Improved *ex vivo* method for microbiocidal activity across vertebrate species

Susannah S. French* and Lorin A. Neuman-Lee

Department of Biology, Utah State University, 5305 Old Main Hill, Logan, UT 84322-5305, USA

*Author for correspondence (sfrench@biology.usu.edu)

Summary

The field of ecoimmunology is currently undergoing rapid expansion, whereby biologists from a wide range of ecological disciplines are increasingly interested in assessing immunocompetence in their study organisms. One of the key challenges to researchers is determining what eco-immune measures to use in a given experiment. Moreover, there are limitations depending on study species, requirements for specific antibodies, and relevance of the methodology to the study organism. Here we introduce an improved *ex vivo* method for microbiocidal activity across vertebrate species. The utility of this assay is that it determines the ability of an organism to remove a pathogen that could be encountered in the wild, lending ecological relevance to the technique. The applications of this microbiocidal assay are broad, as it is readily adaptable to different types of microbes as well as a wide variety of study species. We describe a method of microbiocidal analysis that will enable researchers across disciplines to effectively employ this method to accurately quantify microbial killing ability, using readily available microplate absorbance readers.

© 2012. Published by The Company of Biologists Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial Share Alike License (http://creativecommons.org/licenses/by-nc-sa/3.0).

Key words: Ecoimmunology, Immunity, Bacteria, Complement activity

Introduction

It is becoming increasingly apparent that immune responses play an important role in an organism’s physiological, biochemical, and behavioral responses to its environment and thus have the potential to shape the evolution of life history strategies (Boughton et al., 2011). “Immunocompetence”, an individual’s capacity to mount an appropriate immune response following exposure to a pathogen, is a critical aspect of disease resistance and thus survival (Graham et al., 2011). Therefore, biologists from a wide range of ecological disciplines are increasingly interested in assessing immunocompetence in their study organisms. However, one of the major challenges to researchers is determining what measures to use in a given experiment (Demas et al., 2011). Further there are limitations depending on study species, requirements for specific antibodies, and relevance of the methodology to the study organism.

The microbiocidal assay historically referred to as the bacterial killing assay, measures the capacity to fresh whole blood or plasma to kill microbes *ex vivo* (Millet et al., 2007; Tieleman et al., 2005). However, the utility of this method goes beyond measuring bacterial killing to many different types of microbes and we will therefore refer to it heretofore as the microbiocidal assay. One of the primary benefits of using the microbiocidal assay instead of other measures of immune function is that it determines the ability of an organism to remove a pathogen that could be encountered in the wild. This provides an environmentally-relevant immune response. Additionally, several immune components are measured in this immune challenge. Phagocytes (e.g., macrophages, heterophils, and thrombocytes), opsonizing proteins (complement and acute phase proteins), and natural antibodies (predominantly immunoglobulins M and A, IgM and IgA) can be assessed, depending on the type of microbe and whether whole blood or plasma is used. Consequently, a major advantage to this method of immune function is that a variety of different microbes can be used to test functional responses of different specific immune components. For example, unlike many other immune measures, such as total hemolytic complement activity, the killing of the bacteria *Escherichia coli* also relies on the presence of natural antibodies and phagocytes, providing a more integrative measure of immunity while also providing an indication of complement activity. These benefits are in contrast to many other assays that only assess isolated immune components (e.g., lymphocyte proliferation) or responses to relatively artificial antigens and/or mitogens, (e.g., phytohemagglutinins).

Further advantages to this method are that no specific antibodies are required for this procedure. Therefore, the microbiocidal assay is very adaptable, not species specific, and can be used in a number of species. For example, in the current paper we have validated this assay on non-traditional amphibian (rough skinned newts, *Taricha granulosa*), reptilian (garter snakes, *Thamnophis elegans*), avian (house finches, *Carpodacus mexicanus*), and mammalian (coyotes, *Canis latrans*) species. The selection of this wide range of taxa, with different life histories, from a variety of environments, and with varying blood volumes, helps to demonstrate the applicability of the microbiocidal assay across a range of different taxa.

Additional advantages to the microbiocidal assay are its simplicity, short duration, small sample volume requirements, and that it requires only a minimal amount of specialized applications of this microbiocidal assay are broad, as it is
Microbicidal activity across vertebrates

House finches
Six wild house finches (Carpodacus mexicanus) were passively caught in potter traps from a site near California Polytechnic State University, San Luis Obispo, California. 30 μl blood samples were obtained via puncture of the alar vein with a sterile 26 gauge needle and blood was collected into microhemorheocrit capillary tubes, and transferred to sterile 1.5 ml tubes.

Garter snakes
Thirteen laboratory-housed garter snakes (Thamnophis elegans) were bled via the caudal vein using sterile 26 gauge syringes. 50 μl blood samples were transferred to sterile 1.5 ml tubes.

Side-blotched lizards
Six individual lizards (Uta stansburiana) were captured via noosing and baseline blood samples of 20 μl were collected from the retro orbital sinus using a heparinized capillary tube within 3 minutes of capture. Blood samples were transferred to sterile 1.5 ml tubes.

News
Six laboratory-housed rough skinned newts (Taricha granulosa), were sampled via tail snips with sterile surgical blade, 30 μl of blood was then collected from the caudal vein into microhemorheocrit capillary tubes and transferred to sterile 1.5 ml tubes.

Microbiocidal activity
For validation of this new microbiocidal technique we chose a wide range of environmental conditions, employ different life-history strategies, age, and parasite load). While this variation allows for considerable comparison across different organisms in different contexts, it is necessary to optimize dilutions of the sample and microbe strain prior to conducting the full assay (Buehler et al., 2008; Matson et al., 2006; Rubenstein et al., 2008; Ruiz et al., 2010). The plating of samples on agar plates and manually counting microbial colonies, while standard in immunological research, is time consuming, requires comparatively large amounts of samples, and can be less reliable. In response to these challenges, Liebl and Martin introduced a new method that quantifies microbial colonies using a nanodrop spectrophotometer (ThermoScientific; Wilmington, DE) (Liebl and Martin, 2009). This new approach significantly reduced variation among samples and reduced the amount of necessary sample used in the assay. However, access to nanodrop spectrophotometers is limited at some institutions making it difficult to perform the assay, and the correlation between nanodrop and the traditional agar plate analysis is not ideal (i.e., r=0.458), limiting its utility as a proxy for actual bacterial killing (Liebl and Martin, 2009). Here we introduce a new variation, the microbiocidal assay that is adapted from Liebl and Martin for use on a microplate reader and will enable researchers across disciplines to effectively employ this method to accurately quantify microbial killing ability, using readily available microplate absorbance readers (Liebl and Martin, 2009).

Materials and Methods
Species selection and blood sampling
For validation of this new microbiocidal technique we chose a wide range of species across different taxa. These species were chosen because they inhabit a wide range of environmental conditions, employ different life-history strategies, are a mixture of field sampled and laboratory-housed, and have varying blood volumes. This chosen range of diversity should clearly demonstrate the flexibility and wide applicability of the microbiocidal assay.

Coyotes
Three kennel-housed coyotes (Canis latrans) were manually restrained and 1 ml of blood was collected via the cephalic vein using a sterile 23 gauge syringe and transferred to sterile 5 ml tubes.
broth to all wells, and included the positive and negative controls. We again vortexed (10 s per well) the plate for 1 min and read the plate using the microplate reader (BioRad xMark® Microplate Absorbance Spectrophotometer) at 300 nm for E. coli and S. aureus and 340 nm for C. albicans to determine the background absorbance. Finally, the plates were incubated at 37°C for 12 hours or E. coli and S. aureus, and 30°C for 24 hours for C. albicans.

**Using whole blood**

The protocol for using whole blood was very similar to plasma except during the plating stage. We added 2 µl of the blood sample to 16 µl (1:8 dilution) of CO2-independent media plus 4 mM L-glutamine (media Gibco NO. 18,045; L-glutamine Sigma-Aldrich NO. G3126). For the whole blood procedure CO2-independent media was used instead of PBS to dilute samples. The positive and negative controls each received 2 µl PBS and 16 µl CO2-independent media plus 4 mM L-glutamine. Lastly, we added 6 µl bacteria (prepared as described above) to each sample and positive controls. The negative controls received an additional 6 µl of PBS. The remainder of the protocol is identical to that of the plasma assay.

**Reading Plate**

After the sample/bacteria solution has incubated for the appropriate time (12 h for E. coli and S. aureus and 24 h for C. albicans, optimized as described below), we used a 340 nm absorbance filter was best at measuring absorbance readings from the absorbance readings (i.e., 12 and 24 hour readings). Microbicidal capacity was calculated as one minus the mean absorbance for each sample (samples were run in triplicate, divided by the mean absorbance for the positive controls (wells containing only bacterial broth), and multiplied by 100 (i.e., % bacteria killed relative to the positive control). The negative controls were used to ensure that there was no contamination but not used in the final calculation. Therefore, the negative control absorbance values should not vary between the background and the post-incubation read.

**Optimization of bacterial growth and absorbance**

Prior to testing microbicidal ability of plasma we optimized incubation (interval to log phase growth) and bacterial concentration. Following Liebl and Martin, we used a concentration for E. coli and S. aureus of 10^7 and 10^8 colony forming units (CFU/ml) incubated at 37°C (Liebl and Martin, 2009). Absorbance was measured at 300 nm, 340 nm, 405 nm, 490 nm, and 595 nm, most of which are common filters present on most absorbance readers. We measured absorbance at 2, 4, 6, 12, 18, 24, 29, and 41 hours post-incubation to determine log-phase growth for each bacterial species. Candida albicans was assessed at a concentration of 10^5 CFU/ml and was incubated at 30°C. Absorbance (300, 340, 405, 490, and 595 nm) was read at 2, 4, 6, 12, 18, 24, 29, 41, and 53 hours post-incubation.

**Optimization of different species plasma samples**

We optimized the microbicidal assay using both E. coli and S. aureus for four different species: coyote, house finch, garter snake, and nectar. This range of species should provide an approximate starting point for new researchers utilizing this technique. However, any researcher replicating this protocol should perform species validation. To optimize for different species we plated pooled plasma samples (3 pooled samples of 2 individuals each) for house finches, garter snakes, side-blotched lizards, and newts and individual samples (i.e., not pooled) for coyotes in the top row of 96 well microplates. We serially diluted each sample down the plate (from 1:1–1:128). Specifically, we added 18 µl of pooled plasma sample in triplicate and 18 µl PBS to the first row of the plate and then added 18 µl of PBS to all other wells on the plate (except for positive and negative controls). We mixed the plasma and PBS in row 1 using a multichannel pipette. We then removed 18 µl from row 1 and transfer to row 2 re-mixed the solution and repeated to each subsequent row to serially dilute down the plate (after row 8 the remaining 18 µl can be disposed) for least 8 dilutions. We then followed the same assay procedure as above. All plasma samples were incubated with bacteria (10^5 CFU/ml) for 30 min at 37°C and then for 12 hours at 37°C following the addition of tryptic soy broth. Assay results depict average response across replicate samples for each species.

**Cross-validation**

We performed simultaneous assays using equivalent sample dilutions and microbial concentrations for both the new microplate and the traditional agar plate microbiocidal analysis. We assayed 4 dilutions each of 7 different samples of T. elegans. Samples were not serially diluted for this validation, instead they were prepared independently. For the agar plate assay we followed the traditional, standard methodology (French et al., 2010; Zysling and Demas, 2007). We ran a linear regression to test the new microplate microbiocidal assay against the traditional agar plate method. The significance level statistical test was α=0.05, and was conducted using JMP® IN (v. 8.0.1, SAS Institute Inc., Cary, NC, USA).

**Results and Discussion**

**Optimization of bacterial growth and absorbance**

As found in previous studies, E. coli and S. aureus microbes reached log-phase growth at 12 hours of incubation at 37°C, which is considered optimal (Fig. 1A,B). Concentrations of 10^5 CFU/ml for E. coli and 10^5 CFU/ml for S. aureus were most appropriate. Both concentrations for both microbes exhibited increasing absorbance; however, the time course to reach log-phase growth was slightly different from 10^4 to 10^5 CFU/ml. The coefficients of variation (CVs) for the E. coli and S. aureus plates were 0.019 and 0.016 respectively. C. albicans reached log-phase growth at 30 hours of incubation at 30°C, and we tested a concentration of 10^4 CFU/ml (Fig. 1C). The CV for the C. albicans plate was 0.032.

Different absorbance filters were more effective at measuring microbial growth for the different microbes. For E. coli and S. aureus both 300 and 340 nm filters were most optimal and for C. albicans a 340 nm absorbance filter was best at measuring microbial growth (340 nm filters are found on most standard absorbance readers).

**Optimization of different species plasma samples**

All species samples exhibited decreased killing with increasing dilutions, as would be expected with a serial dilution (Fig. 2A,B). It is however evident that the different species varied greatly in their killing ability among the different microbes. Researchers should therefore optimize for each individual species for each individual microbe prior to using this assay. Optimization should be for sample dilutions that yield approximately 50% killing. Therefore higher plasma volumes than those used in the current protocol should be used when validating for new species to attain a higher percent killing.

**Cross-validation**

The new antimicrobial microplate assay was highly correlated with the traditional agar plate antimicrobial assessment technique (F_{126}=63.19, P<0.01; adj R²=0.71) (Fig. 3). These results suggest that the new method is a good proxy for the traditional, standard agar plate method. The fit appears best within the middle range of killing (Fig. 3), and therefore the assay should be optimized (as in the traditional agar plate method) for a sample dilution that yields an average killing of approximately 50%.

**Conclusions**

Microbicidal activity measured via new microplate analysis was more efficient, yielded less variation than previous methodology, and was more closely related to traditional methods of microbiocidal analysis. We hope this provides a new variation on a powerful ecoimmunological method that will enable researchers across disciplines to effectively employ this method to accurately quantify microbial killing ability.

However the microbial killing assay does not measure the immune function in vivo and thus requires extrapolation. Further this assay also must be optimized following similar procedures to those outlined in the species validation of this manuscript for new species and populations that are assessed in different environmental contexts, such as breeding state and time of year. Finally, samples must be centrifuged (if using plasma), frozen, and analyzed within a relatively short period of time (approximately 20-30 days to analyzing). This may pose a challenge for field researchers who do not have access to the...
Fig. 1. Microbial growth measured as absorbance (nm) over time in (A) *E. coli*, (B) *S. aureus*, and (C) *C. albicans*.

Fig. 2. Microbicidal ability for (A) *E. coli* and (B) *S. aureus* microbes across different plasma dilutions for the non-traditional model species rough skinned newts (*Taricha granulosa*, amphibian), garter snakes (*Thamnophis elegans*, reptilian), side-blotched lizards (*Uta stansburiana*, reptilian), house finches (*Carpodacus mexicanus*, avian), and coyotes (*Canis latrans*, mammalian).
Table 1. Examples of commonly used microbes for analysis of microbiocidal activity in ecoimmunology, immune responses engaged and references.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Immune response engaged</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em> ATCC NO. 6051</td>
<td>Gram-positive bacteria</td>
<td>Nano-particles (Wei et al., 2009)</td>
</tr>
<tr>
<td><em>Candida albicans</em> ATCC NO. 10,231</td>
<td>Complement dependent</td>
<td>Birds (Millet et al., 2007)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ATCC NO. 8739</td>
<td>Gram-negative bacteria</td>
<td>Plants (Duarte et al., 2005; Hammer et al., 1999)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ATCC NO. 51,813</td>
<td>Complement independent</td>
<td>Birds (Millet et al., 2007; Matson et al., 2006)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> NCIMB 12210</td>
<td>Complement dependent</td>
<td>Mammals (Martin et al., 2007)</td>
</tr>
<tr>
<td><em>Salmonella typhi</em> ATCC NO. 19,430</td>
<td>Complement-dependent</td>
<td>Birds (Millet et al., 2007)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> ATCC NO. 6538</td>
<td>Gram-negative bacteria</td>
<td>Nano-particles (Wei et al., 2009; Pourjavadi and Soleyman, 2011)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> ATCC NO. 27,661</td>
<td>Complement independent</td>
<td>Fish (Fernandes et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>Requires phagocytosis and presence of natural antibodies</td>
<td>Mammals (Tagliaube et al., 1985)</td>
</tr>
<tr>
<td></td>
<td>Requires presence of natural antibodies</td>
<td>Birds (Millet et al., 2007; Matson et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Requires phagocytosis</td>
<td>Nano-particles (Wei et al., 2009; Pourjavadi and Soleyman, 2011)</td>
</tr>
</tbody>
</table>

proper equipment. In cases in the field with limited access to equipment researchers may opt for the traditional agar plate method which can be done completely in the field.

Regardless of which ecoinmunology techniques researchers choose to employ, experimental context is paramount to the interpretation of immunological data (Demas et al., 2011). Immune responses are not fixed in nature; they are instead highly variable depending on context and species (Boughton et al., 2011). Microbial killing ability may not be the best method for immune assessment for every system. However, using the microbial killing technique, researchers can optimize ecological relevancy by selecting specific microbes based on the biology of their study organism or scientific question (Table 1). For example, is there a high incidence of a particular pathogen in the system? Are you interested in measuring complement dependent or complement independent immune pathways? With careful consideration for the context of the experiment and the ecology of the organism, microbiocidal activity can be a powerful and versatile tool providing functional and relevant results.

Acknowledgements

We thank Christy Strand, Erika Cologgi, Edmund Brodie Jr., Amber Stokes, Brian Gall, Gareth Hopkins, and Leilani Lucas for help with the various sample collections. Thanks to Greg Demas for providing feedback on this manuscript.

Competing Interests

The authors declare that there are no competing interests.

References


Fig. 3. Relationship between microbiocidal (microplate) assay and traditional agar plate antimicrobial assessment technique using different dilutions of garter snakes (*Thamnophis elegans*) samples, tested via a linear regression (adj $R^2=0.71$).


