Zone of Inhibition Assay
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Overview: This assay measures the antibacterial activity in a sample of haemolymph in vitro. It requires undiluted blood, and since it measures an induced component of insect immunity, it should be attempted first with animals that have received an immune challenge 12-24 hours previously (see Haine et al. 2008). Basically a thin agar plate is poured which has the bacterium *Arthrobacter globiformis* distributed throughout. Holes are punched in the agar and a sample of undiluted haemolymph (0.5 – 1 µl) is placed in each of these wells. The haemolymph will diffuse through the agar and will become more dilute the further from the well it is. At a certain distance from the well the antimicrobial activity of the haemolymph sample will be at the minimum concentration to inhibit the growth of *A. globiformis*. After a few nights of incubation you should be able to see where this minimum concentration has been reached, because there will be a clear circle around the well. Beyond this clear zone the agar is cloudy because the bacteria have grown. The higher the concentration of antimicrobial substances in the haemolymph sample, the larger will be the clear zone around the well. This is a very useful assay for getting a quick measure of how much general antibacterial activity there is in a sample but is no good if you are interested in specific antimicrobial peptides. This is because whatever you have used to challenge your insect, what you are measuring using this assay is the component of the antimicrobial response that stops *A. globiformis* from growing. If you need to know whether the antimicrobial response is effective against different bacteria then I suggest using the liquid killing assay. I have tried to get the zone of inhibition assay working using a variety of different bacteria and haven’t had much luck (but someone else might have better luck than me!).

Materials:
Stock *Arthrobacter globiformis* bacteria
LB agar plates
Agar broth media

Special lab equipment needed:
Incubator
Waterbath
Glass Pasteur pipette and nipple
Plastic bags
Petri dishes

Methods
Preparing bacteria for assay:
Take a scrape of *Arthrobacter globiformis* bacteria from glycerol stock and spread directly onto an LB agar plate (no antibiotics). Incubate 1-2 days at 30C.

Prepare overnight culture of bacteria in broth:
Pick one colony of *A. globiformis*, add to approximately 10 ml of LB broth; incubate overnight at 30C with shaking at 150 rpm.
Prepare agar for ZI plates:
Make 1% agar e.g. add 0.5 g agar to 50 ml broth, autoclave, then put in waterbath at 44C. Wait around 30 minutes for temperature to stabilise. Inoculate with bacteria – I find that adding 100-150 µl of overnight *A. globiformis* culture to 50 ml broth gives plates of the right concentration, but it is a bit trial-and-error (see top tips). Mix bacteria into agar by swirling (do not shake as this can introduce bubbles).

Pour plates and punch holes:
Use 5 ml (autoclaved 5ml pipette tips) solution for each Petri dish. Leave with lid on to solidify for half an hour. Store plates in fridge if not using immediately. Just before using the plates, punch holes using sterile (i.e. dipped in ethanol and waved through Bunsen flame) glass Pasteur pipette and nipple. The maximum number of holes recommended on a Petri dish is 12.

Loading samples:
Use 0.5 – 1 µl of sample per hole. Record which hole contains which sample, but on lid rather than base because you need to see through the base and agar when you measure the diameters. Alternatively you can give each plate a unique code and fill out a corresponding sheet with the template of the plate marked on it. The best thing to do is draw a line from lid to base so you can fix lid-base orientation and then mark lid (also add this to the sheet). Incubate for 48 hours at 30C in a plastic bag with the lids down and you should be able to see clear zones where bacteria have not grown.

Measuring zones:
Either the diameter or the area of the zone of inhibition can be measured. The best way is to use the measure tool in an image analysis programme, but fine-scale callipers can also be used. Bear in mind that the smallest zone may be only 2 mm across and zones can be as large as 30 mm in diameter. I found it useful to take three measures of the diameter of each zone and use the mean of the three measures as my data point.

Top tips:
No zone does not necessarily mean no AMPs, the assay might not be sensitive enough, or you haven’t sampled at the right time after challenge. I would be surprised if there were no AMPs present in insect blood 24 hours after you injured it!
The minimum volume you can put in a well is half a microlitre, more than one microlitre spills out.
Have a few dry runs of the assay to get the right volume of overnight bacterial culture to add to the agar. If you have too few, large colonies, you won’t be able to clearly distinguish where the clear zone starts and finishes. If there are too many colonies then you may find that the bacteria swarm over the surface of the plate, or have a second growth spurt, thus obscuring the zone.
Finally, if you have a sample that has previously generated good zones, it is useful to hold on to it to use as a positive control.

References