Improved ex vivo method for microbiocidal activity across vertebrate species

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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 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.

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.

Key words: Ecoimmunology, Immunity, Bacteria, Complement activity

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
equipment to perform. Ideally, a sterile laminar flow hood is used; however a relatively aseptic enclosure has been effectively used. This assay requires an incubator, plate absorbance reader (with standard filters), and a limited amount of disposables.

Assay Rationale
The traditional bacterial killing assay procedure involved growing a microbe either exposed or not exposed to sample (containing killing elements) on agar plates (Buehler et al., 2008; Matson et al., 2006; Rubenstein et al., 2008; Ruiz et al., 2010). In general, the method typically requires a sample diluted in media or phosphate buffered saline added to a known number of live microbes. In short, the microbes and sample are incubated for a brief period and then added to agar plates. After a longer incubation period, microbe growth is quantified by counting the number of colonies for each sample. By comparing the sample plates to the reference plates, which have only microbes and no sample, the degree of microbial killing is determined. While fresh whole blood is preferable, field work often necessitates the use of frozen plasma. If the frozen samples are used, however, the microbiocidal capability greatly decreases with both freeze-thaws and long periods of storage (over 20 days) (Liebl and Martin, 2009).

It is also critical to note that this measure of immune function varies significantly between species and even individuals in the same population, depending upon a variety of factors (such as sex, 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.

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 microhematocrit 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.

Newts
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 microhematocrit capillary tubes and transferred to sterile 1.5 ml tubes.

Microbe selection and preparation
In the current set of validations we used microbes Escherichia coli (ATCC NO. 8739), Staphylococcus aureus (ATCC NO. 6538), and Candida albicans (ATCC NO. 10,231). These microbes were chosen because 1) they are the most commonly used microbes in ecoinmunology studies (i.e., “the gold standards”) providing abundant data for comparison (Tieleman et al., 2005; Matson et al 2006; Millet et al., 2007; Boughton et al., 2011), 2) they require different functional immune responses to kill (e.g., E. coli—complement dependent; S. aureus—complement independent, requires phagocytosis; C. albicans—killing is mostly by phagocytosis) (Pulendran et al., 2001a; Pulendran et al., 2001b), and 3) they represent different classes of microbes and we wanted to test the range of assay applicability (i.e., E. coli—Gram-negative bacteria; S. aureus—Gram-positive bacteria; C. albicans—diploid fungus/yeast).

Prior to the assay, we autoclaved Tryptic Soy Broth (Sigma-Aldrich NO. T8097; 15 g broth/500 ml nanopure water) and stored it overnight at 4°C. Additionally, we reconstituted the microbes Escherichia coli, Staphylococcus aureus, and Candida albicans in lyophilized pellet form ((Epower Assayed Microorganism Preparation) from Microbiologics Inc., Saint Cloud, MN) in 10 ml of pre- warmed 0.9% Phosphate Buffered Solution (PBS) (37°C for E. coli and S. aureus and 30°C for C. albicans). Using flame-sterilized forceps, we transferred the pellet to the warm PBS and vortexed the solution. We then incubated the microbe solution for 30 minutes at 37°C for E. coli and S. aureus and 30°C for C. albicans. Finally, we vortexed the stock solution until the pellet was completely dissolved and stored the solution for no more than 24 h at 4°C. We used this stock solution to make up a working solution (10^6 colony-forming unit; CFU).

Assay procedures
As a sterile environment was of utmost importance, we worked under an ethanol-sterilized laminar flow hood with ethanol sterilized and/or autoclaved equipment and disposables (such as pipettes, wells, and pipette tips). We washed all of the samples, but ensured that none of the samples had previously been thawed on any occasion. All samples were run in triplicate to enable greater accuracy. For validation on new species, serial dilutions are required to obtain the optimal microbiocidal activity. Here we describe the sample volumes for the side-blotch lizard (Uta stansburiana).

We initially plated positive controls by adding 18 μl of PBS and negative controls by adding 24 μl of PBS only (96 well cell culture round bottom microplates). We then added 2 μl of plasma and 16 μl sterile PBS (1M 10 × PBS; 1:8 dilution plasma) to each of the three wells and add 6 μl of the bacteria working solution to all wells except negative controls. While multi-channel pipettes can be used, we strongly urge that the pipettes be calibrated and regularly maintained to ensure accuracy when pipetting small volumes. The plate was covered while still in the hood for the remainder of the assay. We then vortexed the plate on a plate shaker (150 rpm) for 1 minute gently to ensure solutions are well mixed and that there is no splashing between wells.

After vortexing, plates were incubated for 30 minutes at 37°C for E. coli and S. aureus and at 30°C for C. albicans. Following incubation with microbes we again vortexed the plate gently for 1 minute (150 rpm) and added 125 μl of the sterile
marker ELISA plate reader (BioRad xMark spectrophotometer) to read the absorbance 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 24 h for S. aureus and 24 h for C. albicans) we used a concentration for log phase growth at 30 hours of incubation at 30°C, and we tested a higher percent killing. However, the microbial killing assay does not measure the absolute amount of bacterial killing, and was more closely related to traditional methods of microbiocidal analysis. We hope this provides a new variation 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.

Optimization of bacterial growth and absorbance

Prior to testing microbiocidal 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 106 and 108 colony forming units (CFU/ml) incubated at 37°C (Liebl and Martin, 2009). Absorbance was measured at 300, 340, 405, 490, 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 109 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 microbiocidal assay using both E. coli and S. aureus for four different species: coyote, house finch, garter snake, and newt. 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 transferred to row 2 to re-mix 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 (105 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 104 CFU/ml for E. coli and 105 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 104 to 105 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 104 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 (F1,25=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

Microbiocidal 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 ecoinmunological 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. Microbiocidal 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).
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.

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Competing Interests
The authors declare that there are no competing interests.

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