The use of dried cerebrospinal fluid filter paper spots as a substrate for PCR diagnosis of the aetiology of bacterial meningitis in the Lao PDR

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Abstract

We investigated whether dried cerebrospinal fluid (CSF) conserved on filter paper can be used as a substrate for accurate PCR diagnosis of important causes of bacterial meningitis in the Lao PDR. Using mock CSF, we investigated and optimized filter paper varieties, paper punch sizes, elution volumes and quantities of DNA template to achieve sensitive and reliable detection of bacterial DNA from filter paper specimens. FTA Elute Micro Card™ (Whatman, Maidstone, UK) was the most sensitive, consistent and practical variety of filter paper. Following optimization, the lower limit of detection for *Streptococcus pneumoniae* from dried mock CSF spots was 14 genomic equivalents (GE)/μL (interquartile range 5.5 GE/μL) or 230 (IQR 65) colony forming units/mL. A prospective clinical evaluation for *S. pneumoniae*, *S. suis* and *Neisseria meningitidis* was performed. Culture and PCR performed on fresh liquid CSF from patients admitted with a clinical diagnosis of meningitis (n = 73) were compared with results derived from dried CSF spots. Four of five fresh PCR-positive CSF samples also tested PCR positive from dried CSF spots, with one patient under the limit of detection. In a retrospective study of *S. pneumoniae* samples (n = 20), the median (IQR; range) CSF *S. pneumoniae* bacterial load was 1.1 $\times$ 10^4 GE/μL (1.2 $\times$ 10^5; 1 to 6.1 $\times$ 10^6 DNA GE/μL). Utilizing the optimized methodology, we estimate an extrapolated sensitivity of 90%, based on the range of CSF genome counts found in Laos. Dried CSF filter paper spots could potentially help us to better understand the epidemiology of bacterial meningitis in resource-poor settings and guide empirical treatments and vaccination policies.

Keywords: Bacterial meningitis, cerebrospinal fluid, filter paper, Lao PDR, *Streptococcus pneumoniae*
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Introduction

Bacterial meningitis is a life-threatening disease that carries significant mortality and considerable risk of severe neurological complications. In developing countries, such as the Lao People's Democratic Republic (Laos), the combined mortality rates of all causes of bacterial meningitis are likely to be >20% [1]. Vaccine-preventable diseases account for the major portion of bacterial meningitis cases seen worldwide. As elsewhere in SE Asia, *Streptococcus pneumoniae*, *Streptococcus suis*, *Neisseria meningitidis* and *Haemophilus influenzae* account for the majority of cases of community-acquired bacterial meningitis in Laos (LOMWRU unpublished) [2–5]. An improved understanding of the epidemiology of these diseases would support evidence-based national vaccination policies and empirical treatment guidelines. Vaccination programmes against *H. influenzae* type b, *S. pneumoniae* (seven and thirteen valent) and *N. meningitidis* (serotypes C, A, Y and W135) have had a profound impact on the incidence of bacterial meningitis cases in many regions [6–10].

In Laos, conventional bacteriological and molecular microbiological facilities for the diagnosis of central nervous system disease are confined to the capital city of Vientiane, and

antibiotic treatment is often initiated prior to lumbar puncture [11]. This may reduce the sensitivity of cerebrospinal fluid (CSF) culture by up to 33% [12]. The polymerase chain reaction (PCR) has been shown to be superior to conventional methods, particularly when antibiotics have been administered [13].

Reliable procedures are necessary to preserve, transport and test CSF samples, frequently requiring a challenging and costly cold chain to be in place. Effective disease surveillance is thus severely hampered in resource-limited settings, such as those currently present in rural Laos, where procedures for appropriate laboratory diagnosis are suboptimal. Innovative, simple and inexpensive rapid diagnostic tests for detecting pathogen antigens in CSF hold promise [14–16]. In West Africa, centralized PCR systems for CSF PCR have been developed [17], but it is likely that prolonged transport of CSF at high temperature will reduce sensitivity.

Blood dried onto filter paper to diagnose infectious diseases dates back to as early as 1939 [18] and has proved to be an important tool for diagnosis, epidemiology and monitoring in settings with limited laboratory infrastructure. However, CSF has rarely been collected on filter paper. In the 1970s counter-immunofluorescence was used to detect capsular polysaccharide antigen in dried purulent CSF samples and more recently an enzyme-linked immunosorbent assay used to diagnose neurocysticercosis [19,20]. Peltola *et al.* [21] successfully identified nucleic acids of *S. pneumoniae* and *H. influenzae* type b in CSF-impregnated filter paper strips after 8 months storage at room temperature in sealed plastic bags.

Until laboratory capacity is developed in rural Laos, filter paper could serve as a sample preservation method that can easily be delivered to a central laboratory for CSF PCR and therefore allow better understanding of the geographical epidemiology of bacterial meningitis. We investigated whether dried CSF spots would serve as a sensitive and reliable method for defining three of the major causative agents of bacterial meningitis in Laos: *S. pneumoniae*, *S. suis* and *N. meningitidis*. We investigated different types of filter paper and optimized the processing of dried CSF spots to obtain the maximum possible yield of nucleic acid. A prospective pilot study was conducted utilizing these optimized methods to compare PCR results on DNA extracted from dried CSF spots with routine liquid CSF and conventional bacterial culture.

Methods

Filter paper selection

Three varieties of filter paper were selected for performance evaluation: the Whatman Grade 903 (Cat. no. 10535097; Whatman), the Flinders Technology Associates (FTA) Micro Card™ (Cat. no. WB120210; Whatman) (referred to as FTA hereafter) and the FTA Elute Micro Card™ (referred to as FTA Elute hereafter) (Cat. No. WB120401; Whatman). Whatman 903 requires elution of the organism from the paper followed by lysis and DNA extraction. FTA and FTA Elute papers contain a proprietary mix of reagents that lyse cell walls but stabilize and bind nucleic acids. FTA paper requires a washing protocol, with the paper disc itself being used as a DNA template in the PCR.

A mock CSF was prepared by spiking tryptic soy broth (TSB) with a few discrete colonies of National Collection of Type Culture (NCTC) strains of either *S. pneumoniae* (NCTC 12977) or *N. meningitidis* (NCTC 10025) grown overnight on 5% goat blood agar. This was incubated for 4 h in 5–7% CO_2 at 37°C and then diluted with sterile TSB to achieve a standardized initial concentration (optical density) using the Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA). Ten-fold serial dilutions were prepared and aliquots spotted onto filter paper (Whatman 903 and FTA, 125 μL/1 inch circle; FTA Elute, 40 μL/11 mm circle) and onto blood agar (100 μL) to determine the bacterial load (colony forming units/mL) for each dilution using a micropipette.

Optimization studies were performed using *S. pneumoniae* and to demonstrate inter-species reproducibility final methods were repeated with *N. meningitidis*.

DNA extraction

Discs (3 or 8 mm) were punched out of the centre of a filter paper circle using skin biopsy punches (Stiefel, Maidenhead, UK) and transferred to 1.5-mL microcentrifuge tubes. Three and 8-mm punched discs would contain *c.* 3 μL and 20 μL of CSF, respectively. Biopsy punches were sprayed with 70% ethanol after each punch, dried with tissue paper and then punched into a stack of sterile filter paper a further five times to eliminate cross-contamination [22,23].

The FTA Elute manufacturer's instructions were followed with the following modifications: 1 mL of sterile water was used to wash the disc by pulse-vortexing three times for a total of 5 s. The DNA was eluted with sterile water after 22.5 min at 95°C, the mid-point of the manufacturer-recommended time period. FTA was processed following the manufacturer's instructions, except for the use of a slightly larger disc (3 mm in diameter rather than the recommended 2 mm). Whatman Grade 903 discs were processed following the QIAamp DNA Mini kit Dried Blood Spot Protocol (Qiagen, Crawley, UK) with the following modifications: elution was carried out in 180 μL of buffer ATL with proteinase K incubated at 56°C overnight.

Liquid CSF ($n = 25$) was initially processed using the QIAamp DNA Mini kit Blood or Body Fluids Protocol

