Preparation of Fluoresceisothiocyanate Conjugated IgG (FITC) Anticamel and Antibuffalo

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Abstract: Diagnosis of camel and buffalo diseases that needs the production of specific labeled immunoglobulins (Igs) using ELISA or Immunofluorescein techniques are not available. Pooled blood of camel and buffalo was collected for separation of immunoglobulin using ammonium sulphate, and then specific purification of IgGs isotypes was carried out by using protein-A agarose affinity chromatography. Reduced electrophoresis migration pattern was performed to confirm purity, then 10mg of each fraction was injected subcutaneous in male goats in three different areas weekly for 4 successive injections using complete and incomplete Freund's adjuvant. Goats serum was collected for separation of goat anticamel and antibuffalo IgGs. Conjugation protocol was done and unbound fluorescein dye was removed from the prepared reagent by gel filtration chromatography. Application of indirect immunofluorescein technique on macrophages monolayer culture was performed. [Nature and Science 2010;8(10):342-347]. (ISSN: 1545-0740).

Key words: camelidae, buffalo, immunoglobulin, fluorescien, conjugation.

1. Introduction:

Immunoglobulins(IgGs) are heterotetrameric glycoproteins produced in response to foreign antigens by the immune cells. The primary class of antibody used for diagnosis is the IgG. For most mammals, IgG is a large (160 kDa) protein made up of two pairs of different polypeptide chains: two heavy and two light chains connected by disulphide bonds. Each chain is composed of one variable domain plus either one constant domain for the light chain or three constant domains for the heavy chain. A variable domain from a light chain combines with a variable domain from a heavy chain to form one of two antigen-binding sites, which upon binding helps to neutralize and eliminate pathogens and their toxins (Brockmann et al., 2005).

The distribution of kappa and lambda chains differs in all immunoglobulin classes and subclasses, as well as between different mammalian species. Covalent interchain disulfide bridges join L to H and H to H chains, by participating in the tertiary structure; they confer greater stability to the immunoglobulin molecule (Takatani et al., 2004).

Resistance of buffaloes to some infectious diseases compared to cows pointed out to the difference in immune response against viral and bacterial diseases (Zaghawa, 1998; Helmy, 2000; Molina et al., 2005; Maroudam et al., 2008). This difference between the two species raises the demand for production of specific diagnostic reagents for buffalo.

Camel immunoglobulins (IgGs) violated established rules governing the structures of antibodies. Unlike their conventional IgG1 counterpart the IgG2 and IgG3 subclasses consist of only two heavy chains. The heavy chains lack the first constant domain and thus they fail to pair with light chains, yielding a molecular weight of 110 kDa; meanwhile, the IgG1 subclass maintains the conventional structure 160 kDa. The Heavy chain Antibodies (HcAb) have two antigen-binding sites each formed by only a single variable domain, and thus the binding interaction has only half the normal number of Complementarily Determining CDRs (Hamers-Casterman et al., 1993).

Camels have specific pathogens; suffer from common diseases of ruminants and are resistant to some pathogens (Bishop et al., 1987; Rickard, 1994; Fondevila et al., 1995; Dirie et al., 2003,). Camel heavy chain antibodies (HCAbs) may have distinct roles in fighting infections. Their more discrete antigen-binding domains, extended CDRs, and increased tissue accessibility enabling HCAbs to bind epitopes otherwise inaccessible to conventional antibodies. In addition, HCAbs have been shown to inhibit enzymes (Rivera et al., 1987). The significance of these properties in immune defense and the nature of the stimuli that induce the production of HCAbs are unknown.

Diagnosis of infectious diseases of either camels or buffaloes needs specific reagents for accuracy and sensitivity. Moreover, commercial fluorescein conjugated (FITC) IgGs for diagnosis are

available. Stemmed from the previous mentioned concept the current study was designed to prepare specific buffalo and camel immunoglobulin and aiming at accurate and precise diagnosis of their various diseases.

2. Material and methods

Preparation of immunoglobulin: serum samples from apparently healthy camel and buffalo were collected from Kerdassa abbatoir and immunoglobulins were obtained as described by (Ungar-Waron et al., 1987).

Purification of camel and buffaloes Igs: the immunoglobulins were purified using affinity chromatography, briefly it was loaded on a protein A-agarose column (Sigma Chemical Co.) and the bound fraction was eluted by changing the phosphate buffer saline pH 7.2 to elution buffer 100 mM glycine–HCl pH 2.7, and the fractions volume were collected in vials (Van der Linden et al., 2000; Hamers-Casterman et al., 1993; Daley et al., 2005). Fractions were neutralized immediately using PBS to pH 7.2 and then dialyses against PBS, the antibodies concentration were determined according to the method of (Lowry et al., 1951).

Purity control and characterization of Igs: using reduced polyacrylamide gel electrophoresis SDS-PAGE. Collected fractions were resolved in 12% polyacrylamide minigel-protein II electrophoresis cell (Bio-Rad), and Spectra^{TM} multicolor broad range protein ladder (Fermentra SM1841). Samples were diluted in sample buffer with 2- mercaptoethanol (Sigma Chemical Co) , boiled for 5 minutes before being loaded in the gels and run at 70 volts for 3 hours. Gels were stained with 1% Coomassie blue R-250(Sigma Chemical Co.), then destained at room temperature in 5% methanol and 7.5% acetic acid with shaking for 30 minutes. The different fractions were quantified using Bio-Rad GS 700 imaging densitometer molecular analysis software (Laemmeli, 1970).

Production of polyclonal antisera against camel and buffaloes Igs: two male goats average two years old of native breed were used for the production of hyperimmune sera against each camel and buffaloes IgGs according to the protocol described by (Richard et al., 1970).

Agar gel immune-diffusion test. Sera that obtained from the goat were tested for the production of goat anticamel and antibuffaloes IgGs using 1% Difco agar and 0.85% sodium chloride in phosphate buffer, pH 7.2 according to protocol described by(Mansi, 1958).

Purification of anticamel and antibuffaloes IgGs: was performed by Hamers-Casterman et al., (1993); Daley et al., (2005) and Van der Linden et al., (2000).

Application of conjugation method: fluoresceneisothiocyanide (FITC) 10mgs (molecular Probes, catalog No. F1906) was dissolved in 1 mL anyhydrous dimethylsulfoxide, DMSO, (Aldrich, catalog No. 27,685-5.) immediately before use. Then FITC was added to give a ratio of 40-80 µg per mg of antibody in reaction buffer 500 mM carbonate pH 9.5; mix immediately, wrap the tube in foil; incubate and rotate at room temperature for 2 hours (Barun et al., 2002).

Gel filtration chromatography: the bound protein to FITC and the free dye was separated using sephadex G-50 (Sigma-Aldrich) column by gel filtration and the obtained fraction was stored in 10 mM Tris (sigma aldrich), 150 mM NaCl, 0.1% NaN_3 buffer, pH 8.2 (McGhee and Freeman 1970).

Phagocytosis: blood monocytes collected from buffalo calf average two years old were cultured on cover slides in tissue culture plate (nunc-Denmark), and then phagocytosis of *Brucella melitensis* biotype-3 obtained from brucella lab. Animal Reproduction Research Institute (ARRI) was tested (Liautard et al., 1996).

Application of indirect immuno-florescence assay: was performed on cultured macrophages (Singh et al., 2009).

3. Results

Purification of camel and buffalo Igs: Affinity chromatography, using protein-A agarose was carried out in order to purify IgG1, IgG2 and IgG3 of camel, the eluted protein fraction was 2.53g/dl while that obtained from buffalo 2.56g/dl and eluted fractions collected from goats for each camel and buffalo were 2.58g/dl and 2.53g/dl respectively.

Reducing SDS-PAGE under reducing conditions: camel IgGs was resolved into 52, 46 and 30 KD while that the goat anticamel resolved into 52 and 30 kD. Buffalo IgGs gives pattern of 162, 51, 46 and 28 KD. While the goat antibuffalo give 64, 51 and 27 KD.

Agar gel immune-diffusion test: agar gel double immunodiffusion test from figure (2) show line of precipitation on buffaloes and camel.

Gel filtration chromatography: The conjugated fraction was obtained and the unbound dye was removed, the camel fraction contained 2.1g/dl while that of buffalo averaged 2.3g/dl.

Indirect immuno-florescence: Fluorescence granules were observed in cytoplasm of macrophages (figures, 3-4).

TBLE 1: SDS-PAGE analysis of camel and buffalo IgG

LNE BANDS	Lane 1		Lane 2		Lane 3		Lane 4		Lane 5	
	M.W.	Amount	M.W.	Amount	M.W.	Amount	M.W.	Amount	M.W.	Amount
1	**52.681**	34.414	**52**	49.547	**162.8**	32.418	**64.04**	34.863	**260**	1.2824
2	**46.37**	5.1494	**30.6**	50.5	**51.194**	5.877	**51.194**	41.553	**135**	2.8982
3	**30.646**	60.068			**46.129**	29.423	**27.386**	23.486	**95**	5.7104
4					**28.35**	32.058			**72**	6.2502
5									**52**	11.98
6									**42**	6.6698
7									**34**	12.684
8									**26**	34.009
9									**17**	9.6964
10									**10**	7.9935
Sum in lane	**99.632**		**99.724**		**99.775**		**99.903**		**99.174**	
	100		**100**		**100**		**100**		**100**	

Lane 5: protein marker, Lane 4: goat anti buffalo IgG, Lane 3 : buffalo IgG, Lane 2 : goat anti camel IgG, Lane 1 : camel IgG.

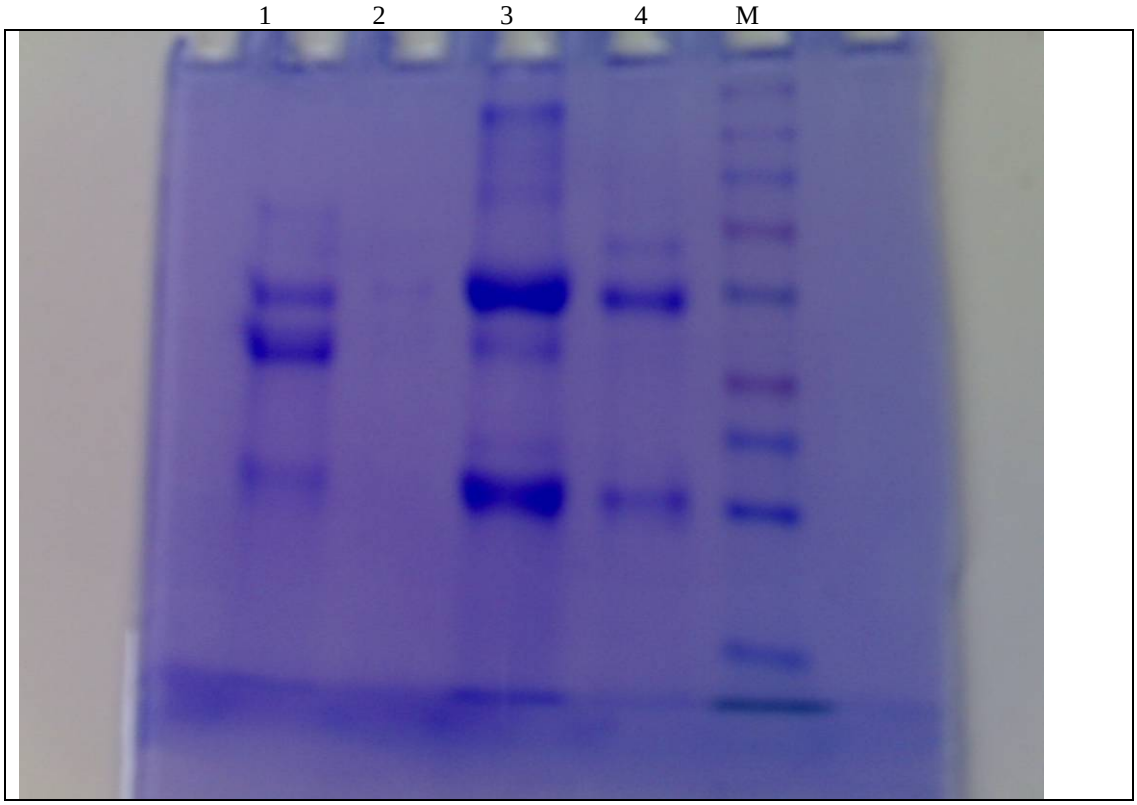


Fig. (1)SDS-PAGE analysis of camel and buffalo IgGs Lane 5: protein marker, Lane 4 , goat anti buffalo IgG, Lane 3 , buffalo IgG, Lane 2 , goat anti camel IgG, and Lane 1 , camel IgG.

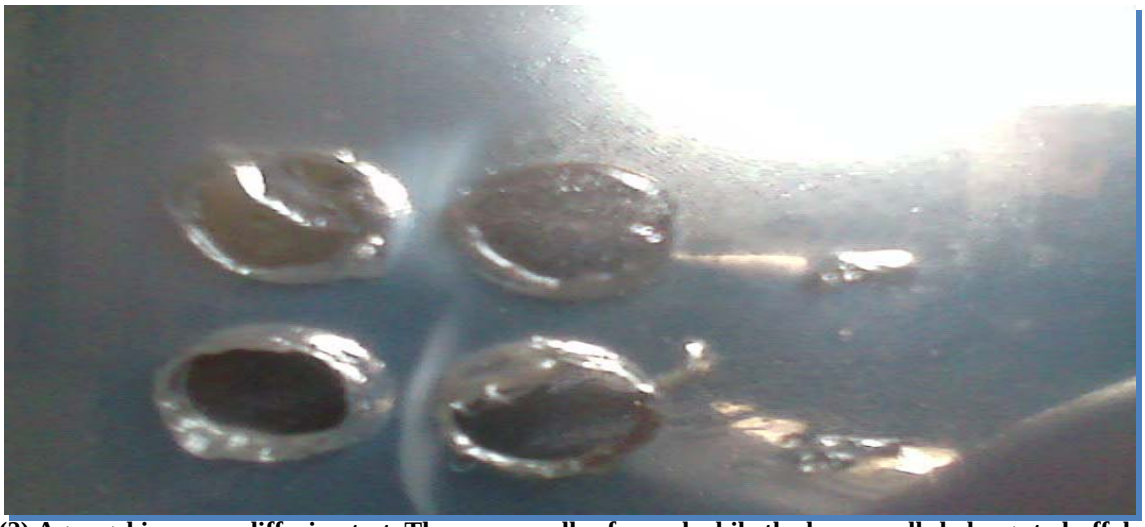
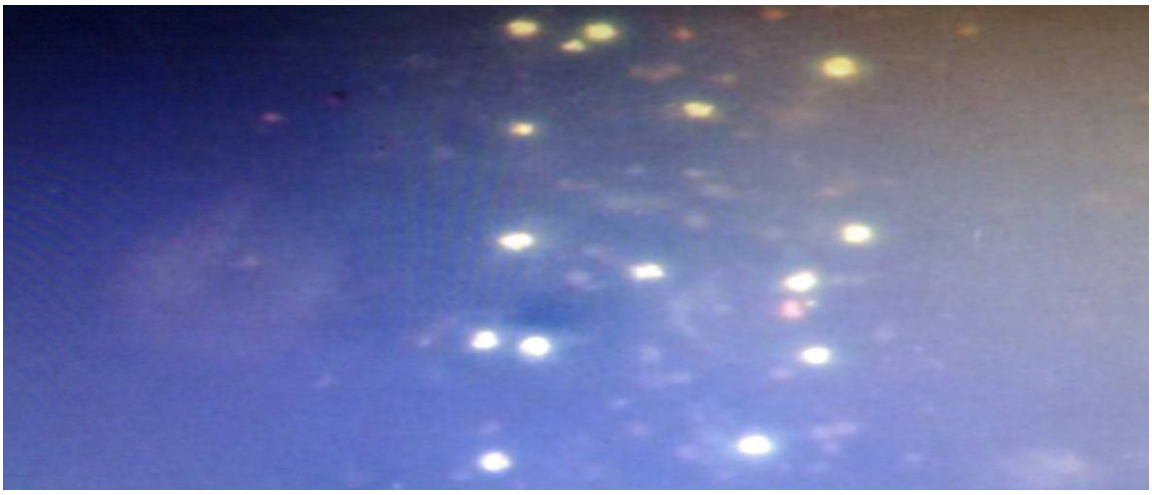


Fig. (2) Agar gel immune-diffusion test. The upper wells of camel while the lower wells belongs to buffalo.



Figures (3, 4): the upper (Fig.3) showing indirect immunofluorescene assay on macrophages culture inoculate *brucella melitensis* biotype-3 using anticamel Igs while the lower (Fig.4) using antibuffalo .

4. Discussion:

Immunoglobulins are fractions of serum protein responsible for protection against infectious diseases in what is called humoral immune response (Brockmann et al., 2005). There are different types of antibodies; IgG, IgM, IgE, IgD and IgA, furthermore some of these types contain isotypes; IgG1, IgG2, IgG3 and differ from species to other.

Separation of Immunoglobulins from serum could be achieved using several methods (Hudson and Hay, 1980). Achieved either by using ammonium sulphate (Harlow and Lane, 1988, and Ajaib and Sat Pal, 1993) or by combination of caprylic acid and ammonium sulphate precipitation (Mohanty and Elaz-hary, 1989).

In the current study, 50% ammonium sulphate precipitates immunoglobulins from camel and buffalo serum, further purification of camel and buffalo Igs isotypes and anti camel and buffalo were done using protein A agarose beads affinity chromatography (Campbell et al., 1970,Thurston, C.F. 1988, and Daley et al., 2005).

Reduced electrophoresis is used for detection of the character of the molecule and in this study was used to detect the pattern of camel and buffalo Igs as shown in table (1) and figure (1) camel IgGs was resolved into 52, 46 and 30 KDa and these finding somewhat similar to that obtained by Daley et al., 2005 as Under reducing conditions, IgG1 was resolved into light chains (22.7 kDa) and one heavy chains (49.4 kDa). The IgG3 preparation was reduced to one dominant heavy-chain species of 42.1 kDa The IgG2 preparation was reduced to one dominant heavy-chain species of 40.9 kDa. While that the goat anticamel resolved into 52 and 30 kDa. Buffalo IgGs gave pattern of 162, 51, 46 and 28 KD. While the goat antibuffalo give 64, 51 and 27 KDa. These findings agree with Kakker and Goel (1993) as buffalo IgG1 and IgG2 had a molecular mass (Mr) of 162.0 and 161.5 kDa, respectively and were found to consist of heavy (H) and light (L) chains. The H and L chains had Mr of 58 and 24 kD, respectively.

Production of hyper-immune serum in goats 10mg of each fraction was injected subcutaneous in male goats in three different areas weekly for 4 successive injections was achieved in the present work which appears the line of precipitation as indicator of production of antispecies (Fig.2).

Diagnosis of camel and buffaloes diseases especially using radio-immunoassays is very important with good accuracy and novel especially in camel viral diseases (Rivera et al 1987).

Diagnosis of camel diseases needs to increase the basic diagnostic tests as ELISA and Immuno-fluorescence for rapid and definite accuracy. On this study, the production of goat anticamel and antibuffaloes Igs obtained by affinity chromatography and the pattern of electrophoresis using reducing gel was definitely detected.

Intra-cellular phagocytosis of *Brucella melitensis* biovar-3 in macrophages culture using indirect immuno-fluorescein assay appear in the cytoplasm of macrophages in either camel and buffalo as a good indicator of phagocytosis.

The products in this study are important for diagnosis of camel and buffalo diseases especially that need immune-fluorescein assay but they lack intensity as it faint rapidly may be covalent of dye or other factor not study in these but can used as of low price and accuracy. It need further study for stability.

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