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Full Article

Detection of cryoglobulins in serum of Caspian miniature horse

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ABSTRACT

Blood samples were collected from 20 healthy miniature Caspian horses at 37 °C. Isolation of cryoglobulin was performed based on a standard method in present study. Harvested sera were kept at 4 °C for two hours and then examined for cryoglubolin. Four serum samples containing precipitate Suspicious of containing cryoglobulin were selected. Subsequently serum protein electrophoresis was performed on normal serum samples and also on four serum samples containing precipitates using an automated electrophoresis system on cellulose acetate strips. In addition Ig isotypes detection (IgG, IgM and IgA) was performed on both precipitates and serum containing precipitates using single radio immunediffusion method (SRID). A narrow-based peak on gamma region of precipitate acetate cellulose strips was observed. Precipitate concentrations were strikingly higher than normal concentration of serum immuneglobulins. It can be suggested that cryoglobulin concentration in a proportion of Caspian miniature horse is higher than other equides may be in relation with animal susceptibility to neoplasias such as lymphoma and leukemia.

Keywords: Caspian miniature horse, cryoglobulin, serum, electrophoresis

INTRODUCTION

Caspian miniature horse is likely one direct ancestor of the Oriental breeds, and of all light horse breeds. Previously it was believed that this tiny horse is pony, but researches cleared that Caspian horse is an ancient breed of miniature horse not pony (Hendricks *et al* 1995). The most important fact about Caspian horse is that probably it is to be the ancestor of all light breeds of horses (Hendricks *et al* 1995, Draper *et al* 1996).

Cryoglobulins are immunoglobulins (Ig) that precipitate as serum is cooled below core body temperatures (Usha *et al* 1999). Isolation of cryoglobulin has a standard method previously reported (discussed in material and methods). For evaluation of cryoglobulinemia two stages may be done. Existence of the cryoglobulins in serum is proven in first stage, in

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which a precipitate is demonstrated to be primarily immunoglobulin and is resolubilized by warming again to 37 °C. Subsequently further analysis to characterization and typing of the cryoglobulin is performed by means of immunofixation and other methods (Usha *et al* 1999). Isolation of cryoglobulins are helpful diagnostic tools for identifying some diseases such as neoplasia e.g. multiple myeloma, macroglobulinemia, lymphoma and lymphocytic lymphoma (Castoulis *et al* 1965, Davie *et al* 1968, Goveric 1995), autoimmune diseases e.g. lupus erythromatous and infectious diseases e.g. subacute bacterial endocarditis (Champion 1992).

Based on immunochemical properties, three types of the cryoglobulin have been identified (Tedeschi *et al* 2007). Simple cryoglobulins (type I) contain only 1 isotype or subclass of immunoglobulin but it is possible to involve immunoglobulin light chain, rarely. Mixed cryoglobulins (type II and III) are immune complexes composed of 2 different types of immunoglobulin. An immunoglobin is the antibody (commonly IgM) and the other antigen (commonly IgG). For type II and III antiglobulin (antibody) are monoclonal and polyclonal, respectively (Usha *et al* 1999, Goveric 1995).

Literature shows that cryoglobulinaemia has occasionally been reported in animals. It has been detected in horses (Traub- dargatz *et al* 1985, Meada *et al* 1991), dogs (Hurvits *et al* 1977, Stein *et al* 1942), mice (Hijman *et al* 1969) and rabbits (Catsoulis *et al* 1965, Daveia *et al* 1968). If blood containing cryoglobulin is collected below body temperature, separation of serum will be difficult. In our experiences with this miniature horse (Atyabi *et al* 2000, Zamani-ahmadmahmudi *et al* 2011). sometimes harvest of serum samples was difficult. After blood collection without anti-coagulant from clinically healthy Caspian horses, some serum samples appeared white and gelatinous, subsequently made serum collection very difficult. This common phenomenon leads us to conduct of a study for finding probable cause(s). By reviewing literature, the most probable cause in horse is cryoglobulin (Traub-Dargatzb *et al* 1985, Maeda *et al* 1991), although previously reported horses with cryoglobuliemia had shown clinical signs of disease. Present study was conducted for isolation and characterization of cryoglobulin from Caspian horse serum.

MATERIALS AND METHODS

Study was performed on 20 healthy miniature Caspian horses in both genders at Khojir animal research station (Tehran National Zoo Park, Tehran, Iran). They were within the age range of 4-20 years old (mean= 8.5 years). None had clinical signs of immunodeficiency disorder.

Isolation of the cryoglubolin was done using a standard method (Meada *et al* 1991). Blood samples of 10 ml were collected by right jugular venipuncture into vacuumed tubes without and with anti-coagulant (0.1 ml of 10% disodium EDTA solution for 5 ml of blood). Before sampling, syringes were prewarmed to the body temperature. Briefly for detection of probable cryoglubolin, blood samples were kept at 37 ^{0}C for 30 min followed by centrifuging at 3500g for 10 min. Harvested serums were kept at 4 ^{0}C for two hours and then examined for cryoglubolin. Precipitates suspected to cryoglubolin were seen in four out of 30 samples. Samples containing precipitate were centrifugated and supernatant was sotred in another tube. Precipitates were suspended in phosphate- base- saline (PBS, pH=7.2) and centrifugated three times. After final washing, precipitates were dissolved in warm electrophoresis buffer. Subsequently precipitates and serum protein electrophoresis were performed using an automated electrophoresis system on cellulose acetate strips (Sartorius GMBH, Munich, Germany) according to the procedure described previously (Zamani-ahmadmahmudi *et al* 2011). In addition Ig isotypes demonstration for both precipitates and serum containing precipitates were performed using single radio immunediffusion method (SRID) (VMRD, Pullman, WA). Using independent t-test, Ig

concentrations of precipitates and sera were compared. A value of P<0.05 was considered significantly.

RESULTS

Four precipitates suspected to cryoglobulin samples were isolated from 20 Caspian miniature horse serum samples. SRID method for determining Ig isotypes and their concentration demonstrated that IgG was main constituent of cryoglobulin in Caspian horse (Figure 2). IgG value of cryoglobulins was markedly higher than serum level (p<o.o5) while IgA and IgM values were lower than normal values (Table 1).

Table 1. Immunoglobulin isotypes concentration (Mean ±SD) of precipitates sample susceptible to cryglobulin (n=4) and total serum samples (n=20)

	1	2	3	4	precipitates	Total samples
IgM	565.06	237.62	199.82	237.62	310±170	66.1±29.4
IgG	3426.59	4521.16	2260.91	3393.41	3400±922[a]	2160.19±940.9
IgA	28.84	18.34	15.41	15.41	19.5±6.4	92.40±99.6

a: IgG concentration of precipitates were markedly higher than immunoglobulin isotype concentration of total samples (p<o.o5) (n=20). IgA and IgM serum values of precipitate weren't significantly differ from normal value

A sharp abnormal peak was notable observation on gamma region of ACE strips in precipitates suspected to the cryoglubolin. The shape of peak was compatible with monoclonal band. Normal serum samples without cryoglobluinemia did not show abnormal band in γ region (Figure 1). It was concluded that probably monoclonal peak in gamma region of cryoglobulin is IgG. It was previously noted, simple cryoglobulins (type I) contain only one isotype or subclass of immunoglobulin resulting in monoclonal peak on ACE strips.

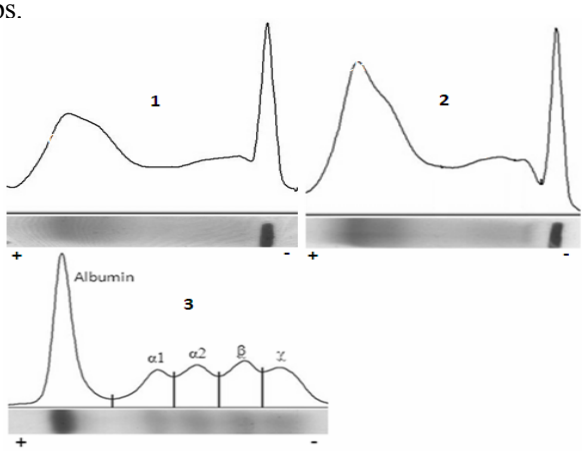

Figure 1. electrophoresis graph from precipitate suspected to the cryoglubulin (1), serum containing cryoglobulin (2) and normal serum without cryoglobulin (3). A sharp band was seen in gamma region of electrophorsis strips of precipitates and serum containing cryoglobulin.

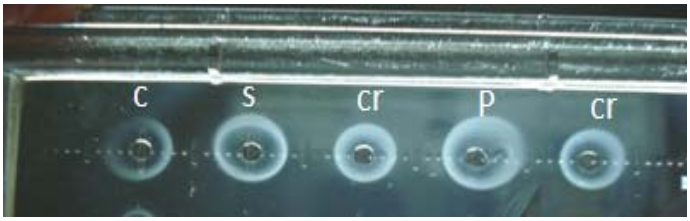

Figure 2. single radio immunediffusion (SRID) test for determination of Ig isotypes and their concentration. Picture show IgG kit. Wells containing precipitate suspected to cryoglobulin (Cr) reacted with antiglobulin antibody. **C**: serum control, **S**: serum containing cryglobulin, **Cr**: precipitate susceptible cryoglobulin, **P**: plasma containing cryoglobulin

DISCUSSION

All above results indicated a normal but high concentration of cryoglobulin (monoclonal IgG) a proportion of Caspian horse population sera. Reviewing literature indicated two reports of cryoglobulin in horse causing pathologic signs in them. But in both cases, horses had clinical sign of disease. Maeda et al (1991) isolated cryoglobulin from a horse which had glomerulo-nephritis and a history of skin ulcers of the limb in winter. They showed that cryoglobulin in serum was type II. Observation of two

sharp peaks in ACE and two distinct bands in immunoelectrophoresis was representative of different molecules.

Traub et al (1985) isolated cryoglobulin from three-year-old male Arabian horse with bilateral uveitis and conjunctivitis sixth month duration. General conditions of the horse deteriorated throughout its subsequent hospitalization (total 20 days). The tip of both ear become dry and subsequently sloughed. Electrophoresis of cryoglobulins resulted in monoclonal gammapathy. After autopsy, important findings included splenomegaly, enlargement of the renal, splenic, and mesenteric lymph nodes. Infiltration of small lymphocytes, plasma cell and occasionally histiocytes to cited organs and follicular pattern of infiltration suggested follicular lymphoma causing cryoglobulin in this horse. Cause of dryness and slough of the limb in this case and skin ulcers of the the limb in previous case was obstruction of terminal vessels of the limbs by cryoglobulin immunocomplex.

Cryogloulin reported in some species normally. For example Usha et al (1991) and Nagata et al (1998) reported normal concentration of the cryoglobulin in human and dogs respectively. Results of present study showed presence of cryoglobulin in Caspian miniature horse serum. But normal value of cryoglobulin in Caspian horse blood is markedly higher than values reported for other species such as human (Usha *et al* 1991), dog (Nagata *et al* 1998), and horse (Traub *et al* 1985). Besides samples were collected from apparently clinically healthy Caspian horse at the time of sampling. Although concentration of cryoglobulin in Caspian horse is higher than horse, but no sign of disease were seen in Caspian horse may be due to the none-pathogenic cryoglobulin (Usha *et al* 1991). Also existence of monoclonal peak in gamma region and significant IgG concentration may indicate type-I croglobulinemia in caspian miniature horse because type I cryoglobulins consists of one isotype of immunoglobulin resulting in monoclonal peak in gamma region of strips. Because of high concentration of cryoglobulin in Caspian horse, this specie is prone to the pathologic consequence of cryoglobulibs. Because average of studied caspain horse age was high (Mean: 6.5 years old), high concentrations of cryoglobulins in Caspian horse also could results from previous diseases. Thus it is suggested to examine Caspian miniature horse for the incidence of some diseases causing pathologic cryoglobulin in other races such as multiple myeloma, macroglobulinemia, lymphoma and lymphocytic leukemia. A pathologic concentration of cryoglobulin has been detected in these diseases (Castoulis *et al* 1965, Davie *et al* 1968, Goveric 1995).

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