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Production of Gentamycin and Ceftiofur Specific Polyclonal Antibodies by Conjugating them with Bovine Serum Albumin

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Abstract

Polyclonal antibodies (pAbs) were produced against gentamycin (GEN) and ceftiofur (CEF) antibiotics in sprague dawley rats (aged 7-8 weeks) by conjugating them with bovine serum albumin (BSA). Total 3 groups (3 rats in each group): one control (without antibiotic) group and two test groups (GEN and CEF) of rats were maintained. Total of four blood samplings were done from each group as follows: First three samples was serially at 15 days' time interval after 1st booster, 2nd booster, 3rd booster and the 4th sampling was one and half month after the third booster. The antibody titres in the antisera of each antibiotic in all the four immunization cycles were determined by an icELISA at various serum dilutions ranging from 1/100 to 1/6400. Analysis of antibiotic conjugates by SDS-PAGE and Coomassie blue staining revealed higher molecular weights when compared to normal BSA (68 KDa). The molecular weights of conjugates were 90 kDa, 78 kDa for GEN-BSA and CEF-BSA respectively. The GEN antisera gave positive antibody titres up to a dilution of 1/1600 in first immunization cycle, 1/6400 in 2nd and 3rd immunization cycles and 1/1600 in 4th sampling. Maximum optical density at 450 nm (OD_{450}) value of 0.928 was obtained at 1/100 antiserum dilution in 3rd immunization cycle. The CEF antisera gave positive antibody titres up to a dilution of 1/800 in 1st immunization cycle, 1/1600 in 2nd immunization cycle, 1/6400 in 3rd immunization cycle and 1/3200 in 4th sampling. Maximum OD_{450} value of 2.072 was obtained at 1/100 antiserum dilution in 3rd immunization cycle.

Keywords: Conjugation of antibiotics; 1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide; Indirect competitive ELISA, Bovine serum albumin

Introduction

The use of antibiotics as prophylactic agents, growth promoters, therapeutic agents, in the treatment of pyrexia, inflammation, wounds and viral diseases in lactating cattle and buffaloes is responsible for their presence in the milk [1]. Animals are exploited for more production and profit with extensive use of antibiotics as growth promoters contributing significantly to drug residues being present in milk [2]. Sub-therapeutic use of antibiotics as growth promoters or to control endemic infections cause development of drug resistance [3]. There is a considerable evidence for the development of antibiotic resistance in bacterial species that cause zoonotic infections like *Salmonella enterica* serotypes, *Campylobacter jejuni*, *Escherichia coli* and *Enterococci* due to the use of antibiotics as growth promoters [4].

Antibiotics residues in milk higher than MRL (Maximum residue levels) are of great concern to dairy farmers, milk processors, regulatory agencies, and consumers due to their possible adverse effects on people allergic to antibiotics and potential build-up of antibiotic-resistant organisms in humans [5]. All these results emphasize the need to have strict control measures on the use of antibiotics in veterinary practice both as therapeutic as well as prophylactic agents and also the need to have rapid and sensitive screening methods to detect antibiotic residues in milk samples.

To develop an antibiotic specific immunologic assay, pAbs against specific antibiotic structure is required [6]. To incite an immune response, the molecular weight of the antigen should be minimum of 10 kDa [7]. Low molecular weight and incomplete antigens (haptens) like antibiotics are conjugated with carrier protein like BSA [8].

This study was undertaken to produce pAbs against GEN and CEF antibiotics and detection of these pAbs by a sensitive icELISA in antibiotic specific antisera. The pAbs produced can be employed for detecting GEN and CEF residues in milk samples.

Materials and Methods

Animals

Sprague Dawley rats aged 7-8 weeks were kept under well lighted experimental house and maintained on standard rat feed with ad *libitum* water. A total of 3 groups were maintained, two test groups (for GEN and CEF) and one control group (without antibiotic) each with 3 rats in a group.

Ethical approval

The experimental protocol was approved by the university animal ethics committee under order no 8/i/10.

Conjugation of GEN

3.5 ml of GEN (40 mg/ml) and 20 mg of BSA were taken into a clean beaker. 580 mg of EDC was dissolved in 2 ml of distilled water

and was added drop wise to the above mixture, accompanied by continuous stirring on a magnetic stirrer. The pH of the solution was adjusted to 5.0-6.0 by adding 0.1N HCl as method described by Haasnoot et al. with slight modifications [9].

Conjugation of CEF

2.5 ml of CEF (100 mg/ml) and 20 mg of BSA were taken into a clean beaker. 580 mg of EDC was dissolved in 2 ml of distilled water and was added drop wise to the above mixture, accompanied by continuous stirring on a magnetic stirrer. The pH of the solution was adjusted to 5.0-6.0 by adding 0.1N HCl as described by Stanker et al. with slight modifications [10].

The above reaction mixtures of GEN-EDC and CEF-EDC were incubated at room temperature (RT) in separate beakers with continuous stirring for 2 hours. After the reaction time of 2 hours, uncoupled antibiotic and EDC were removed by dialysis.

Determination of successful conjugation

The conjugated samples were analyzed by SDS PAGE to confirm successful conjugation [11]. SDS PAGE was performed according to the method described by Bollag et al. [12]. The images of the stained gels were taken in the gel documentation system (G-box-Syngene).

Immunogen preparation for primary immunization

For preparation of GEN immunogen 35 µl of GEN-BSA conjugate was added to 465 µl phosphate buffer saline (PBS) buffer and 500 µl of complete freund's adjuvant (FCA) and for CEF immunogen preparation 40 µl of CEF-BSA conjugate was added to 460 µl PBS buffer and 500 µl of FCA as described by Dykman et al. [13].

Immunogen preparation for booster immunization

For preparation of GEN booster immunogen 35 µl of GEN-BSA conjugate was added to 465 µl PBS buffer and 500 µl of incomplete freund's adjuvant (FIA) and for CEF immunogen preparation 40 µl of CEF-BSA conjugate was added to 460 µl PBS buffer and 500 µl of FIA [13].

The immunogen was mixed thoroughly and 300 µl (150 µl for each site) was injected to each rat (test group) subcutaneously at two different sites according to the immunization schedule as described by Dykman et al. [13].

Collection of blood from rats

The blood was collected by orbital sinus veni puncture method described by Oruganti and Gaidhani [14]. Total of four blood collections were made in each group at different time intervals according to the schedule given by Dykman et al. with slight modifications (Table 1) [13].

Estimation of Total protein, albumin and A/G ratio

The serum samples of each test group (GEN and CEF) and control group animals collected after second booster (third immunization cycle) were analyzed for total protein, albumin and A/G ratio by using Ensure Biotech Total Protein andAlbumin teaching kit.

Preparation of ELISA antigens (casein-antibiotic conjugates)

Casein (0.83 µmol) was dissolved in 2 ml of distilled water in the presence of small amount of sodium-bi-carbonate to maintain alkaline condition. 83 µmol of antibiotic and 83 µmol of EDC were added to the above protein solution. Reaction mixture was stirred on a magnetic stirrer continuously for 2 hrs at room temperature. The pH of the solution was adjusted to 5.0. Reaction mixtures of all the two antibiotics were then incubated overnight at 4°C. Conjugates were dialyzed against distilled water as the method given by Samsonova et al. [15].

Immunization Schedule	Procedure
Day 0	1st Immunization antigen+CFA
Day 15	1st Boost antigen+ICFA
Day 30	1st Test Bleed
Day 37	2nd Boost antigen+ICFA
Day 52	2nd Test Bleed
Day 59	3rd Boost antigen+ICFA
Day 74	3rd Test Bleed
Day 104	4th Test Bleed
CFA: Complete freund's adjuvant ICFA: Incomplete freund's adjuvant,	

Table 1: Immunization schedule.

Indirect ELISA

96 well flat bottom polystyrene ELISA plates (Nunc, Denmark) were coated with 250 µl of antigen (antibiotic-casein conjugate) in 0.01 M carbonate buffer (pH-9.6). The plate was incubated overnight at 4°C. The wells were washed 3 times with PBS that contained 0.05% Tween 20 (PBST), 250 µl/well. The free (unbound) sites were blocked with 2% casein in blocking buffer, 250 µl/well. The plate was incubated at 37°C for 1 hour. The wells were washed three times with PBS, 250 µl/well. 100 µl of diluted antiserum samples (1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200, 1/6400) in PBST were added to each well and the plate was incubated for 1 hr at 37°C. The wells were washed 3 times with PBST (250 µl/well per wash cycle). 100 µl of conjugate of secondary antibodies with horseradish peroxidase in PBST was added to each well and the plate was incubated for 1 hr at 37°C. The wells were washed 3 times with PBST (250 µl/well per wash cycle). 100 µl of the substrate (3, 3', 5, 5' tetramethylbenzidine) in PBST (1 in 20 dilution) was added to each well. Reaction was stopped after 10-15 minutes by adding 50 µl/well of 4M H_2SO_4 as the method described by Samsonova et al. [15]. Optical density was measured by using ELISA microtitre plate reader at 450 nm (Biotech instrument-µquant).

Standardization of indirect competitive ELISA

The serum samples collected after second booster (3^{rd} immunization cycle) were used for the standardization. Checker board titration was performed using different dilutions of antigens against different serum dilutions of test groups and negative control at constant secondary antibody-HRP conjugate dilution of 1/10000 (manufacturer's instruction). Serial antigen dilutions (from 2 x 10^6 ng/ml to 2 ng/ml) were taken from rows B to H in 96 well polystyrene

plates and serial primary antibody dilutions (from 1/50 to 1/1600) were taken from columns 1 to 6 for test group samples and columns 7 to 12 for control group samples. The dilution of antigen which showed, maximum absorbance reading and started to maintain almost a stationary phase was taken as the optimum according to the procedure described by Fan et al. [16].

Testing the antiserum samples for antibody titres

The antibody titres in the serum samples collected from immunized rats were tested by icELISA standardized as described above. The optimum antigen concentrations and primary antibody dilutions obtained for the antibiotics by the above described method were used for the test. The antisera of all the three animals in each group collected during all the three immunization cycles and 4th sampling were tested at various serum dilutions ranging from 1/100 to 1/1600. Each sample was tested in duplicate including the control serum samples. In the reagent blank, PBST was added instead of antiserum. In the negative control wells, serum samples of control group rats were added. The mean OD_{450} of various serum dilutions at each immunization cycle for each group of rats were used to plot a graph with absorbance on y-axis and serum dilutions on x-axis.

Construction of PNT base line

The mean and the standard deviation values of the control group at each dilution ranging from 1/100 to 1/6400 were calculated for each of the two different antigen coated plates used in the present study. Three units of standard deviation were added to the corresponding mean absorbance value and a graph was plotted with values of mean plus three times standard deviation (M+3SD) on y-axis and serum dilutions on x-axis. This was considered as positive negative threshold (PNT) baseline [17]. Separate PNT baselines were constructed for each test group.

Prediction of antibody titres

The positive antibody titres were determined based on the cut off value obtained from PNT base line constructed. The highest OD_{450} value of the PNT baseline rounded off to the nearest single digit decimal was taken as cut off value. The OD_{450} value over and above the cut off value was considered as positive antibody titre [17].

Results and Discussion

Determination of successful conjugation

GEN and CEF antibiotics were conjugated with BSA by using EDC as a crosslinker by employing carbodiimide method. Analysis of antibiotic conjugates by SDS-PAGE and Coomassie blue staining revealed higher molecular weights of antibiotic-BSA conjugates when compared to normal BSA (Figure 1). Before conjugation with antibiotic, the molecular weight of BSA was 68 KDa. After conjugation, the molecular weights of conjugates were 90 kDa, 78 kDa for GEN-BSA and CEF-BSA respectively. These results clearly indicate the successful conjugation of antibiotics with BSA. These results are similar with Jiang et al. [18] Where sarafloxacin-BSA conjugate was analysed on SDS PAGE by observing the increased molecular weights of a conjugate.

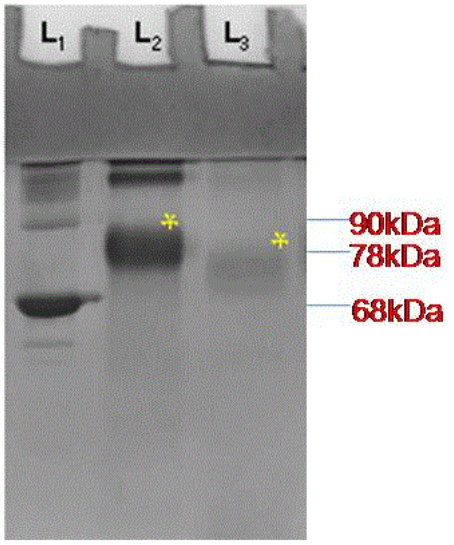

Figure 1: Coomassie blue stained SDS PAGE gel of antibiotic conjugates. L_1-Bovine serum albumin, L_2-Gentamycin conjugate, L_3-Ceftiofur conjugate, (*) conjugates.

Estimation of total protein, albumin and A/G ratio

The mean values of total protein, albumin, and globulin and A/G ratio of serum samples collected during third immunization cycle. The mean total protein concentration was 8.25 ± 0.20 g/dL, 30 ± 1.20 g/dL for GEN and CEF antisera respectively and in the control group it was 6.66 ± 0.01 g/dL. The mean albumin concentration was 2.93 ± 0.14 g/dL, 3.44 ± 0.020 g/dL for GEN and CEF antisera respectively compared 3.36 ± 0.173 g/dL in the control group. The mean globulin concentration was 5.23 ± 0.05 g/dL, 26.56 ± 1.18 g/dL for GEN and CEF antisera respectively, where it was 3.30 ± 0.04 g/dL in the control group. The mean A/G ratio was 0.55 ± 0.023, 0.13 ± 0.020 for GEN and CEF respectively. The mean A/G ratio in the control group serum was 1.02 ± 0.012. Thrall [19] reported that A/G ratio was altered due to increase in total protein concentration and particularly globulin concentration in the serum. Increased globulin concentration and decreased A/G ratio indicates the presence of antibodies in the antisera.

Standardization of antigen concentration and antiserum dilution for Indirect ELISA

In all the checker board titrations the OD_{450} values of the test wells increased suddenly from the coated antigen concentration of 2 ng/ml to 20 ng/ml and continued to maintain a steady phase at higher concentrations. So, 20 ng/ml was chosen as optimum antigen concentration. The absorbance values of the negative control wells dropped suddenly from serum dilution of 1/50 to 1/100 and continued to maintain similar range at higher dilutions and at all antigen concentrations. The test wells continued to maintain higher absorbance values at corresponding dilutions. Hence 1/100 was chosen as optimum serum dilution. The highest mean plus three times standard deviation values (M+3SD) values obtained for negative sera were 0.207 (Table 2) and 0.211 (Table 3) for GEN-casein and CEF-

casein coated plates respectively. Hence the cut off value was selected as 0.3 for antibiotics.

Immunization cycles	Serum dilutions						
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
1st IC	0.739	0.620	0.484	0.386	0.315	0.285	0.255
2nd IC	0.824	0.725	0.586	0.465	0.373	0.334	0.314
3rd IC	0.928	0.815	0.679	0.535	0.436	0.365	0.342
4th Sampling	0.792	0.677	0.527	0.408	0.307	0.281	0.212
Negative control (M+3SD) (M + 3SD)	0.207	0.203	0.191	0.109	0.111	0.097	0.091

Table 2: Mean OD_{450} values of indirect ELISA of gentamycin antisera (Bold numbers indicate 50% titres).

Immunization cycles	Serum dilutions						
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
1st IC	0.905	0.784	0.59	0.428	0.283	0.094	0.068
2nd IC	1.052	0.97	0.807	0.528	0.358	0.198	0.135
3rd IC	2.072	1.865	1.581	1.324	1.239	1.11	1.041
4th IC	1.458	1.282	0.999	0.84	0.633	0.435	0.25
Negative control (M+3SD)	0.211	0.207	0.218	0.175	0.115	0.121	0.110

Table 3: Mean OD_{450} values of indirect ELISA of ceftiofur antisera (Bold numbers indicate 50% titres).

Detection of antibody titres in the GEN antisera

The highest M+3SD value of negative control was 0.207 (Table 2). The cut off value was selected as 0.3 (nearest single digit decimal above 0.207). The mean OD_{450} values of the gentamycin antisera were above the cut-off value up to serum dilution of 1/600 in all the immunization cycles which indicated positive antibody titres (Table 2, Figure 2). The values of 50% antibody titres increased from the antiserum dilution of 1/800 in 1st immunization cycle to 1/6400 in 3rd immunization cycle (Table 2). The gentamycin antisera gave positive antibody titres up to a dilution of 1/1600 in first immunization cycle, 1/6400 in 2nd and 3rd immunization cycles and 1/1600 in 4th sampling. Highest antibody titre was obtained after primary immunization by plate trapped antigen ELISA [20] and after second boosting by indirect ELISA [21] in gentamycin antisera. Gentamycin antisera after 11th booster and streptomycin antisera after 6th booster were used to confirm specific immune response in rabbits by ELISA [9]. Maximum OD_{450} value of 0.928 was obtained at 1/100 antiserum dilution in 3rd immunization cycle (Figure 2) which clearly indicated that the immune response was the highest in 3rd immunization cycle (Table 2). The immune response significantly increased from 1st immunization cycle to 3rd immunization cycle at 1/100 antiserum dilution and decreased in 4th collection (Figure 2).

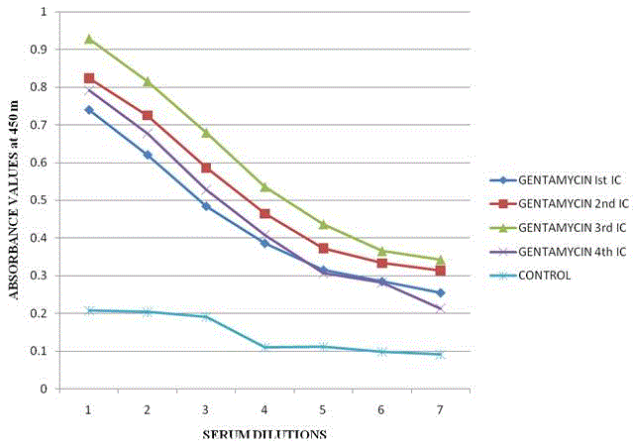

Figure 2: Calibration curve for Indirect ELISA of BSA-Gentamycin antisera at various immunization cycles (IC) (1, 2, 3, 4, 5, 6, 7 represents serum dilutions of 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200 and 1/6400 respectively).

Detection of antibody titres in the CEF antisera

The highest M+3SD values of negative control was 0.211 (Table 3). The cut-off value was selected as 0.3 (nearest single digit decimal above 0.211). The mean OD_{450} values of the serum samples from immunized rats were above the cut-off value up to serum dilution of 1/800 in all the three immunization cycles and fourth sampling which indicated positive antibody titres (Table 3, Figure 3). The values of 50% antibody titres increased from the antiserum dilution of 1/800 in 1st immunization cycle to 1/6400 in 3rd immunization cycle (Table 3). The ceftiofur antisera gave positive antibody titres up to a dilution of 1/800 in 1st immunization cycle, 1/1600 in 2nd immunization cycle, 1/6400 in 3rd immunization cycle and 1/3200 in 4th sampling (Figure 3). Maximum OD_{450} value of 2.072 was obtained at 1/100 antiserum dilution in 3rd immunization cycle (Table 3) which clearly indicated that the immune response was the highest in 3rd immunization cycle. The immune response significantly increased from 1st immunization cycle to 3rd immunization cycle at 1/100 serum dilution and decreased in 4th collection (Figure 3). Stanker et al. [10], Meier and Bianca [22], Chen et al. [23], Thal et al. [24] and Bremus et al. [25] used ELISA to confirm the presence of antibodies against cefteram, cefalexin and ceftiofur, cephalexin, cefquinome and cephalosposin group respectively and all of them obtained positive antibody titres. The results clearly indicated that specific immune response was produced by cephalosporin group of antibiotics which was in accordance with our results.

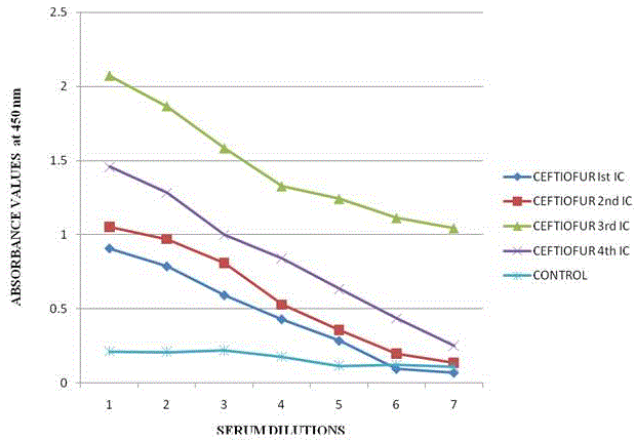

Figure 3: Calibration curve for Indirect ELISA of BSA-Ceftiofur antisera at various immunization cycles (IC) (1, 2, 3, 4, 5, 6, 7 represents serum dilutions of 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200 and 1/6400 respectively).

Comparative immunogenic potency of BSA-GEN and BSA-CEF antisera

Highest immune response was seen in CEF antiserum followed by GEN evidenced by OD_{450} values of 2.072, 0.928 for CEF and GEN antisera respectively at 1/100 serum dilution in 3rd immunization cycle (Figure 4). Least immune response in GEN was due to less efficient conjugation of GEN with BSA compared to CEF which might have resulted in low epitope density of hapten on the hapten-carrier protein conjugate. CEF has free carboxyl groups in its structure for conjugation with BSA whereas gentamycin has free amino groups. The most suitable coupling agents used to couple carboxyl group containing haptens to amines was carbodiimides and amino group containing haptens to amines, thiols, indoles and hydroxyls was glutaraldehyde [26]. But carbodiimides were also suggested to be suitable cross linkers for amino group containing haptens by Mingtao and Jiang [27].

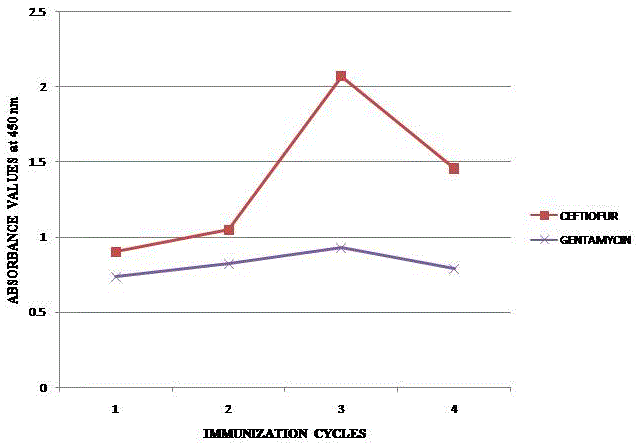

Figure 4: Comparative calibration curves for Indirect ELISA of BSA-antibiotic anisera at 1/100th serum dilution of various immunization cycles (IC) (1, 2, 3, 4 represents 1st, 2nd, 3rd and 4th immunization cycles (IC) respectively at 1/100th serum dilution).

Conclusion

In the present study, the antibiotics (GEN and CEF) were successfully conjugated with BSA by carbodiimide reaction using EDC as a cross linker. These conjugated antibiotics were capable of producing pAbs which was confirmed by icELISA. But the immune response was poor in GEN antiserum when compared to the antisera of CEF. The pAbs produced against GEN and CEF can be used to develop immunoassay based diagnostic tests like lateral flow immunoassay and ELISA kits which can be used to detect antibiotic residues in the biological fluids like milk and urine samples.

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