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)

Biology Open 1, 482–487
doi: 10.1242/bio.2012919

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 relevancy 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

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., phytoheamagglutinin).

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 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.

For all above sample collections, blood samples were stored on ice until further processing could take place, at which time plasma was separated from the cells via centrifugation. For all species, samples of different individuals were pooled and stored at −20℃ until assayed.

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 ecoimmunology 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. T8907; 15 g broth/500 ml nanopure water) and stored it overnight at 4℃. 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℃ for *E. coli* and *S. aureus* and 30℃ 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℃ for *E. coli* and *S. aureus* and 30℃ 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℃. We used this stock solution to make up a working solution (10^5 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 thawed 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℃ for *E. coli* and *S. aureus* and at 30℃ 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 broth to all wells, and included the positive and negative controls. We again vortexed (100 rpm) the plate for 1 min and read the plate using the microplate reader (BioRad xMark^{TM} 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℃ for 12 hours or *E. coli* and *S. aureus*, and 30℃ 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 CO_2-independent media plus 4 mM L-glutamine (media Gibco NO. 18,045; L-glutamine Sigma-Aldrich NO. G3126). For the whole blood procedure CO_2-independent media was used instead of PBS to dilute samples. The positive and negative controls each received 2 µl PBS and 16 µl CO_2-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. aureas* and 24 h for *C. albicans*), we used a microplate absorbance reader (BioRad xMark spectrophotometer) to read the absorbance at 300 nm for *E. coli* and *S. aureas* and 340 nm for *C. albicans* (optimized as described below).

To calculate bacterial killing ability we first subtracted background absorbance readings from the absorbance readings (i.e., 12 and 24 hour readings). Microbiocidal 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 and 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 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 10^4 and 10^5 colony forming units (CFU)/ml incubated at 37℃ (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-inoculation to determine log-phase growth for each bacterial species. *Candida albicans* was assessed at a concentration of 10^4 CFU/ml and was incubated at 30℃. Absorbance (300, 340, 405, 490, and 595 nm) was read at 2, 4, 6, 12, 18, 24, 29, 41, and 53 hours post-inoculation.

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 a 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℃ and then for 12 hours at 37℃ 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 $\alpha=0.05$, 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℃, which is considered optimal (Fig. 1A,B). Concentrations of 10^4 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 and 0.019 and 0.016 respectively. *C. albicans* reached log-phase growth at 30 hours of incubation at 30℃, 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 ($F_{1,26}=63.19$, $P<0.01$; adj $R^2=0.71$) (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 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

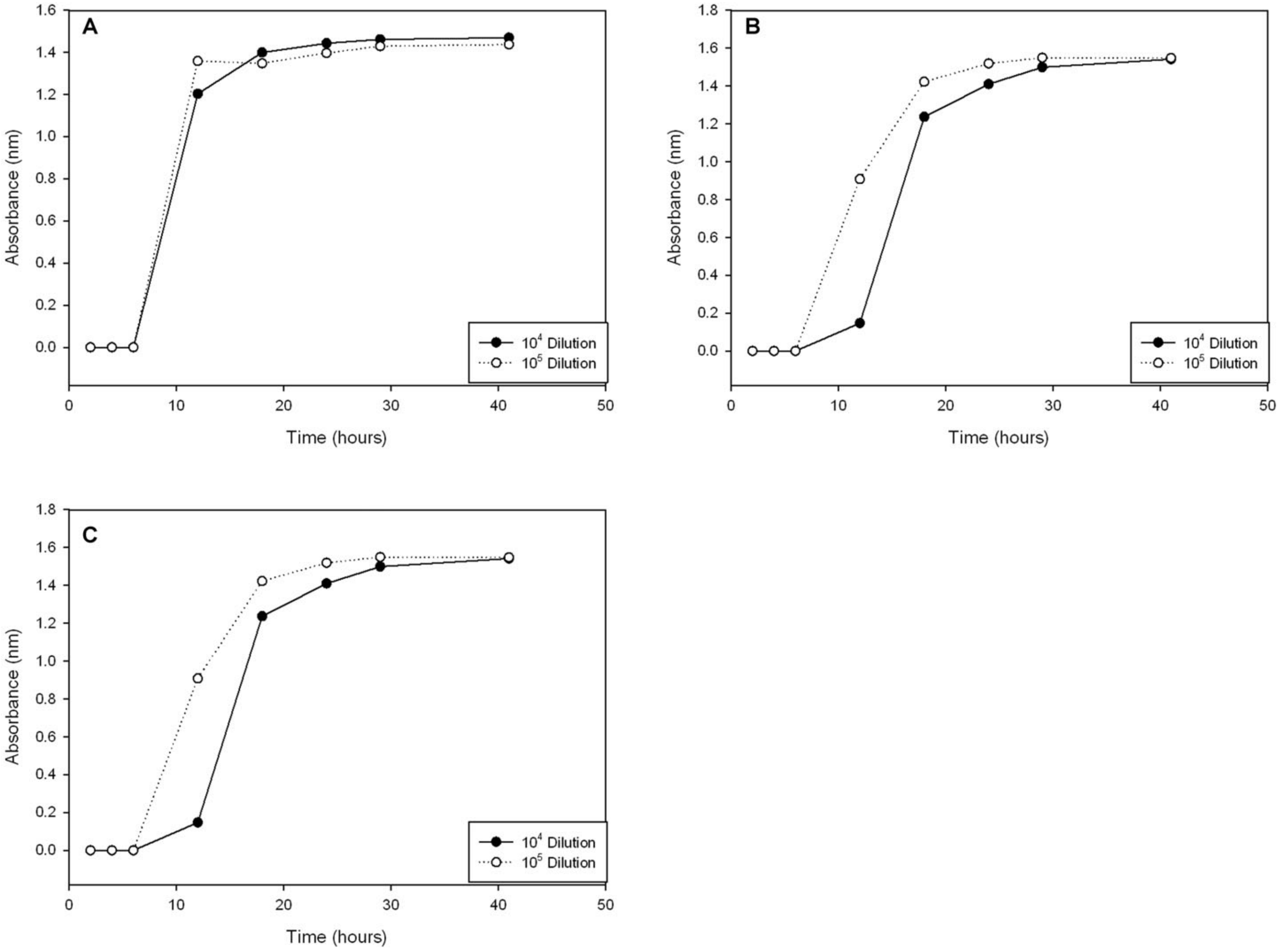

Fig. 1. Microbial growth measured as absorbance (nm) over time in (**A**) *E. coli*, (**B**) *S. aureus*, and (**C**) *C. albicans*.

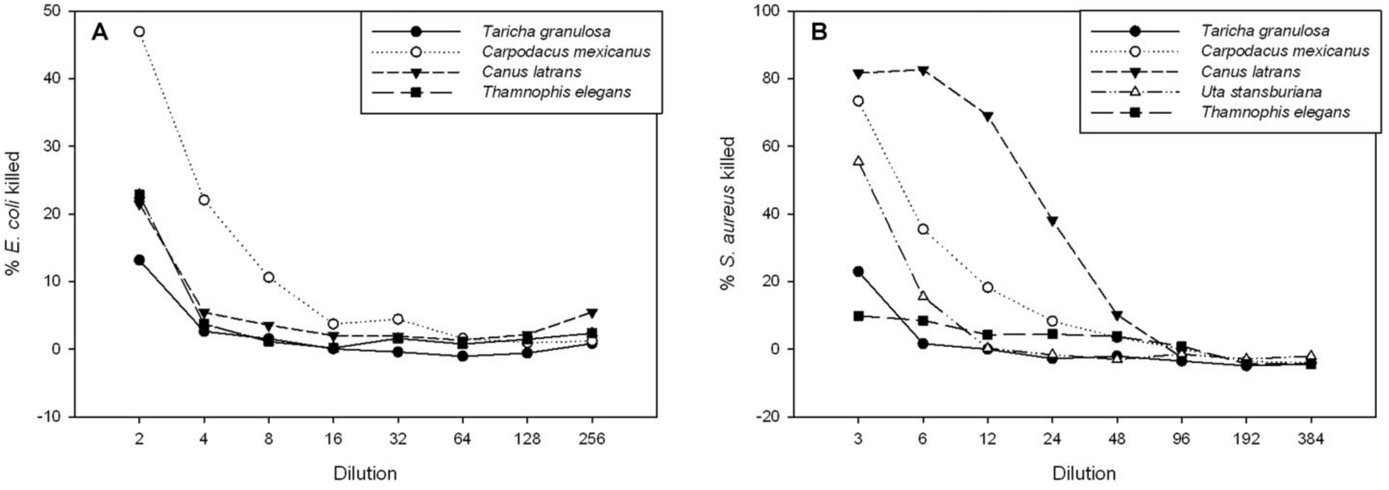

Fig. 2. Microbiocidal ability for (**A**) *E. coli* and (**B**) *S. aureus* microbes across different plasma dilutions for the non-traditional model species rough skinned newts (*Taricha granulose*, amphibian), garter snakes (*Thamnophis elegans*, reptilian), side-blotched lizards (*Uta stansburiana*, 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.

Strain	Immune response engaged	References
Bacillus subtilis ATCC NO. 6051	Gram-positive bacteria Complement dependent	**Nano-particles** (Wei et al., 2009)
Candida albicans ATCC NO. 10,231	Diploid fungus (yeast) Killing is mostly by phagocytosis May also rely on natural antibodies	**Birds** (Millet et al., 2007) **Plants** (Duarte et al., 2005; Hammer et al., 1999)
Escherichia coli ATCC NO. 8739	Gram-negative bacteria Complement dependent Requires phagocytosis and presence of natural antibodies	**Birds** (Millet et al., 2007; Matson et al., 2006) **Mammals** (Martin et al., 2007)
Escherichia coli ATCC NO. 51,813	Gram-negative bacteria Complement independent Requires phagocytosis	**Birds** (Millet et al., 2007) **Nano-particles** (Wei et al., 2009; Pourjavadi and Soleyman, 2011)
Escherichia coli *NCIMB 12210*	Gram-negative bacteria Complement dependent	**Fish** (Fernandes et al., 2002)
Salmonella typhi ATCC NO. 19,430	Gram-negative bacteria Complement-dependent Activates granulomas Requires presence of natural antibodies	**Mammals** (Tagliabue et al., 1985)
Staphylococcus aureus ATCC NO. 6538	Gram-positive bacteria Complement independent Requires phagocytosis and presence of natural antibodies	**Birds** (Millet et al., 2007; Matson et al., 2006)
Staphylococcus aureus ATCC NO. 27,661	Gram-positive bacteria Complement independent Requires phagocytosis	**Nano-particles** (Wei et al., 2009; Pourjavadi and Soleyman, 2011)

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 ecoimmunology 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.

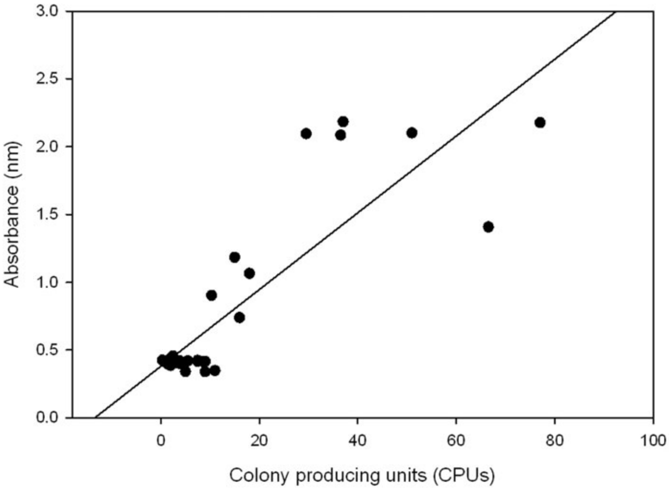

Fig. 3. Relationship between microbiocidal (microplate) assay and traditional agar plate antimicrobial assessment technique using different dilutions of garter snakes (*Thamnophis elegans*) samples, tested via a linear regression (adj R^2=0.71).

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

Boughton, R. K., Joop, G. and Armitage, S. A. O. (2011). Outdoor immunology: methodological considerations for ecologists. *Funct. Ecol.* **25**, 81-100.

Buehler, D. M., Piersma, T., Matson, K. and Tieleman, B. I. (2008). Seasonal redistribution of immune function in a migrant shorebird: annual-cycle effects override adjustments to thermal regime. *Am. Nat.* **172**, 783-796.

Demas, G. E., Zysling, D. A., Beechler, B. R., Muehlenbein, M. P. and French, S. S. (2011). Beyond phytohaemagglutinin: assessing vertebrate immune function across ecological contexts. *J. Anim. Ecol.* **80**, 710-730.

Duarte, M. C. T., Figueira, G. M., Sartoratto, A., Rehder, V. L. G. and Delarmelina, C. (2005). Anti-*Candida* activity of Brazilian medicinal plants. *J. Ethnopharmacol.* **97**, 305-311.

Fernandes, J. M. O., Kemp, G. D., Molle, M. G. and Smith, V. J. (2002). Anti-microbial properties of histone H2A from skin secretions of rainbow trout, *Oncorhynchus mykiss*. *Biochem. J.* **368**, 611-620.

French, S. S., DeNardo, D. F., Greives, T. J., Strand, C. R. and Demas, G. E. (2010). Human disturbance alters endocrine and immune responses in the Galapagos marine iguana (*Amblyrhynchus cristatus*). *Horm. Behav.* **58**, 792-799.

Graham, A. L., Shuker, D. M., Pollitt, L. C., Auld, S. K. J. R., Wilson, A. J. and Little, T. J. (2011). Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology. *Funct. Ecol.* **25**, 5-17.

Hammer, K. A., Carson, C. F. and Riley, T. V. (1999). Antimicrobial activity of essential oils and other plant extracts. *J. Appl. Microbiol.* **86**, 985-990.

Liebl, A. L. and Martin, L. B., I. I. (2009). Simple quantification of blood and plasma antimicrobial capacity using spectrophotometry. *Funct. Ecol.* **23**, 1091-1096.

Martin, L. B., 2nd, Weil, Z. M. and Nelson, R. J. (2007). Immune defense and reproductive pace of life in *Peromyscus* mice. *Ecology* **88**, 2516-2528.

Matson, K. D., Cohen, A. A., Klasing, K. C., Ricklefs, R. E. and Scheuerlein, A. (2006). No simple answers for ecological immunology: relationships among immune indices at the individual level break down at the species level in waterfowl. *Proc. Biol. Sci.* **273**, 815-822.

Millet, S., Bennett, J., Lee, K. A., Hau, M. and Klasing, K. C. (2007). Quantifying and comparing constitutive immunity across avian species. *Dev. Comp. Immunol.* **31**, 188-201.

Pourjavadi, A. and Soleyman, R. (2011). Novel silver nano-wedges for killing microorganisms. *Mater. Res. Bull.* **46**, 1860-1865.

Pulendran, B., Kumar, P., Cutler, C. W., Mohamadzadeh, M., Van Dyke, T. and Banchereau, J. (2001a). Lipopolysaccharides from distinct pathogens induce different classes of immune responses in vivo. *J. Immunol.* **167**, 5067-5076.

Pulendran, B., Palucka, K. and Banchereau, J. (2001b). Sensing pathogens and tuning immune responses. *Science* **293**, 253-256.

Rubenstein, D. R., Parlow, A. F., Hutch, C. R. and Martin, L. B., 2nd. (2008). Environmental and hormonal correlates of immune activity in a cooperatively breeding tropical bird. *Gen. Comp. Endocrinol.* **159**, 10-15.

Ruiz, M., French, S. S., Demas, G. E. and Martins, E. P. (2010). Food supplementation and testosterone interact to influence reproductive behavior and immune function in Sceloporus graciosus. *Horm. Behav.* **57**, 134-139.

Tagliabue, A., Villa, L., Boraschi, D., Peri, G., de Gori, V. and Nencioni, L. (1985). Natural anti-bacterial activity against *Salmonella typhi* by human T4+ lymphocytes armed with IgA antibodies. *J. Immunol.* **135**, 4178-4182.

Tieleman, B. I., Williams, J. B., Ricklefs, R. E. and Klasing, K. C. (2005). Constitutive innate immunity is a component of the pace-of-life syndrome in tropical birds. *Proc. Biol. Sci.* **272**, 1715-1720.

Wei, D., Sun, W., Qian, W., Ye, Y. and Ma, X. (2009). The synthesis of chitosan-based silver nanoparticles and their antibacterial activity. *Carbohydr. Res.* **344**, 2375-2382.

Zysling, D. A. and Demas, G. E. (2007). Metabolic stress suppresses humoral immune function in long-day, but not short-day, Siberian hamsters (Phodopus sungorus). *J. Comp. Physiol. B* **177**, 339-347.