Obtainment of anti chicken IgY reagent in rabbits and its use in IgY Technology

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Key words: rabbit anti chicken IgY, chicken egg yolk immunoglobulins, affinity purification, biotinylated avian antibodies.

RESUMEN. El hecho de que los anticuerpos puedan ser detectados en la yema de los huevos puestos por gallinas inmunizadas, condujo al desarrollo de la tecnología de los anticuerpos de yema (IgY) como método alternativo mucho menos traumático para los animales. En este trabajo se presenta un esquema de inmunización optimizado para obtener un reactivo para el reconocimiento del IgY de pollo. Los animales seleccionados fueron los conejos debido a su lejanía filogenética de las aves, hecho que es predictivo de una buena respuesta inmunológica. A cada animal se le administró una dosis de 250 µg de IgY comercial, emulsificado con adyuvante completo e incompleto de Freund con 15 d de intervalo. La fracción IgG de conejo específica para el IgY de pollo fue obtenida por precipitación con sulfato de amonio del suero de conejo hiperinmune y posteriormente, purificada mediante cromatografía de afinidad. La fracción anti IgY inmunopurificada fue conjugada a la enzima peroxidasa y biotinilada con *N*-hidroxisuccinimidobiotina. La especificidad antes del marcaje fue determinada por técnicas de inmunodifusión e inmunoelectroforesis y el uso en la tecnología IgY del conjugado y el anticuerpo biotinilado fue llevado a cabo mediante ELISA. El conjugado peroxidasa-anti IgY fue comparado con uno similar de la Sigma, obteniéndose una dilución de trabajo de 1/1 800 para ambos, necesario para alcanzar una absorbancia de aproximadamente 1 en el ensayo ELISA.

ABSTRACT. The fact that antibodies can also be detected in the yolk of eggs laid by immunized hens, led to the development of the yolk antibody technology (IgY) as an alternative method less stressful to animals. In this work, an optimal immunization schedule to obtain a reagent for recognition of chicken IgY is presented. Rabbits have been the animals chosen due to their filogenetic distance in relation to birds, fact that is predictive of good immune response. Inoculations were carried out with 250 µg per animal of comercial IgY emulsified in Freund´s complete and incomplete adjuvant at fifteen days intervals. The specific anti chicken rabbit IgG fraction was obtained by ammonium sulphate precipitation of hiperimmune rabbit sera, and further purified by immunoafinity chromatography. The immunopurified anti IgY fraction was conjugated to the enzyme peroxidase and biotinylated with *N*-hydroxysucciniminobiotin. The specificity before labelling was determined by immunodifusion techniques and immunoelectrophoresis and the use of the conjugated and biotynilated fraction in the IgY technology was carried out by ELISA. The peroxidase-anti IgY conjugate was compared with a similar product from Sigma, obtaining a working dilution of 1/1 800 for both in order to have an OD value of approximately 1 in the ELISA test.

INTRODUCTION

Antibody production is often the rate limiting step in many research projects and can be the key to the success or failure of a study.[1-5] For this reason, antibody production in laboratory animals has become an essential part of several investigations.

The basis of all immunological techniques is the detection of antigens and(or) antibodies by the specific reaction between them. The success to develop these detection techniques is keeping specific antibodies to determined antigens. The main goal is to obtain high titer, high affinity antisera in a consistent manner with the welfare of the animals being immunized. Investigators not familiar with antibody (Ab) production require some guidelines to carry out immunizations in an appropiate way. Several decades ago, these procedures consisted in designing of optimal immunization schedules by inoculation of animals with a determined antigen, and later, obtention sera from them. The reagent obtained (polyclonal antiserum) is a broad spectrum of immunoglobulins to several epitopes of the antigen.

In choosing a species for making polyclonal antibodies, an investigator must consider the quantity of Ab or antiserum needed, the phylogenetic relationship between the

recipient and the donor of antigenic protein and the characteristic of the Abs made by the recipient species (such as complement fixing character).[1-5]

A number of vertebrates species (ranging from large farm animals, particularly sheep, goat, and horses to small laboratory rodents, chickens, frogs and fish) have been used over the years, each species offering some advantage for the particular study. The mammalian species used most frequently for polyclonal antibody production in the laboratory setting are mouse, rabbit, guinea pig, rat, goat and sheep. For producing a polyclonal antiserum to antigenic protein, one, generally, chooses a donor-recipient pair whose members are not closely related on a phylogenetic basis. A great phylogenetic distance is predictive of a greater number of aminoacid sequence differences between homologous protein, which translates to a more diverse and potentially higher titer serum Ab response. This is because, in general, the animal´s adaptative immune system responds to individual antigenic sites (epitopes) on a foreign substance and is tolerant to itself; more epitopes yield more Abs.[1-5] On the other hand, the need for a highly specific antiserum directed to a limited number of epitopes demands immunization of a closely related species, or even genetically divergent members of the same species for the production of antiallotype antisera. Mammals hyperimmunized with a large complex protein from a phylogenetically distant species may make as much as 10 mg/mL of specific Ab.[1-5] Due to the fact that egg yolk antibodies laid by immunized hens have been recognized as excellent source of polyclonal antibodies, in the last years an explosion of development of research projects to use chickens as an appropriate biomodel in the production of polyclonal antibodies (IgY) to mammal proteins for their use in the field of diagnostics[11,13-16] as well as therapeutics has been observed.[13,17,20] Therefore, the obtention of polyclonal antibodies from chickens for the specific recognition of these molecules is central to develop this technology, which has been called IgY Technology.[14,20]

For the reasons mentioned above, the main goal of this work has been designing optimal immunization schedules with chicken IgY, to obtain high titers of antibodies that recognize it, purification of these, conjugation of them with the enzyme peroxidase and biotin derivatives and test them in several immunoassays. The chosed biomodel for this purpose was the rabbit lagomorfus that broadly diverges with avian species and so is useful for the production of antibodies to proteins from them as IgY.

MATERIALS AND METHODS

Commercial chicken IgY (Sigma Co. Ltd, St Louis, USA) was used as immunogen. Stocks of 1 mg of this IgY in 1 mL of sterile saline solution were prepared and their concentration determined by ultraviolet (UV) spectrophotometry at 280 nm (Ultrospec 1000 Spectrophotometer from Pharmacia Biotech).

Three female F1 rabbits (New Zealand White x Semigiant White) of 4 months of age and 3 300 g of average weight were used in the experiment. These rabbits were maintained in accordance to Guidelines of Laboratory Animals Care.[21]

Each animal received 250 µg of pure IgY in a volume of 1 mL in Freund Complete Adjuvant (Sigma Co. Ltd.) for first inoculation and Freund Incomplete Adjuvant (Sigma Co. Ltd.) in boosters at 2 weeks intervals. The route of inoculation was subcutaneous in the dorsal region.

Blood samples for antibodies titration were obtained from the central vein of the ear[1,4,22] at intervals of 7 d and the antibodies were titrated by the double immunodiffusion (Ouchterlony)[23,24] technique.

Immunoelectrophoresis[23-26] was performed to verify the specificity of the obtained antibodies to IgY.

The obtained antiserum was purified by ammonium sulfate (BDH Chemicals, England) precipitation[27] and Affinity Chromatography by Sepharose-Protein A[28,29] (Pharmacia, Biotech) and finally by Immunoaffinity Chromatography.[12]

This specific anti IgY fraction underwent a conjugation procedure with the enzyme peroxidase (Sigma) by the periodate oxidation method[30-32] using 1 mg of enzyme for each 2 mg of antibodies and the final product was dialyzed over night (ON) with phosphate buffer (0.01 moL/L; NaCl 0.15 moL/L pH 7,2).

Testing of the conjugate by ELISA

Polystirene ELISA plates (Dynatech Laboratories) were coated with 50 µL of pure preparation of IgY at 1 mg/mL in coating buffer, incubated for 1 h at 37 º C, blocked with 100 µL of 5 % skim milk (Sigma) for 1 h at 37 ºC and several dilutions of the obtained conjugate were applied. Finally, plates were incubated at room temperature in the dark with 100 µ L of a mixture of 1 mg of OPD (Sigma), 1 µL of 30 % H_2O_2 solution per mL of pH 4.5 citrate buffer. The development of the color reaction was stopped by addition of 50 µL of 2 mol/L sulfuric acid. The absorbances at 492 nm were determined with an ELISA plate reader (Organon Tecknika).

Biotinylation of antibodies

Three mg of rabbit IgG anti IgY dissolved in 1 mL of 0.01 mol/L $NaHCO_3$ were added to 10 mg of biotin-*N*-hydroxysuccinimidobiotin (Biotin-NHS) diluted in 300 µL of dimetilformamide and mixed gentle for 1 h at room temperature. The solution was passed through a PD-10 column with Sephadex G-25. Fractions were collected and read at 280 nm (Ultrospec 1000 from Pharmacia). The fractions with absorbances significantly different from the equilibrium-elution buffer and eluted in the void volume were pooled, and tested by ELISA using a comercial avidin-biotin complex (ABC) from VECTASTIN^{R}USA.

Immunoaffinity chromatography[12]

One gram of freeze-dried cyanogen bromide activated Sepharose 4B (Pharmacia) was swelled according to the manufacturer´s instructions, thereby yielding 3.5 mL of resin. Commercial IgY (Sigma) (2.5 mg) was dialyzed against the coupling buffer (0.5 moL/L NaCl, 0.1 moL/L $NaHCO_3$ pH 8.3) and added to the reswollen Sepharose. After end- to end rotation for 2 h at room temperature and centrifugation at 40 g for 5 min, the pellet was incubated with 10 mL of blocking agent (0.2 moL/L glycine, pH 8.0) at 4 ºC for 16 h to block the remaining active groups of the resin. The resin was poured into a column and unspecifically bound proteins were washed out by repeating five times an alternating washing procedure consisting of a washing step with 5 mL of coupling buffer, followed by one with 5 mL of acetate buffer (0.5 moL/L NaCL, 0.1 moL/L ammonium acetate, pH 4.0). After rinsing the column with 50 mL of PBS, the isolated rabbit IgG fraction from Sephorose-Protein A (4 mg of total rabbit IgG), brought to 50 mL with PBS and loaded at a flow rate of 240 µL/min . The column

was washed with 50 mL PBS and bound anti IgY antibodies were eluted with 8.5 mL of elution buffer (0.15moL/L NaCL; 0.2 moL/L glycine, pH 2.2). For immediate neutralization, the eluted fractions (2mL) were collected in tubes containing 8 mL of 1 mol/L Tris- HCL buffer pH 8.0. At the end, the column was neutralized with Tris buffer, washed with PBS, stored in PBS containing 0,05 % NaN_3 at 4 °C, and washed with PBS before re-use.

Determination of IgY by Mancini´s method[11, 23,24,33,34]

Glass plates of (6X10) cm were coated with 3 % noble agar (Difco Laboratories) in Veronal buffer pH 8,6 and dried for 1 h at 60 °C . Mixtures of 10 mL of 1 % agarose (Merck) in Veronal buffer at 56 °C with hyperimmune (final concentration 1, 2 and 3 %) were added carefully to each glass plate until solidification. Wells of 3 mm diameter were punctured in the solidified agarose and 6 µL of IgY solutions at different concentrations (standard curve) were added to each well. The plates were incubated in humid atmosphere at room temperature for 24 h . At the end the diameters of the rings of the antibody-antigen precipitation obtained by the immunodifusion reaction were measured.

Statistical analysis

The main stadigraphs were calculated by using in house computation programs.

RESULTS AND DISCUSSION

The immunization schedule described in this work was useful for the desired proposal. The dynamic of antibody titers from diferent immunized animals are shown in Table 1 and Fig 1. The curves obtained represent classical reportedf reported[23,34,35] immunological responses.

These results agree with reports that have used rabbits and other small mammals as animal models in immunization schedules inoculating heterologous proteins.[1,2,36-40] Optimization of the antigenic dose, injection route and type of adjuvant are similar to the reports mentioned above. On the basis of these results this schedule could be proposed for obtaining this reagent in a reproducible manner.

Doble immunodifusion or Ouchterlony technique (Fig. 2) and immunoelectrophoretic immunoassay demonstrated high specific recognition of the raised antibodies to IgY.

Immunoelectrophoresis (Fig. 3) shows a single precipitation arc recognizing IgY (gamma livetin region) among other proteins from delipidated supernatants of egg yolk (alfa and beta livetins) [6-8,41]

Good results were obtained in the purification process of these sera. Pools of the desorbed bound proteins obtained by immunoaffinity Chromatography of total rabbit IgG performed after ammoniun sulfate precipitation and Chromatography on Protein A-Sepharose were concentrated by ultrafiltration (AMICON, Grace, Beverly, MA) with membranes of 10 kD molecular weight cut off and tested by Ouchterlony obtaining specific reactions to IgY. The concentration of these eluates was determined by Ultraviolet Spectrometry. About 10 % of total rabbit IgG specific to IgY was obtained, which is in agreement

Table 1. Titers of immunoprecipitation (Ouchterlony technique).

Rabbit	Day										
	0	7	14	21	28	35	42	49	56	63	70
1	–	–	–	1/4	1/4	1/8	1/16	1/16	1/32	1/32	1/56
2	–	–	–	1/2	1/8	1/8	1/8	1/16	1/32	1/32	1/50
3	–	–	P	1/4	1/8	1/8	1/16	M	M	M	M

These values represent antibody production as a funcxtion of time in different immunized animals. P Pure serum. M Death.

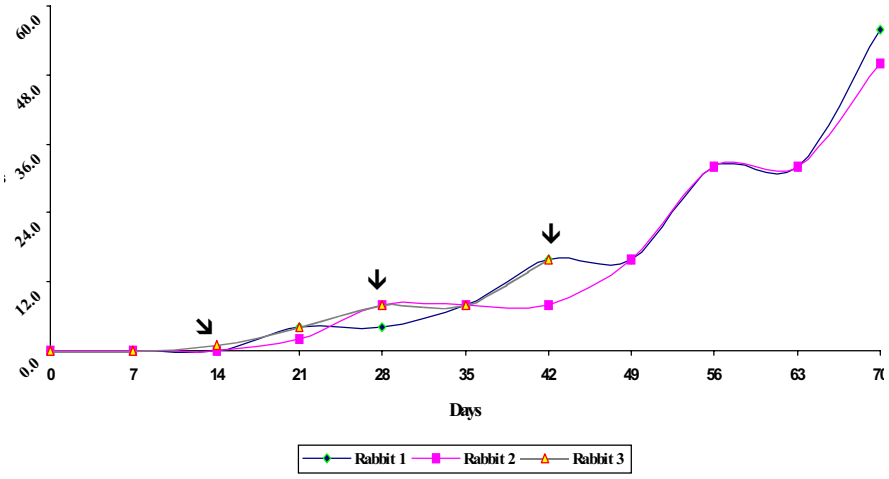

***Fig. 1**. Behavior of titers of antibodies in relation time.*
Arrows indicate inoculations at intervals of two weeks. Weekly blood extractions for screening of the antibodies titers were made before bleeding of the animals.

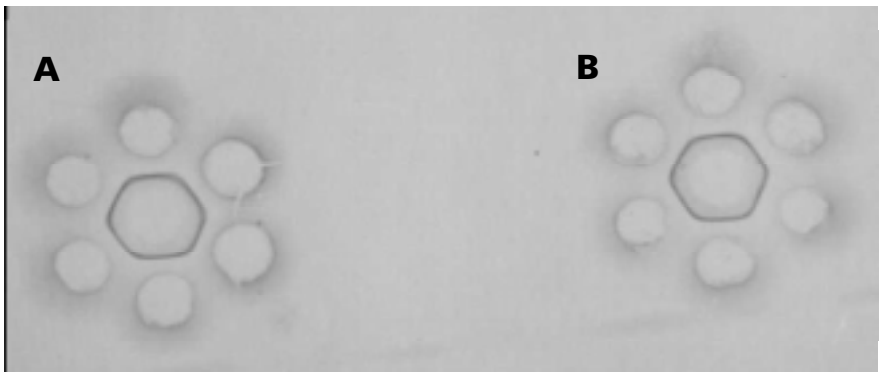

***Fig. 2**. Ouchterlony 's technique of antiserum obtained inone of the three animals in front of pure preparation of IgY.*
A and B are replicates of the same, serial dilutions (from 1:2 to 1:32) of antiserum were made and added to the six small external wells. Pure preparation of IgY (1 mg/mL) was added to the central well.

with other reports[1,6] (approximately 2-10 % of total immunoglobulins specifically recognize the applied antigen).

The absorbance values of the ELISA test of the conjugate obtained with peroxidase as was described in Materials and Methods and a similar conjugate from Sigma Co. Ltd. are shown Tables 2 and 3. To establish the working dilution of the conjugate it is necessary to test it by ELISA coating the plates with 1 µg of antigen per well, performing several serial dilutions of the conjugate, incubating the plate with the specific substrate for the enzyme during 30 min at 37 °C in the dark, and stopping the reaction. The working dilution will correspond to the dilution of the conjugate for which an absorbance value of about 1.0 OD units is obtained.[32]

Results obtained in the test were after 5 min of incubation at room temperature. Thus the working dilution of our conjugate is approximately 1: 8 000, at which we obtained an OD of 0.9701 (mean of 10 replicates). The OD of the control conjugate from Sigma was 1.3345 at the same dilution. This result demonstrates that the obtained conjugate retains antibody and enzyme activity.

The possibility to obtain biotinylated antibodies with the in house biotin-NHS is demonstrated by the results (Table 4). In the table the results of the ELISA test performed with an avidin-biotin peroxidase complex from VECTASTIN, are presented. These biotinylated antibodies might be used to perform immunohistochemical tests to recognize cellular diferentiation antigens in tissues by light microscopy.

One important result in this work was the standardization of the Mancini test to demonstrate the specificity and biological activity of hyperimmune anti IgY sera for the quantitation of IgY in several biological samples as chicken sera and chicken egg yolk. Data of this standardization (Table 5) show that correlation coeficients higher than 99 % were obtained after performing regression analysis of the relation between the square of the diameters of the precipitation rings and the different concentrations of IgY in the calibration curve. Data are in agreement with other authors[11,13,33,34] that have used this method to quantify IgY.

The main goal of this work has been achieved. A reagent that recognizes IgY has been produced by an eficient immunization schedule, a purification scheme from sera to obtain pure antibodies has been established. The sodium periodate oxidation method to conjugate antibodies to enzyme has proven to be useful and a rabbit IgG antiChicken IgY-peroxidase conjugate with a working dilution of 1:8 000, has been obtained.

The recommended method for biotinylation of antibodies in this work is useful as has been efficiently tested in the performed assays using a commercial avidin biotin complex. Mancini´s method as a standardization system to quantify IgY also worked.

CONCLUSION

In this work, a reproducible methodology to obtain reagents that directly recognize IgY (chicken egg yolk immunoglobulin) by immunodifusion or by ELISA tests has been established. These reagents can also be used in assays where primary chicken egg yolk antibodies have been employed as analytical toools in diseases related to veterinary medicine, and human and plant pathologies as well as for detection of several substances in quality control laboratories. This work is the way to produce an universal tool to reveal immunological reactions that use antibodies of the IgY Technology.

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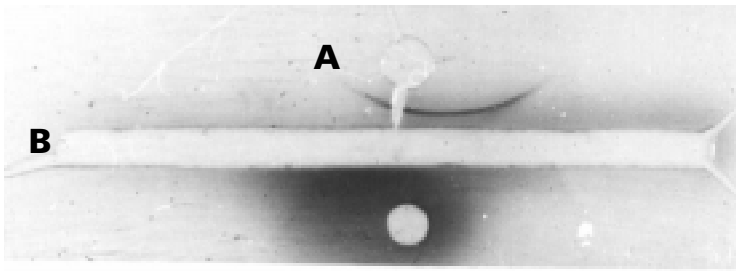

***Fig. 3**. Immunoelectrophoresis of the obtained antiserum to IgY.*
A is a delipidated supernatant of chicken egg yolk.
B is an antiserum of immunized rabbits.

Table 2. Absorbance values obtained by ELISA test using the rabbit IgG antiChicken IgY-peroxidase conjugate obtained in this report.

Rows	Columns											
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.018	1.546	1.551	1.564	1.599	1.576	1.548	1.587	1.585	1.623	1.631	0.021
B	0.015	1.400	1.434	1.381	1.442	1.433	1.349	1.440	1.403	1.457	1.475	0.019
C	0.015	1.208	1.236	1.135	1.242	1.218	1.201	1.262	1.199	1.254	1.273	0.018
D	0.015	1.014	0.974	0.967	0.972	0.982	0.926	0.959	0.952	0.956	0.999	0.015
E	0.014	0.735	0.692	0.693	0.680	0.688	0.667	0.680	0.636	0.681	0.695	0.017
F	0.016	0.498	0.467	0.467	0.462	0.469	0.442	0.458	0.460	0.454	0.467	0.019
G	0.014	0.284	0.275	0.269	0.261	0.260	0.271	0.261	0.264	0.265	0.265	0.018
H	0.013	0.171	0.155	0.159	0.156	0.153	0.156	0.151	0.156	0.156	0.161	0.017

The whole plate was coated with 1.0 mg of pure IgY (Sigma . Co. Ltd. USA) per well. Columns 1 and 12 (A to H) are blanks. Skim milk without conjugate. Dilutions of conjugate (columns 2-11) in the rows: A, 1:1 000; B, 1:2 000; C, 1:4 000; D, 1:8 000; E, 1:16 000; F, 1:32 000; G, 1:64 000; H, 1:128 000.

Table 3. Absorbance values obtained by ELISA test using control rabbit IgG antiChicken IgY-peroxidase conjugate from Sigma. Co. Ltd., USA. The test was peformed as above.

Rows	Columns											
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.027	2.794	2.791	2.783	2.744	2.667	2.609	2.641	2.415	2.639	2.558	0.023
B	0.021	2.508	2.446	2.414	2.407	2.422	2.363	2.343	2.305	2.370	2.393	0.040
C	0.027	2.161	2.059	2.047	2.050	1.930	1.976	1.912	1.855	1.945	1.948	0.031
D	0.015	1.472	1.385	1.337	1.391	1.301	1.305	1.253	1.321	1.246	1.331	0.016
E	0.012	0.883	0.842	0.839	0.826	0.784	0.819	0.758	0.793	0.746	0.798	0.013
F	0.019	0.537	0.503	0.487	0.476	0.452	0.477	0.467	0.475	0.467	0.512	0.021
G	0.017	0.278	0.262	0.268	0.249	0.269	0.243	0.248	0.251	0.260	0.275	0.020
H	0.014	0.159	0.161	0.154	0.147	0.139	0.148	0.145	0.144	0.144	0.151	0.015

The whole plate was coated with 1.0 mg of pure IgY (Sigma . Co. Ltd. USA) per well. Columns 1 and 12 (A to H) are blanks. Skim milk without conjugate. Dilutions of conjugate (columns 2-11) in the rows: A, 1:1 000; B, 1:2 000; C, 1:4 000; D, 1:8 000; E, 1:16 000; F, 1:32 000; G, 1:64 000; H, 1:128 000.

Table 4. Absorbance values obtained by ELISA test using the obtained biotinylated antibodies with an ABC complex from VECTASTIN, USA.

Rows	Columns											
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.324	2.068	2.118	2.033	1.942	1.694	1.250	0.716	0.575	0.421	0.413	0.338
B	0.274	2.058	2.064	2.016	2.068	1.773	1.278	0.880	0.592	0.394	0.361	0.254
C	0.243	2.077	2.020	2.230	1.989	1.752	1.267	0.913	0.563	0.400	0.334	0.251
D	0.201	2.032	2.039	2.086	1.933	1.696	1.268	0.858	0.551	0.387	0.293	0.245
E	0.232	2.254	1.965	2.247	2.063	1.787	1.298	0.844	0.565	0.355	0.255	0.244
F	0.239	2.237	2.132	2.124	2.112	1.773	1.317	0.870	0.544	0.519	0.437	0.229
G	0.217	2.063	2.041	2.057	2.025	1.820	1.333	0.845	0.505	0.556	0.335	0.235
H	0.324	2.339	2.311	2.283	2.088	1.795	1.308	0.924	0.569	0.501	0.364	0.339

Columns 1 and 12 (A to H) Blanks. Coated with 20 mg/mL. Skim milk without biotynilated antibodies. Column 2 to 11: Coated with double serial dilutions of antigen (pure IgY) starting from 20 mg/mL in column 2 until 0,039 mg/mL in column 11. Biotinylated antibodies were used at 1: 10 000 dilution.and ABC complex according to the manufacturer´s instructions. Observe in column 7 the recognition of less than 1 mg/mL of IgY (OD mean 1,28).

Table 5. Correlation coefficients of the linear regression fitting of the data of the relation between the different concentrations of IgY in the calibration curve and their respective precipitation diameters obtained by Mancini´s technique. Results of four replicates of the calibration curve applied to 1 % agarose with immovilized antiserum.

C	DP1	DP3	DP3	DP4
(mg/mL)		(mm)		
1.0	7.90	8.10	7.90	8.05
0.8	7.20	7.5	7.30	7.25
0.6	6.45	6.6	6.35	6.50
0.4	5.40	5.9	5.30	5.40
0.2	4.65	4.7	4.40	4.60
CCr	0.998	0.998	0.998	0.998

DP Diameter of precipitation. CC_r Correlation coeficient.

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