

# COLD HAEMAGGLUTININ DISEASE IN TWO FRENCH BULLDOG PUPS - A CASE REPORT

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Cold haemagglutinin disease (CHD) was diagnosed in two newborn French bulldog pups. Necropsy revealed cyanosis and necrosis, formation of crusts on all anatomic extremities and amputation of the lower parts of the hind limbs. Histologically, cutaneous necrosis was found on the limbs of both animals, with blood vessel thrombosis, hemorrhages and predominantly neutrophilic infiltration, which affected the periosteum of the metatarsal bones. The surfaces of the necrotic areas were covered with thick crusts with Gram Positive bacterial colonies. In the spleen and liver erythrophagocytosis was present. Lung congestion, alveolar edema and thrombosis of pulmonary artery branches. cerebral and chorioidal vessels were present. Immunohistochemically, lymphocytes and plasma cells in necrotic tissue were positive for IgM. Bacteriological examination of swabs from necrotic areas revealed the presence of *Staphylococcus aureus*. The diagnosis of CHD was based on macroscopic and histopathologic findings, which were identical to those described in literature and were supported by immunohistochemical reactivity to IgM.

**Key words** cold haemagglutinin disease, immune-mediated haemolytic anaemia, pathology, pup

## Introduction

Cold haemagglutinin disease (CHO) is a form of autoimmune haemolytic anaemia (AIHA). Classification of AIHA includes warm AIHA, cold agglutinin syndrome, paroxysmal cold haemoglobinuria, mixed-type AIHA and drug-induced AIHA (GEHRS and FRIEDBERG, 2006). The characteristics of the auto-antibodies are responsible for the various clinical entities. Most immunoglobulins implicated in AIHA are reactive at body temperature (warm antibodies) but a smaller portion are more reactive at lower temperatures and may lead to conditions known as cold haemagglutinin disease (cold agglutinin immunohaemolytic anemia) and paroxysmal cold haemoglobinuria (cold haemolysin haemolytic anemia). CHD is caused by so-called cold haemagglutinins, which are IgM antibodies, while paroxysmal cold haemoglobinuria is caused by cold haemolysins IgG antibodies (ASTER, 2005). In animals, cold haemagglutinin immunohaemolytic anemia is usually known as CHO (FRY and McGAVIN, 2007). CHD may be primary (idiopathic) or secondary, caused by bacterial, viral or protozoal infection, systemic autoimmunity, or neoplasm (BUDZA et al., 1976; ZULTY and KOCHIBA, 1990). Primary CHO is associated with monoclonal antibody (FRY and McGAVIN, 2007). Secondary CHO can be associated with either monoclonal or polyclonal auto-antibodies. Cold

agglutinins appear acutely, mostly during the recovery phase of infectious diseases such as mycoplasmal pneumonia, infectious mononucleosis, babesiosis, haemobartolosis, infections with cytomegalovirus, influenza virus, human immunodeficiency virus, in association with certain lymphoid neoplasms and lead poisoning, or as idiopathic conditions (ASTER, 2005; DICKSON, 1990). There are data that transplacental transfer of IgM auto-antibodies from mother to fetus is not possible but there is evidence that IgM is passed through the colostrum (BRIDLE and LITTLEWOOD, 1998). According to this, it is possible that the mother's pathological conditions and cold agglutinins passed through colostrum, can induce CHO in newborn animals. The clinical and pathological signs are the result of IgM binding to red blood cells at sites such as anatomic extremities where the temperature is below 30°C. Intravascular IgM binding agglutinates red cells and fixes the complement on their surfaces (ASTER, 2005; GIGER, 2000; SLAPPENDEL *et al.*, 1975). When blood re-circulates and warms, IgM is rapidly released, usually before complement-mediated hemolysis can occur but sometimes hemolysis may occur (ASTER, 2005; GIGER, 2000). The vascular obstruction caused by the red cell agglutinates results in the clinical signs. Clinical symptoms are similar in humans and animals.

There is a wide range of signs such as pallor, fatigue, dyspnea, poor feeding, anemia, which may be mild, moderate, or severe, and symptoms of hemolysis: jaundice, dark urine caused by haemoglobinuria and acrocyanosis (a purplish discoloration of the anatomic extremities, e.g., the tips of the ears and tail, the nail beds of the toes and fingers) are included (ASTER, 2005; DICKSO, 1990; GIGER, 2000; SLAPPENDEL *et al.*, 1975; BRIDLE and LITTLEWOOD, 1998; GINN *et al.*, 2007; MATHE *et al.*, 1998; SCOTT *et al.*, 2001). Acrocyanosis is due to intraarteriolar agglutination of red blood cells in relatively cool areas of the body. It can lead to gangrene of the anatomic extremities, due to microvasculature occlusion, which can be complicated with secondary infection (SLAPPE DEL *et al.*, 1975; SCOTT *et al.*, 2001). Diagnosis is based on clinical and pathomorphological signs such as acrocyanosis and ischemic necrosis of the anatomic extremities, anemia and jaundice; cytologically by detecting spherocytes and auto-agglutination in the blood smears, or by performing an osmotic fragility test. Positive Coombs test and erythrocyte agglutination on a cooled slide that reverses upon warming support the diagnosis (GEHRS and FRIEDBERG, 2006; GIGER, 2000; GINN *et al.*, 2007; MATHE *et al.*, 1998).

### Materials and methods

The described disease was seen in two female French bulldog puppies delivered in late August by caesarean section. The puppies presented clinically with cyanosis and weakness (shallow breathing, low pulse). They were placed into an incubator with consequent recovery of their general condition, but at the anatomic extremities i.e. tip of the ears and nail beds of the limbs cyanosis upgraded and after a few days gangrene was observed, with amputation of the hind limbs distal from the metatarsal metaphyses. On the fifth day puppies were euthanized with the intravenous application of 0.6 mL T 61<sup>®</sup> (Intervet International, Holland) and delivered for post-mortem examination.

Necropsy was performed on both animals and tissue samples of the distal part of the limbs, the myocardium, lungs, kidney, liver, spleen and brain were fixed in 7% buffered formalin saline and processed by routine procedures for histopathological examination. Histopathological examination with H&E stain was performed on all tissue samples. Histopathological examination of the liver with Sudan 3 stain for lipids was performed. On the sections from distal part of limbs Gram staining was performed. For immunohistochemical analysis paraffin sections (3-4  $\mu\text{m}$ ) of all specimens were deparaffinised in xylene and then rehydrated through graded alcohol. Pre-treatment of tissue with proteinase K and visualization of the IHC staining with DAB was performed. Immunostaining was performed by the avidin biotin peroxidase complex method using LSAB+ kit (Dako, Glostrup, Denmark). Polyclonal rabbit Anti-human IgM (Dako, Glostrup, Denmark) was used for immunohistochemistry, since there is evidence of cross-reactivity between human and canine IgM epitopes (ARCE *et al.*, 2002; BLIXENKRONE-MOLLER *et al.*, 1991). On the same tissue section negative control using an irrelevant IgG antibody (Dako, Glostrup, Denmark) was performed. Smears from necrotic parts of the limbs were taken for routine bacteriological examination.

### Results

At necropsy, in both cases moderate anemia of all mucous membranes was noted. On the distal parts of the hind limbs and to a lesser extent on the front limbs and tips of the ears, cyanosis and ischemic necrosis with loosening of tissue and formation of crusts were seen. The necrosis was very deep and had overtaken the phalangeal and metatarsal bones so that in case No. 1 both distal metatarsal epiphysis and parts below were amputated (Fig. 1). The lungs were moderately congested with pulmonary edema. The liver, spleen, kidneys, brain and meninges were moderately congested. There were no detectable gross pathological changes to the other organs.



Fig. 1. Hind limbs; pups No.1 and 2. Acrocyanosis and ischemic necrosis of hind limbs

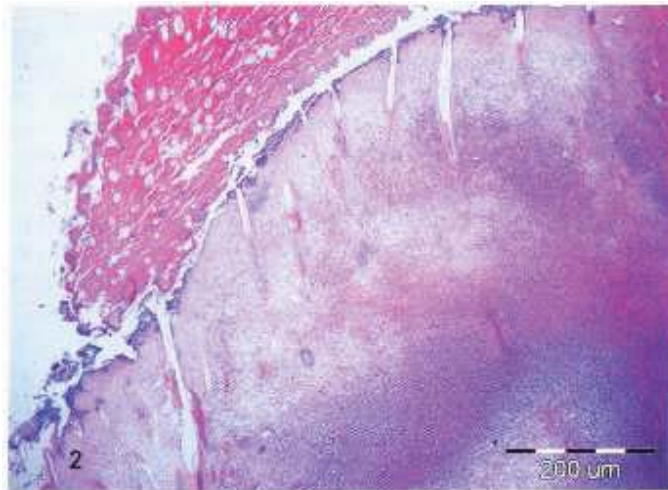


Fig. 2. Skin, hind limb; pup No.1. Necrosis of epidermis, dermis and subcutaneous tissue. H&E; scale bar = 200μm.

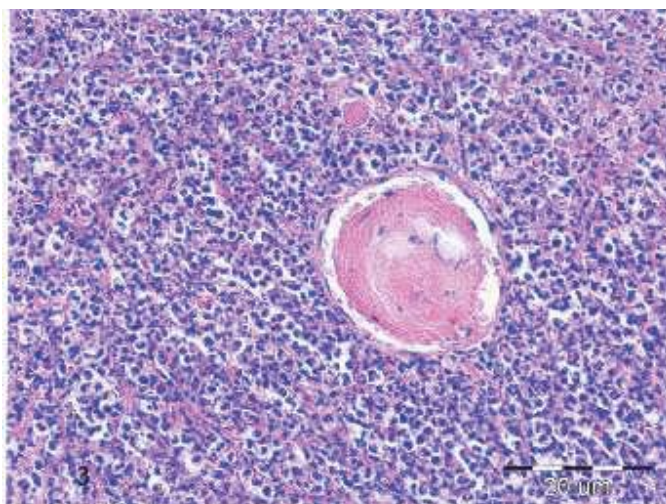


Fig. 3. Skin, hind limb; pup No. 1. Thrombosis of dermal blood vessel, necrosis and neutrophilic infiltration of dermis. H&E; scale bar = 20 μm.



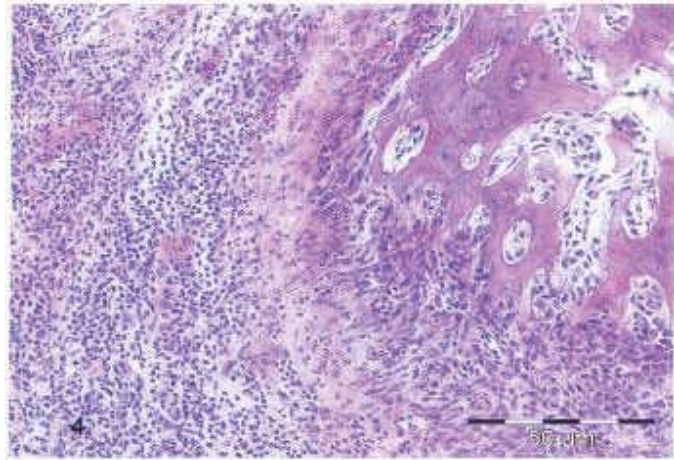


Fig. 4. Metatarsal bone; pup No.1. Neutrophilic infiltration of periost and destruction of metatarsal bone. H&E. scale bar = 50  $\mu$ m.



Fig. 5. Skin, hind limb; pup No. 1. Gram positive coccoid microorganisms on the surfaces of necrotic areas. Gram stain; scale bar = 20 $\mu$ m.

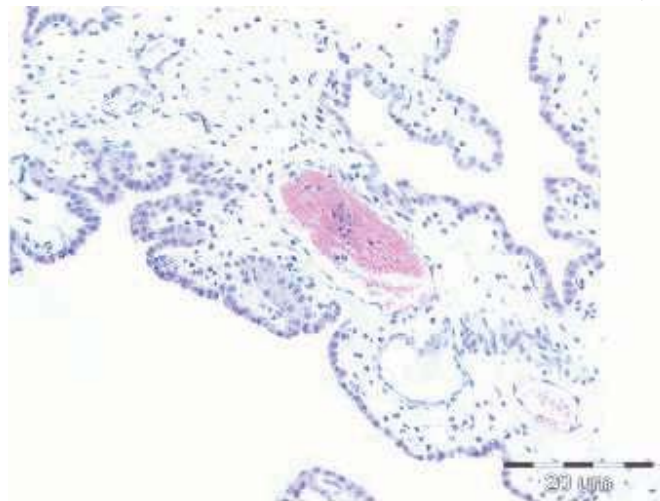


Fig. 6. Brain; pup No.2. Thrombosis of chorioidal plexus vessel. H&E; scale bar = 20  $\mu$ m.

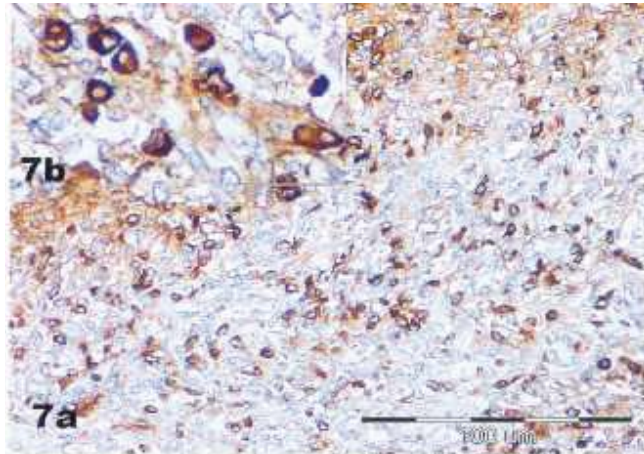


Fig. 7a. Skin, hind limb; pup No. 1. IgM positive lymphocytes and plasma cells in necrotic and inflamed areas. IgM immunostaining. Scale bar = 100  $\mu$ m; Inset, 7b. Skin, hind limb; pup No.1. IgM positive lymphocytes and plasma cells in necrotic and inflamed areas. IgM immunostaining; scale bar = 10  $\mu$ m.

In both animals ungcongestion,alveolaredemaand thrombosisofthe pulmonary artery branches were present. The spleen had highly cellular red pulp, and erythrophagocytosis and extramedullary haematopoiesis was observed. In the liver, erythrophagocytosis, hydropic and multifocal fatty change were present (lipids Sudan 3 positive). On the limbs, focally extensive necrosis of the epidermis, dermis and subcutaneous tissue (Fig. 2) with blood vessels thrombosis (Fig. 3), hemorrhages and predominantly neutrophilic diffuse infiltration, with a high ratio of degenerated neutrophils, infiltrating through the periost of the metatarsal bones (Fig. 4), were observed. The surfaces of the necrotic areas were covered with thick crusts with scattered bacterial colonies. The colonies were composed of large numbers of bluish Gram positive coccoid microorganisms (Fig. 5). In case No. 2 thrombosis of the chorioidal plexus (Fig. 6) and cerebral capillaries was also noted, and in case No. 1, cystic dilatation of the renal tubules, cerebral and myocardial congestion was present. Immunohistochemically, the lymphocytes and plasma cells in necrotic and inflamed areas of the distal limbs were positive for IgM (Figs. 7) and were negative for IgG. Bacteriological examination of swabs taken from the necrotic

areas revealed the presence of *Staphylococcus aureus*.

#### Discussion

Cases of cold antibody induced AIHA, especially idiopathic cases of CHD, are rarely reported. There were no reports described of CHD in the pups from the same litter. In this case diagnosis of the CHD was based on the macroscopical (cyanosis and necrosis with amputation of the anatomic extremities) and histopathological findings (thrombosis of blood vessels, hemorrhages, necrosis and predominantly neutrophilic infiltration, erythrophagocytosis) which are identical to findings described in literature (GEHRS and FRIEDBERG, 2006; ASTER, 2005; FRY and McGAVIN, 2007; DICKSON, 1990; GIGER, 2000; SLAPPENDEL *et al.*, 1975; SCOTT *et al.*, 2001; GREE *et al.*, 1977). The diagnosis was supported by positive immunohistochemical reactivity to IgM. Other laboratory tests which could support the diagnosis (Combs' test, cytological detection of spherocytes and autoagglutination in the blood smears, or osmotic fragility test) were not conducted before euthanasia. Thrombosis of the pulmonary artery branches and thrombosis of cerebral capillaries could also be consequences of cold antibodies

agglutination (BUDZA *et al.*, 1976; KLEIN, 1989). Erythrophagocytosis in the spleen and liver as evidence of haemolysis also support the diagnosis. The presence of colonies of Gram positive coccoid bacteria and isolation of *Staphylococcus aureus* from swabs of the necrotic areas are probably the result of secondary necrotic tissue infection. The most similar condition which could be discussed as a comparative diagnosis would be neonatal isoerythrolysis involving cold-acting agglutinins (BRIDLE and LITTLEWOOD, 1998), but no pathomorphological or pathohistological symptoms like palior, jaundice, haemoglobinuria (FRY and McGAVIN, 2007) were present. In terms of differential diagnostics, a few other conditions may be discussed in our case. Although dogs are not a highly susceptible species, acral cyanosis and necrosis can be found in ergotism (GINN *et al.*, 2007; BLODGETT, 2007). In the case presented this intoxication is less probable, since the puppies were still suckling and just few days old when cyanosis and necrosis occurred, so the only way for intoxication would be transplacental or through the milk. For this reason, it would be probable that the bitch was also affected but there were no clinical signs to support that (BLODGETT, 2007). Similar lesions may be caused by exposure to cold temperatures which can lead to freezing injuries (GIGER, 2000) but this condition can be eliminated because the puppies were delivered in August when the average temperatures in Zagreb are 25-30 °C. Other differential diagnoses such as disseminated intravascular coagulation or dermatomyositis were excluded due to the lack of clinical and pathological evidence.

Although a positive Coombs' test and erythrocyte agglutination on a cooled slide (GINN *et al.*, 2007) would be supportive for confirmation of the diagnosis, according to all the findings the most probable diagnosis for the condition described is idiopathic CHD, because no pathological conditions were found, either in the bitch or the puppies, which cause secondary CHD.

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#### References

- ARCE, C., A. MORENO, Y. MILLAN, J. M. DE LAS MULAS, D. LLANES (2002): Production and characterization of monoclonal antibodies against dog immunoglobulin isotypes. *Vet. Immunol. Immunopathol.* 88, 31-41.
- ASTER, J. C. (2005): Red blood cell and bleeding disorders. In: Robbins and Cotran Pathologic Basis of Disease. (Kumar, V., A. K. Abbas, N. Fausto, Eds.) Elsevier Saunders, Philadelphia. pp. 619-685.
- BLIXENKRONE-MOLLER, M., I. RODE PEDERSEN, M. J. APPEL, C. GRIOT (1991): Detection of IgM antibodies against canine distemper virus in dog and mink sera employing enzyme-linked immunosorbent assay (ELISA). *J. Vet. Diagn. Invest.* 3,3-9.
- BLODGETT, D. J. (2007): Fescue toxicosis. In: *Veterinary Toxicology Basis and Clinical Principles.* (Gupta, R. C., Ed.) Academic Press, Amsterdam, Boston, Heidelberg, London, New York, Oxford, Paris, San Diego, San Francisco, Singapore, Sydney, Tokyo. pp. 907-914.
- BRIDLE, K. H., J. D. LITTLEWOOD (1998): Tail tip necrosis in two litters of Birman kittens. *J. Small. Anim. Pract.* 39, 88-89.
- BUDZA, A., J. H. LUMDSEN, B. J. MCSHERRY, V. E. O. VALLI, E. A. JANZEN (1976): Haemobartonellosis in a dog in association with Coombs' positive anemia. *Can. Vet. J.* 17, 267-270.
- DICKSON, N. J. (1990): Cold agglutinin disease in a puppy associated with lead intoxication. *J. Small. Anim. Pract.* 31, 105-108.
- FRY, M. M., M. D. McGAVIN (2007): Bone marrow, blood cells, and lymphatic system. In: *Pathologic Basis of Veterinary Disease.* 4<sup>th</sup> edn. (McGavin, M.D., J.F. Zachary, Eds) Mosby Elsevier, St. Louis. pp743-832.
- GEHRS, B. C., R. C. FRIEDBERG (2006): Autoimmune hemolytic anemia. *Am. J. Hematol.* 69, 258-271.
- GIGER, U. (2000): Hematology and immunology. In: *Textbook of Veterinary Internal Medicine,* 5<sup>th</sup> ed. (Ettinger S. J., E. C. Feldman, Eds.) Saunders Company, Philadelphia.

- delphia, Pennsylvania. pp. 1793-1797.
- GINN, P. E., L. E. K. L. MANSELL, P. M. RAKICH (2007): Skin and appendages. In: Jubb, Kennedy and Palmer's Pathology of Domestic Animals. Vol.1, 5<sup>th</sup> ed. (Grant Maxie M., Ed.) Elsevier Saunders, Edinburgh, London, New York, Oxford, Philadelphia, St. Louis, Sydney Toronto. pp. 553-781.
- GREEN, C. E., F. KRISTENSENN, E. J. HOFF, M. D. WIGGINS (1977): Cold hemagglutinin disease in a dog. J. Am. Vet. Med. Assoc. 170, 505-510.
- KLEIN, M. K., S. W DOW, R. A. W ROSYCHUK (1989): Pulmonary thromboembolism associated with immune-mediated hemolytic anemia in dogs: Ten cases (1982-1987). J. Am. Vet. Med. Assoc. 195,246-250.
- MATHE, A., K. YOROS, P. YAJDOYICH, I. KOTAI, P. SOOS (1998): Immunohemolytic anemia of dogs - review and case report. Magyar Allatorvosok Lapja. 120,261-267.
- SCOTT, D. W, WH.Jr.MILLER,S.E.GRIFFIN (2001): Cryoglobulinemia and Cryofibrinogellemia. In: Muller & Kirk's Small Animal Dermatology. 6<sup>th</sup> edn. (Scott, D. W, W H. Jr. Miller, C. E. Griffin, Eds.) WB. Sanders Company, Philadelphia. pp. 717-720.
- SLAPPENDEL, R. J, C. L. YAN ERP, J. GOUDSWAARD, M. BETHLEHEM (1975): Cold haemagglutinin disease in a toy Pinscher dog. Tijdschr. Diergeneeskd. 100, 445-460.
- ZULTY, J. C., G. J. KOCHIBA (1990): Cold agglutinins in cats with haemobartonellosis. J. Am. Vet. Med. Assoc. 196, 907-910.

