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Les P. Nagata[3], Fay L. Schmaltz, Carol Balogh[1], A. Rashid Bhatti, John W. Cherwonogrodzky, Lawrence N. Schofield, Victor Gannon[2], and Elaine Fulton

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ABSTRACT

This report details the research that went into the bacterial component of the enzyme-based immunoassays developed for the Mobile Agent Identification Unit (MAGIDU), and were deployed during Operation Friction in the Persian Gulf in 1991. A rapid whole cell enzyme-linked immunosorbent assay (ELISA) was quickly developed for the identification of selected bacterial agents. The early research concentrated on the identification of *Bacillus anthracis* whole cells, and the resulting assays were fielded in the Persian Gulf. Anthrax could be reliably detected in 5.5 hrs at concentrations as low as 4.6 x 10^5 cells/mL (2 µg/mL). An assay with shortened incubation times was later developed (assay run time of 3.0 - 3.5 hr) with a sensitivity of detection of 1.2 x 10^6 cells/mL (5 µg/mL). Technical details in the development of these assays are discussed, as well as recommendations for future work.

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INTRODUCTION

During Operation Friction in the Persian Gulf, the potential threat of BW agents being employed by Iraq against Canadian Forces stationed in Bahrain and Qatar, required a quick and coordinated response from both the Defence Sciences and Defence Technologies Divisions of Defence Research Establishment Suffield [1]. A mobile agent identification unit (MAGIDU) laboratory was produced to carry out the identification of potential BW agents in air samples. The MAGIDU laboratory utilized the aerosol detection and sampling capabilities of a prototype mobile air sampling unit (MASU). This report details the research behind the immunological-based assays developed for use in the MAGIDU which allow the rapid identification of bacterial threat agents. At the time of the Persian Gulf War, only a short window existed for the development of assays to detect suspected BW agents. With the need for a reliable method that could be performed in a mobile field laboratory setting, enzyme-linked immunosorbent assays (ELISAs) were chosen [1].

For ELISAs, in general, a number of assay formats are possible for the immobilization of the analyte antigen and for the generation of signal. In this work, our assay terminology has been derived from *Antibodies: A Laboratory Manual* by Harlow and Lane [2]. The first format examined was the antigen capture sandwich immunoassay which use an immobilized antibody to capture the antigen, and a second antibody to detect the presence of the captured antigen (Annex A, Fig. A-1). Another format evaluated, the antibody capture immunoassay, is simpler than the sandwich format. The antigen is immobilized directly to the surface of the plate and is then detected with specific antibodies (Annex A, Fig. A-2) [2]. Signal generation can be accomplished by either of two general methods: 1) the enzyme used (i.e. horseradish peroxidase, alkaline phosphatase) to indicate the presence of the DAb can be directly conjugated to the DAb (direct detection); or 2) a secondary reagent can be used (i.e. anti-species-enzyme conjugate) to indicate the presence of the DAb (indirect detection). Previous bacterial sandwich ELISAs relied on the extraction of components such as lipopolysaccharide (LPS) [3-7] or outer membrane proteins (OMPs) [6,8,9]. Whole bacterial cells attached to the microtiter plate were used in antibody capture ELISAs [3,9,10]. ELISA assays which had been developed for the detection of anthrax used the protective antigen of the anthrax toxin in a sandwich ELISA [11], or an antibody capture ELISA using whole anthrax cells dried onto the microtiter plate [10]. The technical aspects and problems involved in the development of the anthrax ELISA are discussed.

MATERIAL AND METHODS

Chemicals and reagents were purchased from Sigma Chemicals (St. Louis, MO) and Fisher Scientific (Edmonton, Alta.). Microtitre plates evaluated were from Millipore (Mississauga, Ont.), Nunc (Gibco/BRL, Burlington Ont.). Horseradish peroxidase (HRP) substrate, 2,2'-azinobis(3- ethylbenzthiazolinesulfonic acid) (ABTS)/H2O2 was from Kirkegaard & Perry Laboratories - KPL (Mandel Scientific, Guelph, Ont.). Antibody-HRP conjugates used were purchased from Sigma Chemicals (St. Louis, MO). Anti-anthrax spore goat antibody (Ab) was kindly provided by Dr. E.D. Williamson, Chemical Biological Defence Establishment (CBDE), Porton Down, U.K.

Antigen Preparation

Bacterial strains used included: *Bacillus anthracis* strain 74-402C [field isolate from diseased cow - Animal Diseases Research Institute (ADRI), Lethbridge, Alta.], *B. anthracis* Thraxol Sterne vaccine strain (ADRI, Lethbridge, Alta.), *Brucella abortus* strain 1119-3 (ADRI, Nepean, Ont.), *Brucella melitensis* strain 16M (ADRI, Nepean, Ont.), *Francisella tularensis* strain LVS [American Type Culture Collection (ATCC), Rockville, MD], *Yersinia pestis* strain 19428 (avirulent - ATCC), *Staphylococcus aureus* strain ATCC 12600 and *Escherichia coli* strain JM101 (Stratagene, La Jolla, CA). All strains were initially purified on agar plates to assess purity and bulk cultures were grown in broth for 72 hrs at 37°C with 5% CO_2 and 200 rpm agitation. *Brucella sp.* were grown in *Brucella* broth (Difco, Detroit, MI), *F. tularensis* in Chamberlain's synthetic medium, *Y. pestis* in Brain Heart Infusion broth (Difco, Detroit, MI) with Yersinia Selective Supplement (BDH, Toronto, Ont) and *B. anthracis* and *E. coli* in tryptic soy broth (Difco, Detroit, MI). All were centrifuged, resuspended in 3.5%(v/v) formaldehyde for 2 days at RT with gentle shaking. Formalin-inactivated *B. anthracis* 74.402C was centrifuged, and resuspended in phosphate-buffered saline (PBS - 0.01 M phosphate buffer, 27 mM KCl, 0.137 M NaCl, pH 7.4 @ 25°C) to a final concentration of 250 mg/mL. Aliquots were stored at -70°C. All other bacteria were resuspended to a final concentration of 80 mg/mL, aliquotted at 0.5 mL per vial and lyophilized. All inactivated bacteria preparations were plated to appropriate media (0.1 mL) in triplicate, and all showed no growth (1 mg of wet cells of *B. anthracis* corresponded to 2.3 X 10^8 vegetative cells, 1 mg of *B. melitensis* to 9.4 X 10^9 cells, and 1 mg of *F. tularensis* to 5.4 X 10^9 cells).

Bacterial strains used in extended cross-reactivity assays were: Bacillus cereus strain ATCC 11778, *Pseudomonas fluorescens* strain ATCC 13525, *Arthrobacter globiformis* ATTC 8010, *Flavobacterium odoratum* ATCC 4651 and *Yersinia kristensenii* ATCC 29911. All strains used in extended crossreactivities were obtained from ATCC (Rockville, MD) and grown in ATTC medium #3 at 25 or 30°C.

Animal Inoculation

Antisera were prepared using Nigerian dwarf goats (gt) purchased locally, and New Zealand white rabbits (rb) and Hartley strain guinea pigs (gp) purchased from Charles River Canada (Montreal, Que.). In conducting the research described in this report, the investigators adhered to the "Guide to the Care and Use of Experimental Animals" published by the Canadian Council on Animal Care.

Initial batches of antisera were produced in rabbits and in goats. *B. anthracis* strain 74-402C was utilized for the induction of anthrax antisera, *B. melitensis* for brucellosis antisera and *F. tularensis* LVS for tularemia antisera. Killed, whole cell suspensions in PBS (10^8-10^9 cells/mL) were given intravenously (IV) applying increasing doses of 0.1, 0.2, 0.5 and 1.0 mL to both rabbits and goats. The vaccination schedule consisted of 3 day boost periods followed by 5 day rest periods to a maximum of 40 days. During each period, the dose given was increased incrementally as described to the maximum of 1.0 mL and then continued to the 40th day. Trial bleeds and titres were determined by slide agglutination at 15 and 30 days. Tularemia antiserum was produced only in goats using the above protocol. A second protocol was employed for rabbits and involved inoculation with a reconstituted *F. tularensis* live vaccine strain (U.S. Army Medical Research Institute of Infectious Diseases - USAMRIID, Frederick, MD) given subcutaneously (SC), 0.5 mL in two sites. The vaccination schedule was comprised of 3 day boost periods followed by 5 days of rest to a maximum of 40 days. Titres were checked at 15 and 30 days.

Production of plague and anthrax antibody was accomplished using Hartley strain guinea pigs (Charles River, Montreal, Que.). The guinea pigs were inoculated with 0.2 mL SC using killed whole *Y. pestis* strain 19428 (10^8-10^9 cells/mL) at days 0 and 15. Titres were checked at days 14 and 28. Following the primary immunization schedule, animals were boosted every two weeks until acceptable ELISA titres were obtained. The inoculation of rabbits for the anti-anthrax sera produced on May 27, 1991, followed a similar immunization schedule.

Antisera Purification

Blood was collected by heart puncture and allowed to clot 1-2 hr at room temperature (RT). The clots were then left overnight at 4°C, and the serum removed and stored at -20°C. Caprylic acid-ammonium sulfate precipitation was used to purify the rabbit and goat antisera to anthrax, tularemia and brucellosis [2,12]. Briefly, two volumes of sodium acetate buffer (60 mM, pH 4.0) were added to serum with gentle stirring at RT. The pH was checked and adjusted to pH 4.8 if necessary. For each 10 mL original volume of serum, 0.75 mL of caprylic acid was added dropwise with gentle stirring at RT. The mixture was allowed to stir for 30 min after addition of the caprylic acid. Centrifugation at 5000 X g for 10 min (6000 rpm with JA17 rotor, Beckman J2-21) was followed by transfer of the supernatant to dialysis tubing (12,000-14,000 MW exclusion limit). The supernatant was dialyzed against PBS with three changes over 24 hr. For ammonium sulfate precipitation [13], the pH of saturated ammonium sulfate solution was adjusted to pH 7.8 by the addition of 2.0 N NaOH. With constant stirring, one-half volume of saturated ammonium sulfate

was slowly added (dropwise) to one volume of antibody dialysate from the caprylic acid purification. During the first stages, the precipitate was allowed to redissolve before the addition of more ammonium sulfate. Eventually the precipitate persisted and the suspension was stirred for an additional 2-3 hr at RT. The suspension was centrifuged at 1400 X g for 30 min at RT. The precipitate was dissolved in PBS to the original volume of dialysate, and the ammonium sulfate precipitation repeated two more times. After the third ammonium sulfate precipitation, the precipitate was dissolved in borate-buffered saline [5 parts borate buffer (6.184 g boric acid, 9.536 g sodium tetraborate - $Na_2B_4O_7.10H_2O$ and 4.384 g NaCl made up to 1000 mL and adjusted to pH 8.4- 8.5) was diluted with 95 parts saline - 0.85 %(w/v) NaCl] (BBS) to a volume of less than one-half the original volume. The antibody solution was dialyzed against BBS for several days at 4°C with two changes of BBS per day. A sample of the dialysate was checked for the presence of sulfate ions by the addition of 10% barium chloride. If a white precipitate formed, dialysis was continued. When no sulfate ions were present, the dialysate was collected, 0.0001% (w/v) thimerasol was added, and the purified antibody was aliquotted and store at -70°C. Protein concentrations were determined by absorbance at 280 nm or by the Bradford assay (Bio-Rad Laboratories, Mississauga, Ont.)

Protein A immobilized to cross-linked agarose beads (Pierce, Rockford, IL) was used to purify small batches of antisera, including the gp α-anthrax and α-plague sera. Immobilized protein G (Pierce, Rockford, IL) was used to purify the gt α-anthrax spore sera and a batch (May 27, 1991) of rb α-anthrax serum. The protocols supplied with the kits were explicitly followed (Pierce, Rockford, IL). Purified antibodies were adjusted to 0.0001 % (w/v) thimerasol, aliquoted and stored at -70 °C.

Antisera absorbed with anthrax or *F. tularensis* cells were prepared as follows: *Francisella* or anthrax cells were diluted to 10 mg/mL in PBS and boiled 30 min. The cells were washed twice with PBS and the final pellet was resuspended in PBS to the original volume. To 0.5 mL of purified rb α- anthrax and rb α-tularemia antibodies (Abs), 0.5 mL of the washed bacterial cells was added to the appropriate tube. The α-anthrax was absorbed with *Francisella* cells (α-anthr tular-minus) and the α-tularemia was absorbed with anthrax cells (α-tular anthr-minus). The samples were allowed to incubated for 60 min at RT. Cells were pelleted 5 min in a microfuge, and the absorbed Ab saved in a new tube.

Antigen Capture Sandwich ELISA (indirect)

A sandwich ELISA was performed [1] as follows: Nunc Maxisorb™ flat bottom 96-well microtitre plates (Gibco, Burlington, Ont.) were coated with capture antibody (CAb) by the addition of 200 µL of an appropriate dilution of antibody in PBS. Plates were sealed and left overnight at 4°C. The next day, plates were washed five times on an automated plate washer (Molecular Devices, Menlo Park, CA) with 300 µL wash buffer (WB) [0.1 % (w/v) bovine serum albumin (BSA), 0.05% Tween 20 in PBS]. Plates were blocked with the addition of 300 µL of blocking buffer (BB) [2% (w/v) BSA, 0.05 %(v/v) Tween 20 in PBS] for 1 hr at 37°C. Plates were washed five times with WB, then 200 µL of antigen (formaldehyde-treated anthrax, sonicated or boiled 15 min) diluted in PBS was added and samples were incubated 30-60 min at 37°C. Plates were washed five times followed by the addition of 200 µL of detector antibody diluted in filter-sterilized WB, and incubation was continued for 30-60 min at 37°C. Plates were washed five times and 200 µL of an appropriate anti-species HRP-conjugated indicator antibody (IAb) was added and plates incubated 30-60 min at 37°C. Plates were washed five times with WB and 200 µL of freshly prepared ABTS/H_2O_2 HRP substrate (KPL - Mandel Scientific, Guelph, Ont) prewarmed to RT was added and incubation continued for up to 30 min at RT. After addition of the substrate, the plate was read in a Molecular Devices plate reader (Molecular Devices, Menlo Park, CA) set at a wavelength of 405 nm with the automix on. The absorbance of the wells was read every 10 min for up to 30 min against a substrate blank. Controls included an antigen-minus, DAb-minus and IAb-minus samples. Apart from the CAb, all antisera and conjugates were diluted in filter-sterilized wash buffer. All samples were set up in triplicate.

Antibody Capture ELISA (indirect)

Aliquots of 100 µL of antigen was added to 100 µL of 2X carbonate buffer [2X CB - 0.1 M sodium carbonate/bicarbonate (pH 9.6), 0.0001% (w/v) thimerasol] per well, Nunc Maxisorb™ flat bottom 96-well microtitre plates. The antigen (sonicated or boiled formaldehyde-treated anthrax) was allowed to adsorb for 1-2 hr at 37°C. Plates were washed five times with WB, followed by the addition of 200 µL of BB and plates incubated 1 hr at 37°C. Plates were washed 5 times with WB, then 200 µL of rb α- anthrax DAb diluted in filtered WB to 10 mg/mL was added and plates incubated 30-60 min at 37°C. Plates were washed five times, followed by the addition of 200 µL of gt α-rb HRP-conjugated IAb (Sigma, St. Louis, MO) diluted 1/2500 with WB. Plates were incubated 30-60 min at 37°C, followed by washing five times with WB. Freshly prepared ABTS/H_2O_2 substrate (200 µL, 1:1 mixture) was added and plates were incubated up to 30 min at RT, then read against a substrate blank. All samples were run in triplicate, with readings averaged by the software provided with the microplate reader. Also included on the plate were antigen-minus, DAb-minus and IAb-minus negative controls and 10 and 50 µg/mL inactivated anthrax positive controls (Annex A, Fig. A-3). Antisera and conjugates were diluted in filter-sterilized wash buffer.

Variations to the method were employed when membrane-backed plates were used. Washes were performed with a vacuum filtration device (Millipore, Mississauga, Ont.) or manually, using a multichannel pipettor and a 8-well vacuum manifold. Plastic trays and templates were used under the plates to minimize leakage when incubating or reading the plates. Plates used include nitrocellulose (HA, 0.45 m), Duropore^{TM} GV and Milliscreen^{TM} HV (Millipore, Mississauga, Ont.). The same incubation temperature and times were used, as well as the same number of washes.

RESULTS

Antigen Preparation

Preparation of the antigen for the anthrax ELISA was a problem with the formaldehyde-treated bacteria. Severe clumping was observed with the inactivated anthrax field isolate that was used. Samples were disrupted by sonication using several 1 min. bursts from a microprobe sonicator, or dispersed by boiling 1-2 mL samples 15 min in screw-capped 7 mL scintillation vials. Sonication or boiling of the sample 15 min gave similar results in an antibody capture ELISA and seemed effective in the break-up of clumps (Fig. 1). Boiling was chosen as the method of antigen preparation for use in the antibody capture ELISA. In the assessment of boiling times required, comparable results were obtained after 5, 10 or 15 min boiling for anthrax, *Francisella tularensis* and *Brucella melitensis* (data not shown). Almost no signal was obtained for untreated *Brucella*, and a reduced signal was observed for untreated *Francisella* cells, in contrast to samples boiled 5, 10 or 15 min for these bacteria (data not shown). On the basis of these results a standard boiling time of 5 min was selected. The results with *Brucella* and *Francisella* indicated antigenic determinants are "unmasked" by this treatment, similar to the removal of interference by M antigen in the serotyping of *Salmonella* isolates [14].

Antibody Purification

Rabbit and goat antisera were initially purified by caprylic acid - ammonium sulfate precipitation [2,12,13]. After this treatment, relatively pure antibody preparations were obtained as indicated from countercurrent immunoelectrophoresis results (data not shown). The antibody concentrations obtained were 2.6 mg/mL for the purified gt α-anthrax Ab (Dec. 19, 1990), 3.8 mμg/mL for rb α-anthrax Ab (Dec. 19, 1990) and 1.08 mg/mL for the rb α-anthrax Ab (Feb. 23, 1991) (see Table I). The purified gt α-anthrax Ab showed almost no reactivity to the killed anthrax cells, as compared to good reactivity obtained with the purified rb α-anthrax in the antibody capture ELISA (indirect). The crude goat sera showed good reactivity as a DAb in the antibody capture ELISA at a 1/100 dilution. The reactivity was approximately 55-70% of that obtained with purified rb α-anthrax at 20 μg/mL (data not shown). Nevertheless, for the sake of simplicity, rabbit sera was used in the optimization of additional agents (*Brucella* and *Francisella*) during and after the Gulf War. In this manner, only one IAb (α-rb-HRP) would be needed. A second batch of rb

α-anthrax sera was produced on May 27, 1991 (protein G purified, 2 mg/mL), and a batch of gp α-anthrax sera (protein A purified, 2.9 mg/mL) was produced on June 4, 1991. Both were very weak in ELISA titration (Table I), and were unsuitable for use even when the [DAb] was increased (data not shown). The gp α-plague Ab showed good reactivity at a 1/100 to 1/200 dilution, but was not characterized further (Fig. 2). The initial results indicated plague could be detected down to at least 5 μg/mL, and possibly lower (Fig. 2). Data on ELISA titres of different batches of sera are summarized in Table I.

Anti-anthrax-HRP conjugate was prepared from the purified rb α-anthrax, Dec. 19, 1990 (J.D. Biologicals, Etobicoke, Ont.), in an attempt to develop the antigen capture sandwich ELISA. With direct detection, the purified rb α-anthrax could be used for capture (CAb), and the rb α-anthrax-HRP used as detector/indicator (DAb/IAb) (Note: indirect detection requires the CAb and DAb be from two different species). The preparation was likely contaminated with high levels of unbound HRP as very high backgrounds were obtained in the DAb/IAb controls (data not shown). The unbound HRP was not expected to interfere in the assays [2], however, the results suggested the need to purify the conjugate by gel-sieve or affinity (protein A or G) chromatography.

Antigen Capture Sandwich ELISA

Attempts to develop the more sensitive, indirect antigen capture sandwich ELISA yielded disappointment. With anthrax, both goat (crude and purified) and purified rabbit Abs were tried in all combinations. The lack of activity with the purified gt α-anthrax Ab was a contributing factor, but the substitution of crude gt α-anthrax sera did not allow for the resolution of this problem (data not shown). Anthrax is probably too large an antigen to be used in an antigen capture ELISA, and troubles with formaldehyde-induced clumping compounded the difficulties. However, surprising results were obtained after the boiling of anthrax samples, and analyzing both the supernatant and the cell pellet by antibody capture ELISA. A strong signal was found associated with the cell pellet. In addition, an equally strong signal was obtained from Ag released into the supernatant (data not shown). These results indicate that it should be possible to detect this soluble antigen by antigen capture sandwich ELISA. Antigen extractions for cell wall components were not considered [3-9] once the antibody capture assay was deemed feasible, so as to avoid complications once the assays were in the field.

Antibody Capture ELISA

The previous bacterial whole-cell ELISA for anthrax required the drying down of the sample onto the plate overnight and then heat-fixing for 1 hr at 60°C [10]. Because of the requirements to quickly identify an alarm situation, other methods to immobilize the bacterial cell to a solid support were examined. Membrane-backed plates were seen as a possibility since they could be used immediately to immobilize the bacteria by filtration, reducing the time required to bind the Ag onto the plate. The second advantage of these plates is

their very high protein binding capacity (100-1000 fold higher then plastic plates) [15]. Nonetheless, in the antibody capture ELISA, two problems were encountered with the use of membrane-backed plates. First, a severe leakage problem was apparent with the Duropore™ membrane-backed GV plate, and to a lesser extent, with the nitrocellulose HA plate (Millipore, Mississauga, Ont.). The second problem encountered was a very high level of background in the control wells (data not shown). Possible explanations for these results could be insufficient blocking of the plates or aggregates of HRP-conjugates nonspecifically binding to the membranes during vacuum filtration washing steps. The HV Milliscreen™ plate did not have leakage problems, but yielded high levels of background when washed with the plate washer (data not show) instead of the vacuum filtration device.

Immobilization of the antigen using reduced drying times was considered. Polysorb™ plates (Nunc - Gibco/BRL Burlington, Ont,) were initially used as recommended by the manufacturer for binding antigen. With time requirements for rapid identification, overnight drying of the plate was ruled out, although it was used as the standard to which other methods of Ag were compared (Fig. 1). By reducing the volume of antigen to 50 µL, a hairdryer could be used to dry down a sample in 1-2 hr (Fig. 3). The greatest problem with this method was inconsistant drying from well to well, nevertheless, the results obtained were reliable.

Direct attachment of the antigen from solution to the microplate was examined with Maxisorb™ plates (Nunc - Gibco/BRL Burlington, Ont,). Aliquots of anthrax antigen (200 µL) bound for 1 hr in solution onto Maxisorb™ plates showed an ELISA signal equivalent to that obtained by drying down 50 µL of anthrax Ag onto Polysorb™ plates (see Fig. 3). In the optimization of the binding of the anthrax from solution onto Maxisorb™ plates, two major variables were identified. First, the buffer used was significant in determining the amount of anthrax antigen that could be bound in a limited time-frame. PBS as a binding buffer was slightly better than dH_2O (data not shown). However, when PBS was compared to carbonate buffer (2 hr incubation at 37°C), it bound only 40-60% of the anthrax Ag which was diluted in carbonate buffer (Fig. 4). The second determinant in the binding of anthrax Ag to the plate was the length of incubation of antigen binding. The results for 0.5, 1 and 2 hr in carbonate buffer are shown in Fig. 4. The reduction in binding in carbonate buffer from 2 hr to 1 hr, reduced the ELISA signal by up to 50% (data not shown).

The optimal amounts of DAb were 20 µg/mL for rb -anthrax Ab (Dec. 19, 1990), 10 µg/mL for the rb α-*Brucella* Ab and 30 µg/mL for rb -tularemia Ab (data not shown). The IAb used was anti-rabbit HRP-conjugated Ab (Sigma, St. Louis, MO), which was originally optimized as a 1/2500 dilution for antigen capture sandwich ELISAs developed for the detection of botulinum toxins and for Staphylococcal enterotoxin B [1]. Results with the anthrax antibody capture ELISA indicated a higher dilution of IAb could be used (up to 1/5000). However, to maintain consistency with the toxin sandwich ELISAs, a 1/2500 dilution of the anti-rabbit HRP (IAb) was used and no significant increase in background levels was observed (data not shown).

Sensitivity- Antibody Capture ELISA (indirect)

The sensitivity of the antibody capture ELISA for the detection of anthrax whole cells was determined. As mentioned previously, one of the major determinants of the sensitivity of the assay was the amount of time allowed for the binding of the boiled anthrax Ag (in carbonate buffer) to the Maxisorb^{TM} plate (Nunc, Burlington Ont.). A second determinant of sensitivity was the length of time allowed for binding of DAb (rb α-anthrax Ab) to the anthrax Ag, and the binding of the IAb (anti-rabbit HRP) to the DAb. Conditions were initially optimized for a DAb concentration of 20 µg/mL, and using 2 hr anthrax Ag binding, 1 hr blocking, 1 hr DAb incubation, 1 hr IAb incubation, and 30 min substrate development (assay time of 5.5 hr). A positive result was indicated when the signal obtained was greater than two times the background levels [Blk or controls (-DAb, -Ag, -IAb)]. Sensitivity of the assay was down to 2 µg/mL (4.6 x 10^5 cells/mL) of anthrax Ag using the above conditions (see Fig. 4,5).

While the MAGIDU was in service in Qatar, a request was received from the military medical technologists operating the station to shorten the running time of the assay. Reduced incubation times were evaluated, and implemented. The times for a shortened anthrax antibody capture ELISA (indirect) were changed as follows: 1 hr antigen binding, 30 min blocking, 30 min DAb incubation, 30 min IAb incubation and 30 min substrate development. The sensitivity of the assay was roughly halved to 5 µg/mL (1.2 x 10^6 cells/mL), with the running time reduced to 3.0-3.5 hr (Fig. 5).

Live agent testing was performed to examine whether the assay conditions developed for the anthrax antibody capture assay would also work with live anthrax cells, and anthrax spores. The assay was performed in level 3 biological containment using conditions identical for the original anthrax antibody capture assay (5.5 hr assay), with the exception that visual examination of the plate was used in place of the ELISA plate reader. Results indicated that a 5- 10 fold increase in signal was attained with both live anthrax cell or spore preparations. In comparison, values obtained using heat-killed (1 hr @ 60°C) or formalin-fixed cells (virulent assay test strain or Thraxol strain) were significantly lower as shown in Fig. 6. The rb α-anthrax Ab was more sensitive at the same concentration of Ab (20 µg/mL) than a specific gt α-anthrax spore Ab which was obtained from the UK (Fig. 6). These results, taken together indicated that the anthrax ELISA would be more sensitive in the field against live agent than against the formalin-killed anthrax, and would also be able to detect anthrax spores.

Specificity- Antibody Capture ELISA (indirect)

Two problems anticipated for the assays were potential interference from sand and cross-reactivity to other bacteria. Sand was thought to be a potential problem in the assay, and it was incorporated into several unknowns to determine its effect on the anthrax antibody capture ELISA. The net effect of the sand at a concentration of 1% (w/v) or supernatant from the sand [supernatant from 1% (w/v) sand diluted 1/5 with sample unknowns] was a drastic reduction in sensitivity (Fig.7). The reduction in ELISA signal was approxi-

mately 30-50% with the sand supernatant and up to 70% with 1% (w/v) sand. The experimental conditions used were too extreme a scenario in terms of the amount of sand spiked into the assay, compared with the design of the air sampling unit which had a 15 µm cutoff for the dicotymous filter samples. Fortunately, sand interference was not a problem during operation of the MASU/MAGIDU in the field setting in Qatar or during analysis of samples from the MASU in Bahrain. It was encouraging to see that anthrax was identified (20 and 50 µg/mL) under the excessive experimental conditions used for interference with sand (Fig. 8).

A second problem that could arise with the anthrax antibody capture ELISA was cross-reactivity to other bacteria. Cross-reactivity to selected bacteria (*S. aureus, E. coli*) was tested in unknown samples (Fig. 7), and later in antigen titration curves. To control for cross-reactivity, a control rb antibody (purified, preimmune rabbit serum) was added to the assay. The ELISA sample was split into two, with half tested against α-anthrax and the other half tested against the control rb Ab or other detector Ab (Annex A, Fig. A-3). The format of a multiple agent plate had the sample being tested against anthrax, brucellosis, tularemia and plague on a single plate. Each antiserum would act as a control for each of the other antisera used (Annex A, Fig A-4). Results of crossreactivity with *E. coli* and *S. aureus* were illustrated when they were spiked into unknown samples (Fig. 7). An increased ELISA signal was obtained from the control serum (rb α-tularemia Ab used instead of control rb Ab) to both *E. coli* and *S. aureus*, over the background levels in the ELISA. Twice the background levels were indicated for the assay controls (Blk and Negative Controls) and for the control antiserum. Using the criteria of a positive signal being greater than twice the background, test results were evaluated relative to two times the negative control level and two times the control antisera (Fig. 7). The results suggest that without the use of control serum, false positives could result if significant amounts of these nonpathogenic bacteria are collected in the sampler. The levels of *E. coli* and *S. aureus* used were unrealistically high, as biological analysis of the sand indicated a very low cell count (J. Cherwonogrodzky, unpublished results). Nevertheless, the control antisera was effective in picking up false positives due to cross-reactivity, and was incorporated into the design of the template for the anthrax, as well as the four bacterial agent template (Annex A, Fig. A-3 and Fig. A-4).

An unexpected result was uncovered during the evaluation of the purified rb α-anthrax Ab for unknown samples. The rb α-anthrax Ab proved to be as sensitive as the rb α-tularemia Ab for detection of *Francisella tularensis*. Titration of rb α-anthrax Ab and rb α-tularemia Ab indicated that they were equivalent in detecting *Francisella* Ag (Fig. 8). Sensitivity of the rb α-anthrax Ab was around 5 µg/mL in the detection of *F. tularensis*. Absorbed Ab samples were prepared to investigate whether the reactivity to tularemia in the α-anthrax Ab could be selectively removed. The results of antibody capture ELISAs to the α-anthrax absorbed with Fransicella cells (α-anthr tular-minus) and α-tularemia absorbed with anthrax cells α-tular anthr-minus) are shown in Fig. 9. The tularemia activity was almost completely removed in the α-anthr tular-

minus, without drastically reducing its reactivity to anthrax. The rb α-tularemia Ab showed slight cross-reactivity to anthrax (data not shown). The rb α-anthrax Ab was sent to the Persian Gulf without absorption, as the sensitivy of the assay was reduced following absorption (see Fig. 9). This was seen not as a problem but as an advantage for the assay would be able to pick up both agents of interest, and that a second ELISA using rb α-tularemia could confirm the presence or absence of tularemia. The format for the future incorporation of four bacterial assays on one plate would also be able to differentiate anthrax from tularemia (Annex A, Fig A-4).

Antigen titrations were performed with the rb α-anthrax Ab at 20 µg/mL against a number of bacteria. These included *F. tularensis*, *B. melitensis*, *Y. pestis* and *E. coli*. Results of the titration found *E. coli* to give significant background levels to rb α-anthrax Ab (Fig. 8). Extended cross-reactivities were performed with a number of potential soil bacteria, including: Bacillus cereus, *Pseudomonas fluorescens*, *Arthrobacter globiformis*, *Flavobacterium odoratum* and *Yersinia kristensenii*. None of these bacteria crossreacted with the rb - anthrax Ab at 20 µg/mL as shown by the antigen titration curves (below twice the background - data not shown).

DISCUSSION

In the development of the anthrax antibody capture ELISA, many problems were overcome in the limited period of time available. The first was finding a suitable method to "prepare" the bacterial cells. By borrowing from a procedure for the typing of *Salmonella* strains, whereby boiling was used to destroy interfering capsular material, we were able to have a simple procedure to disperse the anthrax cells, and also to "unmask" *Brucella* and *Francisella* antigens. The results with anthrax were comparable to sonication, thus boiling eliminated the danger of aerosols being generated and the need for an additional piece of equipment in the field setting. U.K. scientists from CBDE had noticed similar problems with their ELISA and suggested irradiation to inactivate the antigen (personal communication). Also, the noted problem was inherent only to the formalin-killed anthrax cells used to optimize the assay, and was not a problem with live Thraxol strain of anthrax.

In the preparation of polyclonal antisera, rabbits and goats were used. This allowed for the potential development of sensitive antigen capture sandwich ELISAs. The lack of reactivity of the purified gt α-anthrax Ab was puzzling, since good reactivity of the crude gt α-anthrax Ab was obtained. One interpretation of the results was that the caprylic acid-ammonium sulfate precipitation was too harsh and denatured the goat antibody. The caprylic acid-ammonium sulfate precipitation method had an advantage in that it was a cheap and convenient method for large volumes of sera, and was stated as being useful for the concentration of antibody from all species [2]. However,

the amount of purified antibody recovered varied considerably, even from the same batch of antiserum (i.e. protein concentrations from rbα-anthrax Ab. from Dec. 19, 1990 and from Feb. 23, 1991). The recommended alternative purification would be chromatography on immobilized protein A or protein G. These were used to purify small batches of gt (anti-anthrax spore Ab) and gp (anthrax, plague) sera, and could be scaled up for larger batches. It should be noted that proteins A and G have varying affinities for immunoglobulins (Igs) from different species. Thus, it is recommended by suppliers (Pierce, Rockford, IL) that protein A is used for gp sera and protein G for gt sera.

In the development of an ELISA, several requirements were made. The assay must be sensitive, specific and able to be performed in a relatively short time-frame. An antigen capture sandwich ELISA was initially chosen since the capture antibody and the blocking steps could be precoated onto the plate the night before. Futhermore, the antigen capture time would only be 1hr, leaving a total assay time of 3.5 hr. Other advantages of this type of assay are that it has good specificity (2-5 times more sensitive than other immunoassays) and can be used with less pure antigens [2]. UK scientists at CBDE used a sandwich ELISA to detect the anthrax spores, which they found to be about 10 times more sensitive than an antibody capture assay (personal communication). Whole anthrax cells are likely too large an antigen for the sandwich ELISA, which was developed for use with soluble Ags [2]. An alternative was to extract the major antigenic component (i.e. polysaccaride, OMPs). Nonetheless, it would still have require extensive research to identify such a component, and apply this extraction methodology for anthrax (Gram positive bacteria) to other agents (*Brucella*, *Y. pestis* and *F. tularensis* are all Gram negative). Instead, the effort was placed on refining the antibody capture ELISA for whole cells.

The whole cell antibody capture ELISA to anthrax was based initially on a previous anthrax ELISA where whole cell samples were dried down overnight on the plate [10]. The assay time could be decreased by drying down minimal volumes of anthrax (50 µL) in 1 to 2 hr with a hair dryer. Nevertheless, comparable or better results were obtained by binding and immobilizing the anthrax antigen from solution (200 µL volume) onto Maxisorb^{TM} plates (Nunc, Burlington, Ont.) as shown in Fig. 3. The choice of plates and buffer for binding the anthrax proved critical. Nunc Polysorb^{TM} plates demonstrated almost no binding of the antigen from solution in 1-2 hr. Furthermore, the use of membrane plates should be reevaluated despite the poor performance obtained in these studies. The background levels of reactivity may be reduced by better blocking of the plate, and with the use of improved plates and vacuum washing systems (Millipore, Mississauga, Ont.). Carbonate buffer was another important factor in the binding of the anthrax to the plate, with sufficient binding being acheived in 1 hr (Fig. 4). PBS and distilled water were not as efficient in binding of anthrax Ag to the plate.

Total assay time of 5.5 hr included 2 hr antigen binding, 1 hr blocking, 1 hr DAb, 1 hr IAb and 30 min substrate incubation. Antibody concentrations were optimized at a [DAb] (rb α-anthrax Ab) of 20 µg/mL and [IAb] (gt α-rb-

HRP) dilutions of 1/2500. The sensitivity of the assay for anthrax was 4.6 x 10^5 cells/mL (2 g/mL). An assay with shortened incubation times was later developed (assay run time of 3.0 - 3.5 hr) having a sensitivity of 1.2 x 10^6 cells/mL (5 µg/mL). Although adequate binding was obtained in 1 hr, 2 hr binding would be recommended to ensure that the bulk of the antigen is bound. Other incubation times for the assay would remain as stated in the reduced time assay (3.0-3.5 hr).

Specificity of the anthrax antibody capture ELISA (indirect) was tested in respect to sand interference and cross-reactivity to other bacteria. Interference from sand resulted in reduced sensitivity of the assay. However, the amounts of sand used in the assay were excessive. During the operation of the MASU/MAGIDU complex in the field in Qatar, no problems were reported with interference from sand. Furthermore, large particles of sand should be excluded by the 15 m cutoff of the MASU dicotymous air sampler.

In examination of cross-reactivity to other bacteria, it is not surprising that the assay showed significant cross-reactivity to *S. aureus* and *E. coli*. The polyclonal antisera used would be expected to contain Abs to common ubiquitous bacteria. Again, an excessively high concentration of bacteria was used in the cross-reactivity tests (10 µg/mL), and it is unlikely that levels of contaminating bacteria would approach these concentrations in real samples. Further, extended cross-reactivities to number of soil saphrophytes (including: *Bacillus cereus, Pseudomonas fluorescens, Arthrobacter globiformis, Flavobacterium odoratum* and *Yersinia kristensenii*) proved negative, with cross-reactivity being taken as any signals greater than twice the background levels. Absorption of the antisera (to *E. coli*, *S. aureus*), or the production of affinity (anthrax) purified polyclonal antisera [2] should eliminate cross-reactivity and the need for serum controls in the assay.

A surprising result was obtained in the testing of the α-anthrax rabbit sera for specificity. A very strong signal was obtained when this antisera was tested against *F. tularensis* cells. The rb α-anthrax Ab at 20 µg/mL was as good as the specific rb α-tularemia Ab at 30 µg/mL. This signal could be completely absorbed out by incubation of the antisera with killed *F. tularensis* cells that had been inactivated by boiling for 30 min and washed. The reactivity of the rbα-anthr tular-minus was also reduced for anthrax, but not as strongly (Fig. 9). Our speculation was that some of the rabbits immunized to anthrax became infected with the live vaccine strain (LSV) of *F. tularensis* (used in the production of rb α-tularemia). It will be interesting to see if any of the other rabbit antisera (α-SEB,α-botulinum toxin) exhibit the same cross-reactivity. A second possibility is a mix up in samples during purification. The rb α-Brucella Ab exhibited only slight cross-reactivity to *F. tularensis,* which is to be expected in view of the reported antigenic cross-reactivity between the two bacteria [16]. Nevertheless, the rb α-anthrax Ab could detect F. tularensis cells down to a concentration of 5 µg/mL (2.7 X 10^7 cells/mL).

The evaluation of the whole cell antibody capture ELISA to anthrax in the Persian Gulf led to a number of findings. Overall, the anthrax ELISA performed as expected with no false positives reported. The assay was labour

intensive, and a request was made to DRES to reduce the overall assay time. A reduced time assay was implemented with a corresponding decrease in sensitivity of approximately 50%. During the short training period at DRES, the military technologists were found to be highly proficient at their jobs, with excellent backgrounds in medical laboratory technology to carry out the task at hand. The mobile field laboratory (MAGIDU) could be stocked with additional microbiological tests such as API strips, plates for culture and Gram stain (with a 1000X microscope). This would allow simple, confirmatory microbiological tests to be carried out immediately on suspected samples or alarms from the MASU. An example of this type of a test is a Gram stain for bacteria. Anthrax cells form distinct chains of Gram-positive boxcar-shaped cells under the microscope [14]. Optional rotation of military technologists through DRES on a 6 month to 2 year basis would familarize them with the research conducted at DRES. Intensive short-term training would not be required, and direct military input could be supplied to products under development. The period of training of 9 days was barely adequate. Better training would allow the military technologists to trouble shoot the assays on their own, if the communications link was ever lost. In addition, communications between DRES and the Persian Gulf was very good, with DRES able to provide continuous advice to the military technologists, and directly monitor the results sent back (raw data). However, some minor improvements could be made in terms of providing better assay detection and identification.

CONCLUSIONS

In the development of bacterial immunoassays for the MAGIDU during the Persian Gulf War, a whole cell antibody capture ELISA was developed for anthrax/*F. tularensis*. The assay used 5 min boiling of the sample for processing the antigen, which was then immobilized onto Maxisorb^{TM} (Nunc, Burlington, Ont.) microtiter plates in carbonate buffer. Variables important to the assay were the coating buffer used, the microtiter plates used, the length of time of Ag binding, and the total reaction time. The initial anthrax ELISA was optimized for 5.5 hr, and yielded sensitivities as low as 4.6×10^5 cells/mL (2 µg/mL). An assay with reduced incubation times (total assay time of 3.0-3.5 hr) had a decreased sensitivity of 1.2×10^6 cells/mL (5 µg/mL) for anthrax.The sensitivity of the assay for F. tularensis was 2.7×10^7 cells/mL (5 µg/mL).

The cross-reactivity of polyclonal antisera was monitored by the inclusion of a rabbit serum control in the anthrax assay used in the Persian Gulf. Neither cross-reactivity to other bacteria, nor interference from sand was a problem encountered during the operation of the MAGIDU for the detection of anthrax or *F. tularensis*. Preliminary analysis indicated that the same assay conditions developed for anthrax could be applied in the detection of brucellosis, plague and a specific tularemia assay, and used on a single microtiter plate for all agents (Annex A, Fig. A-4).

The whole cell antibody capture ELISA to anthrax proved to be reliable in the field, but very labour-intensive. Immunoassays under development employing new technology should alleviate these problems. Nevertheless, a program developed to allow greater liaison and exchange of ideas between military medical technologists and DRES personnel should be explored. An optional 6 month to 2 year rotation in the DSD research laboratories at DRES would be ideal.

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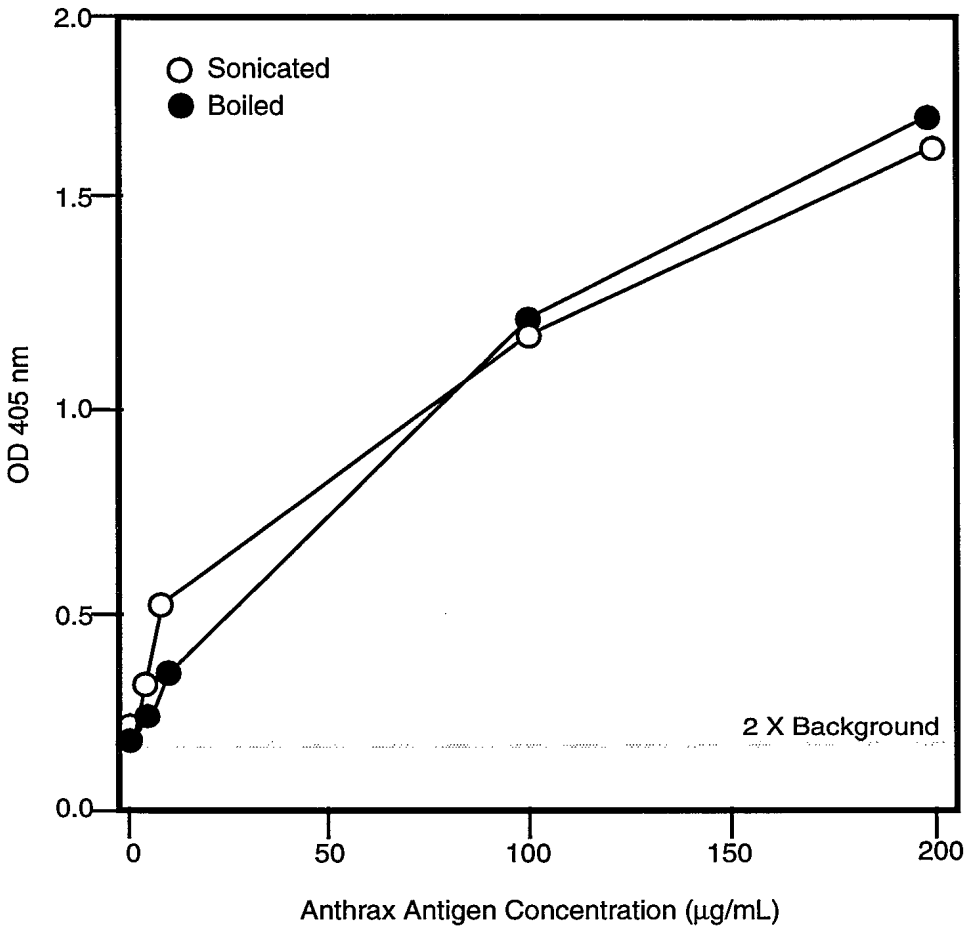
Table I- Specifications of Antisera Against Bacteria

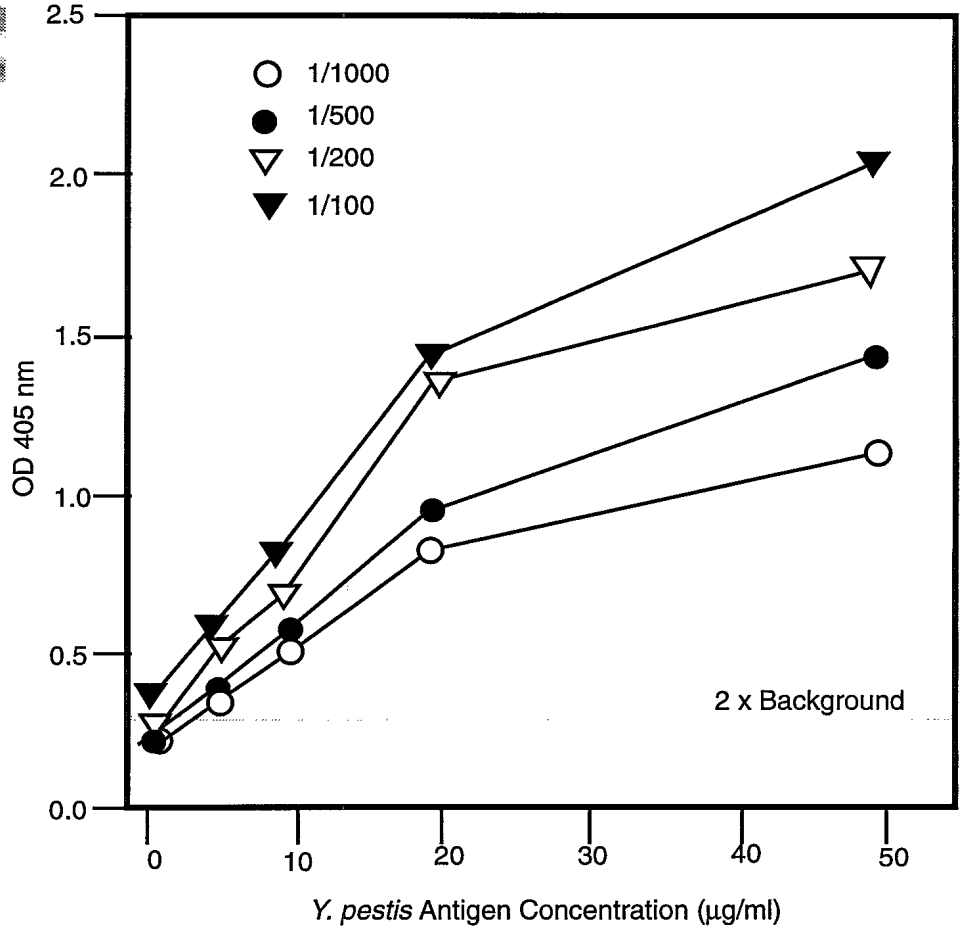
Antibody species/specificity	Date	Method of Purification	Protein (µg/mL)	Titre (reciprocal)	Comments
rb α-anthrax (Frans)	Dec.19,1990	capr. acid/$(NH_4)_2SO_4$	3.8	10,240	good @ 20 µg/mL, 55 mL
	Feb. 23,1991	capr. acid/$(NH_4)_2SO_4$	1.08		good @ 20 µg/mL, 85 mL
rb α-anthrax-HRP		capr. acid/$(NH_4)_2SO_4$	3.8	10,240	from Dec. 19 batch, not purified
rb α-amthrax	May 27, 1991	Protein G	2.0	320	poor discard
gt α-anthrax	Dec. 19, 1990	capr. acid/$(NH_4)_2SO_4$	2.6		inactive, discard, 48 mL
		crude sera	-		good @ 1/100 dilution
gt α-anthrax spore (UK)	Jan. 24,1991	protein G	6.4	-	UK, good-anthrax spores, 19 mL
gp α-anthrax	June 4, 1991	protein A	2.9	320(wk)	poor, discard
rb α-tularemia	Dec. 19, 1990	capr. acid/$(NH_4)_2SO_4$	3.1	6,400 to 12,800	good @ 30 µg/mL, 65 mL
	Feb. 23,1991		1.87		not evaluated, 85 mL
rb α-tularemia	July 29, 1991	crude sera	-	5,120	not purified
gt α-tularemia	Dec. 19, 1990	capr. acid/$(NH_4)_2SO_4$	2.1	-	not evaluated
rb α-brucellosis	Dec. 19,1990	capr. acid/$(NH_4)_2SO_4$	3.5	>25,600	good @ 10µg/mL, 83 mL
	Feb. 23, 1991		1.67		not evaluated, 85 mL
rb α-brucellosis	May 27, 1991	crude sera	-	>102,400	not purified
gt α-brucellosis	Dec. 19,1990	capr. acid/$(NH_4)_2SO_4$	2.6	-	not evaluated
gp α-plague (Y. pestis)	May 22,1991	protein A	0.67	5,120	good @ 1/100 to 1/200 dilution
			(3.6)		use Sigma a-gp-HRP, 15 mL
gp α-plague	May 15, 1991	crude sera	-	5,120	10 mL

Figure 1



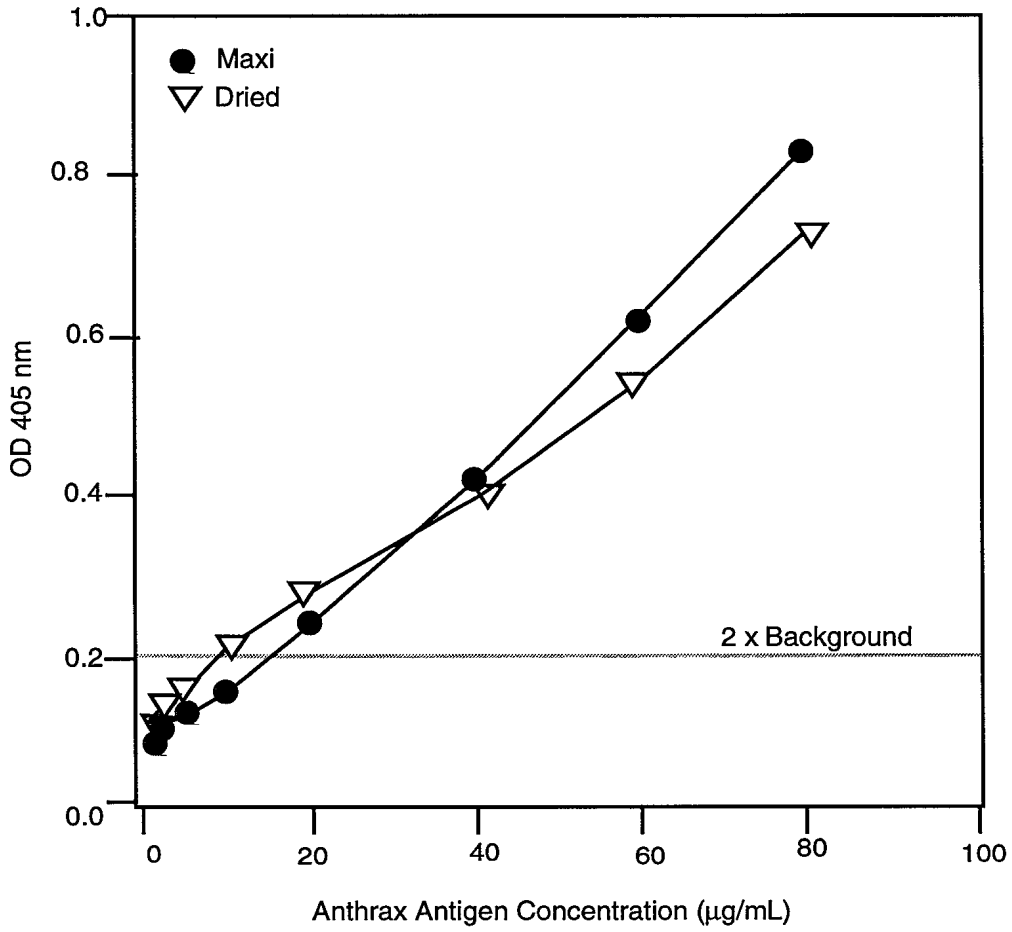
Preparation of anthrax antigen. For anthrax antibody capture ELISA (indirect), 100 µL of inactivated anthrax antigen in PBS was air-dried overnight onto Polysorb^{TM} microtitre plates, followed by heating 30 min at 60°C. Incubation times were: blocking, 60 min; rb α-anthrax (20 µg/mL), 60 min; α-rb-HRP (1/ 5000 dilution), 60 min; ABTS/H_2O2, 30 min. The antigen was sonicated (O) or boiled 15 min (●) as described.

Figure 2



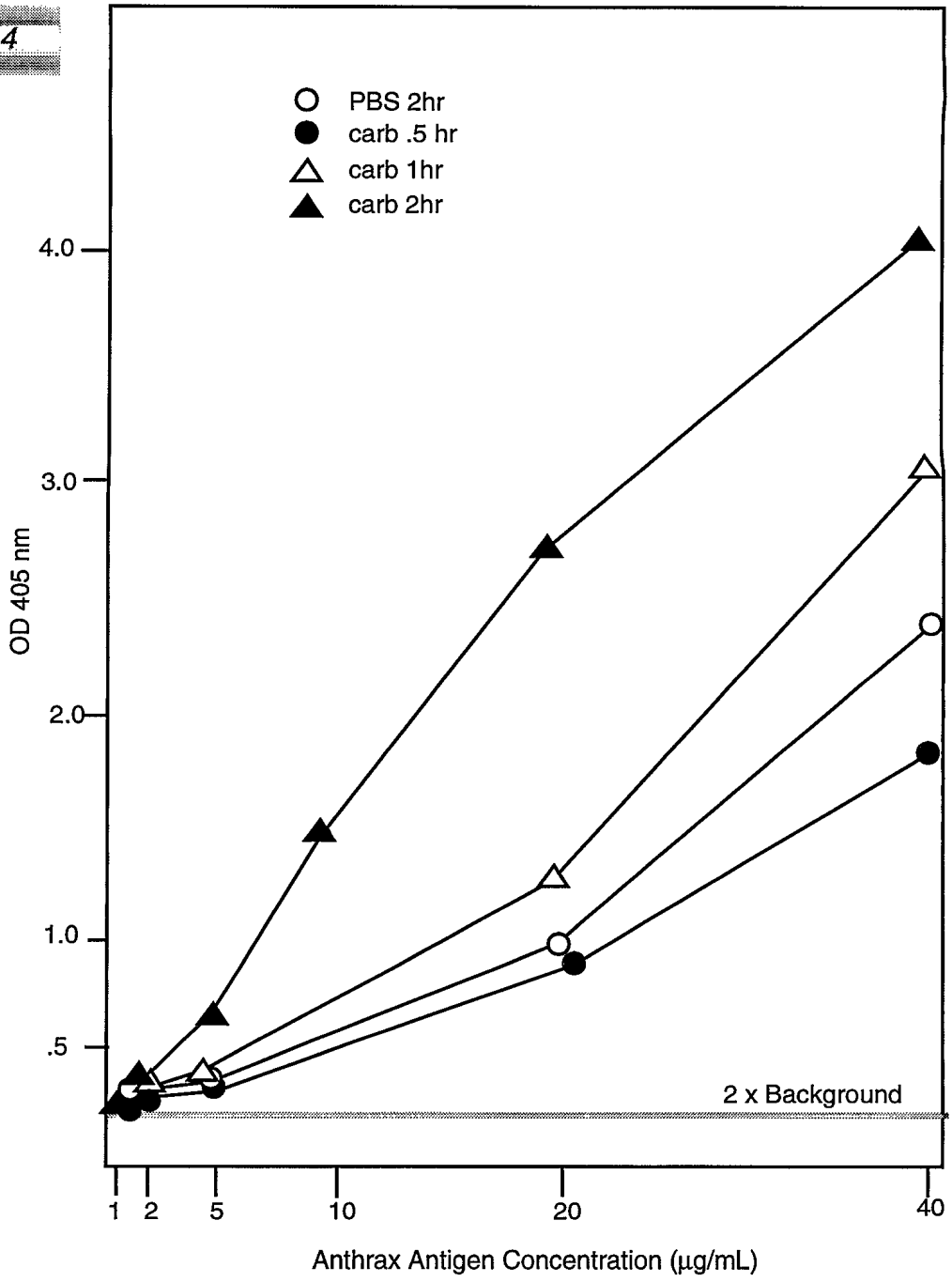
Titration of anti-plague antibody. A plague antibody capture ELISA was as described for the shortened incubation time protocol. The protein A purified gp α-plague Ab was tested at 1/100 (▼), 1/200 (▽), 1/500 (●) and 1/1000 (○) dilutions in filtered WB. The IAb used was α-gp-HRP (Sigma, St. Louis, MO) at a dilution of 1/1000.

Figure 3



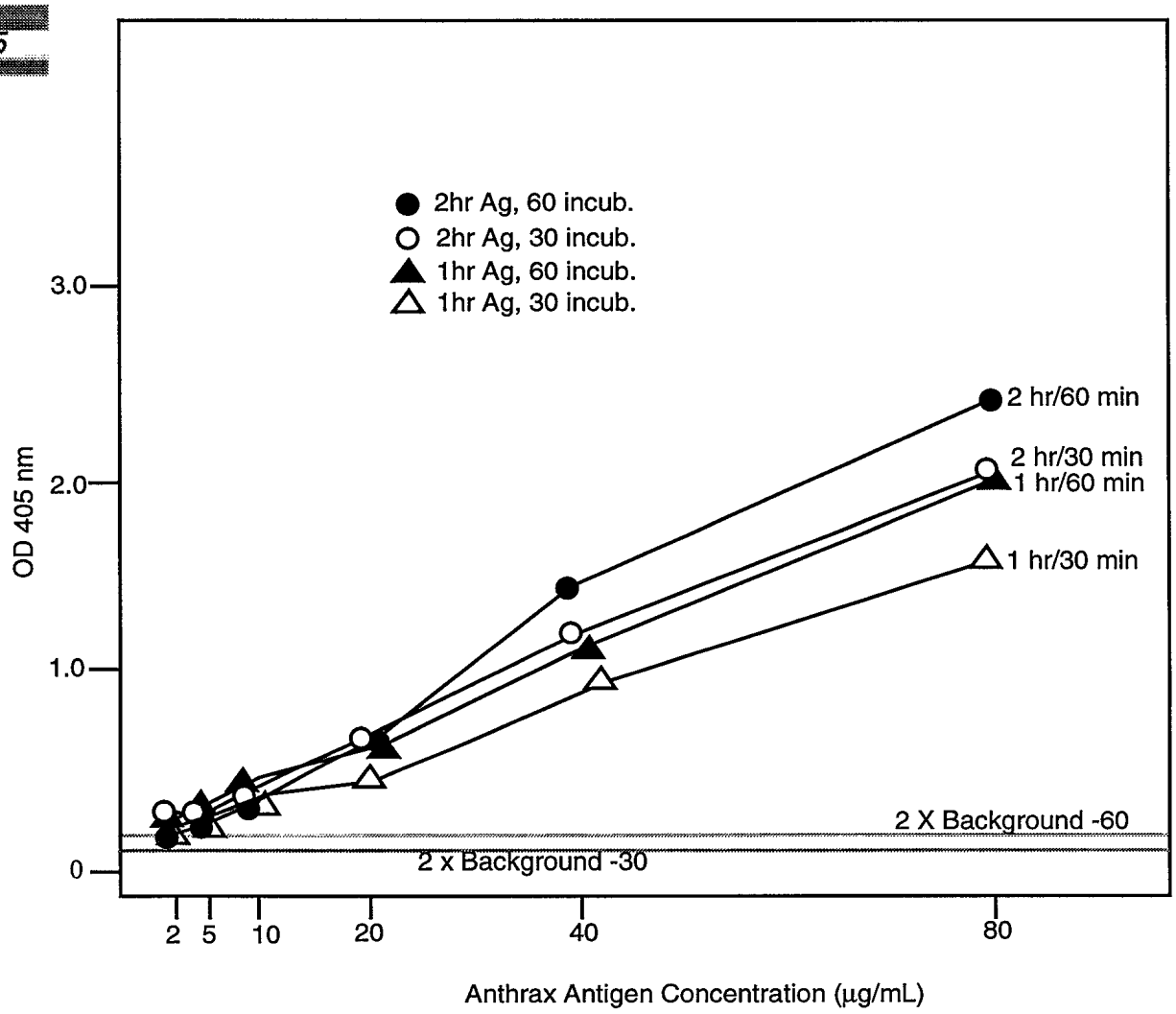
Immobilization of anthrax antigen onto microtitre plate. Boiled inactivated anthrax antigen in dH_2O, was dried down (50 µL) onto a Polysorb^{TM} plate using a hairdryer (approximately 60 min), or was allowed to attach from solution (200 µL) onto a Maxisorb^{TM} plate. Assay conditions were for the 5.5 hr antibody capture ELISA protocol, except the α-rb-HRP was at a 1/5000 dilution.

Figure 4



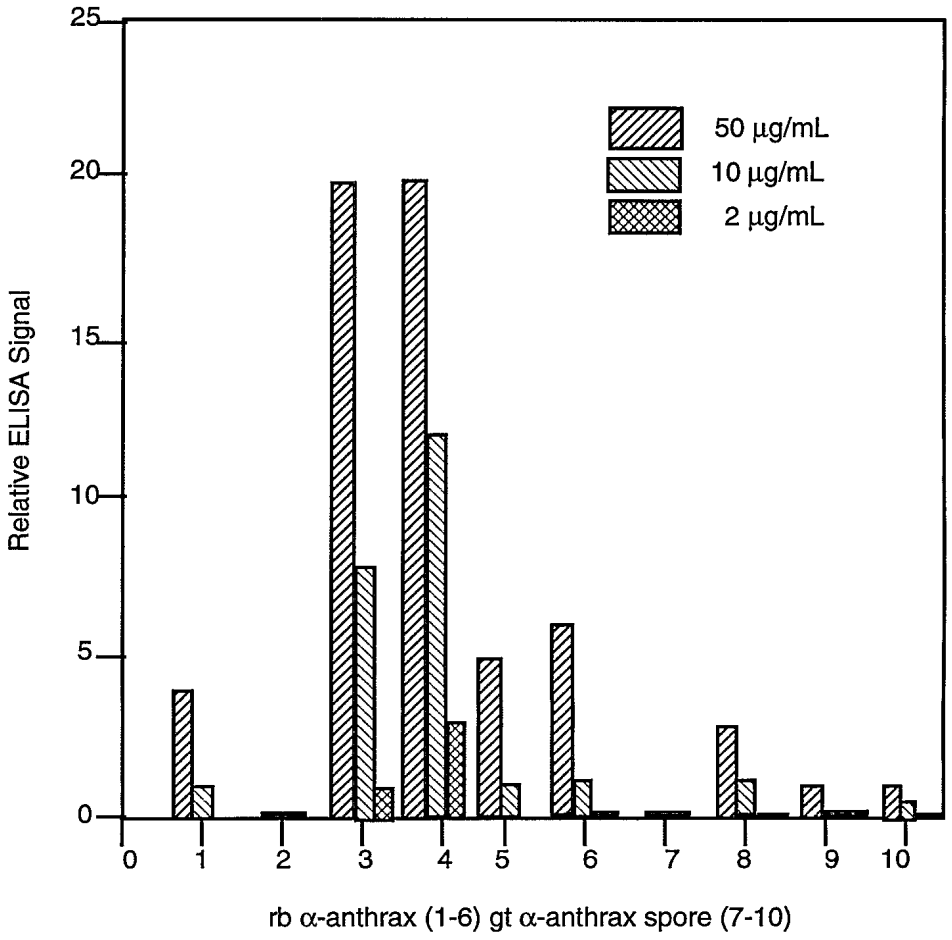
Effect of buffer on antigen immobilization. Anthrax antibody capture ELISA conditions for standard 5.5 hr assay used with the changes noted. The inactivated anthrax was diluted in PBS for and allowed to attach to Maxisorb^{TM} plates for 2 hr (○); or was diluted in carbonate buffer and allowed to attach for 0.5 (●), 1 hr (△) and 2 hr (▲).

Figure 5



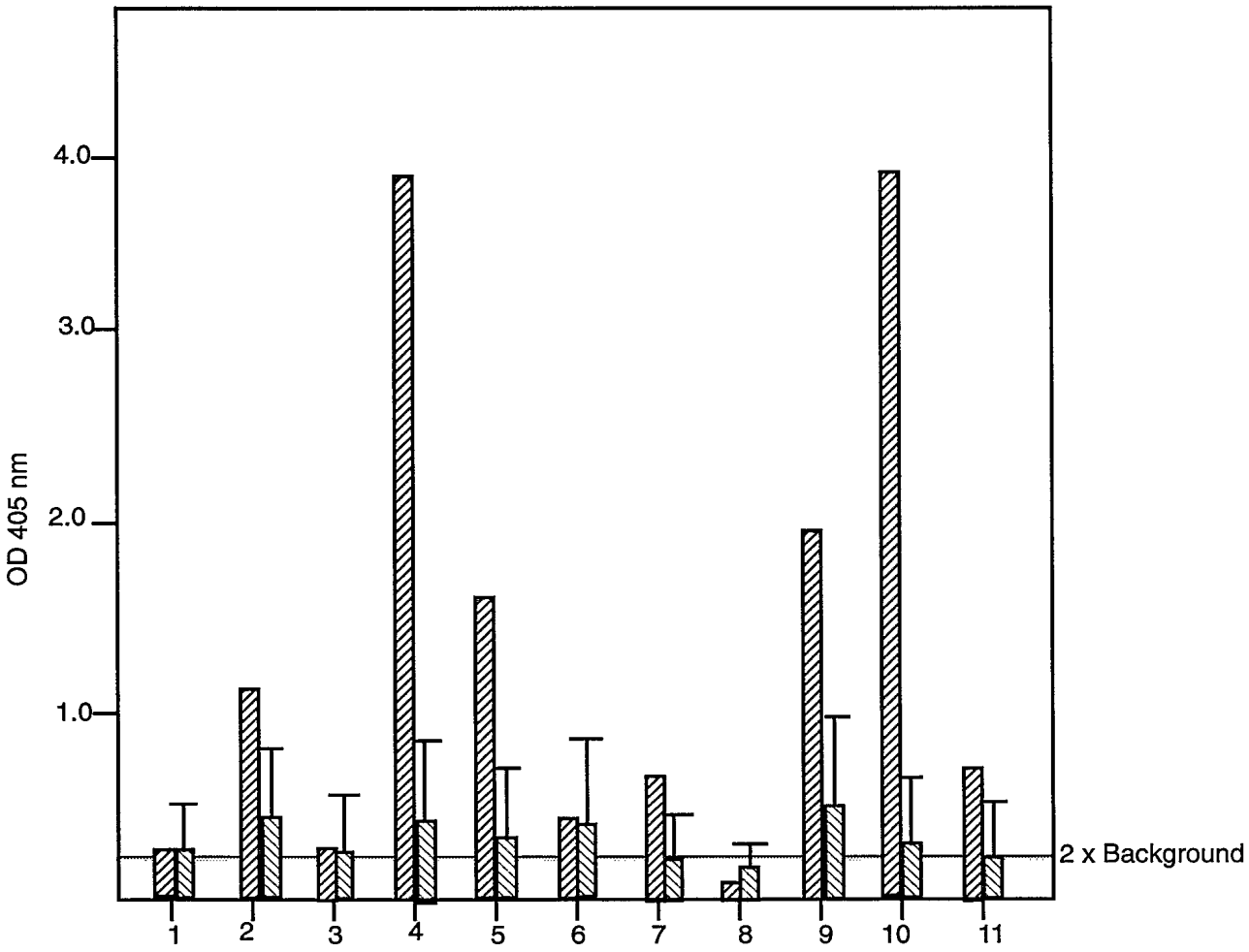
Reduced incubation times in anthrax ELISA. The anthrax antibody capture ELISA was assessed for: 1) incubation time of anthrax antigen attachment (1 or 2 hr); or incubation times of reaction (30 min or 60 min each for blocking, DAb and IAb). All other conditions were identical for the standard anthrax ELISA (5.5 hr). The following conditions were used: 2 hr antigen binding and 60 min incubation (●); 2 hr antigen binding and 30 min incubation (○); 1 hr antigen binding and 60 min incubations (▲); and 1 hr antigen binding and 30 min incubations (△).

Figure 6



Live agent testing of anthrax. The antibody capture ELISA was performed under standard conditions, except that the results were determined by visual comparison of samples (range of 0-20 used). Samples 1-6 used rb α-anthrax (20 µg/mL); samples 7-10 used gt α-anthrax spore (20 µg/mL). Samples: 1) inactivated anthrax Ag; 2) *E. coli*; 3) & 7) live Thraxol cells; 4) & 8) live Thraxol spores; 5) & 9) ciprofloxacin/heat inactivated; and 6) & 10) 1 % (v/v) formalin-treated Thraxol. Antigen concentrations used were 2, 10 and 50 µg/mL as indicated in the figure legend.

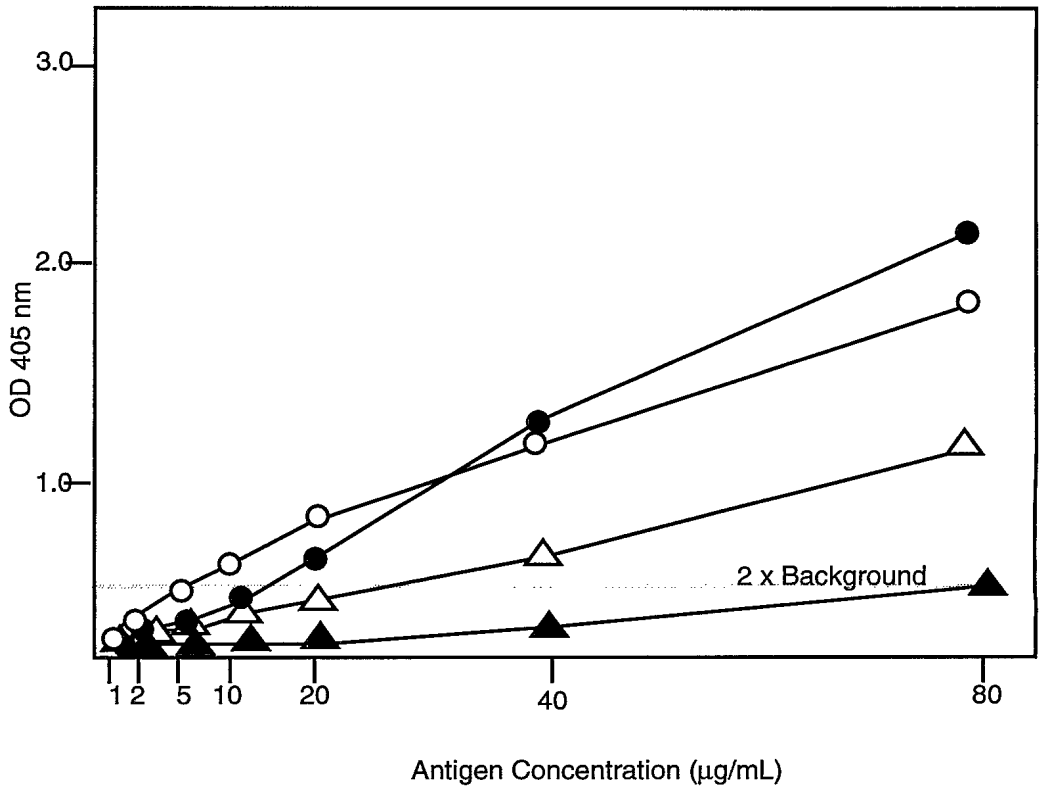
Figure 7



1	2	3	4	5	6	7	8	9	Unk.
+	+	+	+	+	+	+	-	+	N.C.
-	+	–	+	+	-	+	-	+	C.S.

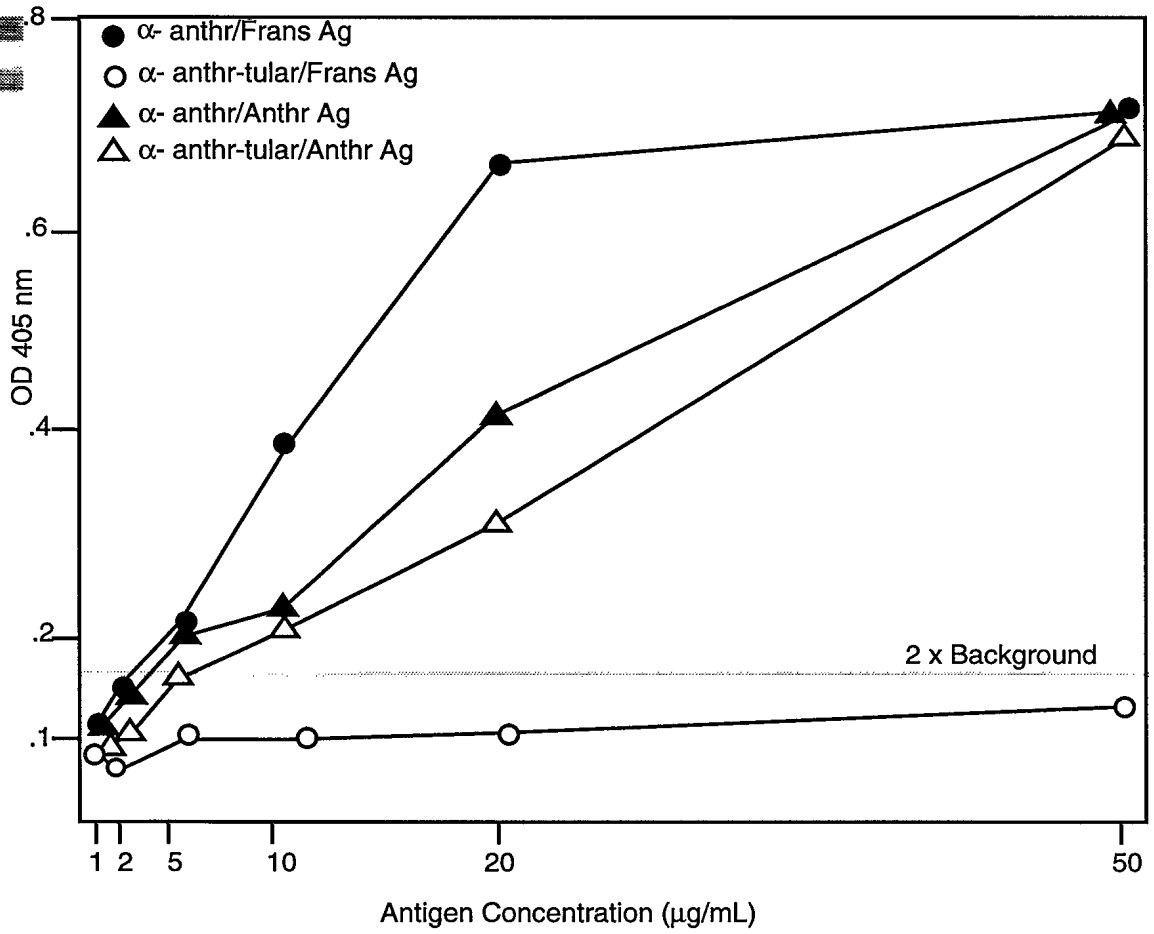
Testing of unknown samples. Conditions used were for the standard anthrax antibody capture ELISA, except only 1 hr Ag binding was used. The control serum was rb α-tularemia (20 µg/mL). Unknowns: 1) S. aureus 10 µg/mL; 2) anthrax 20 µg/mL + 1/5 sand supernatant; 3) anthrax 20 µg/mL + sand 1% (w/v); 4) anthrax 50 µg/mL + *E. coli* 10 µg/mL; 5) anthrax 20 µg/mL + *S. aureus* 10 µg/mL; 6) *E. coli* 10 µg/mL; 7) anthrax 5 µg/mL; 8) sand 1% (w/v); 9) anthrax 50 µg/mL + 1/5 sand supernatant; 10) anthrax 50 µg/mL; 11) anthrax 10 µg/mL. N.C. (negative control), C.S. (control serum).

Figure 8



Cross-reactivity of rabbit anti-anthrax antibody. Conditions for the shortened incubation time anthrax ELISA were used. Antigens were anthrax (●), F. *tularensis* (○), *B. melitensis* (▲) and *E. coli* (△).

Figure 9



Results from whole cell absorbed antisera. Conditions used were for the standard anthrax antibody capture ELISA, except additional DAbs were used with both *F. tularensis* and anthrax Ag. The following combinations of DAb and Ag were used:1) rb α-anthrax (20 µg/mL) + *F. tularensis* Ag(●); 2) rb α-anthr tular-minus (20 µg/mL) + *F. tularensis* Ag(○); 3) rb α-anthrax (20g/mL) + anthrax Ag(▲); and 4) rb α-anthr tular-minus (20g/mL) + anthrax Ag(△)

Annex A

Figure A-1

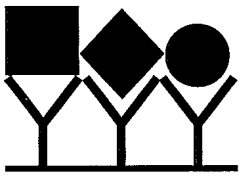
Amplified "Sandwich" Antibody

1

✦ COAT WITH "CAPTURE" ANTIBODY

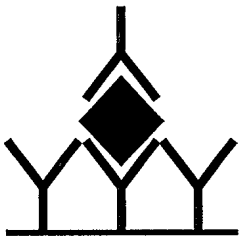
✦ WASH
✦ BLOCK UNREACTED SITES

2



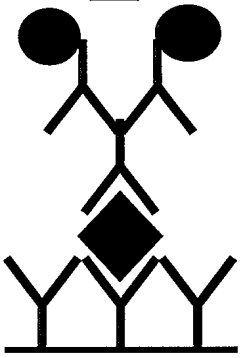
✦ INCUBATE WITH TEST SAMPLE
✦ WASH

3



✦ INCUBATE WITH "DETECTOR" ANTIBODY
✦ WASH

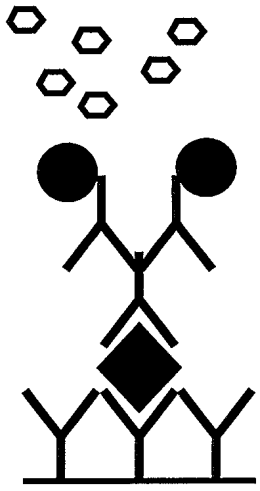
4



✦ LABELLED "INDICATOR" (ANTI-SPECIES) INCUBATE WITH HORSERADISH PEROXIDASE ANTIBODY

✦ WASH

5



✦ INCUBATE WITH ABTS (CHROMOGENIC SUBSTRATE FOR THE ENZYME)

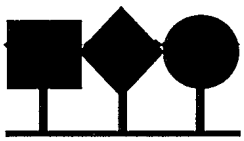
6

✦ MEASURE PRODUCT SPECTROPHOTOMETRICALLY (OR BY EYE

Figure A-2

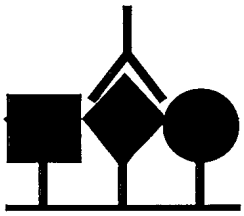
Direct Method

1



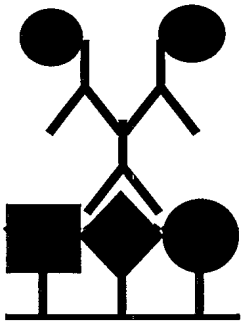
✦INCUBATE WITH TEST SAMPLE
✦WASH

2



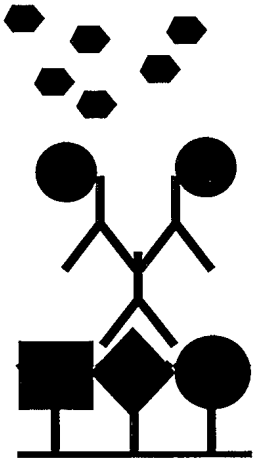
✦INCUBATE WITH "DETECTOR"
WASH
✦ANTIBODY

3



✦LABELLED "INDICATOR"
(ANTI-SPECIES) INCUBATE WITH
HORSERADISH PEROXIDASE
ANTIBODY
✦WASH

4

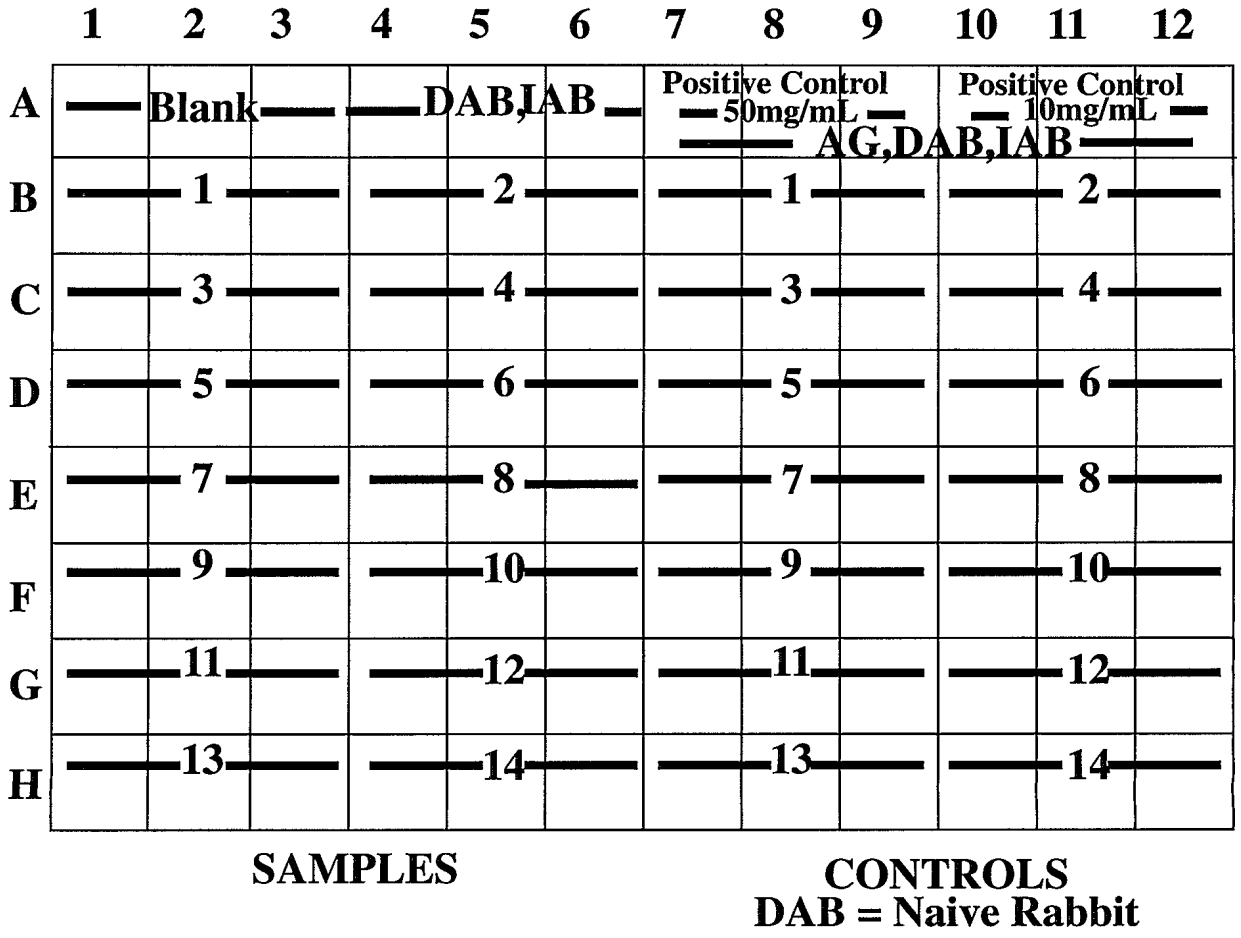


✦INCUBATE WITH ABTS (CHROMOGENIC
SUBSTRATE FOR THE ENZYME)

5

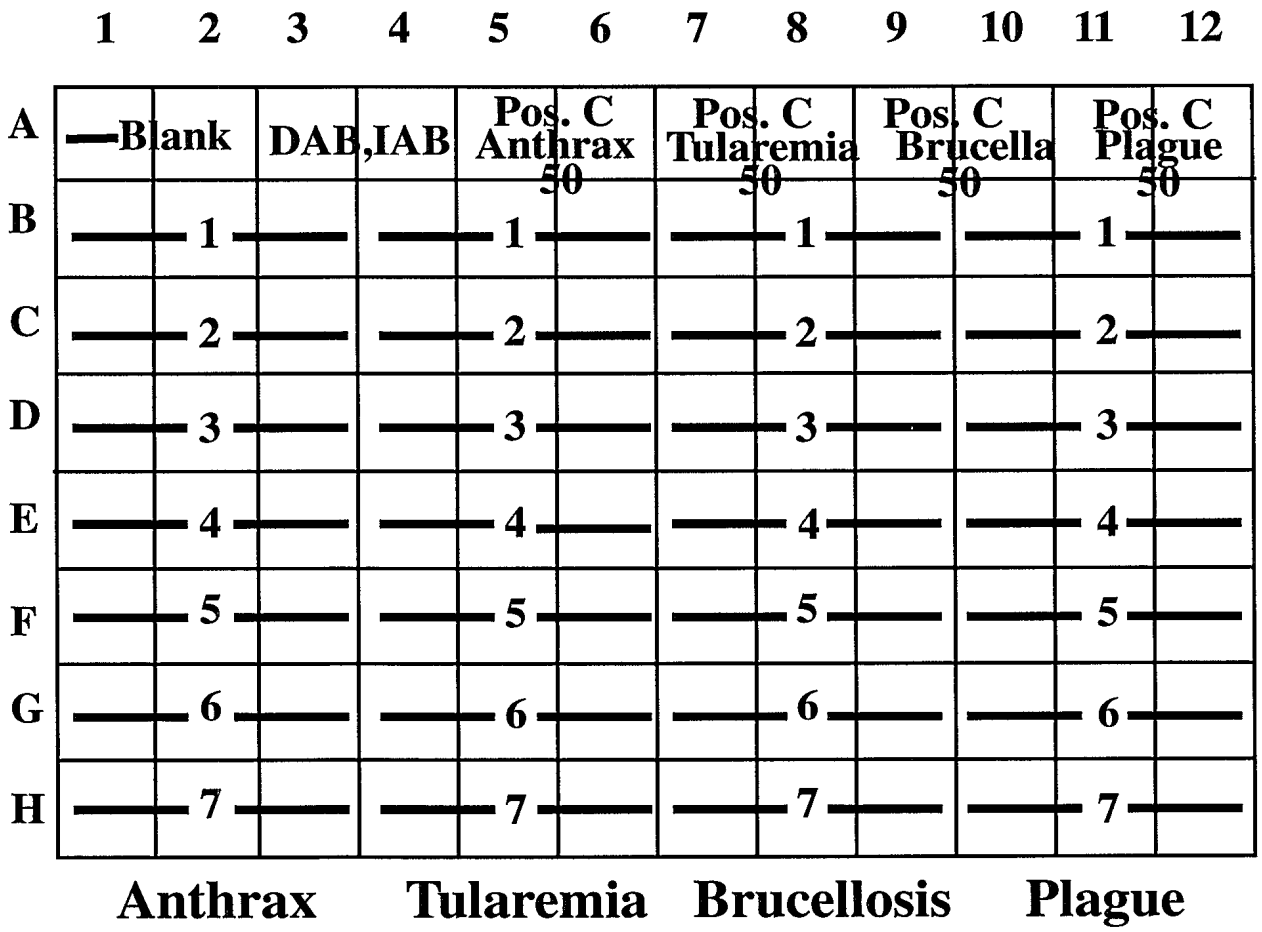
✦ SPECTROPHOTOMETRICALLY (OR BY EYE)

Figure A-3



Anthrax Immunoassay Template

Figure A-4



Four Bacterial Agent Template

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