African Journal of Pharmacology and Therapeutics Vol. 1 No. 2 Pages 41-45, 2012
Open Access to full text available at http://www.uonbi.ac.ke/journals/kesobap/

Research Article

Potential Animal Sources of Antibodies for the Development of a Chloramphenicol Enzyme-Linked Immunosorbent Assay

Jesca O. Wesongah [a,*] and Anastasia N. Guantai [b]

[a] *Department of Medical Laboratory Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya*
[b] *Department of Pharmacology and Pharmacognosy, School of Pharmacy, University of Nairobi, Kenya*

* **Corresponding author:** Department of Medical Laboratory Sciences, Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62000-00200, Nairobi, Kenya; **Tel**: +254-67-52711; **Fax**: +254-67-52164; **Email**: sokwara@jkuat.ac.ke

Background: Chloramphenicol (CAP) is a cheap and effective broad-spectrum antibiotic that has been used in veterinary practice to treat septicaemia, pulmonary, urinary and digestive tract infections. However it has been found to be toxic to humans and may lead to an irreversible aplastic anaemia. Due to these side effects CAP was banned from use in food-producing animals.

Objective: The objective of the present study was to produce and select a suitable anti CAP antibody from camels, donkeys or goats for development of a CAP Enzyme Linked Immunosorbent Assay.

Method: The methods employed were immunological for immunization of experimental animals and conjugation for preparation of CAP- HRP (Horseradish Peroxidase) conjugate. Ammonium sulphate was used for purification of the antibodies.

Results: Antibody production improved with subsequent boosters in camels whereas in donkeys initial immunization yielded significantly ($P<0.05$) higher titres of anti-CAP antibodies. The anti-chloramphenicol antibody produced in camels following the 11th booster (797 days after immunization) was found to be more specific and sensitive than that produced in donkeys and goats.

Conclusion: From this study it was concluded that anti-CAP antibodies from camels were more suitable for the development of a chloramphenicol ELISA.

Key words: anti-chloramphenicol antibodies, camels, goats, donkeys

Received: February, 2012
Published: July, 2012

1. Introduction

The use of chloramphenicol (CAP) in veterinary medicine in the European Union (EU) is currently limited to pets and non-food-producing animals (European Decision 2003/181/EC). It was banned in 1994 from use in any food-producing animals in the EU and also in other developing countries including Kenya. This was due to the fact that it causes bone-marrow depression leading non-dose related aplastic anaemia in human. A minimum required performance limit (MRPL) of 0.3 µg/kg has been established. A typical testing scheme for the presence of the antibiotics in samples from food producing animals employs a rapid and low cost screening assay followed by confirmatory analysis using an analytical chemical method. CAP-ELISA was

 ISSN 2303-9841

compared to three other types of screening assays; a four plate bioassay, a commercial radioimmunoassay and a commercial enzyme-linked immunosorbent assay (ELISA) by Lynas et al, 1998. These workers found that ELISA delivered the highest level of sensitivity. Since then other screening tests have been developed employing ELISA (Posyniak et al, 2003)

A suitable antibody is the common feature of all immunoassays systems (Price et al, 1991). Previously, antibodies for development of immunoassays have often been raised in rabbits because they are cheap and easily available all over the world. However, in this study, anti-CAP antibodies were raised in camels, donkeys and goats in order to harvest a large volume of antisera for distribution to other countries. The aim of this study was to produce and select a suitable antibody for the development of a sensitive and specific CAP ELISA for the determination of CAP in serum and residues in edible animal tissues. The antibody production was carried out over a period of two years and monitored for titres using an appropriate CAP Horseradish peroxidase (CAP HRPO) conjugate. The antibody produced in the present study was also purified using ammonium sulphate in order to improve the sensitivity and specificity of the CAP ELISA developed for serum (Wesongah et al, 2007).

2. Materials and Methods

2.1 Reagents and Chemicals

Isobutylchloroformate (17,798-9) was obtained from Aldrich (Poole, Dorset, UK) and N-methyl morpholine was obtained from Fluka (Poole, Dorset, UK). Chloramphenicol succinate (C-3787), Freunds adjuvant complete (F-5881), Dimethyl formamide (DMF) and incomplete (F-5506) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) (Sigma E7750-5G) were obtained from Sigma (Poole, Dorset, UK).

2.2 Experimental Animals

Two male animals from each of three selected species, camels, goats and donkeys, were purchased and housed in a barn at the Kenya Agriculture Research Institute – Trypanosomiasis Research Centre (KARI-TRC). On arrival at the KARI-TRC all the animals were ear-tagged. They were sprayed with a solution containing 12.5% w/v amitraz (Triatix ®Coopers (K) Ltd.) for tick control. The animals were given albendazole ® (Norbrook Ltd, Newry, United Kingdom) orally for deworming. They were left to acclimatize for three weeks before the actual treatment commenced in accordance with the Guide for the care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). The animals were maintained on hay, lucerne and had water *ad libitum*.

2.3 Preparation of CAP immunogen

CAP immunogen was obtained from Agri-Food and Biosciences Institute (AFBI), Veterinary Surveillance Division, Belfast, Northern Ireland. The immunogen was prepared as described by Fodey et al, 2007. Briefly, 1 mg of CAP succinate was dissolved in 500 µL of dry dimethyl formamide (DMF). N-methyl morpholine was added and the mixture cooled at –20 °C for 10 min. Isobutylchloroformate was added and the mixture stirred for 10 min (**Figure 1**). Precipitation occurred, so a further 1.5 ml of DMF was added to regain solubility. Human Serum Albumin (HSA) was dissolved in 4 ml of 1mM sodium acetate solution. The activated CAP succinate was added slowly with stirring to the HSA solution. The mixture was allowed to incubate for 1 hr at 4 °C followed by 1 hr at room temperature. The conjugate was then purified by extensive dialysis against saline.

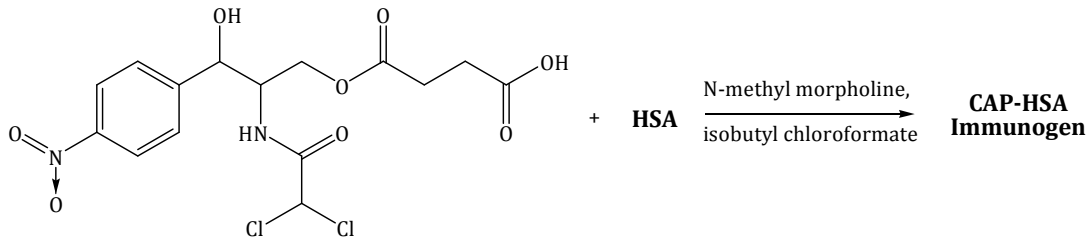

Figure 1: Preparation of CAP HSA immunogen

Figure 2: Conjugation of CAP to Horseradish peroxidase enzyme (HRPO) using EDAC or EDAC/NHS activation

 ISSN 2303-9841

2.4 Preparation of immunogenic emulsion using Freunds adjuvant

The immunogen (in sterile saline 0.9 %) was added slowly in 200 µl amounts to an equal volume of Freund's complete adjuvant with vortexing to produce an emulsion. For the first injection, complete Freunds adjuvant containing heat-killed *Mycobacterium tuberculosis* was used. Subsequent injections were performed using incomplete adjuvant. The emulsions were thickened by passing them through a syringe several times.

2.5 Immunization of experimental animals

The CAP immunogen was administered at an initial dose of 3 mg for camels and donkeys and 2mg for goats. Subsequent inoculations were dosed at 1 mg and 0.5 mg, respectively. The emulsions were injected subcutaneously into four sites of each animal (left and right front-quarters and left and right hind-quarters). Blood samples were collected from the jugular vein for all immunized animals immediately before each booster, and the anti-CAP antisera prepared and stored at -20 °C for analysis.

Immunization of experimental animals and collection of hyperimmune sera from the animals was carried out in two phases: In phase I all the experimental animals were immunized and then given boosters monthly and test bled every two weeks for 5 months.

Phase II of the immunization was initiated 114 days after the 4th booster of phase 1. The experimental animals were given boosters starting from the 5th booster , 6th, 7th, 8th, 9th and 10th booster at two-monthly intervals followed by monthly test bleeding for a period of 16 months. The final booster (11th) was carried out 797 days following the 10th booster and test bled after 2 weeks. This was done to allow the antibody to mature.

Serum samples were prepared from the test bleeds and stored at -20 °C for analysis later.

2.6 Conjugation of CAP to horseradish peroxidase (HRPO)

Figure 2 shows the basic conjugation principle of CAP to HRPO (Sigma P8375). Three different conjugation procedures were used to prepare CAP HRP conjugate.

1: CAP (Standard drug) conjugation to HRPO using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) (Sigma E7750-5G) as the coupling agent and N-hydroxysulfosuccinimide (NHS) as the catalyst; **2**: CAP (Standard drug) conjugation to HRPO using EDAC as the coupling agent and tin chloride as a reducing agent for CAP; and **3**: CAP sodium succinate conjugation to HRPO using EDAC/NHS activation and dissolving of the drug in 2-(N-morpholino) ethanesulfonic acid (MES) buffer and DMF.

The proportions for the different reactants and reagents were 1:1:1 (w/w) for CAP:HRPO:EDAC or 1:4:2 (w/w) CAP:HRPO:EDAC/NHS. All reactants were dissolved in $TDDH_2O$ before mixing, except for the CAP, which was derivatized using acetic acid and tin chloride. The solutions were mixed and left stirring overnight at 4 °C.

2.7 Conjugate Purification

A six centimetre Visking® dialysis tubing size 2.18/32" (Medicell International, London) was boiled in $TDDH_2O$ for 15 min. The conjugate was then cleaned, first by dialysis against 0.9% sodium chloride for 7 hr with two sodium chloride changes at equal intervals. Further purification of the conjugate was done by adding 0.2 ml dextran-coated charcoal to the dialysed conjugate and subsequent centrifugation at 10,000 rpm for 30 min. Further purification of the conjugate was carried out using ammonium sulphate. The supernatant was aspirated and an equal volume of glycerol (Sigma G7893) added. It was then stored at –20 °C until required.

2.8 Evaluation of anti-CAP antibody raised in camels, goats and donkeys

The anti-CAP antibody raised in the 3 different animal species was assessed using checkerboard titrations. 96-well microtitre plates (Immulon 4®, Dynatech Labs, Chantilly, USA) were coated horizontally (12 columns) with doubling serial dilutions of antibody starting from either 1/100, 1/200, 1/400 or 1/800 depending on the strength of the antibody. After an overnight incubation at 4 °C, the plates were kept at –20 °C. When required for use the plates were thawed and washed five times and a doubling serial dilution of conjugate added vertically (8 rows) starting from either 1/100, 1/400, 1/500, 1/800, 1/1k, 1/2k or 1/16k depending on the strength of the antibody. The plates were developed and optical densities (OD) determined using CAP ELISA (Wesongah et al, 2007). The ODs obtained from antisera collected on different days after booster immunizations and between and within the different animal species were compared. The anti-CAP antibody from the final booster (797 days post immunization) from each of the three animal species that gave high ODs were purified and characterized.

2.9 Data and statistical analysis

Individual optical densities obtained from different conjugate and antibody dilutions were compared between and within the 3 different animal species by applying the ANOVA test using StatView statistical software (Version 5.0). The significance of changes in antibody production titres with subsequent boosters was tested by simple regression analysis using StatView.

2.10 Ethical considerations

Permission to carry out the study was obtained from the Institutional Animal Care and Use Committee (IACUC) of Trypanosomiasis Research Centre. The study was conducted in accordance with the Guide for the care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

3. Results

Anti-CAP antibodies produced in camels, donkeys and goats were evaluated and compared. Antibody titres were observed to increase with subsequent boosters in camels and goats but in donkeys the initial response was very strong and declined with subsequent boosters (**Table 1**). The donkeys responded strongly during the

first three boosters as indicated by significantly (P<0.05) high ODs obtained compared to camel and goat antisera. In contrast, the reaction in camels and goats was initially weak with antibody titres building to high levels with subsequent booster immunizations.

The antibody profiles (**Table 1**) show that goat 1 and camel 1 had significantly (P<0.05) higher ODs than goat 2 and camel 2, while donkey 2 had significantly (P<0.05) higher ODs than donkey 1 indicating individual differences in antibody production. Goat 1 antisera following the final (11th) booster gave significantly (P<0.05) higher optical densities than the two camels and the two donkeys indicating a species difference in antibody production.

Anti-CAP antibodies of the final (11th) booster, 797 days post immunization from the three animal species were selected for antibody purification because it was more specific than the antibody obtained during the first ten boosters as indicated by significant (P<0.05) high ODs obtained during checkerboard titration.

Table 1: A comparison of optical densities recorded for camels, donkeys and goats following immunological boosting.

Booster Number	Optical densities from camel antisera		Optical densities from donkey antisera		Optical densities from goat antisera	
	C1	C2	D1	D2	G1	G2
1	0.01	0.01	0.05	1.4	0.3	0.2
2	0.01	0.02	0.8	1.6	ND	ND
3	0.8	0.6	0.4	2.0	2.0	0.5
4	1.2	0.9	0.4	1.5	1.5	0.5
6	0.3	0.3	0.2	0.6	ND	ND
8	0.8	0.6	ND	ND	ND	ND
10	2.0	0.9	ND	ND	2.0	0.5
11	2.5	2.0	0.1	0.1	0.01	0.01

ND= *not done*
C1=*Camel 1;* C2= *Camel 2;* D1=*Donkey 1;* D2=*Donkey 2;* G1=*Goat 1;* G2=*Goat 2.*

4. Discussion

The results of the present study indicate that antibody production improved with subsequent boosters except in the donkeys, which responded to the initial immunization by producing significantly (P<0.05) higher titres of anti-CAP antibodies than goats and camels. This is the first observation of this kind in donkeys as, previously, antibodies to CAP have been raised in sheep (Gaudin and Maris, 2001) and rabbits (Posyniak et al, 2003).

Differences within species in antibody production were also observed in this study, which may be attributed to genetic differences. Previous studies on effects of expression of immune response genes in inbred strains of guinea pigs and mice have indicated that genetic loci which are closely located or part of the Major Histocompatibility Complex (MHC) control the immune response (Dorak, 2006).

Purified antibody from Camel 1 following the final (11th) booster was selected for the development of the CAP ELISA for the following reasons: it gave significantly (p<0.05) higher titres compared to the purified antibody from the other animal species tested. Similar observations were made by Fodey et al, 2007 who reported high IC_{50} values of 0.7 - 1.4 ng/ml in camels compared to 0.4 - 1.3ng/ml in donkeys using the same polyclonal antisera but different boosters. However, this difference was not significant. This could be attributed to the different boosters used.

The high optical densities obtained in the present study indicate that camel 1 purified antibody was more sensitive to CAP compared to the antibody produced in the other animals. In addition, the selection of camel antibody was because of their ability to produce large volumes of antisera. This antibody can also be used in the detection of CAP in more animal species such as cattle, sheep and goats that are a preferred source of proteins and are of economic importance worldwide. However Fodey et al, 2007 reported that a comparison of the sensitivity of individual antibodies indicates that there is not substantial variance across species. This could be because they used antisera from earlier boosters. Fodey et al, 2007 also reported low sensitivity of antisera produced in two donkeys using ELISA technology. This observation is similar to the one made in the present study that antisera from donkeys was less sensitive to CAP compared to the other two animal species.

5.0 Conclusion

This study has demonstrated that camel antibodies were more specific and sensitive to CAP compared to donkey and goat antibodies. This study recommends further characterization of these antibodies and the possibility of using camel antibodies to develop ELISAs for other commonly used veterinary drugs.

 ISSN 2303-9841

Conflict of Interest declaration

The authors declare no conflict of interest

Acknowledgements

Funds for the study were provided by International Atomic Energy Agency (IAEA) and Kenya Agriculture Research Institute - Kenya Agriculture Productivity Programme (KARI-KAPP). The authors wish to acknowledge Dr. Murilla G.A. Center director of Trypanosomiasis Research center for the scientific input and logistic facilitation she provided during this work, and Dr. Judy Chemuletti of the Epidemiology Division of Trypanosomiasis Research Centre for her assistance during collection of blood samples from experimental animals. The authors also wish to acknowledge the staff of Residue Analysis Department of the Trypanosomiasis Research Centre for their professional and technical assistance.

References

Hoyle B (2004) This pack of camels smokes cancer. *Nation review of medicine.* Vol **1**. http://www.nationalreviewofmedicine.com/issue/2004_06_15/clinical01_12.html (Accessed 31 May 2012).

Daley LP, Gagliardo LF, Duffy MS, Smith MC and Appleton JA (2005) Application of monoclonal Antibodies in functional and Comparative Investigations of Heavy –Chain Immunoglobulins in New World Camelids. *Clin. Vaccine Immunol.* **12:** 380-386.

Dorak MT, Shao W, Machulla HKG, Lobashevsky ES, Tang J, Park MH and Kaslow RA (2006). Major histocompatibility complex (MHC), conserved extended haplotypes update. *Genes Immun.* **7:** 450–467.

European Commission (2003) Commission Decision 2003/181/CE. Official Journal of the European Communities. **L71**: 17-18.

Fodey T, Murilla G, Cannavan A and Elliot C (2007). Characterisation of antibodies to CAP, produced in different species by ELISA and biosensor technologies. *Anal. Chim. Acta*, **592**: 51-57.

Maria H, Guillermo L, Alvaro S, Fabricio M, Bruno L, Jean Philippe C and Jose Maria G (2005). Factors associated with adverse reactions induced by caprylic acid fractionated whole IgG preparations comparison between horse, sheep and camel IgGs. *Toxicon*, **46**: 775-781.

Institute of Laboratory Animal Resources (1996). Guide for the Care and Use of Laboratory Animals. National Academy Press, Washington, D.C.

Lynas L, Currie D, Elliott CT, McEvoy JDG and Hewitt A (1998). Screening for chloramphenicol residues in the tissues and fluids of treated cattle by four plate test, charm II, Radioimmunoassay and Ridascreen, CAP-Glucuronid enzyme immunoassay. *Analyst* **123:** 2773.

Lange IG, Daxenberger A and Meyer HHD (2001). Studies on the antibody response of Lama glama-evaluation of the binding capacity of different IgG subtypes in ELISAs for clenbuterol and BSA. *Vet. Immunol. Immunopathol*. **83:** 1-9.

Mohanty JG and Elazhary Y (1989). Purification of IgG from serum with caprylic acid and ammonium sulphate precipitation is not superior to ammonium sulphate precipitation alone. *Comp Immunol. Microb. Infect. Dis.* **12:**153-60.

Murilla GA (1996). Studies on trypanocidal drug, homidium, development and use of ELISA for its detection and quantification in cattle. PhD Thesis. University of Glasgow, United Kingdom.

Omidfar KR, Modjitahedi MJ, Forouzandeh H, Taghikhan M, Bakhtiari A, Paknejad M and Kashanian S (2004). Production and characterization of a new Antibody Specific for Mutant EGR receptor, EGFRvIII, in Camelus bactrianus. *Tumor Biol.* **25**:179-187.

Posyniak A, Zmudzki J and Niedzielska J (2003). Evaluation of sample preparation for control of chloramphenicol residues in porcine tissue by enzyme Linked Immunoassay and Liquid Chromatography. *Anal. Chim. Acta* **483**: 307-311.

Price PC and Newman JD (1991). Principles and Practices of Immunoassay. Macmillan Publishers, United Kingdom.

Gaudin V and Maris P (2001). Development of a biosensor-based immunoassay for screening of Chloramphenicol Residues in milk. *Food Agric. Immunol*. **13:** 77.

Wesongah JO, Murilla GA, Guantai AN, Elliot C, Foddey T and Cannavan A (2007). A competitive enzyme-linked immunosorbent assay for determination of chloramphenicol. *J. Vet. Pharmacol. Ther.* **30:** 1-6.

 ISSN 2303-9841