Antibacterial activity of lysozyme and complement in canine blood sera

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Introduction:
Complement and lysozyme are important non-specific humoral components of antibacterial immunity of vertebrates. Complement lyases G- bacteria and lysozymes G- bacterial cell wall mainly.

Complement system is composed of approx. thirty proteins in soluble or cell-associated form. In the blood plasma the complement components are in inactive form. First component C1q is activated by binding of IgG or IgM to the target cell in the classical activation pathway or by contacting with the surface structures of foreign particles or altered own cells in alternative pathway. C1q then starts a cascade of reactions of particular complement components which leads to membrane attack complex (MAC) formation. MAC kills G- bacteria by perforating its cell wall. In mammals also exists lectin pathway of complement activation, which is activated by lectins exclusively.

Lysozyme (EC 3.2.1.17, muramidase) is an enzyme which hydrolyses β-1,4-glykosidic bonds between N-acetylmuramic acid and N-acetyl-D-glucosamin in peptidoglycans. That’s why it touches mainly G- bacteria. Lysozyme appears e.g. in blood plasma, tears or saliva.

The first aim of this work was to optimize luminescence method using recombinant bacterial strain for complement activity determination in canine blood sera. The second aim was to use this method to determine total bacteriolytic activity of complement in different samples of canine blood sera and in the same samples determine lysozyme concentration. The hypothesis was that there is a difference between sexes and groups of different age and weight in measured parameters.

Complement bacteriolytic activity in canine blood sera:

Materials:
Canine blood sera were collected from Central clinical laboratory of Veterinary and Pharmaceutical University in Brno. The sera were stored one week in 4°C than in -80°C until use.

Recombinant Escherichia coli K12gfGFPluxBAmp bacteria were used. The bacteria harbors recombinant plasmid pGFPuvKlux which encodes enzyme luciferase and its substrate aldehyd (likewise Nikokselainen et al., 2002). This way modified bacteria express their own bioluminescence.

Method:
The bacteriolytic activity of complement was quantified by luminescence method based on bioluminescence of recombinant bacteria. Light emission is a result of the reduction of FMNH2 catalysed by luciferase. This reaction requires ATP which is produced by live bacteria only, thus the intensity of the light emission corresponds to bacteria viability. Efficient concentration (minimal concentration which lysed 50% of bacteria) 60µl/ml of sera in reaction mixture was determined previously range concentration. Reaction kinetics was measured for 3 hours at 37°C in Lumitester LMT 01 (Immunotech, CZ). Complement bacteriolytic activity value was set as the time period in which 50% of bacteria were killed. If 50% of bacteria were not killed, the value of bacteriolytic activity was 3 hours. It means that shorter period of time corresponds to higher bacteriolytic activity. This value was taken from time luminescence dependency graph.

Results:
Bacteriolytic activity in blood sera was measured in 115 male and 190 female samples. In age categories and male and 16 female samples in weight categories as follows in the graphs:

**Fig 1:** Bacteriolytic activity of complement in canine blood sera according to dog’s age. (mean ± S.D.)

**Fig 2:** Bacteriolytic activity of complement according to dog’s weight. (mean ± S.D.)

According to Kruškal-Wallis ANOVA test statistically considerable differences were found between discrete female weight categories and age categories as well (p=0.046 or p=0.0077 resp.) In males categories no statistically considerable differences were found.

**References:**

Lysozyme concentration in canine blood sera:

Materials and method:
The lysozyme concentration was measured in vitro by zone inhibition assay in agarose. 0.12% agarose gel was prepared with addition of Micrococcus luteus (CM 169). This mixture was boiled and filled in glass dishes. Wells of diameter of 4mm were cut into the gel. In each well 1µl of sera or calibration solution of lysozyme (Lysozym, Sigma, EC3.2.1.17) was applied. Incubation run in the wet chamber at room temperature for 24 hours. The size of inhibition zones was measured with HP-SEAVAC scale. The diameter of inhibition zone is directly proportional to lysozyme concentration in the sample. The concentration was calculated according to calibration curve in mg/ml (Hyltěl & Simek, 2005).

Results:
Lysozyme concentration in blood sera was measured in 103 male and 116 female samples in age categories and 90 male and 101 female samples in weight categories as well. The results were shown in the graphs:

**Fig 3:** Heat inactivation of complement in canine blood sera according to dog’s age. (mean ± S.D.)

**Fig 4:** Heat inactivation of complement – specification (mean ± S.D.)

**Fig 5:** Lysozyme concentration in canine blood sera according to dog’s age (mean ± S.D.)

According to Kruškal-Wallis ANOVA test statistically considerable differences were found between discrete male age categories (p=0.038). In males weight categories and females weight and age categories no differences were found. Also statistically considerable difference was found between males and females in age category of 14 and more years (Mann-Whitney U test, p=0.01603).

Discussion and conclusion:
We used original luminescent method for determination of complement activity, probably this method was used firstly for canine blood sera. Data of another recombinant bacterial strains are published with human blood sera (Virta et al., 1997) and fish sera (Nikokselainen et al., 2002)only.

Benefits of this method are quite low price, on the other hand for precise measurement is essential maintain level of luminescence of the bacteria. Appropriate cultivation conditions are essential.

Because we had not complete information about dogs health state we can not determine the reason of differences between particular categories. After consultation with veterinarians, differences in complement activity between age female categories can be due to hormonal cycle during bitch’s life. According to Sugirua et al. (2004) estral cycle affects activity of monocular cells in peripheral blood, what could also influence changes in complement activity. Activity of complement is influenced with the actual health state of the dog (Wolfe et Hallwell, 1980), so it would be interesting to repeat the experiment with sera of clinically examined healthy dogs.

No information was published about concentrations of lysozyme in different weight and age categories of healthy dogs. Alterations of lysozyme concentration were found during bacterial infections (Faldyna et al., 2008), neoplastic changes (Borst et Coutoune, 1985) or hiscocytic diseases (Marchal et al., 1997). Same as mentioned above it would be interesting to repeat the experiment with sera of clinically examined healthy dogs.

To conclude, data about complement activity and lysozyme concentration should be used as helpful diagnostic marker of certain diseases as described before (Borst et Coutoune, 1985).