FILTER- CAPTURE DNA ISOLATION: A FILTER STERILIZATION AND NUCLEIC ACID PREPARATION METHOD FOR THE DETECTION OF *BURKHOLDERIA PSEUDOMALLEI* IN URINE

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Abstract (Revised)

The filter-capture DNA isolation (FCDI) method is a DNA preparation method for the rapid and sensitive detection of *Burkholderiapseudomallei*(Bp) by PCR in human urine samples (Michel 2017, Peddayelachagiri 2018). In the U.S., laboratory work with cultures of Bp must be performed in BSL3 facilities, however Bp DNA lysates are often transferred to BSL2 for PCR analysis after confirmation of lysate sterility. The objectives of the current study were to evaluate: (1) the sterilization performance, (2) total operational time, and (3) PCR detection range and sensitivity of FCDI. As a surrogate for clinical samples, we spiked human urine with the non-virulent Bp laboratory strain Bp82 and eight wild-type strains. FCDI lysates were prepared from the spiked urine samples by centrifugation using 0.1 µm pore-size spin filter microfuge columns for cellular capture and lysis of the captured organisms directly on the filters. Some FCDI lysates (100% volume) were cultured on agar media for 5 days at 35°C to evaluate the exclusion of viable bacterial cells. Additionally, FCDI lysates were used as templates for PCR to determine Bp82 detection range and sensitivity. The FCDI method was completed in less than one hour. No growth was observed from 45 cultured lysates after 5 days incubation on agar, and PCR yielded a detection range and sensitivity of 4.1 X 10^6 to 410 CFU/ml. FCDI is a rapid and effective laboratory method for removal of viable Bp82 from spiked human urine. Minimal sample volume (450µl) and common laboratory equipment are required. FCDI reagents are also shelf-stable at room temperature. The FCDI method yields DNA lysates of Bp that are free of viable bacterial cells. For labs that perform routine PCR detection of Bp, downstream DNA analysis of FCDI lysates could occur at BSL2.

Materials & Methods

Biosafety: All procedures involving virulent Bp strains were performed in a Class II Type A2 biological safety cabinet located in a BSL-3 laboratory registered by the U.S. Select Agent Program. All procedures were performed by trained personnel wearing personal protective equipment including a powered air-purifying respirator and protective laboratory clothing.

Strains: Bp82, an avirulent, select agent-excluded, ΔpurM mutant of Bp 1026b (deficient in adenine and thiamine biosynthesis) (Propst 2010) and wild type Bp strains: 1162, 1618, 1620, 1624, 1625, 1626, 1628, and 1634 were included in this investigation.

Growth & bacterial quantification: Strains were cultured overnight at 35 ± 2°C on Trypticase Soy Agar with 5 % sheep's blood (SBA). For strain Bp82, growth media were supplemented with adenine. Plate count estimates (CFU/ ml) were determined per cell suspension per strain for every experiment.

Spiking of pooled human urine: Bacterial suspensions of Bp were prepared in sterile saline and spiked into pooled human urine (BioIVT) at final concentrations of ~10^6, 10^4 ,or 10^2 CFU/ml.

Filter Capture DNA Isolation: For each spiked urine sample, Bp DNA was isolated using the method described in Michel et al, 2017. See Figure 1A. Briefly, an equal volume of 4% Triton Buffer was added per sample and transferred to a 0.1 µm PVDF spin filter device (Millipore). Bp cell were captured by centrifugation and 450 µl of 2 % Triton Buffer was added to each filter device, then centrifuged. Residual buffer was removed by another centrifugation. Bp cells were lysed on the filter using 65 µl of 0.05 N NaOH (in 250 mM NaCl ,1mM EDTA) and incubated at 35°C for 15 min. Bp DNA lysates were collected as flowthough after a final centrifugation. Each lysate was pH neutralized with 10 µl of 0.5 M Tris-Cl, pH 7.5.

Verification of DNA lysate sterility: For a subset of FCDI lysates (n=45 total), 100 % of the Bp DNA solution was spread plated on SBA. For lysates used for PCR analysis, 10% of Bp lysate was cultured on SBA. The SBA plates were incubated at 35°C/ 5 days to verify the absence of cultivable Bp cells. For a subset of spiked urine (n=15 total), cellular Bp material was directly removed from the 0.1 µm filters at steps 3 and 5 of FCDI, Gram stained and observed using a Nikon Eclipse Ni microscope.

Real-time PCR: Bp DNA was detected using the TTS1 real-time PCR targets described by Novak et al. 2006. TaqMan PCR using DNA lysates (5 µl/reaction) was added to a 20 µl reaction (25 µl final volume) of PerfeCTa Multiplex qPCR ToughMix with Low ROX (Quantabio, Beverly, MA, USA) with 5 mM $MgCl_2$. Thermocycling conditions consisted of 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec to 59°C for 2 min, performed in "Standard" mode. All real-time PCR results generating a positive amplification curve with a cycle threshold (Ct) value < 45 have been reported.

Results

FCDI Method Steps: 1 - 5

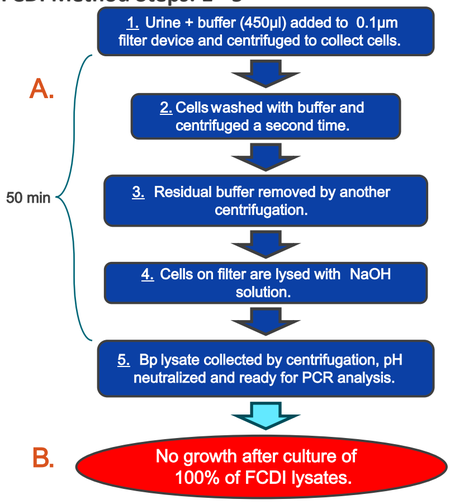

Figure 1. Sterilization performance and operational time of the FCDI Method (A.) The method requires ~50 min to complete. (B.) Eight wildtype strains and Bp82 were used to spike human urine and sterility was verified by culturing 100% of the lysates for 5 replicates per strain. No visible growth was observed in 45/45 total lysates cultured for 5 days on SBA.

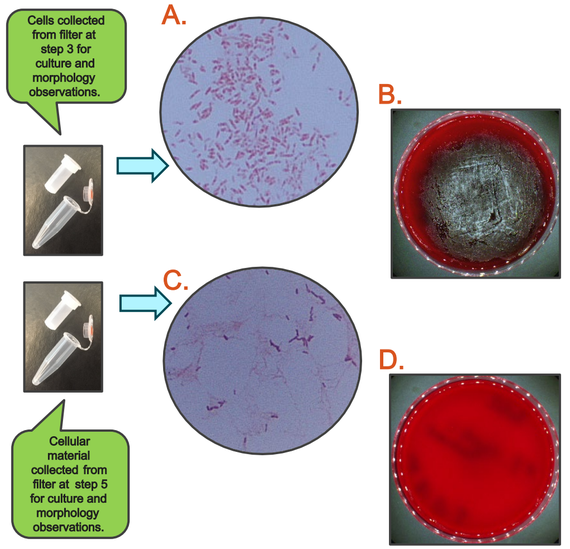

Figure 2. Assessment of Bp82 from spiked urine (~10^6 CFU/ml) before and after the FCDI alkaline lysis (Steps 3 and 5). (A.) Gram stain of Bp82 collected prior to lysis (100X oil). (B.) Growth on agar of Bp82 collected prior to lysis. (C.) Gram stain of Bp82 after lysis on filter (100X oil). (D.) No growth on agar of Bp82 cultured after lysis.

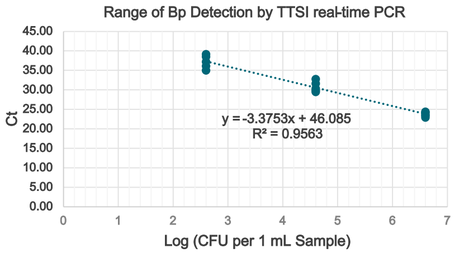

Figure 3. Bp DNA detection from FCDI lysates by PCR. Linear regression of real-time PCR results. Bp82 was consistently detected by TTS1 PCR (in 5/5 reactions per concentration) in urine spiked at ~10^6, 10^4 and 10^2 CFU/mL.

Concentration of Bp82 Spiked into Human Urine CFU/mL	Average C_t of Bp82 (5/5) Detected by TTSI PCR
4.1 X 10^6	23.7
4.1 X 10^4	30.8
410	37.2

Table 1. Sensitivity of Bp DNA by real-time PCR using FCDI lysates. Bp DNA was detected in urine spiked with Bp82 (5/5 PCR reactions per concentration tested).

Conclusions

- Viable Bp could not be recovered from FCDI lysates prepared from human urine spiked with Bp at concentrations of ~ 10^6 CFU/mL.
- Prior to the alkaline lysis step of FCDI, Bp cells captured on the 0.1µm filter are viable. Treatment of 0.1µm filters with 0.05N sodium hydroxide rendered lysates sterile.
- Up to 20 urine samples can be safely processed by the FCDI method in under 1 hour.
- By real-time PCR, FCDI lysates yield a detection range and sensitivity of 4.1 X 10^6 to 410 CFU/ml for Bp82.

Discussion

From less than 1 mL of a urine sample, lysates that are free of viable organisms and immediately available for PCR can be rapidly generated using the FCDI method. The FCDI method reduces laboratory hands on time and utilizes shelf stable reagents for extraction. The FCDI method demonstrates a robust approach to the detection of Bp in human urine with consistent sensitivity (≤ 1.0 x 10^3 CFU/ml) at clinically relevant concentrations (Wuthiekanun V 2007, Limmathurotsakul D 2005). A suspected melioidosis urine sample can be processed for detection of Bp in a BSL3 laboratory and then safely moved to a lower containment level laboratory for PCR testing with results the same day.

References and Acknowledgements

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