Comparative Biochemistry and Physiology, Part A 191 (2016) 115–118

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Bacteria-killing ability of fresh blood plasma compared to frozen blood plasma

Anne C. Jacobs [a,*,1], Jeanne M. Fair [b]

[a] Department of Biology, University of California, Riverside, CA 92521, USA
[b] Los Alamos National Laboratory, Global Security–Emerging Threats, Mailstop K404, Los Alamos, NM 87545, USA

ARTICLE INFO

Article history:
Received 12 June 2015
Received in revised form 2 October 2015
Accepted 2 October 2015
Available online 9 October 2015

Keywords:
Ecological immunology
Microbicidal ability of blood
Repeatability

ABSTRACT

In recent years, the bacteria-killing assay (BKA) has become a popular technique among ecoimmunologists. New variations of that assay allow researchers to use smaller volumes of blood, an important consideration for those working on small-bodied animals. However, this version of the assay requires access to a lab with a nanodrop spectrophotometer, something that may not be available in the field. One possible solution is to freeze plasma for transport; however, this assumes that frozen plasma samples will give comparable results to fresh ones. We tested this assumption using plasma samples from three species of birds: chickens (*Gallus gallus*), ash-throated flycatchers (*Myiarchus cinerascens*), and western bluebirds (*Sialia mexicana*). Chicken plasma samples lost most or all of their bacterial killing ability after freezing. This did not happen in flycatchers and bluebirds; however, frozen plasma did not produce results comparable to those obtained using fresh plasma. We caution researchers using the BKA to use fresh samples whenever possible, and to validate the use of frozen samples on a species-by-species basis.

1. Introduction

Eco-immunology, or the study of how immune function trades off with other aspects of life history, has become a popular field (Sheldon and Verhulst, 1996; French et al., 2009). To properly study these trade-offs, however, researchers must have reliable ways of measuring immune function in captive and wild animals. Several techniques are available, particularly for birds, each with benefits and drawbacks (Norris and Evans, 2000; Salvante, 2006). Recently, measuring the antimicrobial capacity of blood has become a popular field technique in eco-immunology (Matson et al., 2006; Millet et al., 2007; Forsman et al., 2008; Merrill et al., 2014). This assay, also known as the bacteria-killing assay (BKA), provides a measure of innate immune response by quantifying the ability of proteins in the plasma (such as complement, natural antibodies, and lysozyme) and/or phagocytic cells to kill bacteria (Matson et al., 2006; Millet et al., 2007). Some positive aspects of this assay are that it is relatively simple to carry out, requires only small volumes of blood, and is easy to interpret.

The BKA was adapted from an assay used in humans to assess the bactericidal ability of leukocytes (Keusch et al., 1975), and was modified to measure immune response in birds by Millet et al. (2007). These methods have been readily adapted for other vertebrate taxa as well, including amphibians (e.g. Venesky et al., 2012; Gervasi et al., 2014) and mammals (e.g. Schneeberger et al., 2013). The original form of the assay involves mixing blood or plasma with a common microbe that has the potential to cause disease (e.g. *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*) and plating out the resulting mixture on agar plates (Millet et al., 2007). Researchers then measure the number of microbes killed by comparing the results to a control plate in which microbes were not mixed with blood. However, Liebl and Martin (2009) devised an alternative version of the assay, which uses a nanodrop spectrophotometer to measure bacterial growth in lieu of counting bacterial colonies on plates. The advantage of this modified assay is that it requires smaller blood volumes; the disadvantage is that it requires access to laboratory equipment, such as the nanodrop, and therefore cannot be carried out by field researchers working in remote areas with little or no access to electricity.

When describing the modified version of the antimicrobial assay, Liebl and Martin (2009) also conducted a test to determine if frozen samples could be used in the assay. They reported that, after approximately three weeks in the freezer, the antimicrobial capacity of the samples dropped to zero. However, other studies have used frozen plasma samples and reported viable results (e.g. Morrison et al., 2009; Merrill et al., 2014). For example, Morrison et al. (2009) used nestling tree swallows plasma that had been frozen at −80 °C for 6 months. They stated in their Methods that the results obtained using frozen plasma were similar to those obtained using fresh plasma. The Morrison et al. (2009) study used the plate method to measure bacterial growth rather than the nanodrop method; however, the two assays should measure

* Corresponding author.
E-mail address: ajacobs@allegheny.edu (A.C. Jacobs).
[1] Present Address: Department of Biology, Allegheny College, Meadville, PA 16335, USA.

http://dx.doi.org/10.1016/j.cbpa.2015.10.004
1095-6433/

the same thing, albeit by different means. Thus, the question of whether frozen plasma can produce usable results must be further addressed.

Here, we examine the repeatability of the bacteria-killing (BKA) assay described by Liebl and Martin (2009) using fresh and frozen samples to determine whether frozen samples can still yield useful results. We took plasma samples from three different species of birds, ran the BKA using the fresh samples, and then froze the remaining plasma for several weeks before running the assay again. We predicted that frozen plasma would produce results comparable to fresh plasma.

2. Materials and methods

We collected fresh blood from domestic chickens (*Gallus gallus*; $n = 11$), ash-throated flycatchers (*Myiarchus cinerascens*; $n = 7$), and western bluebirds (*Sialia mexicana*; $n = 22$). Adult chicken samples came from a backyard poultry collection in northern New Mexico. Bluebird and flycatcher blood was obtained from wild birds that were monitored as part of the Los Alamos National Laboratory's Avian Nestbox Network. This network includes several hundred nestboxes placed on trees at various sites throughout the laboratory's property and the surrounding areas. Ash-throated flycatchers and western bluebirds are the two most common species inhabiting these boxes (Fair et al., 2003). All work was approved by the Los Alamos National Laboratory Animal Care and Use Committee and conducted in accordance with the Guidelines to the Use of Wild Birds in Research (Fair et al., 2010). For further details on the system, see Fair et al. (2003) and Jacobs et al. (2013).

All chicken and flycatcher blood samples were collected during the summer of 2012; bluebird blood samples were collected in the summers of 2011 ($n = 6$ samples) and 2012 ($n = 16$ samples). During the breeding season, adult bluebirds ($n = 11$) were captured using mist nets placed in front of active nestboxes. We also obtained samples from nestling bluebirds between the ages of 9 and 21 days while they were still in the nest. For further details about bluebird sampling, see Jacobs et al. (2015). All flycatcher samples came from nestling flycatchers between 9 and 14 days old, and all the chicken samples came from adult females. To collect blood from the bluebirds and flycatchers, we first swabbed the collection site with 70% ethanol, then used a sterile needle to puncture the wing vein and collected 20–50 μL in a heparinized microhematocrit tube. On chickens, we used a sterile syringe to draw blood from the wing vein. We then transferred the blood into a heparinized tube until processing.

Samples were briefly stored on ice before processing. All blood samples were taken back to the laboratory for processing on the same day as collection. We spun blood down in a centrifuge for five minutes and collected the plasma for use in the assay. We used enough fresh plasma to run the initial bactericidal assay (see below), then immediately placed the remaining plasma in individually marked tubes in the freezer at −80 °C. After samples had been in the freezer for a minimum of 19 days (average time frozen = 30 days, range 19–53 days), we removed and allowed them to thaw in a bucket of ice and reran the assay. We chose this length of time based on data from Liebl and Martin (2009), which suggested that plasma loses its ability to kill *E. coli* after roughly 20 days in the freezer. Assay protocols were identical for both fresh and frozen samples.

2.1. The bacteria killing assay

To measure the ability of plasma to kill bacteria, we used the methods described in Liebl and Martin (2009). We used *Escherichia coli* (ATCC #8739) supplied in pellets containing 1×10^6 to 1×10^7 microorganisms per pellet (Epower Microorganisms, MicroBioLogics, St. Cloud, MN). Each pellet was reconstituted in 40 mL of sterile 1× phosphate buffered saline (PBS) at 37 °C. This stock solution was then diluted down to make a working solution of 2×10^5 microorganisms per mL. We made a fresh stock solution every week and kept all bacterial solutions at 4 °C at all times.

A total of 1.5 μL of plasma was mixed with 34.5 μL PBS and 12.5 μL of the bacterial working solution. This mixture was then incubated for 30 min at 37 °C. After the initial incubation, we added 250 μL of sterile tryptic soy broth. We also prepared a sterile blank, which contained 48.5 μL of PBS with 250 μL of tryptic soy broth, and a positive control, which contained 36 μL PBS, 12.5 μL of the bacterial working solution, and 250 μL of tryptic soy broth. All samples were incubated for 12 h at 37 °C after the addition of the tryptic soy broth. We used a Tecan Infinite 200 (Tecan Group Ltd., Männedorf, Switzerland) to measure the absorbance of the samples at 300 nm. We calculated the antimicrobial activity of the plasma as 1 − (absorbance of sample/absorbance of positive control). We ran all samples in triplicate when we had sufficient plasma volumes and in duplicate when we did not and we averaged the results from each replicate to get a single value for each individual.

2.2. Statistical analysis

All analyses were performed using JMP 11 (SAS Institute, 2008). We tested all data for normality using a Shapiro–Wilks test. Frozen BKA values were non-normally distributed and could not be transformed to meet assumptions of normality due to a large number of zero values. We had bluebird samples from two years (2011 and 2012); however, bacteria killing ability of the fresh plasma did not differ between years ($F_{1,21} = 1.24$, $P = 0.28$). Likewise, we had bluebird samples from both adult birds and nestlings; however, BKA values did not differ significantly with age ($F_{1,21} = 0.19$, $P = 0.66$). We therefore pooled samples for all analyses.

To calculate repeatability for the individual replicates for both fresh samples and frozen samples, we used the methods described in Lessells and Boag (1987). We then tested whether results obtained from frozen samples correlated with results obtained from fresh samples using a Spearman rank correlation. We also compared the average BKA values of fresh and frozen plasma using a Wilcoxen signed rank test.

Different species showed different patterns in terms of the ability of the frozen plasma to kill bacteria (see below). We therefore reran the analysis on each species separately. To determine whether storage time affected our results, we used a linear regression to test for a relationship between how many hours we had stored the fresh blood before processing it and the BKA of fresh plasma. We were missing data on collection time for three individuals; thus our sample size was reduced to $n = 37$ for this test. We also ran this analysis on bluebirds only; we did not test for a relationship between assay timing and BKA in chickens because all samples were collected and processed at roughly the same time, and we did not run this test for flycatchers because missing data reduced our sample size to $n = 4$. Alpha levels are set at $\alpha = 0.05$, and all values are reported as means ± standard deviation.

3. Results

When examining the repeatability of the replicates used, both fresh and frozen replicates showed significant variation among rather than within individuals and moderate to high repeatability (fresh plasma: $P < 0.001$, $R = 0.40$; frozen plasma: $P < 0.001$, $R = 0.70$). However, we found no correlation between the BKA of fresh plasma and the BKA of frozen plasma when pooling all species ($r_s = 0.06$, $n = 40$, $P = 0.70$). The number of days a sample had been frozen did not affect the magnitude of the difference between fresh and frozen samples ($r_s = -0.18$, $n = 40$, $P = 0.25$). BKA values for frozen samples tended to be lower than those of fresh samples (fresh BKA mean = 0.29 ± 0.21; frozen BKA mean = 0.13 ± 0.16; $z = 3.58$, $n = 40$, $P = 0.0002$). However, this was due to a large number of frozen samples in which the BKA value dropped to zero (33%), particularly among chickens (see below).

3.1. Within-species comparison of fresh and frozen plasma

In chickens, the BKA of frozen plasma dropped close to zero in most samples (Fig. 1A), and there was no correlation between the killing