HEMAGGLUTINATION ASSAY

INTRODUCTION

Agglutination is demonstrated either using red blood cells or latex beads as the carrier which increases agglutination visibility, hence sensitivity.

The direct haemagglutination (HA)

If the antigen interacts directly with the carrier, red blood cells causing hemagglutination.

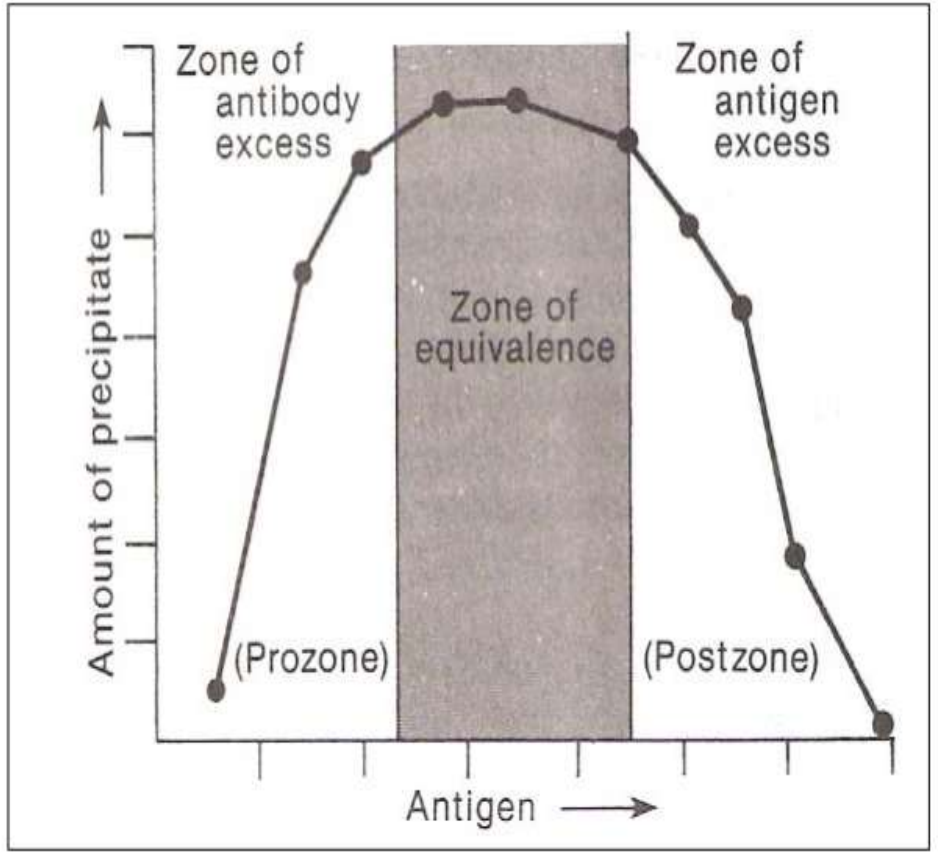

Figure-2 Effect of increasing amounts of antigen on the total immune precipitate obtained in a mixture of soluble antigen and its homologous antibody.

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Haemagglutination Inhibition assay (HI) :

Meijer et al (2006) use the ability of antigen, avian influenza virus (H7N7) to bind and agglutinate turkey or horse red blood cells to investigate presence of antibodies to H7N7 in patient's serum which prevents the hemagglutination of red blood cells by the virus

"**The HI-titre** is defined as the last dilution of serum that inhibits completely hemagglutination – exact opposite of the hemagglutination titer.

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The indirect haemagglutination (IHA):

If the carrier, either latex beads or red blood cells, is first “sensitized” coated with antigen or antibody, this is indirect agglutination (IA) or The specimen containing cognitive antibody or antigen cross links with sensitized carrier causing agglutination.

For example, Sundar et al (2005)3 coated latex beads with polyclonal antibodies against leishmanial antigen and tested for leishmanial antigen in the urine of patients. Ninety-five percent of patients with visceral leishmaniasis demonstrated agglutination.

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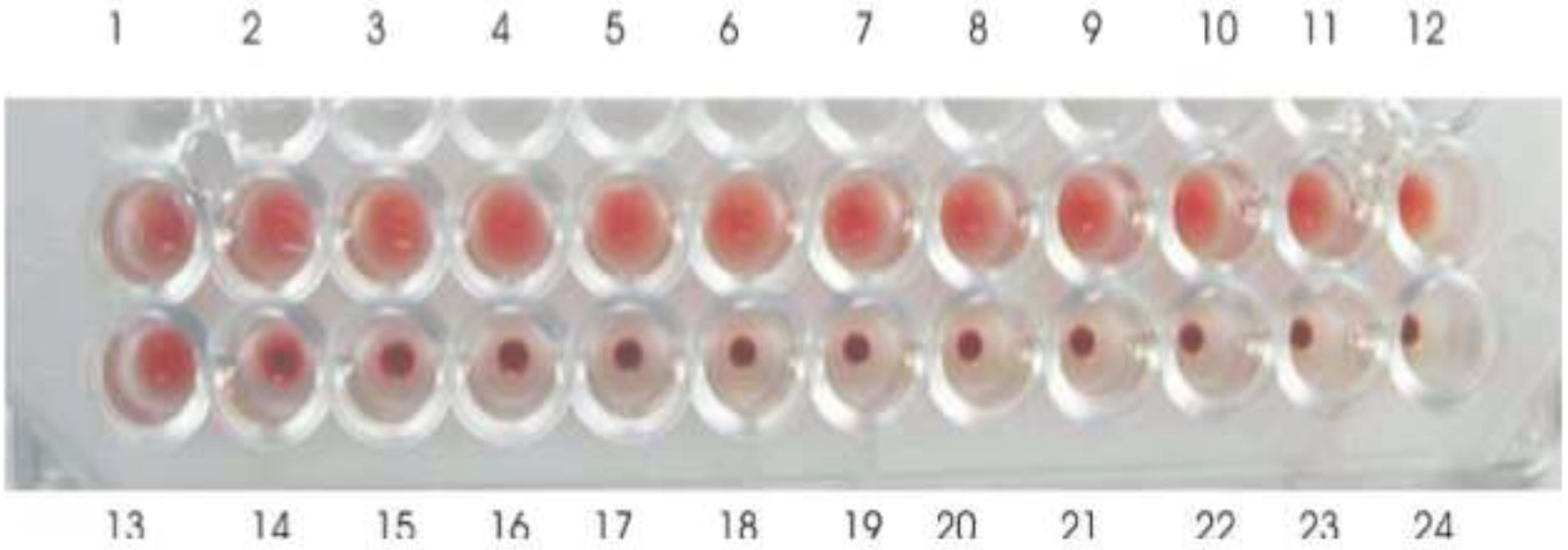
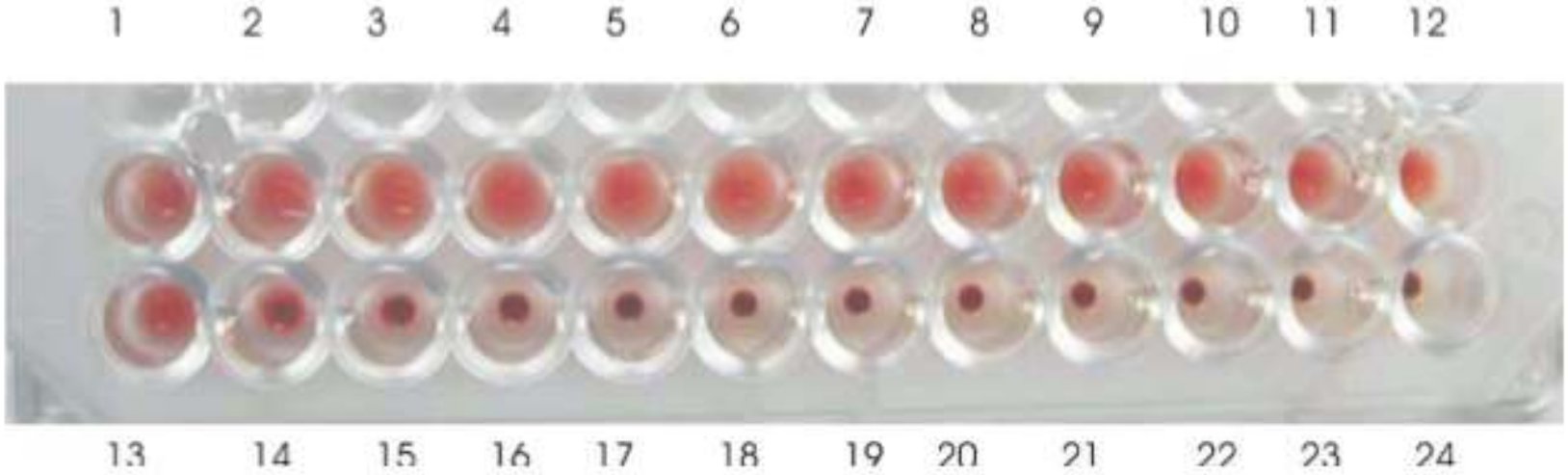
Hemagglutination titer is the reciprocal of the greatest dilution of the solution that still has a strong detectable amount of antibody (agglutination). Agglutination appears as a mat of red blood cells covering the bottom of the well.



HEMAGGLUTINATION ASSAY

Analysis of the following hemagglutination assay demonstrates titer determination.



- The reaction is **strongly positive in the wells 1 to 13**. **Wells 14 and 15** are weak reactions (considered negative). The solid button of settled red blood cells in wells 16 to 23 are negative reactions. Well 24 is the control negative well. **The titer well is 13**. If the solution that is being titered is diluted 2 fold starting well 1 then the titer is $2^{13} = 8192$.
- On occasion, the first few wells may not form mats; this is due to too much Ab and should be ignored for titre calculation.

An Example of Direct Haemagglutination (HA)

Usage of *Helix ponatia* lectin, a glycoprotein which binds and non-covalently cross-links red blood cell via specific surface carbohydrate determinants.

Diagram of 96 well hemagglutination plate design and the specific wells used (shaded). A1 to B12 assay and duplicate E1 to F12.

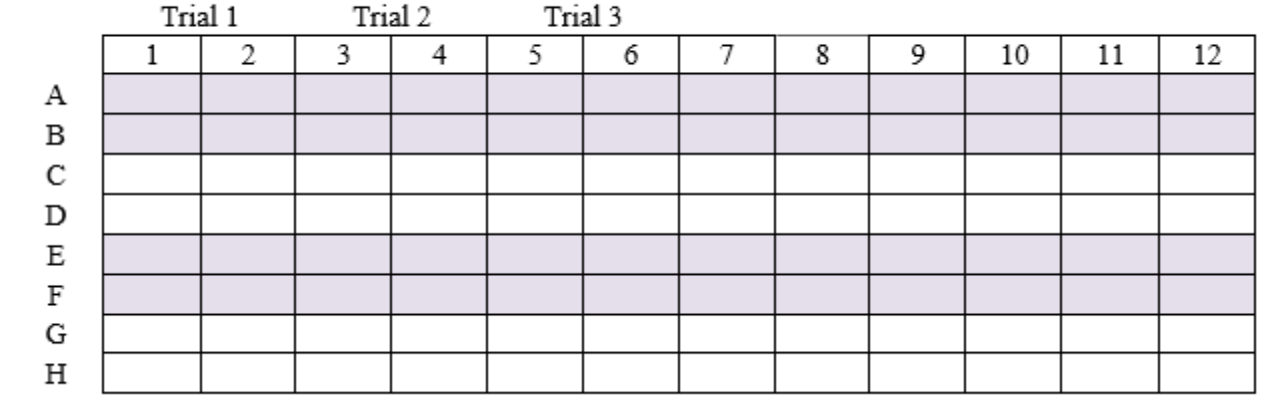


Table 1. Titre and concentration determination at titre of lectin, *Helix ponatia*, by direct hemagglutination.		
Trial	Titre well	lectin, *Helix ponatia*, concentration at titre (µg/ml)
1 (A1-B11)		
2 (E1-F11)		

INDIRECT HAEMAGGLUTINATION ASSAY (IHA)

for MELIOIDOSIS

(*Burkholderia pseudomallei*)

INDIRECT HAEMAGGLUTINATION ASSAY (IHA)

- Introduction

The **indirect haemagglutination assay (IHA)** 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*.

INDIRECT HAEMAGGLUTINATION ASSAY (IHA)

Preparation

A . Preparation of antigen: Pooled antigens are separately prepared from two clinical *B. pseudomallei* isolates (strains 199a and 207a).

1. Streaks each isolate from the –80^{o}C freezer vial directly onto separate Columbia agar or TSA plates to obtain single colonies.
2. Incubate aerobically at 37 ^{o} C in air for 24-48 hours. Check purity by visual inspection of colonies.
3. Using a sterile disposable loop, touch 6 colonies and inoculate into 50ml volumes of Rice medium in 100 ml glass bottles.
4. Incubate loosely capped at 37 ^{o} C in air for 14 days, agitating the culture twice daily.
5. Subculture each 14-day-old culture onto Columbia agar plate to check for purity (If not pure, discard).
6. **Autoclave cultures at 121 ^{o} C for 15 minutes.**
7. **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.

INDIRECT HAEMAGGLUTINATION ASSAY (IHA)

B. Collection and storage of sheep red cells:

1. Collect sheep blood under aseptic conditions into an equal volume of sterile Alsever's solution.
2. Label with date and store at 4°C for no longer than one month.
3. Just prior to use in an assay, cells require washing and suspension to give an initial working dilution of 10% washed cells. 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)

INDIRECT HAEMAGGLUTINATION ASSAY (IHA)

C. Determination of optimal bacterial antigen dilutions:

The optimal bacterial antigen dilution is established for each new batch of bacterial antigen.

The known positive control is pooled serum from 3 patients with culture proven melioidosis who have an established IHA titre of > 1:160 (aim for pooled IHA of around 1:1280).

The antigen from strains 199a and 207a are tested separately at this stage.

INDIRECT HAEMAGGLUTINATION ASSAY (IHA)

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).

INDIRECT HAEMAGGLUTINATION ASSAY (IHA)

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

INDIRECT HAEMAGGLUTINATION ASSAY (IHA)

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).

INDIRECT HAEMAGGLUTINATION ASSAY (IHA)

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. 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.

INDIRECT HAEMAGGLUTINATION ASSAY (IHA)

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.

INDIRECT HAEMAGGLUTINATION ASSAY (IHA)

Diagram 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)													

Controls:

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

Row H should contain serum and non-sensitized red cells.

INDIRECT HAEMAGGLUTINATION ASSAY (IHA)

Picture showing the IHA plate