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Short Communication

Ammonium improves elution of fixed dried blood spots without affecting immunofluorescence assay quality

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Abstract

OBJECTIVE To solve the problem of fixed dried blood spot elution without damaging IgG antibodies.
METHODS The minimum effective concentration of liquid ammonium (NH_3) in a PBS solution, which was found to elute fixed blood, was determined. By using a dilution series, the effects of NH_3 on IgG antibody quality were assessed using immunofluorescence assays.
RESULTS The minimum effective concentration of 0.2% NH_3 has no detectable effects on IgG quality.
CONCLUSION Ammonium greatly improves blood elution from fixed DBS while maintaining IgG antibody quality. These results are encouraging and provide a basis for further testing of the efficacy of ammonium in different situations as well as its effect on other compounds.

keywords capillaries, filter paper, heat-fixed, immuno-fluorescence assays, serology, Whatman

Introduction

Blood samples are collected on absorbent paper as dried blood spots (DBS) for many medical and other purposes and for various reasons. Dried blood spots can preserve the quality of nucleic acids, proteins (Parker & Cubitt 1999) and amino acids (Levy *et al.* 1985) and are frequently used for newborn screening (Levy *et al.* 1985), toxicology (Burnett 2011) and serology (Mercader *et al.* 2006). Because of the high quality of DBS, a lower blood volume is generally needed, which in the case of animal studies reduces animal stress (Burnett 2011). The use of DBS also facilitates logistics, as centrifuging and refrigeration are not necessary, which is an important reason for the frequent use of DBS in field-work settings, especially in the tropics where it can be challenging to arrange a field laboratory (Andriamandimby *et al.* 2013). Another advantage is the small size of the paper, which can make storage in freezers more efficient than when working with vials.

Dried blood spots are sensitive to sampling and storage conditions, and a number of guidelines should be followed. As stated by the Clinical and Laboratory Standards Institute, 'blood should be applied only to one side of the filter paper', and 'both sides of the filter paper should be examined to assure that the blood uniformly penetrated and saturated the paper' (NCCLS 2003). The sample should then be air dried for a minimum of 2 h in a dry environment, at temperatures of 15–22 °C (NCCLS 2003; Burnett 2011). Storage is best in a low-humidity (<30%) environment at ambient or low (4 °C) temperature for storage up to 2 years, and at -20°C for periods longer than 2 years (Therrell *et al.* 1996).

Storage conditions are, however, not always ideal (especially in the tropics), and a number of problems can arise that result in fixation of blood and prevent proper elution in a buffer. Probably, the most common and serious problem is heat-fixing, where high temperatures somehow fix the blood to the absorbent paper and prevent proper elution of the blood sample, possibly rendering the sample useless (NCCLS 2003).

This short note presents a possible solution to the problem that allows elution while preserving the quality of IgG antibody and possibly other compounds.

Methods and results

In March–April 2012, blood samples were collected from multimammate mice (*Mastomys natalensis*) at the University of Antwerp, as part of a serological study on African arenaviruses (Borremans *et al.* 2011). In laboratory conditions, blood samples (six per animal, ca. 10 μl each) were administered to pre-punched cellulose filter paper (Whatman grade 3, untreated, thickness 390 μm,

28 mm^2 punches, distributed as Serobuvard© by LDA[22], Zoopole, France), were left to air-dry for 2 h and were stored away from direct sunlight in an air-conditioned room (10 °C) in a zip closure bag containing silica desiccants. But, some filter papers were accidentally left in a closed zip closure plastic bag in direct sunlight behind a window, causing overheating. The effects of heat, UV and/or humidity caused the blood samples to become fixed and not elute in a phosphate-buffer saline (PBS) solution, which was necessary for the immuno-fluorescence assays (IFA) for the detection of anti-MORV IgG antibodies (Günther *et al.* 2009). Several unsuccessful attempts were made to elute the blood without damaging the antibodies: using Tween 20/80, sodium azide, hydrogen peroxide, increased/decreased elution temperatures, violent shaking and strong pestling. The addition of

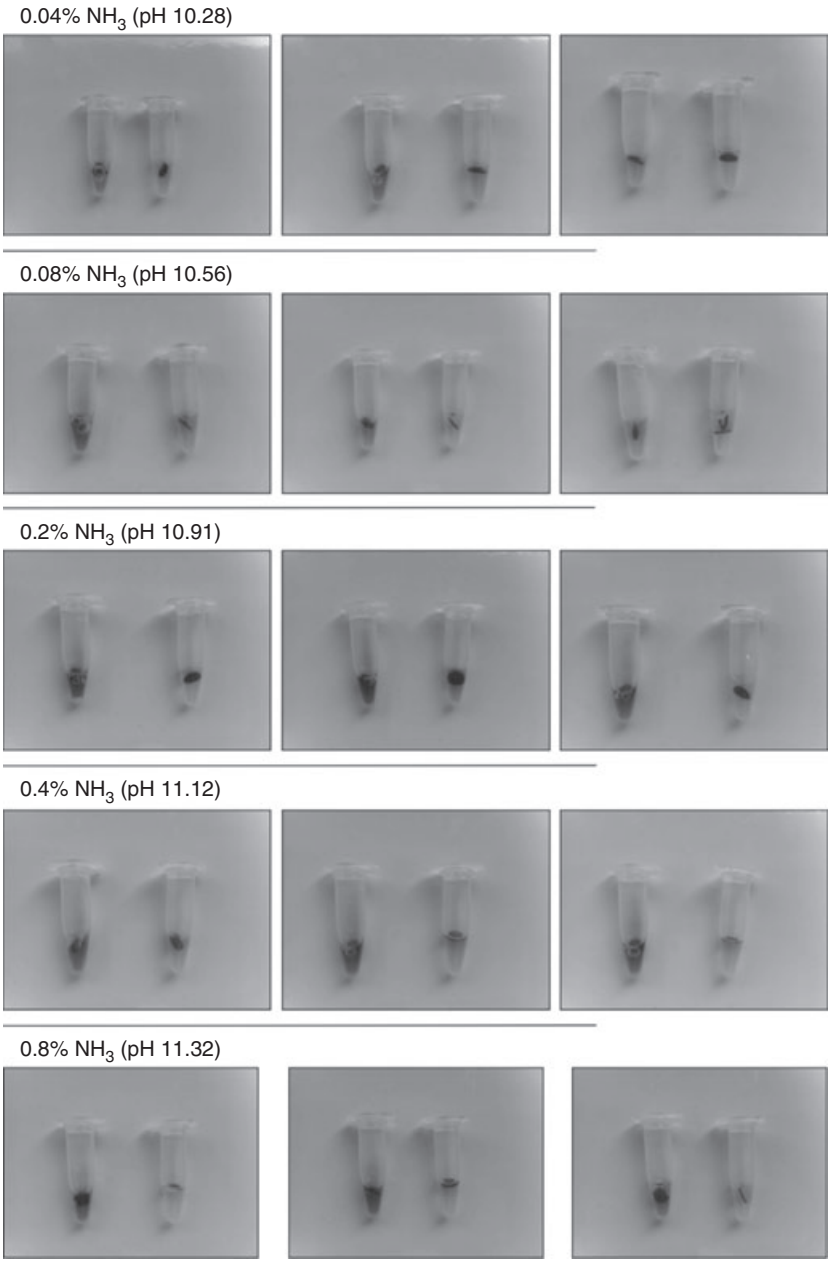

Figure 1 For each of an increasing range of concentrations, two non-eluting blood spots were taken from three animals (left, middle and right). Liquid ammonium was added to one of the blood spots in PBS (left in each picture), while the other blood spot served as a control (right in each picture).

liquid ammonium (NH_3, a weak base) turned out to elute the blood successfully, and basic tests were performed to determine the minimum effective concentration necessary for elution and to assess the effect on IgG quality.

Minimum effective concentration

For each of an increasing range of five concentrations, two blood spots originating from the batch of non-eluting samples were taken from three animals. To each blood spot, 300 μl PBS (ambient temperature - ±22 °C; Gibco® - Life Technologies tablets, containing 10 mM phosphate and 150 mM sodium chloride, pH 7.3–7.5) were added, following the standard IFA protocol. Next, liquid ammonium was added to one of the blood spots in PBS, while the other blood spot served as a control. Samples were left on a slow shaker (70 rpm) for 1 h and then left to elute for 17 h. NH_3 dilutions used and corresponding pH values are shown in Figure 1.

As can be seen in Figure 1, no ammonium concentrations induced complete blood elution (which would render the filter paper white), but an ammonium concentration of 0.2% (2.5 μl of a 25% NH_3 solution) was sufficient for a much improved blood elution, allowing the samples to be analysed. Elution in PBS, the pH-level of which was raised to 10.85 using NaOH, was compared to elution in normal PBS (pH 7.43) and PBS-NH_3 (pH 10.85), and was found to slightly improve elution compared to normal PBS, but much less than NH_3-containing PBS.

Effect of ammonium on antibody quality

The effect of 0.2% ammonium on IgG quality was tested through antibody titre comparison. For each of 5 individuals known to have DBS of good quality (complete elution) and known to be IgG-positive, two blood spots from the same blood sample were used. Both samples were eluted in 300 μl PBS, and one sample was used as a control, while 0.2% NH_3 was added to the second sample. Each sample was then diluted with PBS in a number of increasing dilution steps (1:30, 1:15000, 1:30000, 1:45000, 1:60000, 1:75000 and 1:90000). The highest dilution at which the sample tested IgG-positive was taken as the titre of that sample. IgG presence was determined indirectly through binding of fluorescein isothiocyanate-tagged secondary antibodies detected using fluorescence microscopy. Each sample was blind-checked by two people.

All ammonium-treated samples had the same antibody titre as the control samples (titres 1:60000, 1:30, 1:75000, 1:30000, 1:60000). This method was then used on fixed blood samples originating from a controlled infection experiment, in which it was known which *M. natalensis* individuals had to be anti-MORV IgG-positive (B. Borremans, N. Hughes, R. Vossen, S. Gryseels, S. Günther & H. Leirs, in preparation). All fixed samples that were eluted using 0.2% NH_3 and were expected to contain IgG antibodies indeed tested positive, proving the applicability of the method.

Conclusion

The tests carried out here are very basic, and many aspects remain to be tested such as the effects of ammonium on other blood-borne compounds and on different assays. Even so, the preliminary results are encouraging, and provide a much-needed solution to the problem of non-eluting dried blood spots.

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