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Development of Polyclonal Antibodies

Dipankar Das and Mavanur R. Suresh
Faculty of Pharmacy and Pharmaceutical Sciences
University of Alberta

Contract Scientific Authorities: H. Gail Thompson & R.E.K. Fulton, DRDC Suffield

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Defence R&D Canada

Contract Report

DRDC Suffield CR 2009-210

October 2009

Canada

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Dipankar Das
Mavanur R. Suresh
Faculty of Pharmacy and Pharmaceutical Sciences
University of Alberta
Edmonton, AB T6G 2N8

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Contract Scientific Authorities: H. Gail Thompson (403 544-4517)
R.E.K. Fulton (403 544-4630)

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Defence R&D Canada – Suffield

Contract Report

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DEVELOPMENT OF POLYCLONAL ANTIBODIES

DRDC Suffield contract # W7702-04R003/001/EDM

Dipankar Das and Mavanur R. Suresh
Faculty of Pharmacy and Pharmaceutical Sciences
University of Alberta
Edmonton, Canada
T6G 2N8

Title of the project DEVELOPMENT OF POLYCLONAL ANTIBODIES

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Abstract

The Defence Research and Development Canada (DRDC) have identified four biological warfare agents such as Vibrio cholera, Coxiella burnetii, Yellow fever and Yersinia pestis for the development of polyclonal antibodies for diagnostic application. However, DRDC Suffield has provided VEE antigen in place of yellow fever antigen due to lack of yellow fever antigen. The specific polyclonal antibodies against each of the above four inactivated agents has been developed in goats and rabbits. The specific IgG fraction of the total immunoglobulins was separated by protein G chromatography and ELISA method has been established for diagnostic immunoassays. We have delivered hyperimmune serum as well as purified IgG for each of the antigen as described in Table 1.

1. Introduction

The Defense Research and Development Canada – Suffield (DRDC Suffield) have identified the potential biological threat agents for the development of polyclonal antibodies in Goats and Rabbits. The agents relevant to the proposal are

i) Vibrio cholera, ii) Coxiella burnetii, iii) Yellow fever virus and iv) *Yersinia pestis*

But due to lack of Yellow fever virus antigen supply, DRES has supplied VEE antigen to replace the yellow fever antigen. The above antigens for immunization have been provided in sufficient amounts by DRDC Suffield and the Technical Authority (Ms. Gail Thompson). The specific polyclonal antibodies against each of the above four inactivated agents has been developed, purified by Protein G agarose chromatography and ELISA method has been established. Polyclonal antibodies are easily made in rabbits and goats as a specific diagnostic reagent. The advantages of polyclonals are that its production is generally straight forward consisting of a standard time tested immunization protocol with periodic bleeds to collect the antiserum. The specific IgG fraction of the total immunoglobulins are also separated by protein G chromatography and characterized for specificity and utility in diagnostic immunoassays.

We have immunized goats and rabbits using a standard immunization protocol as follows.

i) Immunized 6 rabbits and 3 goats with Vibrio *cholera*

ii) Immunized 6 rabbits and 3 goats with Coxiella *burnetii*

iii) Immunized 4 rabbits with VEE (only rabbit polyclonal requested by Gail Thompson)

iv) Immunized 4 rabbits and 3 goats with *Yersinia pestis*

2. Immunization

2.1 Immunization protocol (rabbit)

DATE OF PROCEDURE	ANIMAL I.D.	PROCEDURE	ANESTHETIC OR ANALGESIC	VOLUME	MISC. PROCEDURE DETAILS
DAY -1	Rabbit (NZW)	Pre-immune bleed		5 ml	
DAY 0	Rabbit (NZW)	Intradermal/Subcu inj	ID:25 µl /site x 2 sites ; 200, 200, 150 µl SC in 3 sites	600 µl	300 µl antigen +300 µl CFA
DAY 14	Rabbit (NZW)	Subcutaneous inj	200, 200, 200 µl SC in 3 sites	600 µl	300 µl antigen +300 µl IFA
DAY 28	Rabbit (NZW)	Subcutaneous inj	150 µl /site X 2 sites	300 µl	150 µl antigen +150 µl IFA
DAY 42	Rabbit (NZW)	TEST BLEED		3-5 ml	
DAY 56	Rabbit (NZW)	Subcutaneous inj	150 µl /site X 2 sites	300 µl	150 µl antigen +150 µl I IFA
DAY 84	Rabbit (NZW)	Subcutaneous inj	150 µl /site X 2 sites	300 µl	150 µl antigen +150 µl IFA
DAY 112	Rabbit (NZW)	Terminal Bleed			

2.2 Immunization protocol (goat)

DATE OF PROCEDURE	ANIMAL I.D.	PROCEDURE	ANESTHETIC OR ANALGESIC	VOLUME	MISC. PROCEDURE DETAILS
DAY -1	Goat	Pre-immune bleed		5 ml	
DAY 0	Goat	Intramuscular inj	250 µl /site X 2 sites	500 µl	250 µl antigen + 250 µl CFA
DAY 14	Goat	Intramuscular inj	250 µl /site X 2 sites	500 µl	250 µl antigen +250 µl IFA
DAY 28	Goat	Intramuscular inj	250 µl /site	250 µl	125 µl antigen +125 µl IFA
DAY 42	Goat	TEST BLEED		3-5 ml	
DAY 56	Goat	Intramuscular inj	250 µl /site	250 µl	125 µl antigen +125 µl IFA
DAY 84	Goat	Intramuscular inj	250 µl /site	250 µl	125 µl antigen +125 µl IFA
DAY 112	Goat	Terminal Bleed			

3. Results

3.1 Antibody titer by ELISA

The preimmune serum and test bleeds were evaluated by ELISA on antigen coated plate for the titer of the antibodies against each antigen. Each of the four antigens was coated separately on the ELISA plate (1 µg/well) overnight, blocked with 1% bovine serum albumin (BSA) for 1 h and washed with phosphate buffer saline. The respective test bleeds at various dilutions along with pre-bleed serum samples as controls were incubated at room temperature for 2 h. Following a wash, the anti-rabbit or anti-goat IgG labeled with peroxidase (HRPO) were added and incubated for 2 h. After final wash, the substrate tetramethyl benzidine (TMB) solution was added to monitor colour development and subsequently estimated the titer of the antibody.

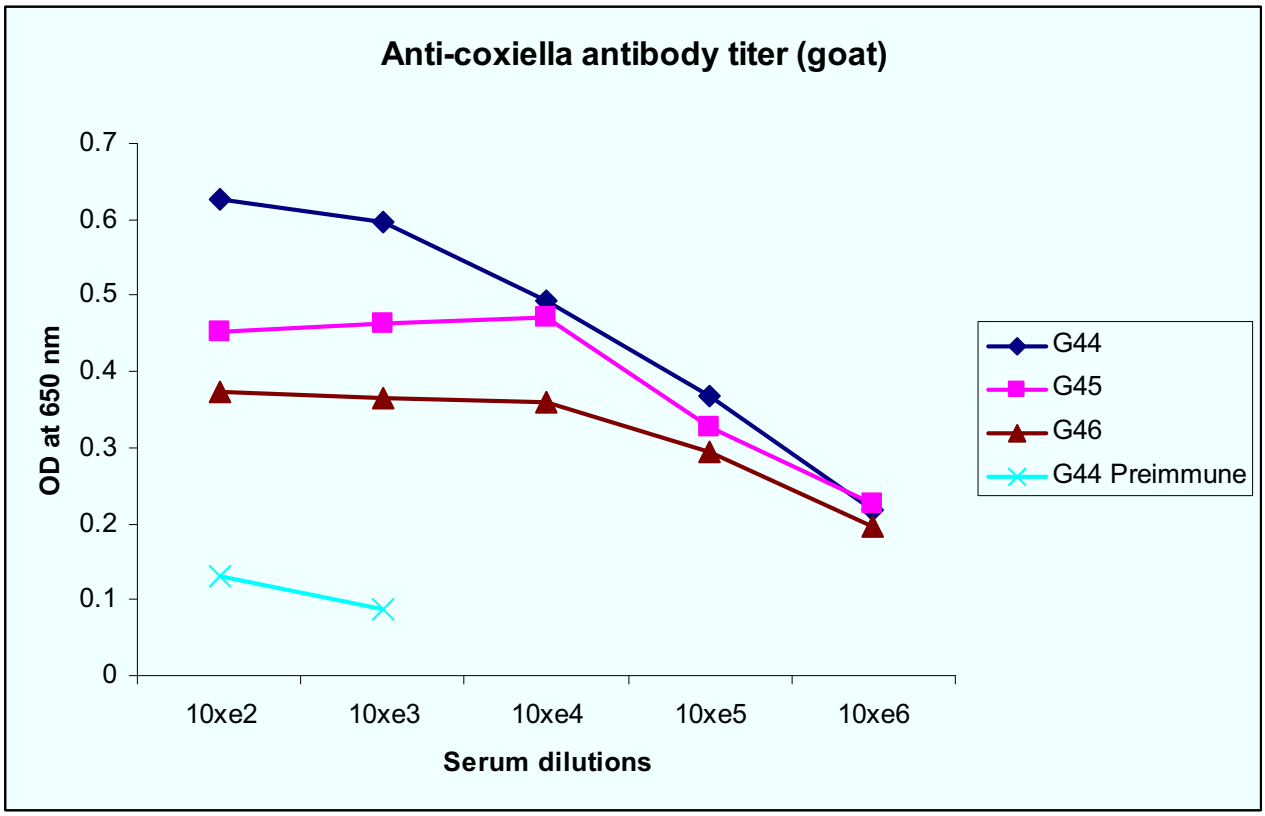


Fig. 1 Antibody titer against anti-coxiella in goat

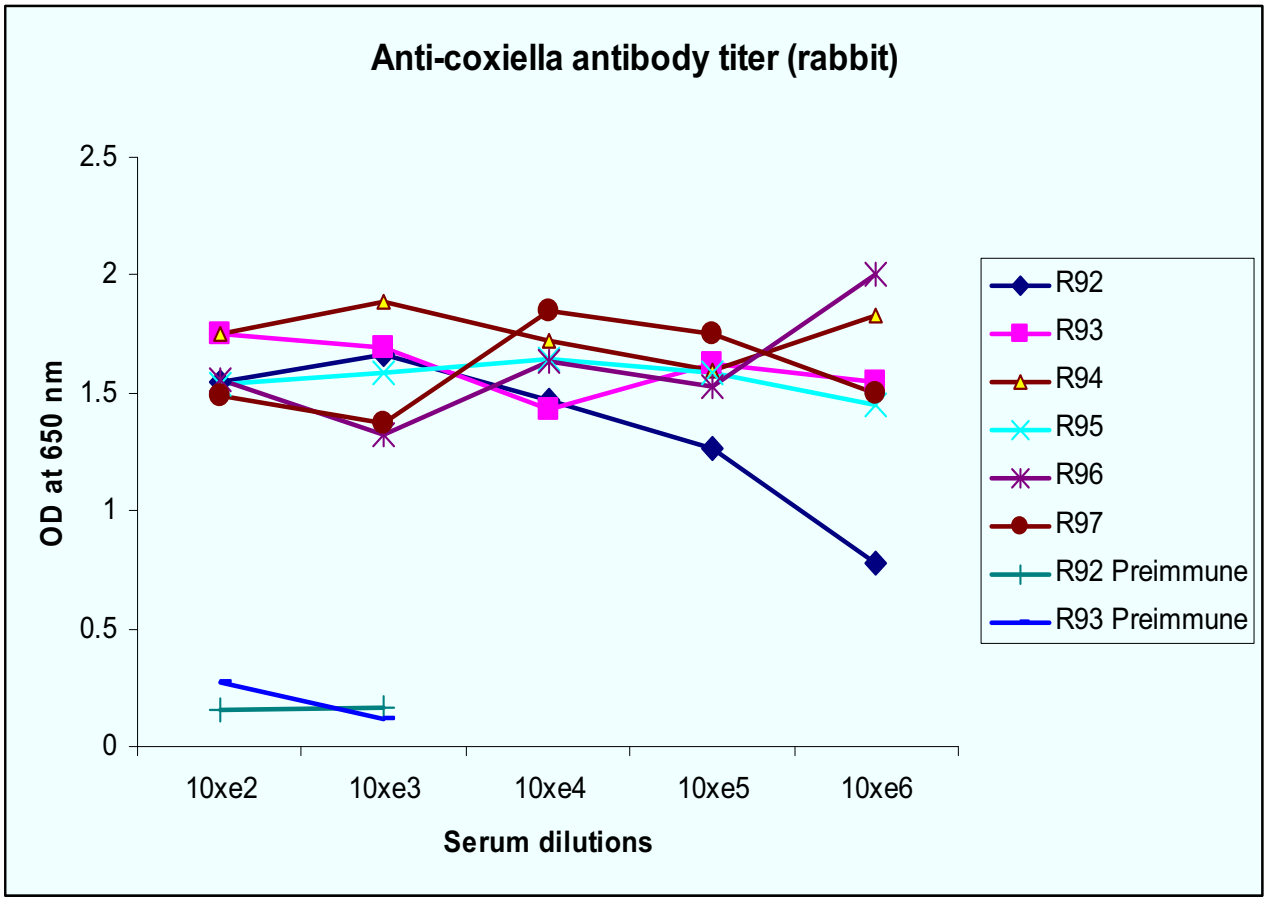


Fig. 2 Antibody titer against anti-coxiella in rabbit

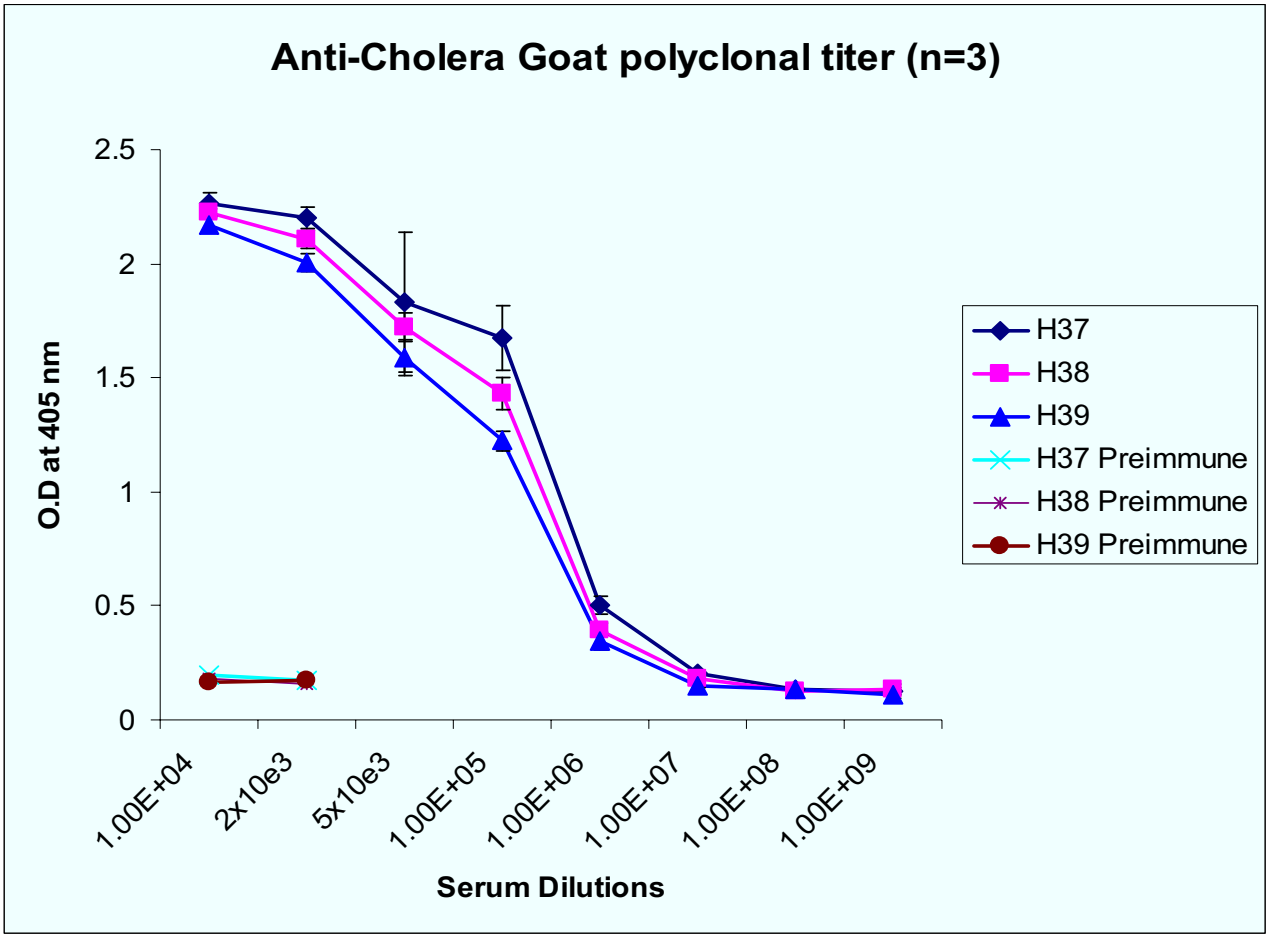


Fig. 3 Antibody titer against anti-cholera in goat

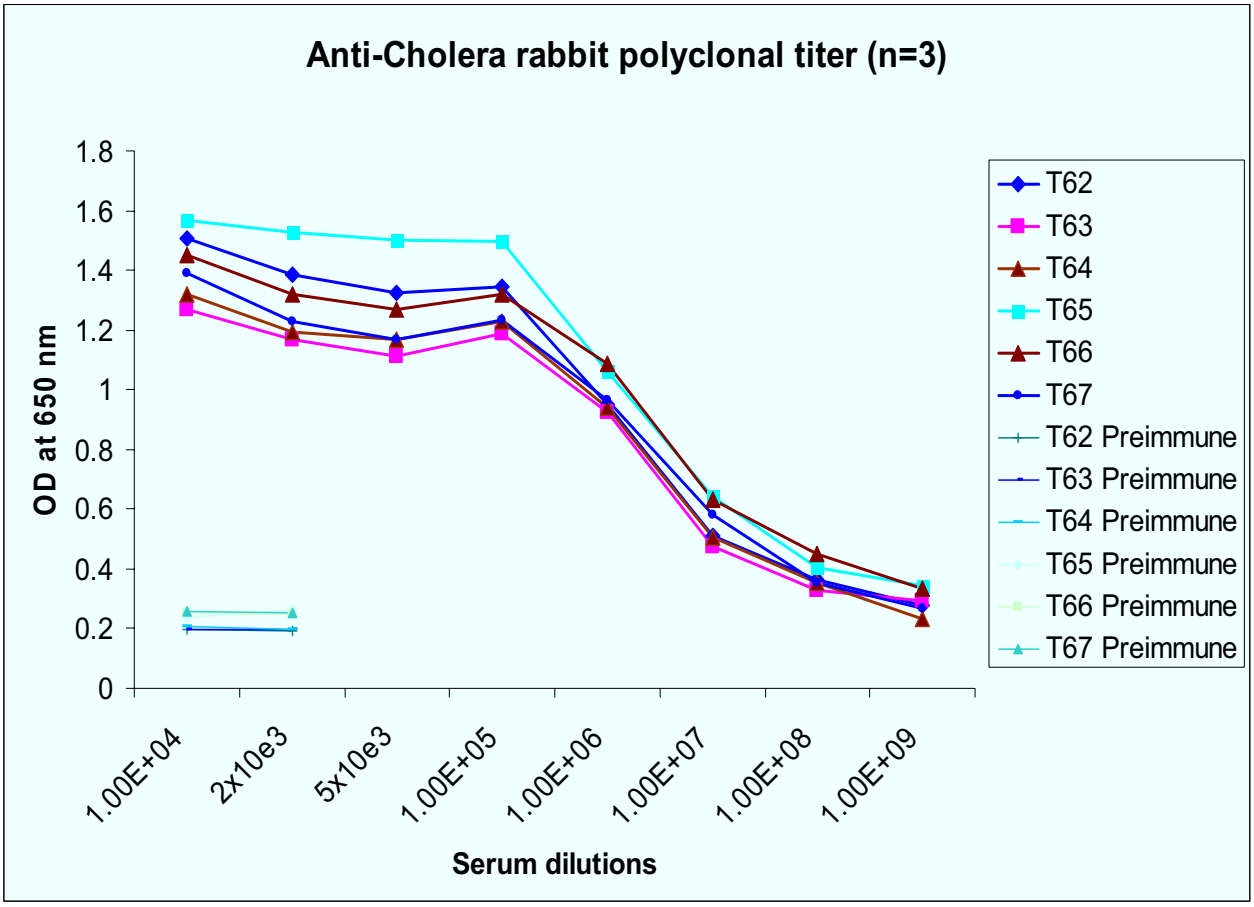


Fig. 4 Antibody titer against anti-cholera in rabbit

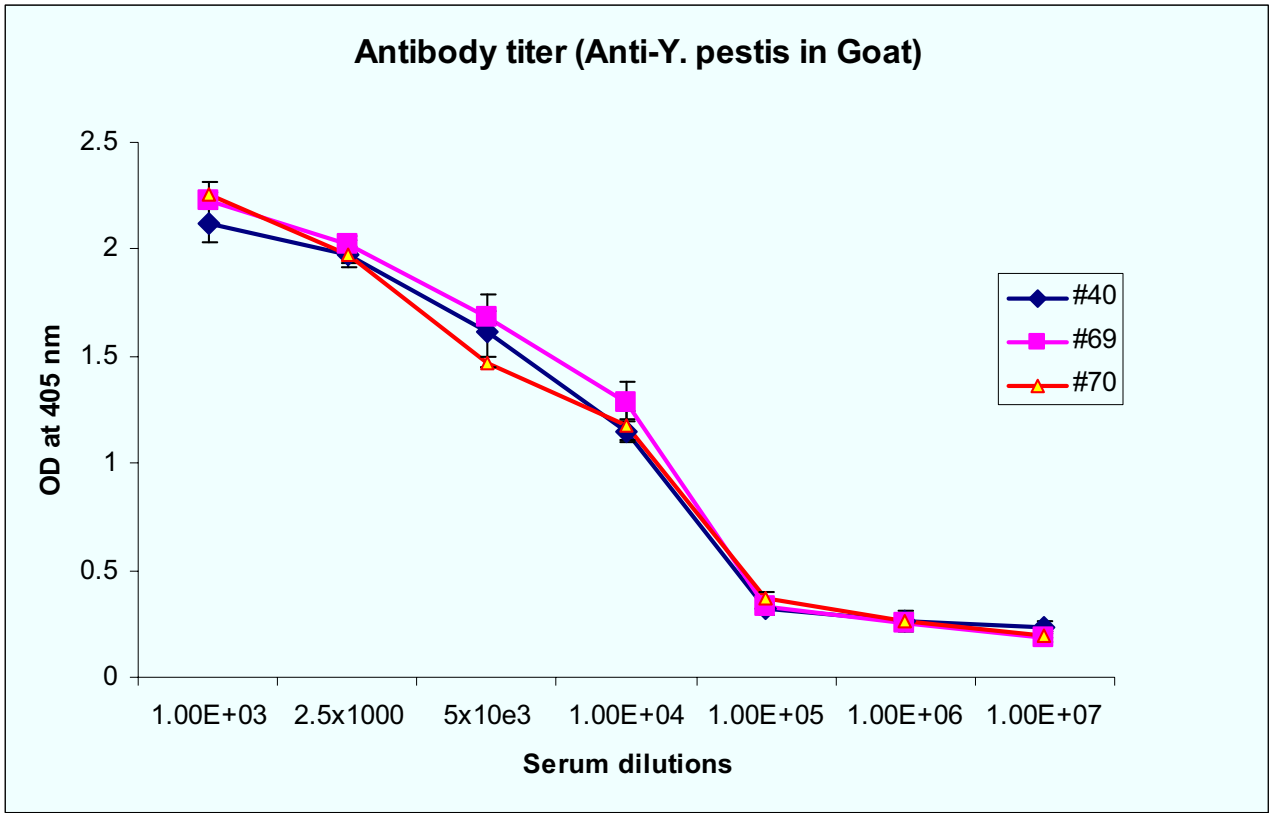


Fig. 5 Antibody titer against anti-Y.pestis in goat

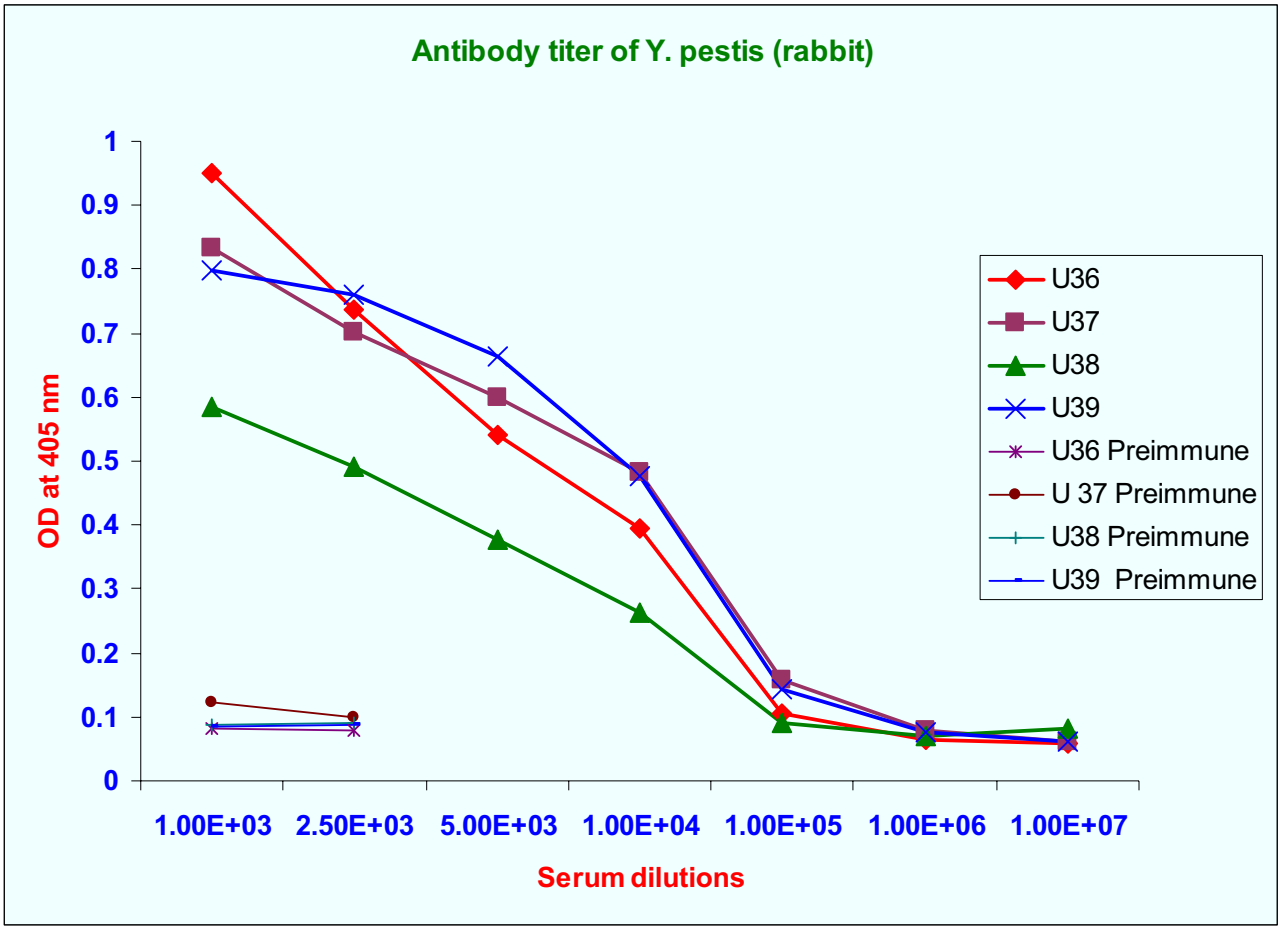


Fig. 6 Antibody titer against anti-Y.pestis in rabbit

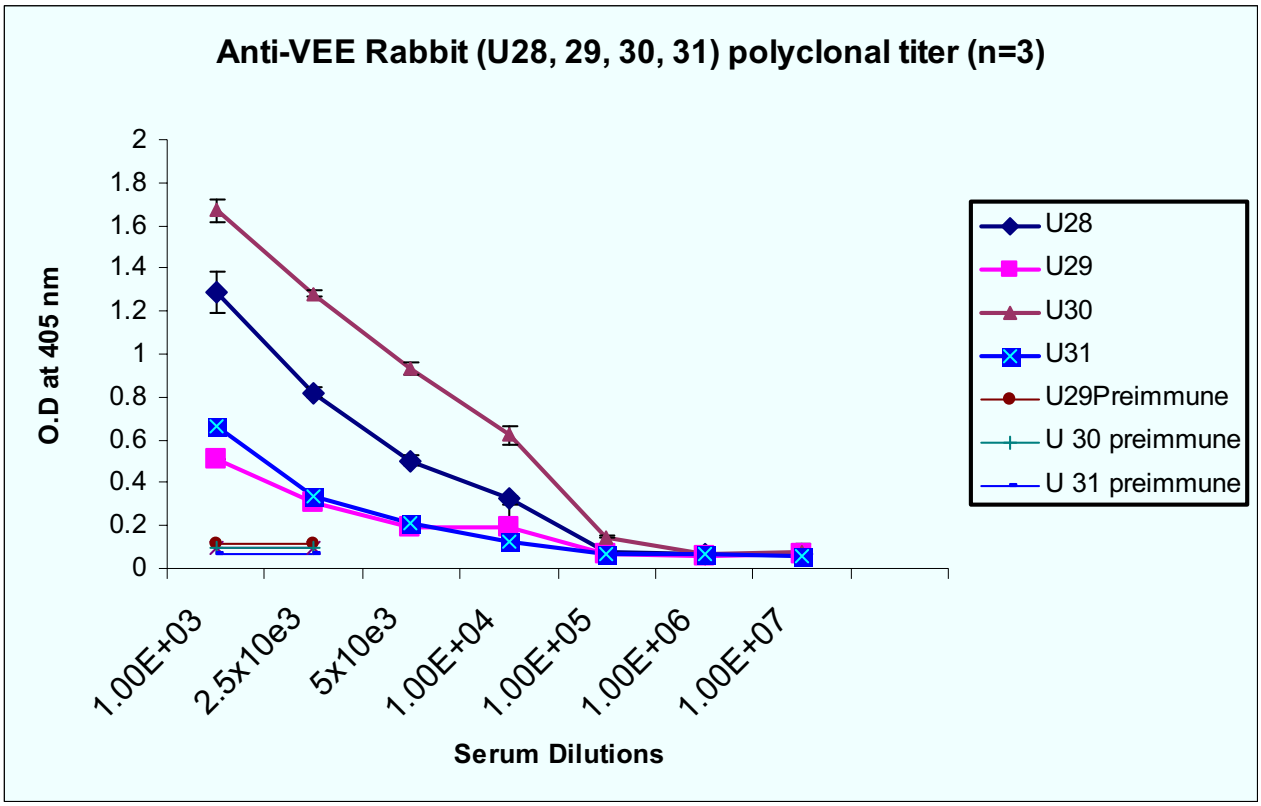


Fig. 7 Antibody titer against anti-VEE in rabbit

3.2 Terminal bleeding, collection of serum, purification by Protein G agarose and protein assay to determine the concentration.

Animals exhibiting good titers of antibody following booster injections were terminally bled to collect blood. Sera with good titers to the respective antigens were pooled and fractionated for purification of IgG. The purification of the IgG was accomplished by protein G affinity chromatography and purified by automated protein purification system (Biologic Duo Flow system, BioRad).

The purified antibody fractions were pooled and protein concentration was determined using the BioRad protein detection reagent. In case of coxiella and cholera antibody, we have established the ELISA heterosandwich assay and provided the complete test report to DRES. The homosandwich assay is sensitive and takes advantage of the universal repeat architecture of the pathogens exhibiting multiple identical epitopes.

3.3 Total amount of neat hyperimmune serum as well as purified IgG provided to DRES during the project.

Table 1

Antigen	**#Goat**	**#Rabbit**	**Pooled goat serum**	**Pooled rabbit serum**	**Purified Goat IgG**	**Purified rabbit IgG**
Coxiella	3	6	2000 ml	900 ml	151 mg	91.6 mg
Cholera	3	6	2500 ml	2800 ml	150 mg	72 mg
Y.pestis	3	4	1250 ml	250 ml	215.6 mg	59.4 mg
VEE		4	-	250 ml	-	68.8 mg

4. Discussion and Conclusion

The Defense Research and Development Canada (DRDC) identified four biological warfare agents such as Vibrio cholera, Coxiella burnetii, Yellow fever and Yersinia pestis for the development of polyclonal antibodies in goats and rabbits. The above antigens have been provided in sufficient amounts by DRES for immunization and screening. We have successfully developed goat and rabbit polyclonal antibodies against cholera, coxiella, Y. pestis and VEE antigens using a standard immunization protocol. The hyperimmune serum from each group has been successfully affinity purified and ELISA method has been established for the diagnostic application. Finally we have delivered hyperimmune serum as well as purified IgG to DRES for each of the antigen as described in Table 1.

Sandwich ELISA for Coxiella antigen

Dipankar Das and Mavanur R. Suresh
Faculty of Pharmacy and Pharmaceutical Sciences
University of Alberta
Edmonton, Canada
T6G 2N8

Objectives

- Determination of optimal concentration of capture antibody (CAb)
- Determination of Sandwich assay format
- Determination of optimal concentration of capture antigen (Ag)
- Determination of optimal concentration of detector antibody (DAb)
- Determination of optimal dilution of indicator antibody-HRPO conjugate (IAb)
- Determination of assay sensitivity for Coxiella antigen

Reagents:

ELISA plate: NUNC
Coating buffer: Carbonate-Bicarbonate buffer (Sigma, C-3041)
Wash buffer: 0.1 % BSA (w/v), 0.1% Tween-20 (v/v) in PBS pH 7.3
Blocking buffer: 2% BSA (w/w) in PBS pH 7.3
Diluent buffer: 0.1% (v/v) Tween-20 in blocking buffer

Antibody: Anti-Coxiella Rabbit IgG and Anti-Coxiella Goat IgG, Protein-G purified.

Antibody stock: Anti-Coxiella Rabbit IgG (800 µg/ml in PBS pH 7.3)
Anti-Coxiella Goat IgG (1100 µg/ml in PBS pH 7.3)

Antigen: Coxiella burnetti Henzerling Phase (Formalin treated in PBS), Panbio Inc. 1 mg/ml

Indicator antibody: Goat anti-Rabbit IgG HRPO and Rabbit anti-Goat IgG HRPO (Sigma)

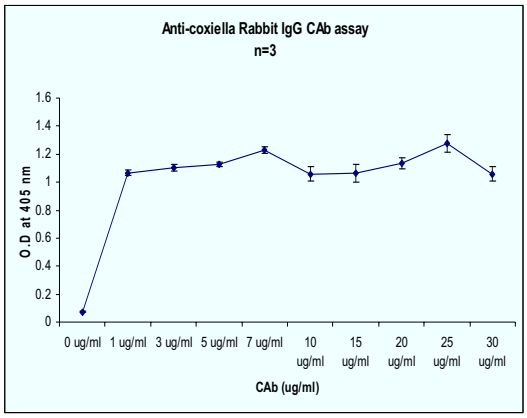
Substrate: ABTS + H_2O_2 (KPL)

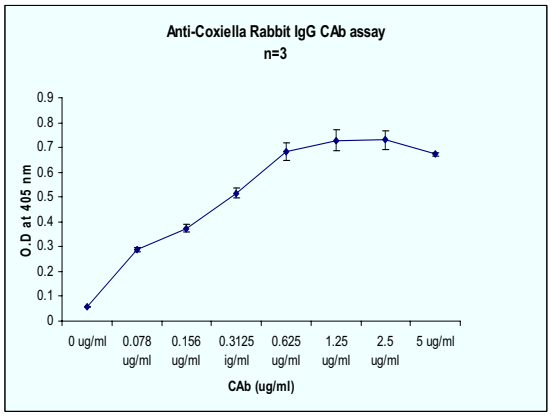
Optical Density: 405 nm (ELISA plate reader, Molecular Devices)

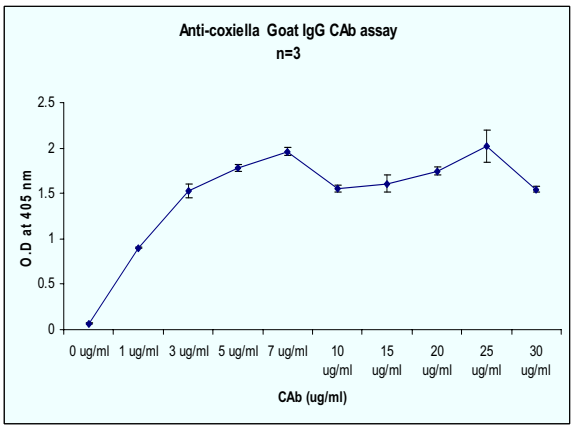
Determination of optimal concentration of capture antibody (CAb)

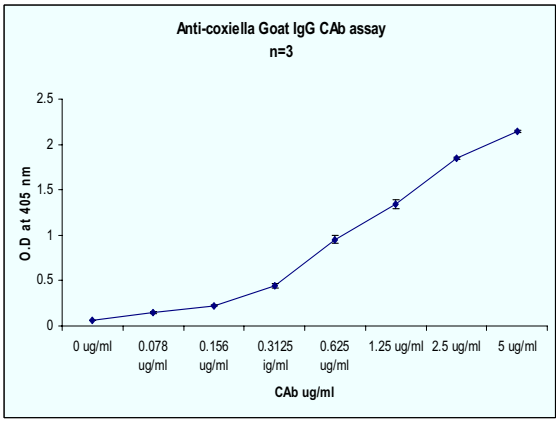
Steps:

- Capture antibody concentration (Anti-Coxiella Rabbit IgG and Anti-Coxiella Goat IgG: 1, 3, 5, 7, 10, 15, 20, 25, 30 µg/ml respectively in coating buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Block, 37°C /1hr
- Wash (5X)
- Indicator antibody (Goat anti-Rabbit IgG HRPO and Rabbit anti-Goat IgG HRPO) 1:10000. 37°C /1hr
- Wash (5X)
- Substrate







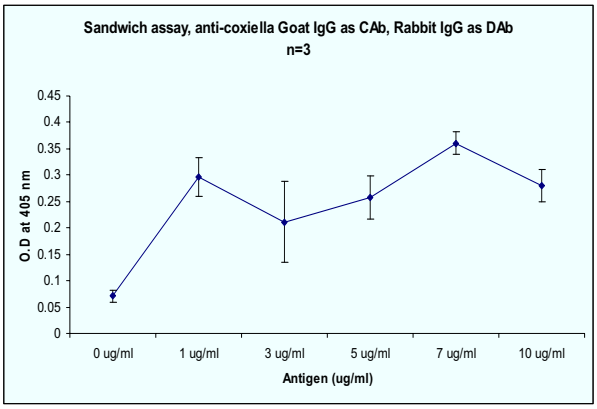


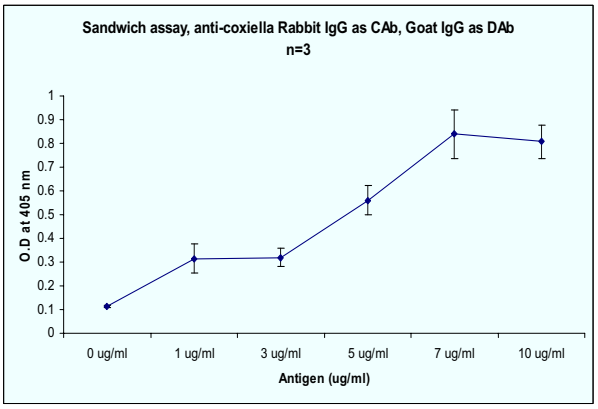
Conclusion: 5 µg/ml of capture antibody (both anti-Coxiella Goat IgG and anti-Coxiella Rabbit IgG) is optimum to coat the plate.

Determination of Sandwich assay format (Anti-Coxiella Goat IgG as capture Ab, Anti-Coxiella Rabbit IgG as detector Ab and vice versa)

Steps

- Capture antibody concentration (Anti-Coxiella Rabbit IgG and Anti-Coxiella Goat IgG: 5 µg/ml in coating buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Block, 37°C /1hr
- Coxiella antigen (1, 3, 5, 7, 10 µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Detector antibody concentration (Anti-Coxiella Goat IgG and Anti-Coxiella Rabbit IgG, 5 µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Indicator antibody (Goat anti-Rabbit IgG HRPO and Rabbit anti-Goat IgG HRPO) 1:10000 37°C /1hr
- Wash (5X)
- Substrate



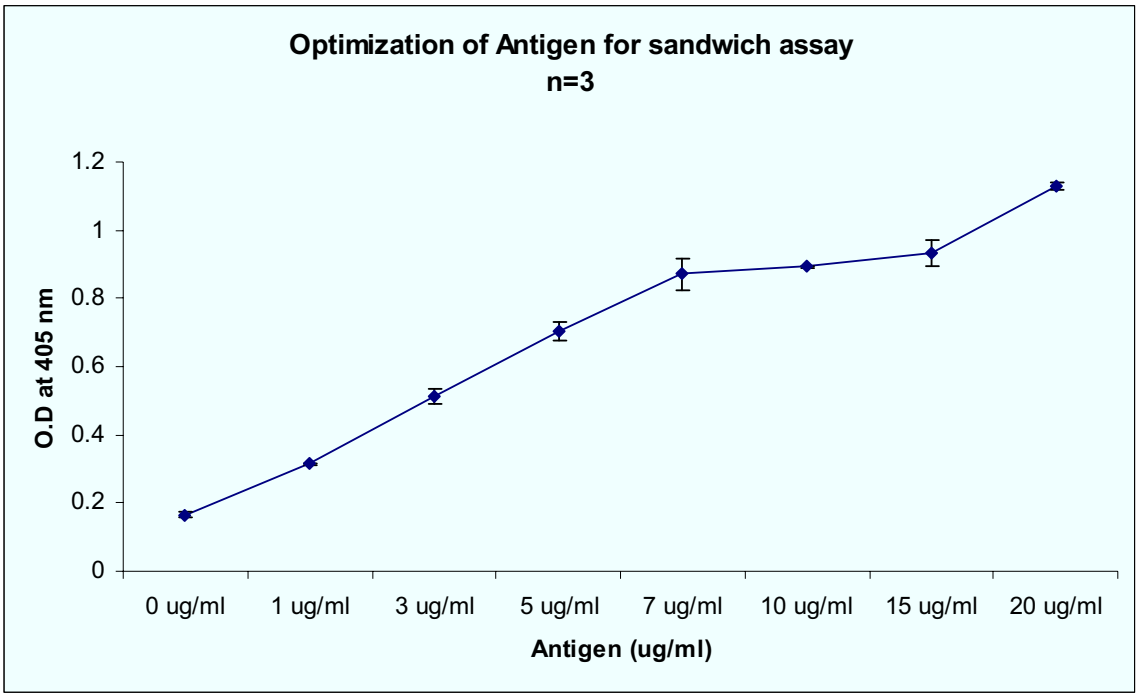


Conclusion: Anti-Coxiella Rabbit IgG as capture Ab, Anti-Coxiella Goat IgG as detector Ab forms a better sandwich.

Determination of optimal range of capture antigen (Ag)

Steps

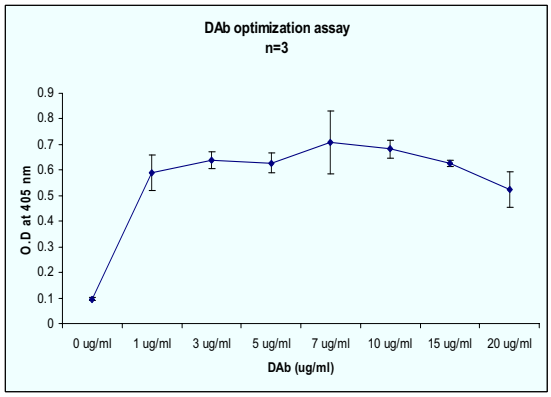
- Capture antibody concentration (Anti-Coxiella Rabbit IgG, 5 µg/ml in coating buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Block, 37°C /1hr
- Coxiella antigen (1, 3, 5, 7, 10, 15, 20 µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Detector antibody concentration (Anti-Coxiella Goat IgG, 5 µg/ml in diluent buffer,100 µl/well). 37°C /1hr
- Wash (5X)
- Indicator antibody (Rabbit anti-Goat IgG HRPO) 1:10000. 37°C /1hr
- Wash (5X)
- Substrate

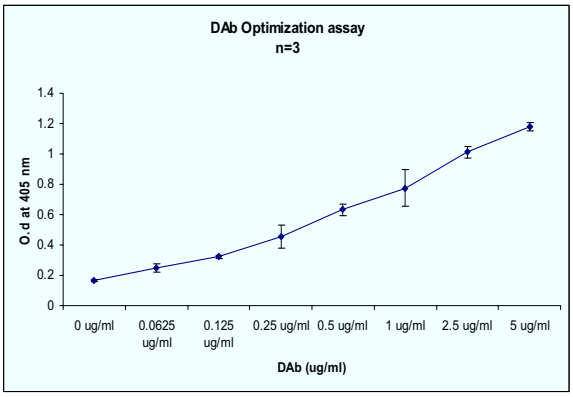


Conclusion: 7 µg/ml of Coxiella antigen is optimum to form a Sandwich

Determination of optimal concentration of detector antibody (DAb)
Steps

- Capture antibody concentration (Anti-Coxiella Rabbit IgG, 5 µg/ml in coating buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Block, 37°C /1hr
- Coxiella antigen (7µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Detector antibody concentration (Anti-Coxiella Goat IgG: 1, 3, 5, 7, 10, 15, 20µg/ml and 0.0625, 0.125, 0.25, 0.5, 1.0, 2.5, 5 µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Indicator antibody (Rabbit anti-Goat IgG HRPO) 1:10000. 37°C /1hr
- Wash (5X)
- Substrate



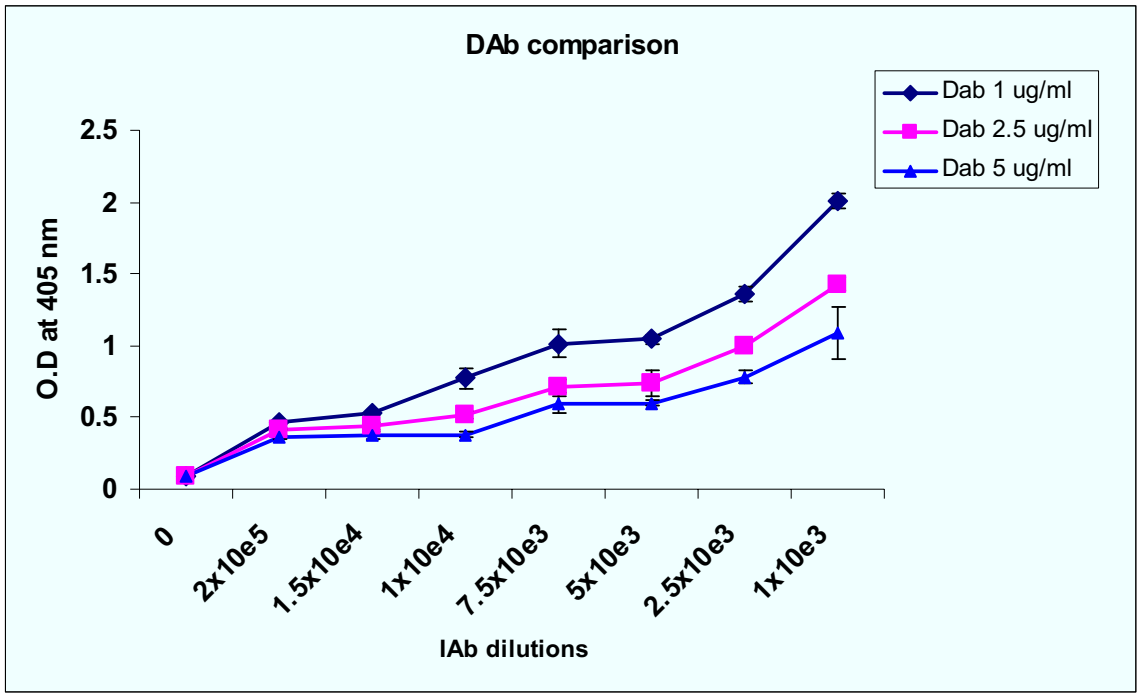


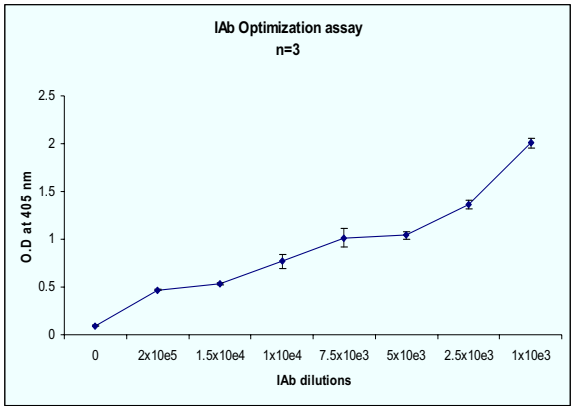
Conclusion: 1 µg/ml of detector antibody (Anti-Coxiella Goat IgG) is optimum for detection of captured Coxiella antigen.

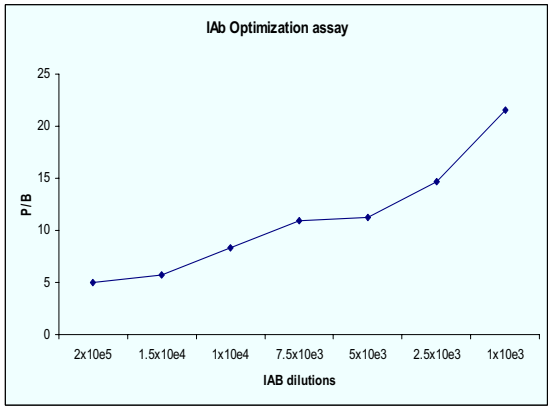
Determination of optimal dilution of indicator antibody-HRPO conjugates (IAb)

Steps

- Capture antibody concentration (Anti-Coxiella Rabbit IgG, 5 µg/ml in coating buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Block, 37°C /1hr
- Coxiella antigen (7µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Detector antibody concentration (Anti-Coxiella Goat IgG, 1, 2.5 and 5 µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Indicator antibody (Rabbit anti-Goat IgG HRPO) 1:1000, 1:2500, 1:5000, 1:7500, 1:10000, 1:15000 and 1:20000 dilutions. 37°C /1hr
- Wash (5X)
- Substrate



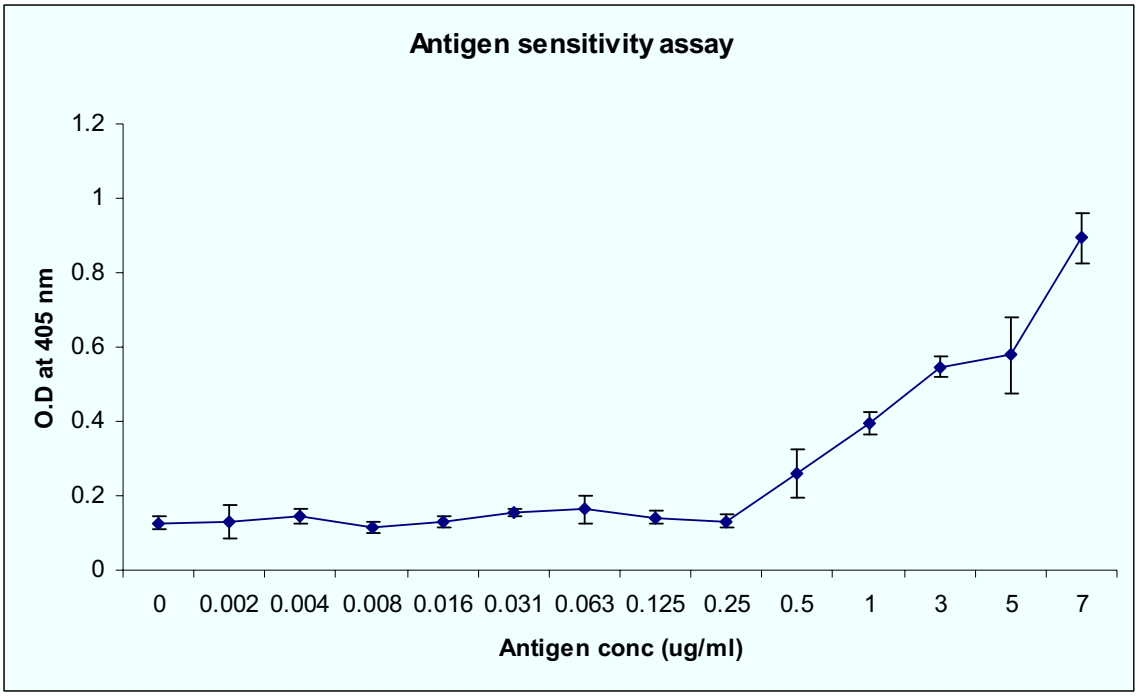




Conclusion: 1:7500 dilution of Indicator antibody conjugate (Rabbit anti-Goat IgG HRPO) is optimum for detection of captured Coxiella antigen.

Determination of assay sensitivity for Coxiella antigen
Steps

- Capture antibody concentration (Anti-Coxiella Rabbit IgG, 5 µg/ml in coating buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Block, 37°C /1hr
- Coxiella antigen (7, 5, 3, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.0156, 0.0078, 0.0039, 0.00195 µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Detector antibody concentration (Anti-Coxiella Goat IgG, 1 µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Indicator antibody (Rabbit anti-Goat IgG HRPO) 1:7500 dilution. 37°C /1hr
- Wash (5X)
- Substrate



Conclusion: 50 ng of Coxiella antigen in 100 µl volume could be detected by heterosandwich ELISA using ABTS as substrate.

Overall conclusions of Coxiella antigen heterosandwich assay:

- CAb :5 µg/ml Anti-coxiella Rabbit IgG
- Coxiella Antigen :1-20 µg/ml range
- DAb :1 µg/ml Anti-Coxiella Goat IgG
- IAb 1:7500 (Rabbit anti-Goat IgG HRPO conjugate)
- 50 ng of Coxiella antigen in 100 µl volume could be detected by heterosandwich ELISA using ABTS as substrate.

Sandwich ELISA for Cholera antigen

Dipankar Das and Mavanur R. Suresh
Faculty of Pharmacy and Pharmaceutical Sciences
University of Alberta
Edmonton, Canada
T6G 2N8

Objectives

- Determination of optimal concentration of capture antibody (CAb)
- Determination of Sandwich assay format
- Determination of optimal concentration of capture antigen (Ag)
- Determination of optimal concentration of detector antibody (DAb)
- Determination of optimal dilution of indicator antibody-HRPO conjugate (IAb)
- Determination of assay sensitivity for Cholera antigen

Reagents:

ELISA plate: NUNC
Coating buffer: Carbonate-Bicarbonate buffer (Sigma, C-3041)
Wash buffer: 0.1 % BSA (w/v), 0.1% Tween-20 (v/v) in PBS pH 7.3
Blocking buffer: 2% BSA (w/w) in PBS pH 7.3
Diluent buffer: 0.1% (v/v) Tween-20 in blocking buffer

Antibody: Anti-Cholera Rabbit IgG and Anti-Cholera Goat IgG, Protein-G purified.

Antibody stock: Anti-Cholera Rabbit IgG (720 µg/ml in PBS pH 7.3)
Anti-Cholera Goat IgG (1250 µg/ml in PBS pH 7.3)

Antigen: V. cholera OI Inaba classic strain, heat inactivated ca. 2.5 $x10^{10}$ vibrios
V. cholera OI Inaba El Tor strain, formalin inactivated ca. 2.5 $x10^{10}$ vibrios
V. cholera OI Ogawa classic strain, heat inactivated ca. 2.5 $x10^{10}$ vibrios
V. cholera OI Ogawa classic strain, formalin inactivated ca. 2.5 $x10^{10}$ vibrios
Total ca. $1x10^{11}$ vibrios
Recombinant cholera toxin B subunit (rCTB) 1 mg

Indicator antibody: Goat anti-Rabbit IgG HRPO and Rabbit anti-Goat IgG HRPO (Sigma)

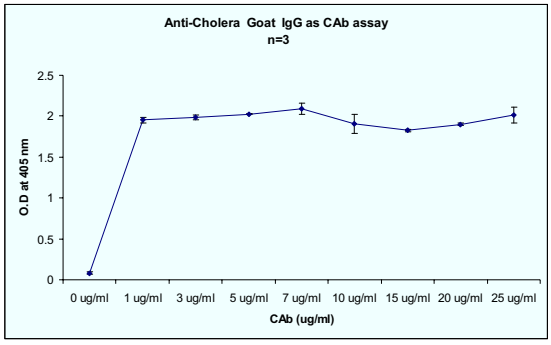
Substrate: ABTS + H_2O_2 (KPL)

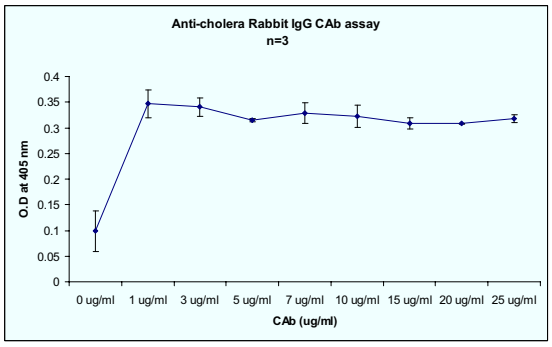
Optical Density: 405 nm (ELISA plate reader, Molecular Devices)

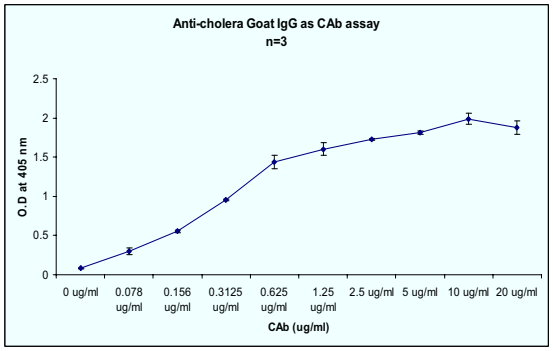
Determination of optimal concentration of capture antibody (CAb)

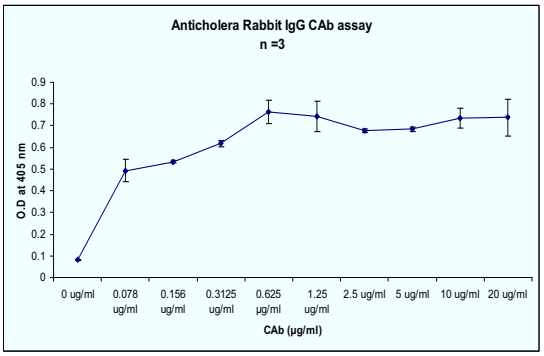
Steps:

- Capture antibody concentration (Anti-Cholera Rabbit IgG and Anti-Cholera Goat IgG: 1, 3, 5, 7, 10, 15, 20, 25 µg/ml and lower scale 20 ……0.0078 µg/ml respectively in coating buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Block, 37°C /1hr
- Wash (5X)
- Indicator antibody (Goat anti-Rabbit IgG HRPO and Rabbit anti-Goat IgG HRPO) 1:10000. 37°C /1hr
- Wash (5X)
- Substrate







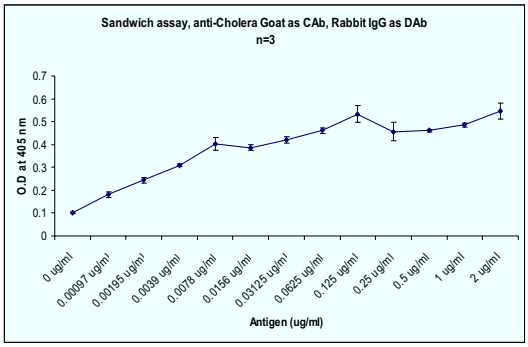


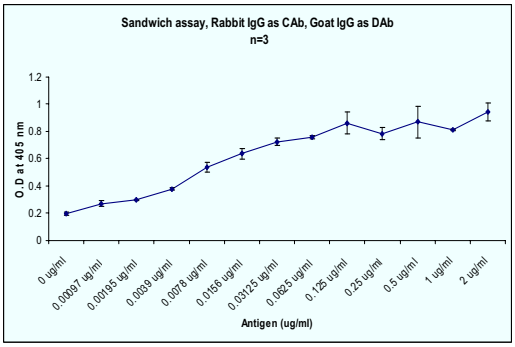
Conclusion: 1 µg/ml of capture antibody (both anti-Cholera Goat IgG and anti-Cholera Rabbit IgG) is optimum to coat the plate.

Determination of Sandwich assay format (Anti-Cholera Goat IgG as capture Ab, Anti-Cholera Rabbit IgG as detector Ab and vice versa)

Steps

- Capture antibody concentration (Anti-Cholera Rabbit IgG and Anti-Cholera Goat IgG: 1 µg/ml in coating buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Block, 37°C /1hr
- Cholera antigen (2……..0.00097 µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Detector antibody concentration (Anti-Cholera Goat IgG and Anti-Cholera Rabbit IgG, 1 µg/ml in diluent buffer,100 µl/well). 37°C /1hr
- Wash (5X)
- Indicator antibody (Goat anti-Rabbit IgG HRPO and Rabbit anti-Goat IgG HRPO) 1:10000 37°C /1hr
- Wash (5X)
- Substrate



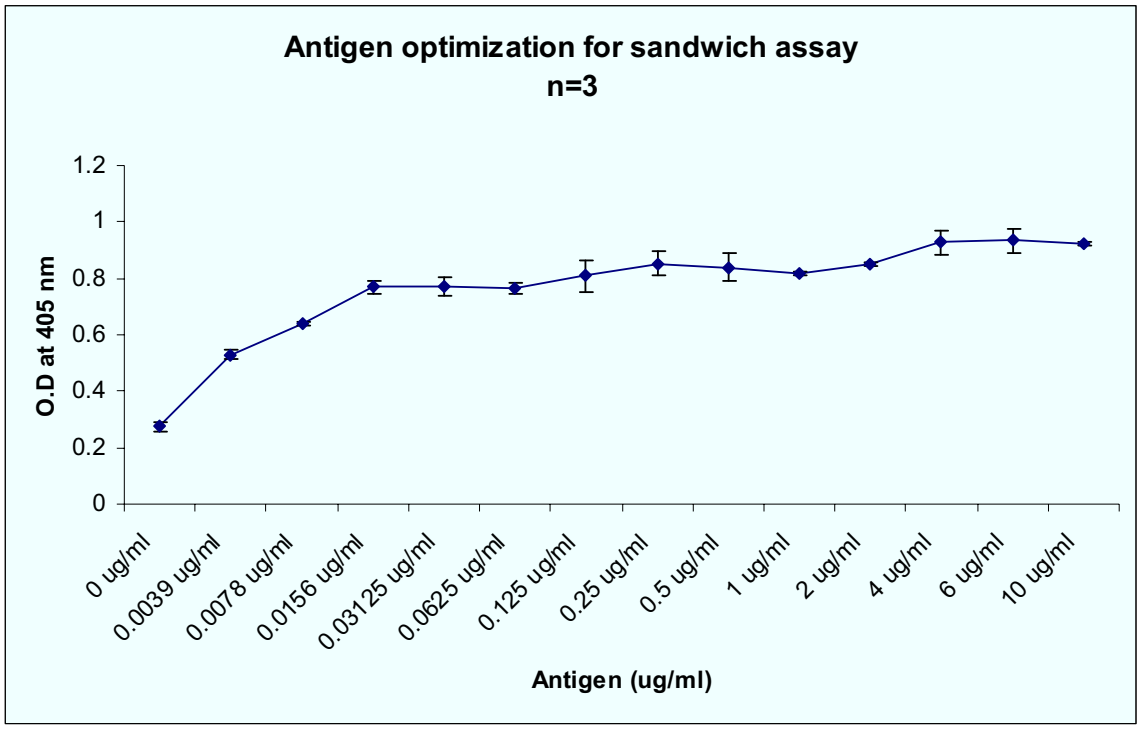


Conclusion: Anti-Cholera Rabbit IgG as capture Ab, Anti-Cholera Goat IgG as detector Ab forms a better sandwich of the two orientations.

Determination of optimal range of capture antigen (Ag)

Steps

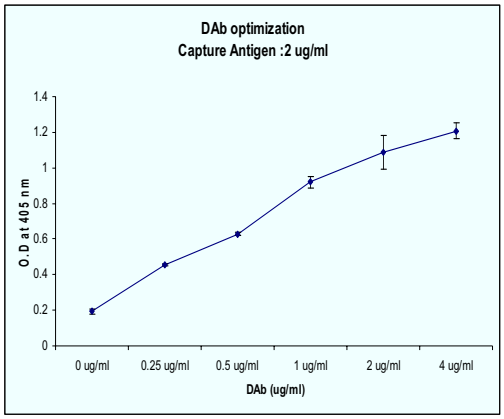
- Capture antibody concentration (Anti-Cholera Rabbit IgG, 1 µg/ml in coating buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Block, 37°C /1hr
- Cholera antigen (10.00 ……0.0039 µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Detector antibody concentration (Anti-Cholera Goat IgG, 1 µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Indicator antibody (Rabbit anti-Goat IgG HRPO) 1:10000. 37°C /1hr
- Wash (5X)
- Substrate

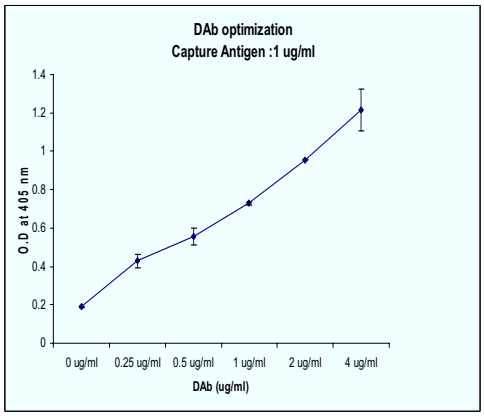


Conclusion: Cholera antigen is in the optimum range approximately 30 to 300 ng/ml.

Determination of optimal concentration of detector antibody (DAb)
Steps

- Capture antibody concentration (Anti-Cholera Rabbit IgG, 1 µg/ml in coating buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Block, 37°C /1hr
- Cholera antigen (7µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Detector antibody concentration (Anti-Cholera Goat IgG: 0.25, 0.5, 1.0, 2.0, 4 µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Indicator antibody (Rabbit anti-Goat IgG HRPO) 1:10000. 37°C /1hr
- Wash (5X)
- Substrate



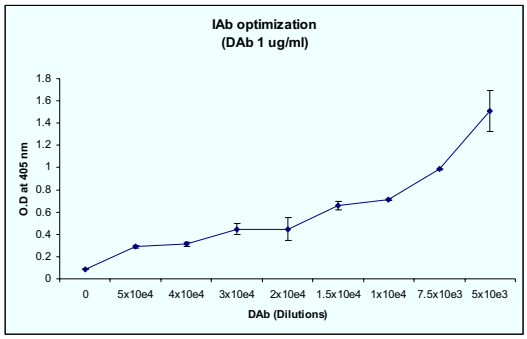


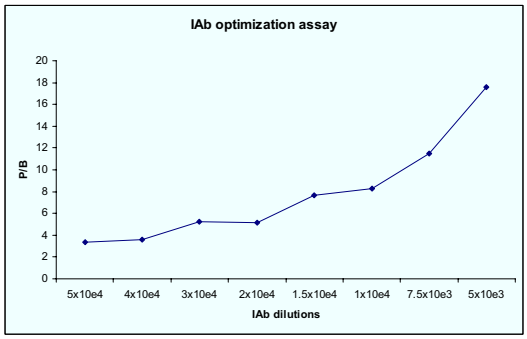
Conclusion: 1 µg/ml of Detector antibody (Anti-Cholera Goat IgG) is optimum for detection of captured Cholera antigen.

Determination of optimal dilution of indicator antibody-HRPO conjugates (IAb)

Steps

- Capture antibody concentration (Anti-Cholera Rabbit IgG, 1 µg/ml in coating buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Block, 37°C /1hr
- Cholera antigen (1 µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Detector antibody concentration (Anti-Cholera Goat IgG, 1 µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Indicator antibody (Rabbit anti-Goat IgG HRPO) 1:5000, 1:7500, 1:10000, 1:15000, 1:20000, 1:30000, 1:40000 and 1:50000 dilutions. 37°C /1hr
- Wash (5X)
- Substrate

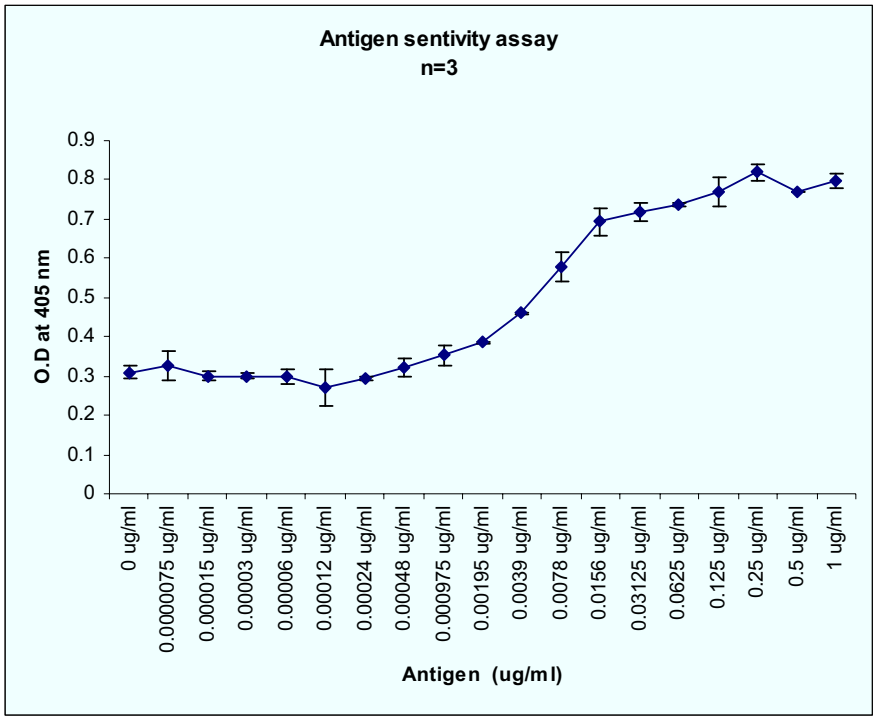




Conclusion: 1:7500 dilution of Indicator antibody conjugate (Rabbit anti-Goat IgG HRPO) is optimum for detection of captured Cholera antigen.

Determination of assay sensitivity for Cholera antigen
Steps

- Capture antibody concentration (Anti-Cholera Rabbit IgG, 1 µg/ml in coating buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Block, 37°C /1hr
- Cholera antigen (1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.0156, 0.0078, 0.0039, 0.00195 ……0.0000075 µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Detector antibody concentration (Anti-Cholera Goat IgG, 1 µg/ml in diluent buffer, 100 µl/well). 37°C /1hr
- Wash (5X)
- Indicator antibody (Rabbit anti-Goat IgG HRPO) 1:7500 dilution. 37°C /1hr
- Wash (5X)
- Substrate



Conclusion: 0.00195 µg/ml of Cholera antigen could be detected by heterosandwich ELISA using ABTS as substrate.

Overall conclusions of Cholera antigen heterosandwich assay:

- CAb :1 µg/ml Anti-Cholera Rabbit IgG
- Cholera Antigen :30-300 ng/ml
- DAb :1 µg/ml Anti-Cholera Goat IgG
- IAb 1:7500 (Rabbit anti-Goat IgG HRPO conjugate)
- 0.00195 µg/ml of Cholera antigen could be detected by heterosandwich ELISA using ABTS as substrate ($p<0.05$).

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13. ABSTRACT

The Defense Research and Development Canada (DRDC) have identified four biological warfare agents such as Vibrio cholera, Coxiella burnetii, Yellow fever and Yersinia pestis for the development of polyclonal antibodies for diagnostic application. However, DRES has provided VEE antigen in place of yellow fever antigen due to lack of yellow fever antigen. The specific polyclonal antibodies against each of the above four inactivated agents has been developed in goats and rabbits. The specific IgG fraction of the total immunoglobulins was separated by protein G chromatography and ELISA method has been established for diagnostic immunoassays. We have delivered hyperimmune serum as well as purified IgG for each of the antigen as described in Table 1.

14. KEYWORDS, DESCRIPTORS or IDENTIFIERS

Polyclonal antibodies
Vibrio cholerae
Coxiella burnetii
Yersinia pestis
Venezuelan Equine Encephalitis
VEE
rabbit antibodies
goat antibodies
Enzyme Linked Immunoassay
ELISA

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