observed in most Coombs' positive cattle. Patients with IMHA commonly have regenerative anemia.[12,26] However, a nonregenerative immune-mediated hemolytic anemia may occur when antibodies or complement are directed against erythroid precursors in bone marrow.[27] Therefore, absence of reticulocytosis does not rule out a diagnosis of IMHA.[28] Only 25.00% of cattle (three cases) and 16.66% of buffaloes (one case) with IMHA in the current study had a regenerative response (MCV > 50 fL). In the remaining cases, immune-mediated destruction of immature erythroid cells and ineffective erythropoiesis may have occurred; however, further studies are needed to support this hypothesis.

In our study, four examined cattle and three examined buffaloes suffered from both IMHA and thrombocytopenia. Immune-mediated thrombocytopenia was previously reported in dogs and cats with primary IMHA.[3,29]

In the present study leukopenia and neutropenia was noted in both cattle and buffaloes with IMHA. The WBC count typically exhibits a mild leukocytosis in cattle IMHA, representing predominantly neutrophils, which is usually ascribed to inflammatory disease or tissue necrosis secondary to anemic hypoxemia.[30] However, neutropenia may also be seen. Concurrent leukopenia and/or thrombo-cytopenia, as in the case of this study, are probably associated to an immune-mediated process which suppresses the bone-marrow.[31] Also, in this study, MCF was increased in anemic cattle with IMHA. Due to the lack of central pallor in erythrocytes of ruminants, the osmotic fragility test can be used as an indirect measure of spherocytosis in these species considering that spherocytes are more fragile than normal RBCs.[32] The rise in RDW of Coombs' positive anemic cattle indicates a high variation in the size of erythrocytes, which confirms this morphologic feature.

Hyperbilirubinemia, most likely due to increased breakdown of hemoglobin,[5] was present in cattle with IMHA. The liver enzyme activities were also increased in Coombs' positive anemic buffaloes which can be attributed to cellular hypoxia due to low liver blood volume in anemia that leads to hepatocyte injury.[33]

In conclusion, IMHA may occur in a significant proportion of anemic cattle and river buffaloes in southwest of Iran. The occurrence of IMHA in both cattle and buffaloes is mostly secondary to infectious diseases especially theileriosis and anaplasmosis. Coombs' positive anemic animals exhibit a combination of clinical and laboratory symptoms which highly rely on the underlying cause of the anemia. The mechanisms of primary or idiopathic and secondary IMHA in cattle and buffaloes deserve further study.

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Conflict of interest

The authors declare that there is no conflicts of interest.

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