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WELLCOME TRUST - MAHIDOL UNIVERSITY - OXFORD
TROPICAL MEDICINE RESEARCH PROGRAMME

STANDARD OPERATING PROCEDURE (SOP) of

INDIRECT HAEMAGGLUTINATION ASSAY (IHA)

for MELIOIDOSIS

Prepared by

Senior Microbiologist

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Change History:

Version 1.1, July 2008
Prepared by Senior Microbiologist *Vanaporn Wuthiekanun* /**June 2008**
Approved by Head of Microbiology *Sharon Peacock* /**July 2008**

Version 1.2, Oct 2008
Modified by *Mindy Glass and Alex Hoffmaster*, National Center for Zoonotic, Vector-borne, and Enteric Diseases, CDC /**Oct 2008**

Version 1.3, Dec 2011
Modified by Senior Microbiologist *Vanaporn Wuthiekanun* /**Dec 2011**

Limitation on use of this SOP and disclaimer

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Introduction

The indirect haemagglutination assay (IHA) is a simple serological test that can be used to detect antibodies raised by humans to *Burkholderia pseudomallei*, the cause of melioidosis. The IHA is currently the most common test used worldwide to quantify the human antibody response to *Burkholderia pseudomallei*. It has been used extensively during serosurveys, but it should be noted that the diagnostic sensitivity and specificity of the IHA in regions endemic for melioidosis are low because background seropositivity in the healthy population is high.

Preparation

Preparation of antigen

Pooled antigens are separately prepared from two clinical *B. pseudomallei* isolates (strains 199a and 207a), that were originally isolated from patients with melioidosis in northeast Thailand. The value of using two strains versus one strain has not been determined, but is done on the basis that there may be antigenic variation between strains.

1. Streaks each isolate from the -80^0C freezer vial directly onto separate Columbia agar or TSA plates to obtain single colonies. Incubate aerobically at 37^0C in air for 24-48 hours. Check purity by visual inspection of colonies.
2. Using a sterile disposable loop, touch 6 colonies and inoculate into 50ml volumes of Rice medium (see appendix) in 100 ml glass bottles.
3. Incubate loosely capped at 37^0C in air for 14 days, agitating the culture twice daily.
4. Subculture each 14-day-old culture onto Columbia agar plate to check for purity (If not pure, discard).
5. Autoclave cultures at 121^0C for 15 minutes.
6. Centrifuge at 4,000 rpm (16,000 xG) for 30 minutes, filter the supernatants using a 0.2µ Millipore filter with a 10ml syringe (Sartorius 16534 minisart 0.20µm CE non-pyrogenic sterile-EO), add phenol (final concentration 0.5%), and store at 4°C until use. Antigen is good for 2 years.

Collection and storage of sheep red cells

1. Collect sheep blood under aseptic conditions into an equal volume of sterile Alsever's solution (see appendix). Label with date and store at 4°C for no longer than one month.
2. Just prior to use in an assay, cells require washing and suspension to give an initial working dilution of 10% washed cells. Calculate volume of red cells required and wash three times in PBS (see appendix). Do not use if supernatant is still coloured after second wash. Prepare these fresh each time cells are to be sensitized. Example:
    a. Spin 20 ml Alsever's blood 12,000 x g 10 min
    b. Remove supernatant (should be clear – not lysed)
    c. Wash with 20 ml PBS. Mix (invert) and centrifuge as above (3 times).
    d. Use RBC pellet from 3rd wash to make 10% solution in PBS (i.e., 2 ml blood cells in 18 ml PBS)

Determination of optimal bacterial antigen dilutions

The optimal bacterial antigen dilution is established for each new batch of bacterial antigen. This represents a process of standardisation of antigen concentration by reference to previous batches. This is determined by testing the agglutination of a known positive serum with red cells sensitized with a dilution series of the bacterial antigen. The known positive control is pooled serum from 3 patients with culture proven melioidosis who have an established IHA titre of $\geq$ 1:160 (aim for pooled IHA of around 1:1280). The antigen from strains 199a and 207a are tested separately at this stage.

I. *Preparation of positive control serum (to remove complement and non-specific sheep cell agglutinins)*
   a. Inactivate 300µl of positive control serum in a closed eppendorf tube for 30 minutes in 56°C water bath.
   b. Add 900µl PBS-BSA and 300µl non-sensitized 10% sheep red blood cells and mix thoroughly.
   c. Incubate at room temperature for 1 hour, gently mixing every 15 minutes.
   d. Centrifuge in micro-centrifuge for 3 minutes (13,000 rpm) and retain supernatant, which represents a 1 in 5 dilution of serum. Store at 4°C for up to 24 hours or freeze at -80°C (good for up to 5 years).

II. *Preparation of bacterial antigen dilution series and sensitization of red cells.*
   a. Make 1/20 dilution of each antigen in 3.0 ml of PBS (150µl antigen plus 2.85 ml PBS) as the starting tube
   b. Set a row of 8 tubes for each antigen; make the following final dilutions of antigen in a total volume of 1ml.

Tube (5ml plain tubes)	1	2	3	4	5	6	7	8
Antigen (µl)	1000	500	333	250	200	167	143	0
Buffer (µl)	0	500	667	750	800	833	857	1000
Final dilution	1/20	1/40	1/60	1/80	1/100	1/120	1/140	0

   c. To each tube add 0.1ml of 10% washed sheep red cells in PBS, mix thoroughly (invert) and incubate at 37 °C for 1 hour, carefully mixing every 15 minutes.
   d. Centrifuge for 5 minutes at 4,000 rpm (16,000 x g) and discard supernatant (can pour off supernatant – has tight pellet).
   e. Wash the cells 3 times in 2ml PBS, and then carefully remove all supernatant.
   f. Re-suspend (invert) red cells completely in 990µl PBS-BSA to give final concentration of 1% sensitized red cells. Store at 4°C until used in titration assay (within 24-48 hours).

III. Titration

a. Using 2 x 96 U well microtitre plates (one for each strain), add 50µl of PBS/BSA into all wells of columns 1 to 12.
b. Add 50µl of absorbed positive control serum to all wells in column 1.
c. Use a multi-channel pipette to make 1:2 dilutions from column 1 to 11 for each row, discarding the final volume. The final dilution of serum should be 1:10 to 1:10,240 from column 1 to 11. Pipette up and down to mix prior to transfer of 50 ul.
d. Add 25µl of red cells from each of the antigen dilution tubes into each well of the adjacent row from column 1 to 12 (see figure below, e.g.: 25µl of cells from antigen dilution 1:20 is added to each well of row A, 25µl of cells from antigen dilution 1:40 is added to each well of row B, etc). Note that red cells in rows A to G are sensitized whilst row H contains non-sensitized red cells.
e. Tap the plate gently on each of the 4 edges to mix thoroughly, cover with aluminium foil, and leave on the bench at room temperature for 2 hours, then at 4°C overnight. Plates can be reliably read at either time point.

IV. Reading

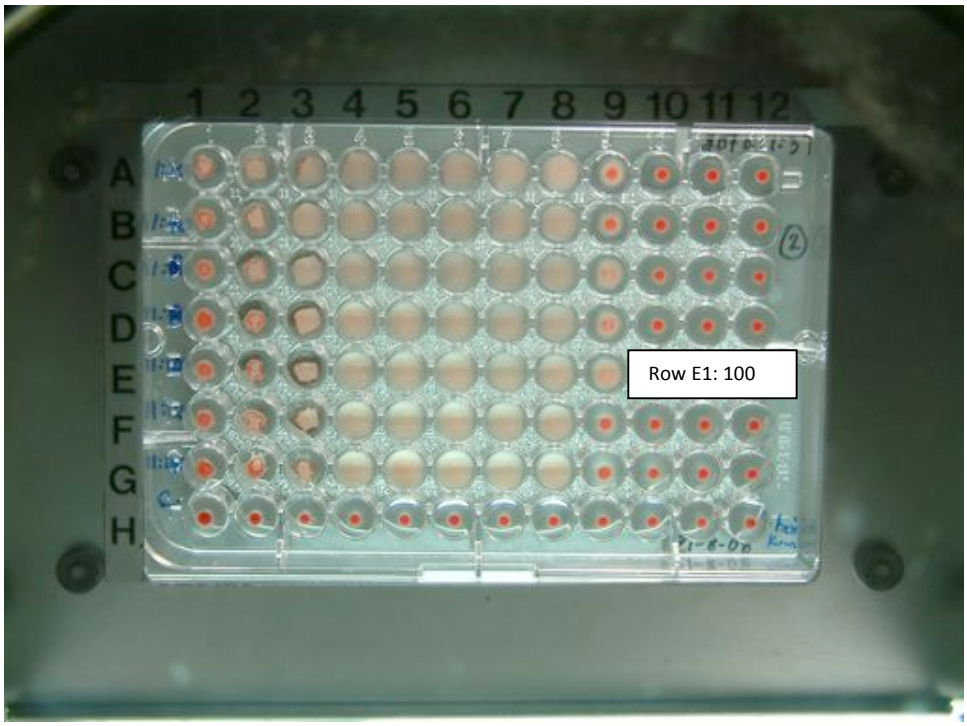
Negative wells (no red cell agglutination) have an intact button at the bottom of the well. Positive wells (red cell agglutination) demonstrate red cells settled as a fine carpet or appearing as a loose button with ragged or folded edges. The plates can be read with a reading mirror for microtitre plate with transmitted light from below. The titre recorded is the first clearly positive well; indeterminate results are recorded as equivocal and not used to define titre. There should be no agglutination in column 12 and row H.

The optimal antigen concentration is identified as the one, which gives the correct (previously known) IHA titre for the pooled serum. The optimal concentration of antigen is usually in the range of 1:80-1:120, but this may vary between bacterial antigens.

Controls: Column 12 should contain PBS-BSA and sensitized red cells but no serum (with the exception of the last well which contains non-sensitized cells). **NC (Negative control).** Row H should contain serum and non-sensitized red cells.

Diagram and Picture showing the IHA plate

Ag Dilution		1:10 1	1:20 2	1:40 3	1:80 4	1:160 5	1:320 6	1:640 7	1:1280 8	1:2560 9	1:5120 10	1:10240 11	NC 12
A 1:20													
B 1:40													
C 1:60													
D 1:80													
E 1:100													
F 1:120													
G 1:140													
H Control (buffer)													



Testing unknown serum samples

At this stage, the bacterial antigens are pooled (from 2 strains). The ratio of the volume of each antigen is adjusted so that the optimal antigen concentration of each is maintained after dilution. This is important when the two antigens do not have identical optimal concentrations.

Preparation of 10% sheep red blood cells

Centrifuge an appropriate volume of sheep red cell stock solution at 3,000 rpm 10 minutes, then discard supernatant and wash cells 3 times with PBS. Re-suspend the red cells in PBS to give approximately a 10% suspension. Do on day of use (Note: can use for a 1-2 days. Spin and make sure supernatant is clear – cells not lysed).

Preparation of sera (to remove complement and non-specific sheep cell agglutinins)

1. Inactivate 50µl serum samples in Eppendorf tubes at 56°C for 30 minutes in water bath. Add 350µl PBS/BSA and 100µl 10% non-sensitized sheep cells (final dilution 1:10).
2. Incubate at room temperature for 1 hour, mixing every 15 minutes.
3. Spin in microcentrifuge for 3 minutes (13,000 rpm) and retain supernatant (Note: can just leave pellet undisturbed – no need to remove the supernatant to separate tube).
4. Store as described previously.
5. Include known positive and negative controls if not already prepared. The negative control is pooled sera from 3 patients with no detectable IHA titre. The positive control is pooled sera from 3 patients with known positive IHA (aim for control positive value of around 1:1280).

Preparation of antigen and sensitization of red cells

The antigen solutions are mixed as follows. Make the dilution of two antigens in an appropriate volume, for example: 25µl of antigen A (optimal 1:100) and 21µl of antigen B (optimal 1:120) in total volume of 2.5ml (one plate) of PBS. Add 0.25ml of 10% washed sheep red cells in PBS, mix thoroughly and incubate at 37 °C for 1 hour mixing every 15 minutes.

During Training we did:

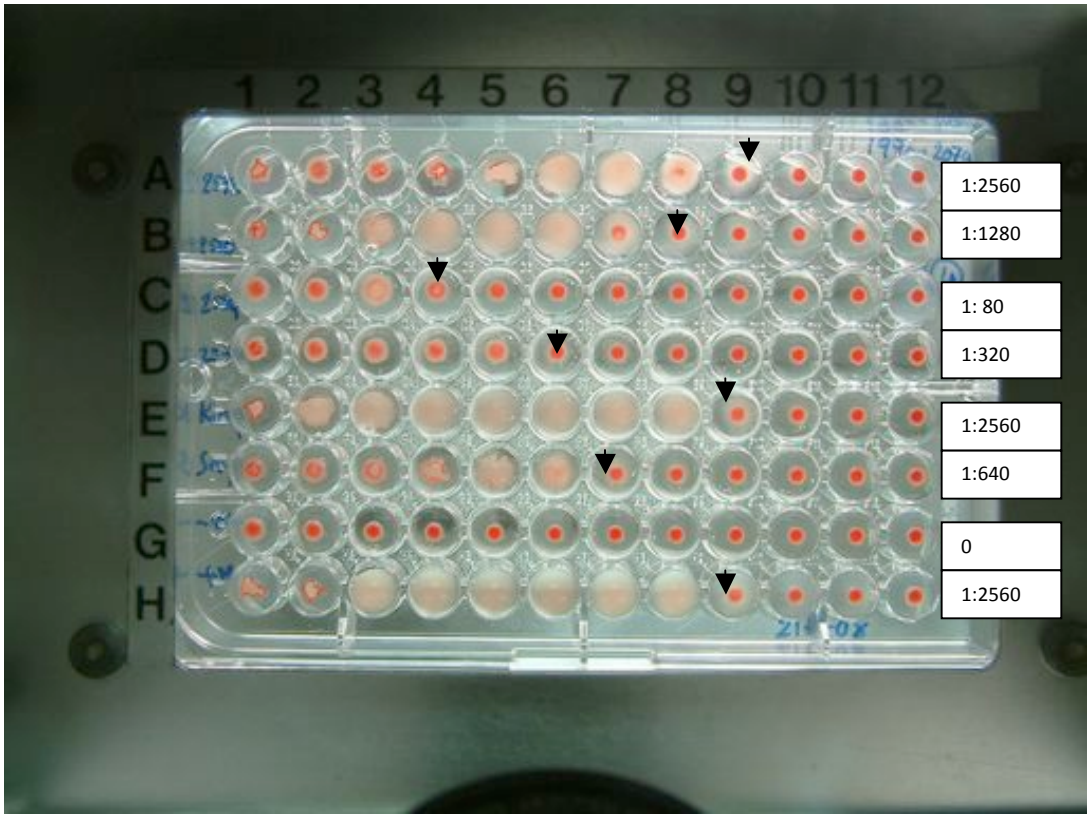
1. 1% non-sensitised cells = 18 ml PBS and 2 ml or 10% RBC
2. 1% sensitised cells = 18 ml PBS, 2ml of 10% RBC, 167 µl Antigen A (optimal 1:120) and 143 µl antigen B (optimal 1:140)
3. Centrifuge for 5 minutes at 4,000 rpm (16,000 x g) and discard supernatant. Wash three times in PBS and after third wash; completely remove supernatant by blotting on tissue, but taking care not to disturb the red cell pellet. Can pour off the majority of supernatant and remove remainder with a Pasteur pipette. Re-suspend red cells to a final volume of 2.5ml in PBS-BSA and a final concentration of 1% sensitised red cells. The control of red cell without antigen prepare the same way to get a final concentration of 1% non-sensitised red cells. Store at 4°C until use (24-48 hours).

Performance of test

1. Add 50µl of PBS/BSA into all wells of columns 2 to 11 of a 96 well microtitre plate.
2. Add 50µl of 1:10 serum into all wells of columns 1, 2 and 12. Note that the last 2 rows are the negative and positive control sera, respectively. If multiple plates are used in a given assay, controls are only required on one plate.
3. Make a two-fold dilution series from column 2 through to column 11 (using 50µl volumes) and discard remainder. The final volume in all wells should be 50µl. The serum dilution achieved should be as shown below. (Note: Try not to make bubbles)
4. Add 25µl of 1% sensitized cells to all wells of columns 1 – 11.
5. Add 25µl of non-sensitized cells to column 12.
6. Tap plate to mix, cover with aluminium foil, and incubate 2 hours at room temperature and overnight at 4°C.
7. Read agglutination patterns at either time point (they should be identical at 2 hours and overnight), and record titres.

Controls: Column 12 should show no agglutination. This is a control for non-specific red cell agglutination. Check rows containing negative control (no agglutination) and positive control. The positive control result should be identical with or no more than one dilution different from the known IHA result (1:2560).

Serum dilution	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	(control)
Column	1	2	3	4	5	6	7	8	9	10	11	12
Patient serum A												
Patient serum B												
Patient serum C												
Patient serum D												
Patient serum E												
Patient serum F												
Known negative control												
Known positive control												



Appendix

A. Rice medium

L-asparagine	0. 7	g	(Sigma A.0884)
Monopotassium dihydrogen phosphate	0 .2	g	(Sigma P5379)
Anhydrous citric acid	0 .2	g	(Sigma C0759)
Magnesium sulphate hetahydrate	0.205	g	(Sigma M5921)
Ammonium ferric citrate	0.0075	g	(BDH27163)
Sodium citrate	0.2	g	(BDH102425)
Dextrose	1	g	(DIFCO 721047)
Glycerol	5.6	ml	(Sigma G6279)

Dissolve in 100ml-distilled water and adjust pH 7.4 (1MNaOH). Autoclave at 115ºC for 15 minutes

B. Alsever s solution

Glucose	2.05	g	(Sigma G8270)
NaCl	0.42	g	(BDH102415)
Sodium citrate dehydrate	0.8	g	(BDH102425)
Anhydrous citric acid	0.055	g	(Sigma C0759)
Distilled water to	100	ml	

Sterilize at 121 ºC for 15 minutes. Check pH 6.1

C. PBS (phosphate buffered saline)

Use Dulbecco 'A' Tablets (Oxoid code BR0014g) as per instructions.

D. PBS/BSA (phosphate buffer saline containing bovine serum albumin)

Dissolve 0.06 grams BSA (Sigma A4503) in 100ml PBS as above

References

1. Ileri, S. Z. 1965. The indirect hemagglutination test in the diagnosis of melioidosis in goats. *Brit. Vet. J*. 121:164-170
2. Rice, C. E., H. Koust, and R. C. Duthie. 1951. Studies by complement-fixation methods of mulleins produced in broth and synthetic media. I. Relative immunizing activities in horses and rabbits. *Can. J. Comp. Med*. 15:284-291
3. Alexander, A.D., D.L. Huxsoll, A.R.J. Warner, V. Shepler, and A. Dorsey. 1970. Serological diagnosis of human melioidosis with indirect hemagglutination and complement fixation tests. Appl. Microbiol. 20:825-833

COSHH risk assessment - University of Oxford COSHH Assessment Form

Department Microbiology **Location of work** Bangkok	**Persons involved** Sayan Langla/ Premjit Amorchai/Vanaporn Wuthiekanun
Description of procedure Serological diagnosis of melioidosis	**Substances used** Serum or plasma
Quantities used Serum or plasma at least 50 μL	**Frequency of use** As designed
Hazards identified Human serum/ plasma could contain blood borne viruses, which could pose a risk. Wearing goggles while working on the bench.	**Could a less hazardous substance be used instead?** No **Justify not using it**

What measures have you taken to control risk? Risk is minimal

Engineering controls:

i. Transport specimens using IATA 650 specifications
ii. Work in Biological Safety Level II area
iii. Centrifuge samples in bio-contained buckets

PPE:

i. Designated white gown for BSL II work
ii. Gloves and goggles

Checks on control measures

Annual assessment of BSL2

Is health surveillance required? No	**Training requirements** i. Centrifuge **ii.** Waste disposal
Emergency procedures: Spills	**Waste disposal procedures:** 1% Virkon for 15 minutes then autoclave 121C/15

Outside BSCII – clean up using 1% Virkon	minutes

Name and position of assessor:

Dr Stuart Blacksell, Biological safety officer

Signature:

Name of supervisor (student work only)

Signature:

Name of head of department or nominee

Signature: