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# The Getting, the Purification and the Characterization of Species' Immunoglobulin G (IgG) and Anti-immunoglobulin G Serum for Dog, Goat and Rabbit

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**Abstract.** The research aimed at preparing, purifying and characterizing the immunoglobulin G, for dog, goat and rabbit, and the anti-IgG serum for the same species. The IgG species purification was done through precipitation, using ammonium sulfate solution and ion exchange chromatography (DEAE-cellulose). Checking the IgG purity was done by immunoelectrophoresis (IEF), for the corresponding anti-species serum, and by polyacrylamide gel electrophoresis in denaturant system (SDS-PAGE). Using IEF, we obtained a precipitation arch with cathodic migration, whereas using SDS-PAGE, we obtained a fraction with a molecular mass of 150 KDa. Dog and goat anti-IgG serum was prepared on rabbit, and the rabbit anti-IgG serum on goat, by IgG hyperimmunisation concealed in Freund adjuvant at a 1 to 1 ratio. Sensitivity and specificity testing of antisera was performed by immunoenzymatic test (ELISA). There were highlighted the high titers of antibodies to the dilution of 1:500 for anti-IgG rabbit and goat sera, and 1:400 for anti-IgG dog serum. Specificity testing of dog and rabbit anti-IgG in the heterologous system showed results which are in the negative range. The DO values were high for goat anti-IgG serum, sheep IgG serum and sheep anti-IgG serum and goat anti-IgG serum, due to antigenic kinship between these species. The reagents prepared will be used in great specificity and sensitivity immunodiagnostic tests.

**Keywords:** immunoglobulin G, chromatography, ELISA, anti-immunoglobulin G sera

## INTRODUCTION

Immunoglobulin G is the main immunoglobulin class in humans as well as in animals, first of all because its high concentration in blood serum (0,7 – 2,2 g/dl for cows, 0,8 – 2,1 g/dl for pigs, 0,9 – 2,1 g/dl for dogs (Vior, 2000). The IgG synthesis resides in structural genes, represented for C and V regions of those two types of chains by independent sets. Five subclasses of IgG have been described for pigs: $IgG_1$, $IgG_{2a}$, $IgG_{2b}$, $IgG_3$ and $IgG_4$ (Novotna *et al.,* 2005). Bovine IgG contains the following subclasses: $IgG_1$ and $IgG_2$. $IgG_1$ is the main component of milk and saliva secretion, and $IgG_2$ is found in blood serum, being main serum immunoglobulin. $IgG_1$ has a molecular mass of 163.000 KDa and $IgG_2$ of 150.000 KDa (Wernicki, 1988). Based of immunoelectrophoretic aspects and column separation with DEAE-Sephadex, the presence of a third IgG subclass has been hyphotesized (Jeffrey, 2007). For dogs, the existence of sub-subclasses of IgG was reported: two from $IgG_1$ subclass ($IgG_{1a}$ and $IgG_{1b}$), three from $IgG_2$ subclass ($IgG_{2ab}$, $IgG_{2a}$, $IgG_{2b}$) (Koichi *et al.*, 2006). For species IgG separation and purification, most of the researchers used the chromatographic techniques such as ion exchange chromatography and gel filtering (Murakami and Kazu, 2005). Ox IgG was purified by mixing the serum with caprylic acid, followed by ammonium sulfate precipitation and by ion exchange chromatography. The analysis of the obtained fractions

using SDS-PAGE and Western blot confirmed the IgG purity (Walker and Jhon, 2002). Murakami and Kazu (2005) have isolated the IgG from the pig serum using the precipitation technique with ammonium sulfate, and chromatography on DEAE cellulose using phosphate buffer with different molarities (0,01M - 0,04M). The IgG was diluted with the first two buffers (0,01M and 0,02M). The fractions were then put together and then diluted by columns with CM – cellulose and Sephadex G-200. Within their research, Laxmi *et.al* (2001) have purified the rabbit anti-IgG goat serum by affinity chromatography, using an agar column, on which they put rabbit IgG. The purified antibody was tested using the immunodiffusion technique and then used as the second antibody in the immunoenzymatic reaction. Bokhout *et al*. (1986) used affinity chromatography for the pig IgG subclasses purification and the results obtained showed two peaks having antigenic determinants partially different for $IgG_1$ and $IgG_2$ subclasses. The analysis of the two subclasses was done by immunodiffusion tests and ELISA tests (Bokhout *et al*., 1986; Kim *et al*., 1994). A series of researchers purified the IgG by affinity chromatography using columns with Sepharose based immunosorbents mixed with species' IgG. Lastras *et al.* (2000) tested the crossed reactions between IgG from phylogenetic related species (sheep ang goat IgG) and noticed that they presented identity reactions with deer, stag and mouflon IgG. Also, the pig IgG presented identity reaction with the wild bore IgG serum towards the anti-pig serum using the immunodiffusion test in agar gel. For the preparation of anti-IgG species sera, rabbit, goat, horse and sheep are commonly used. Usually, a more distant taxonomic species from that which the IgG originates from is used. Within the same animal species, there are significant differences between individuals concerning their ability to respond to the IgG. The synthesized antibody titre during the immune response depends on many factors such as IgG path of administration, dose, administration rhythm and mode (Kim *et al.,* 1994; Vior, 2000). The most efficient way of IgG administration is the intradermic one because of its lasting persistence in the inoculation area and thus, the cells implicated in the immune response elaboration accumulate. (Jeffrey, 2007). For anti-IgG species sera quantification, a series of authors recommend the usage of immunoenzymatic technique (ELISA), due to its high sensitivity and specificity (Laxmi and Prakash, 2001; Jeffrey, 2007; Turcu *et al*., 2009a; Turcu *et al*., 2009b). In this research paper, we present the results obtained concerning the preparation, purification and characterization of IgG and anti-IgG species sera, that will be used in immunodiagnostic tests.

## MATERIALS AND METHODS

**Total gamma globulin preparation from normal species serum (dog, goat and rabbit).** The obtaining of ox total gamma globulin was done by bovine normal serum precipitation with ammonium sulfate saturated solution neutralized to a 7,0 pH. Three consecutive precipitations were performed, and the final deposit of gamma globulin was mixed with a small amount of distilled water. The obtained gamma globulin was undergoing dialysis at 4oC against 0,15 M NaCl solution.

**The IgG purification from total species gamma globulin solution on DEAE cellulose.** The ox IgG purification was done using ion exchange chromatography (DEAE – cellulose). For the obtaining of IgG, a chromatographic column (K 2,5/30 cm) was used with DEAE – cellulose (with an exchange capacity of 0,09±0,1mEq/g) balanced with phosphate buffer 0,075 M and 6,3 pH. For establishing the gammaglobulin solution volume deposited in the column, we took into account that at 3-4 g DEAE – cellulose we added 200 mg of protein. The sample dilution was done using phosphate buffer 0,0175 M, 6,3 pH and we gathered 2 ml

per column. A part from the gathered fractions were put together, concentrated and then tested for purity and specificity using SDS–PAGE and immunoelectrophoresis (IEF) technique.

**Electrophoresis in polyacrylamide gel.** The electrophoresis of fractions in polyacrylamide gel was done in denaturant conditions (SDS – PAGE), using a SCIE – PLAS TV 100 according to the technique described by Laemmli. The protein separation was done in two gels of different concentrations and pH: one for proteins' concentration, using 4% acrylamide gel in Tris – HCl buffer 0,5 M, 6,8 pH, and another one, for their separation, using 7,5% acrylamide gel in Tris – HCl 1,5 M, 8,8 pH. After the gels migrated, they were colored with a 0,1% solution of Coomassie Brilliant Blue G-250. The molecular mass markers used for the protein separation by SDS – PAGE were: ovalbumin (45 KDa), bovine seric albumin (66 KDa), galactozid (116 KDa) and myosin (205 KDa).

**The immunoelectrophoresis.** The immunoelectrophoresis was performed in 1,2 % agarose gel preparated in veronal buffer 8,6 pH (with an ionic force of 0,05) laid on a glass microscopic slide of 7,5 cm long and 2,5 cm wide. The fractions that had to be studied were put in the gel wells. After their electrophoretic migration, in the split created between the two fractions we put the rabbit serum ox anti-serum in the gel wells. The microscopic slides were maintained in a wet room, at the lab temperature for 18-24 hours. The precipitation arch was seen using a down-up highlight source. The proteins were colored using a 0,1 % Amido Black 10B solution.

**The anti-IgG species preparation.** Three batches of rabbits were used for the sheep, dog and goat anti-IgG species sera preparation. The rabbits were immunized with 4mg/mL IgG from the mentioned species, put in Freund complete adjuvant in a ratio of 1:1. Three subcutaneous inoculations were done, in several places such as the dorsal area and on the body sites. The bleeding was done after seven days, after the last inoculation. The quantification of species antibodies anti-IgG level was made using the immunoenzymatic technique. For obtaing the rabbit anti-IgG goat serum we used an adult goat in good health which was being immunizated with rabbit IgG (6mg/mL) put in Freund complete adjuvant. The inoculations were done in the same manner and in the same way like the rabbit technique.

**The immunoenzymatic technique**

**The antigen**: to catch and to quantize the ox anti-IgG sera we used as antigen ox IgG diluted at 10μg/mL in a 0,1N NaOH solution. In the coated stage we added 100μL IgG in every hole. After the plates were put in the incubator for two hours at 37ºC, they were washed using PBS/Tween in a Mindray MV-12A plate cleaner. The ox anti-IgG sera was diluted in a ratio of 1/25, 1/50, 1/200, 1/400, 1/800, 1/1600, and 1/3200 with a PBS/Tween buffer with an addition of 0,5% bovine seric albumin. The plates incubation was done at 37ºC, for 60 minutes.

**The conjugate**: we used an ox anti-IgG conjugate marked with peroxidase. It was diluted in a ratio of 1/100 in PBS/Tween buffer to which we added 1% bovine seric albumin and the we put 100μL in each well.

**The substratum**: it contained 0,005 hydrogen peroxide and 0,6mg/mL ABTS in citrate buffer of 4 pH. We used 100μL substratum in each well and after one hour the reaction was stopped with 50μL 1,5% sodium fluoride.

**The reading**: the optical densities (DO) were read at 405nm with a multichannel plates ELISA Apollo LB 911 spectrophotometer (Berthold Technologies).

## RESULTS AND DISCUSSION

The total gamma globulin fraction on a DEAE-cellulose column showed only one protein pick. The fractions that are in the picks' maximal area were put together, concentrated and analyzed for their purity using IEF against the species anti-serum serum. In the diluted part the IgG appeared after collecting 30mL buffer. From the total gamma globulin, the IgG was diluted in the next 20-25mL buffer. The elution of the others proteins left in the column was done with a 0,25M NaCl solution.

Using the electrophoretic analyses for the fractions suitable to the pick obtained, we have seen a precipitation arch characteristic to IgG with electrophoretic migration in the cathodic area of the microscope slide (Fig. 1).

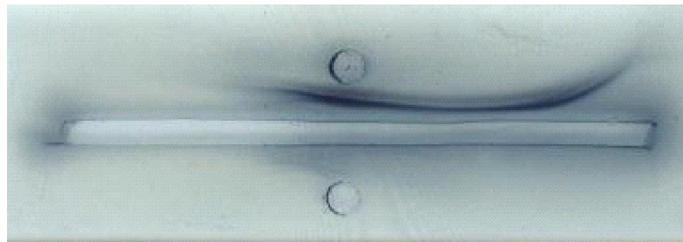


Fig. 1. The purity checking of species IgG using immunoelectrophoresis towards an anti-species rabbit serum

From the electrophoregamas' imagine showed in Fig. 3 appeared that IEF is a qualitative method very useful in estimating the G immunoglobulin purification degree. Using the SDS-PAGE electrophoretic study of the molecular fractions obtained through separation on DEAE cellulose we have seen the presence of a single band with the molecular mass of 150KDa (Fig. 2).

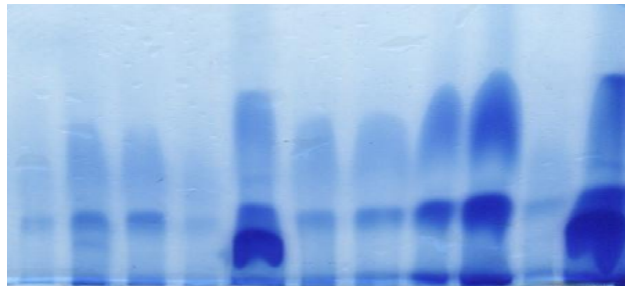
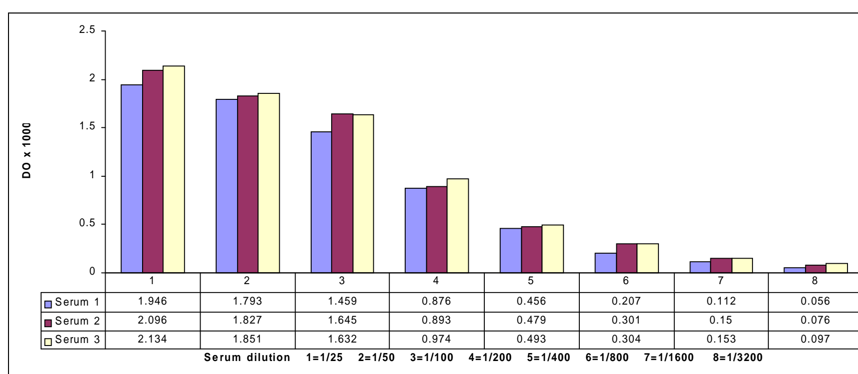


Fig. 2. Electrophoretic analysis of species IgG using SDS-PAGE

In Fig. 2 it is clearly seen the IgG corresponding line (arrow, samples 1, 2, 3, 4, 6, 7, 10) and the species gamma globulin (sample 8, 9) which shows that using the DEAE-cellulose fraction technique we can provide a good separation in a pure state.

In Fig. 3 we showed the values for the optical densities (DO) of dog anti-IgG sera at a dilution of 1:25 to 1:3200 obtained using the ELISA test.




| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Serum 1 | 1.946 | 1.793 | 1.459 | 0.876 | 0.456 | 0.207 | 0.112 | 0.056 |
| Serum 2 | 2.096 | 1.827 | 1.645 | 0.893 | 0.479 | 0.301 | 0.15 | 0.076 |
| Serum 3 | 2.134 | 1.851 | 1.632 | 0.974 | 0.493 | 0.304 | 0.153 | 0.097 |

Fig. 3. The value for the OD of dog anti-IgG

It can be seen high titres of antibodies until 1:400 dilution (serum 1=DO 0,456; serum 2=DO 0,479, serum 3=DO 0,493). This value was chosen as being the optimal dilution for the dog anti-IgG sera to which positive reactions are obtained.

The dog anti-IgG serum specificity determination was performed by immunoenzymatic test towards the following species immunoglobulins: pig IgG, ox IgG, sheep IgG, rabbit IgG (10μg/mL). Negative DO values were recordered for dog anti-IgG sera in comparison with the pig IgG (0,020 DO), ox IgG (0,025 DO), sheep IgG (0,027 DO) and also a weak reaction in comparison with the rabbit IgG (0,206 DO). The results are synthetized in Fig. 4.

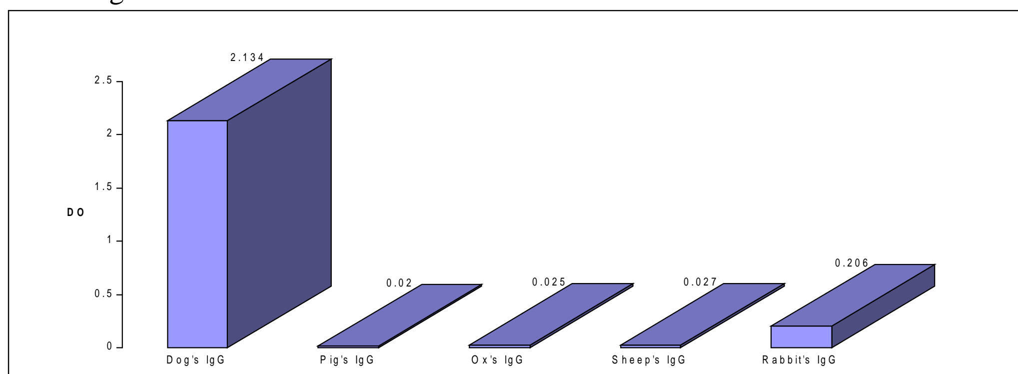



Fig. 4. The dog anti-IgG serum specificity testing towards the heterologous species IgG

In Fig. 5 it is shown the results obtained using ELISA technique for goat anti-IgG rabbit sera titration from 1:25 to 1:3200 sera dilution. It is noticed that the optimum goat anti-IgG antibodies concentration is up to 1:800 sera dilution.

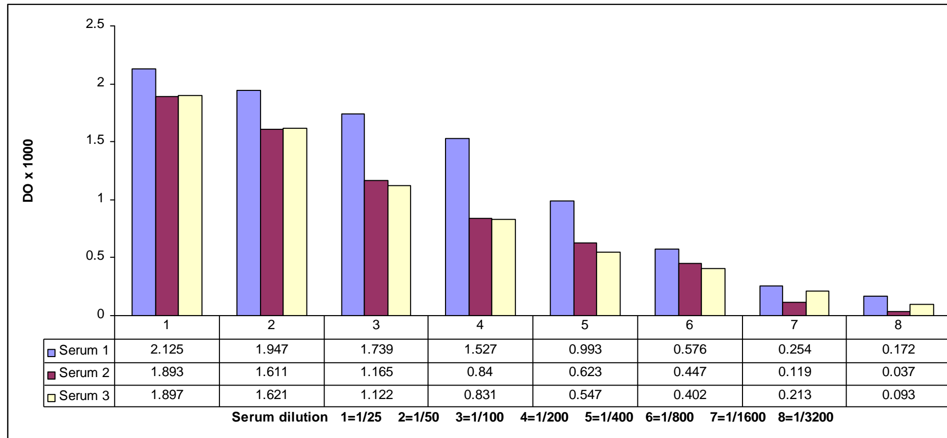



Fig. 5. Goat anti-IgG rabbit sera testing using the immunoenzymatic test (ELISA).

The goat anti-IgG rabbit sera specificity was demonstrated by evaluation of antibodies reactions from others species IgG antisera such as: pig, ox, sheep and dog. The DO values obtained were negative in comparison with pig IgG (0,035 DO), ox (0,321 DO) and dog (0,021 DO). It can be noticed the cross-reactivity between goat anti-IgG sera and sheep IgG because of their close antigenic kinship (Fig. 6).

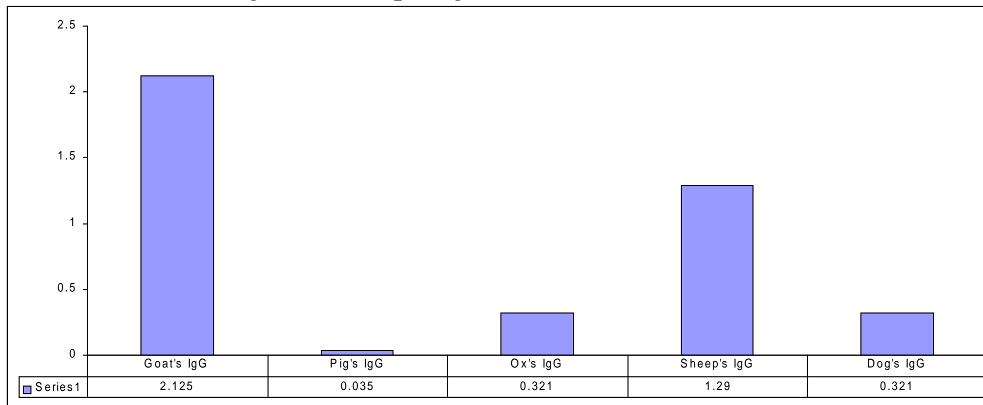



Fig. 6. The goat anti-IgG rabbit serum specificity testing towards the heterologous species

The rabbit anti-IgG goat, in corresponding system, showed a specific reaction towards the rabbit IgG (2,030 DO) and in heterologous system, the reactions were located in the negative values towards the pig IgG (0,026 DO) and dog IgG (0,076 DO) (Fig. 7).

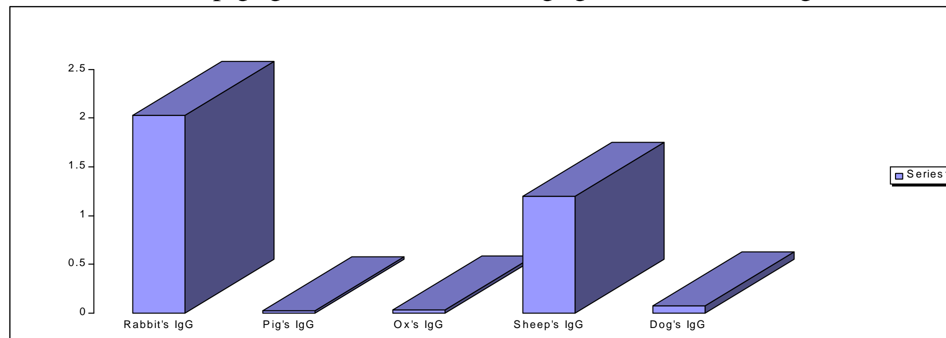



Fig. 7. The rabbit anti-IgG goat serum specificity testing towards the heterologous species IgG

It can be seen the high value of 1,202 DO resulting in the reaction between the goat and the sheep anti-IgG serum, because of their antigenic kinship between these species (Fig.8).

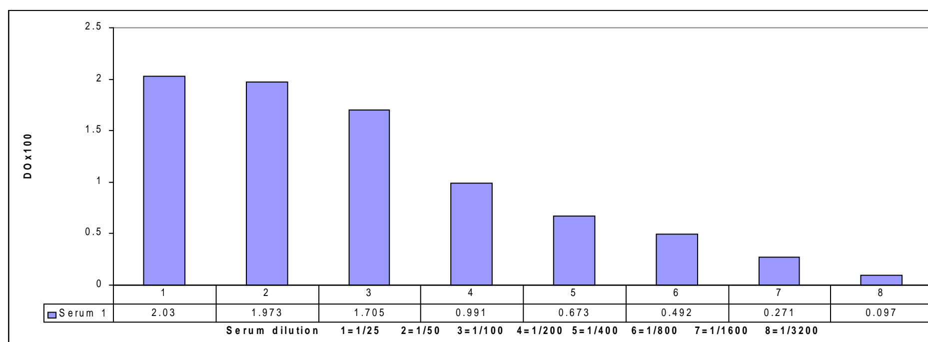



Fig. 8. The rabbit anti-IgG goat sera specificity testing using the immunoenzymatic technique

It highlights the quality of reagent of the goat anti-IgG rabbit serum both in terms of sensitivity and specificity. The goat anti-IgG serum will be absorbed using sheep IgG to eliminate cross-reactions between species.

## CONCLUSIONS

The species immunoglobulin G (IgG) (dog, rabbit, goat) purification was done using the precipitation reaction with ammonium sulfate and ion exchange chromatography (DEAE).

The species IgG purity control was done using the IEF technique towards the coresponding antiserum and also using SDS-PAGE. By using IEF we obtained a single precipitation arch located in the cathodic area and by using SDS-PAGE we obtained a fraction with a molecular mass of 150KDa.

Dog and goat anti-IgG sera were prepared by rabbit hyperimmunization, and the rabbit anti-IgG sera was obtained on goat by rabbit IgG hyperimmunization.

Overall data obtained showed the reaction sensitivity of species anti-IgG sera, obtaining high titre of 1:800 for goat and rabbit anti-IgG sera and of 1:400 for dog anti-IgG sera.

Testing the rabbit and dog anti-IgG sera in heterologous system showed results that are in negative values area. In the case of goat-IgG-sheep-IgG serum and of sheep-IgG goat anti-IgG serum the DO values were high because of the antigenic kinship between these species.

The prepared reagents will be used in immunodiagnostic tests of high specificity and sensitivity.

## REFERENCES


1. Bokhout B.A., Vandesten Noor Dijk, Stok W. (1986). Porcine IgG isolation of two IgG subclasses and anti-IgG class and subclass specific antibodies. Molecular Immunology, 23 (6), 675-683.
2. Jeffrey K.A. (2007). Integrated Immunology and Microbiology, Mosby Elsevier, Houston, Texas, 73-80.
3. Kim J.W., Koh S.Y., Sho S.H. (1994). ELISA for mesurement of IgG concentration in porcine serum. Korean Journal of Animal Sciences, 35 (5), 453-458.

4. Koichi O., Uehara M., Okano S., Watanage K. (2006). Purification and characterization of canine serum Ferritin binding proteins. Biometals, 19, 3, 315-322.
5. Lastras M.E., Pastor J., Vinas L., Marco I., Lavin S. (2000). Immunoglobulin G class identification from wild ungulates by cross reactivity with antisera to domestic animal. Journal of veterinary Medicine, Serie B, 47 (6), 429-432.
6. Laxmi N.A., Prakash B.S. (2001). Production and purification of goat antirabbit IgG. Indian Journal of Dairy Science, 54 (6), 332-334.
7. Murakami J.J., Kazu O. (2005). Purification of immunoglobulin IgG and IgM from porcine serum and sows colostrum. The Japanese Journal of veterinary Science, 37, (3), 199-208.
8. Novotna J., Frantisek F., Frantisek S. (2005). Large scale issolation, characterization of pig immunoglobulinK-chain. European Journal of Immunology, 2 (2), 127-130.
9. Turcu D., Oporanu Mariana, T nas R., C lin V., Petru T., Ciocârlie Nicoleta, (2009a). The purification and characterization of pig's immunoglobulin G (IgG) and also of pig's anti-immunoglobulin G serum for using them in the diagnosis tests. Scientific works, C series, Veterinary Medicine LV (1), 209-216.
10. Turcu D., Mariana Oporanu, C. Coman, V. Calin, E. Vlase (2009b). The getting, the purifying and the characterization of the bovine's G immunoglobuline and bovine's anti G immunoglobuline serum for the immunodiagnosis tests. Rev. Rom. Med. Vet. 3/2009, 69-77.
11. Vior C. (2000). Biotehnologii medicale. Editura Funda iei România de Mâine, Bucure ti, 250-262.
12. Walker M.M., Jhon M. (2002). The protein protocol hand book. Humana Press, second Edition, Hatfield, U.K.
13. Wernicki A. (1988). The level of immunoglobulins in sera whey of colostrum and milk of cows in periparturient period. Annales of University Marie curie Sklodowska, 39, 241-249.