JOURNAL OF CLINICAL MICROBIOLOGY, June 2007, p. 1920–1926 Vol. 45, No. 6
0095-1137/07/$08.00+0 doi:10.1128/JCM.00147-07

Assessment of Fluorescent In Situ Hybridization and PCR-Based Methods for Rapid Identification of *Burkholderia cepacia* Complex Organisms Directly from Sputum Samples▽†

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Received 20 January 2007/Returned for modification 2 March 2007/Accepted 17 April 2007

Several species within the *Burkholderia cepacia* complex (BCC) have emerged as significant opportunistic pathogens of patients with cystic fibrosis (CF). BCC infection is typically associated with a poor clinical prognosis and decreased survival. These factors, combined with the existence of highly transmissible epidemic strains, have resulted in strict segregation of BCC- and non-BCC-infected patients to minimize cross infection. Accurate and rapid diagnosis of infections is essential to enable appropriate patient management. However, the rapidly evolving taxonomy of BCC poses a considerable challenge to diagnostics. In the present study, we assessed a commercially available fluorescent in situ hybridization (FISH) assay (seaFAST Cystic Fibrosis I kit) and a novel rRNA gene-based PCR assay for the rapid identification of BCC-positive sputa, irrespective of the BCC species. We report that, while the FISH assay fails to identify all BCC species, it does identify the majority of species, including the two most clinically relevant species, *B. multivorans* and *B. cenocepacia*. The sensitivity of the assay applied to sputum was limited by nonspecific background fluorescence. While sputum processing was optimized to minimize background, the resulting sensitivity for BCC detection was 8×10^5 CFU/ml. In contrast, the novel PCR assay reported herein exhibits 100% sensitivity and specificity for all BCC species and can detect 10^4 CFU/ml when applied to sputum. This novel rRNA gene-based assay is currently the most sensitive BCC-specific PCR assay for the detection of BCC direct from clinical samples and as such is a valuable addition to the field of BCC diagnostics.

The *Burkholderia cepacia* complex (BCC) is a diverse and highly adaptable group of bacteria, currently comprising 10 closely related species. Originally identified as plant pathogens (3), over the last 20 years BCC organisms have emerged as significant opportunistic pathogens of patients with cystic fibrosis (CF) and chronic granulomatous disease. There is considerable variability in the clinical outcome following BCC infection. However, infection in individuals with CF is typically associated with a poor clinical prognosis, with increased hospitalization and decreased survival (7, 12, 21). Additionally, a proportion of BCC-colonized patients succumb to "cepacia syndrome," a necrotizing pneumonia with septicemia that results in a rapid and invariably fatal clinical deterioration (12).

The rapidly evolving taxonomy of the BCC poses a considerable challenge to the diagnosis of infections within CF patient lungs (18). Originally a member of the *Pseudomonas* genus, *B. cepacia* was transferred to the *Burkholderia* genus alongside six other pseudomonads in 1992 (30). However, it was rapidly apparent that considerable diversity existed among "*B. cepacia*" strains. Five years later, a polyphasic taxonomic study resulted in the identification of the BCC, initially comprising five distinct genomovars (24), now known as *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, and *B. vietnamiensis*. In recent years, the BCC has expanded following the inclusion of *B. dolosa* (27), *B. ambifaria* (5), *B. pyrrocinia* (23), *B. anthina* (23), and *B. ubonensis* (24a). The majority of BCC infections of CF patient lungs are caused by *B. multivorans* and *B. cenocepacia*. However, with the exception of *B. ubonensis*, all BCC species have been identified within CF patient lungs, indicating that the ability to cause infection is not species dependent. Additionally, while *B. cenocepacia* is arguably the most virulent species within the BCC, there are several reports of dramatic clinical deterioration and/or decreased survival following infection with other BCC species, including *B. multivorans*, *B. stabilis*, and *B. dolosa* (2, 8, 13, 14). The significant clinical burden imposed by BCC infection has resulted in the implementation of strict segregation measures in order to reduce cross infection between BCC-infected and noninfected CF patients. Currently, the segregation policy does not discriminate between BCC species. Therefore, diagnostic assays that encompass all members of the BCC are crucial to the effective management of CF patients. The challenge facing BCC diagnostics is compounded by the ability of closely related non-BCC *Burkholderia* species to infect CF patient lungs, including *B. gladioli* (1, 4) and more recently *B. pseudomallei* (11, 19, 28). Currently, commercially available bacterial identification systems are insufficiently reliable for the identification of BCC species and their discrimination from closely related organisms.

To facilitate the accurate identification of BCC species, numerous PCR-based assays have been developed, typically based on rRNA gene sequences or the *recA* gene, both of which are either species or BCC specific. Many of these assays are now routinely used in reference laboratories for the iden-

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† Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 25 April 2007.

tification of BCC. Typically, presumptive BCC organisms are subjected to a BCC-specific *recA* PCR assay with subsequent HaeIII restriction fragment length polymorphism (RFLP) analysis of the PCR product, enabling tentative identification to the species level (15). Speciation is then confirmed by the application of the relevant species-specific PCR assay. This process is routinely applied to bacterial cultures. However, culture of BCC organisms from sputum typically requires 48 to 72 h of incubation before the appearance of colonies on selective media (22). Thus, reliance on culture introduces inherent delays to the identification of the infecting organism, particularly if bacterial counts within the sputum are low or isolates atypical. Several BCC PCR assays have been successfully applied directly to sputum, bypassing the need for culture and thus enabling rapid identification of the organism. However, the sensitivities of such assays differ dramatically. For example, while the BCC-specific *recA* PCR assay identifies all species within the BCC, a high bacterial count must be present in order for it to be successfully applied to sputum (17). In contrast, rRNA gene-based assays typically have better sensitivity when applied to sputum (29). However, to the authors' knowledge, no single rRNA gene-based PCR assay that reliably detects all BCC members and discriminates them from closely related non-BCC species has been developed. As the current segregation policy for BCC-infected patients is not species dependent, we have assessed two methodologies, a commercially available fluorescent in situ hybridization (FISH) assay and a novel 16S rRNA gene-based PCR assay, for their ability to rapidly identify BCC-positive sputa irrespective of the BCC species present.

MATERIALS AND METHODS

Bacterial strains and culture conditions. To determine the specificity and sensitivity of the diagnostic assays in the present study, a panel of relevant strains was assembled against which each assay was tested. This panel comprised the previously published BCC experimental strain panels (6, 16), together with *B. gladioli* ($n = 3$), *B. caledonica* ($n = 3$), *Pseudomonas aeruginosa* (mucoid and nonmucoid strains and representatives of major epidemic strains) ($n = 34$), other pseudomonads ($n = 6$), *Stenotrophomonas maltophilia* ($n = 4$), *Ralstonia* spp. ($n = 6$), *Pandoraea* spp. ($n = 4$), *Achromobacter xylosoxidans* ($n = 5$), and *Acinetobacter baumannii* ($n = 2$). The BCC experimental strain panels, obtained from the BCCM/LMG Bacteria Collection (Ghent, Belgium), comprise 8 *B. multivorans* isolates, 10 *B. cenocepacia* isolates, 3 *B. ambifaria* isolates, and 4 isolates each of *B. cepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. anthina*, and *B. pyrrocinia*. All other strains were clinical isolates from the Edinburgh Strain Repository, identification of which was previously confirmed by relevant API and/or species-specific PCR. Subsequent to the analysis of the strain panel described above, the diagnostic assays were also applied to *B. ubonensis* LMG20358^T (BCCM/LMG Bacteria Collection, Ghent, Belgium), five presumptive novel species of the BCC (24a), and 37 clinical BCC isolates received by our laboratory in the course of this study (24 *B. multivorans* isolates, 11 *B. cenocepacia* isolates, and one isolate each of *B. cepacia* and *B. vietnamiensis*), bringing the total number of strains tested to 88 BCC isolates and 67 non-BCC isolates. All strains were cultured on nutrient agar with overnight incubation at 37°C.

Microbiological analysis of sputum. Sputum samples received by our laboratory were initially mixed with an equal volume of Sputolysin (Calbiochem) and vortexed thoroughly until liquefied. Tenfold serial dilutions of this sputum-Sputolysin mixture were prepared in sterile saline prior to plating on the following media: chocolate blood agar (10^{-2} and 10^{-4} dilutions), blood agar (10^{-2} and 10^{-4} dilutions), *Pseudomonas* isolation agar (Difco, Becton Dickinson) supplemented with glycerol (2% final concentration) (neat and 10^{-2} and 10^{-4} dilutions), and *Burkholderia cepacia* medium supplemented with *B. cepacia* Selectatab (Mast Diagnostics) (neat and 10^{-2} and 10^{-4} dilutions). By this protocol, the lower limit of detection by culture is 10^2 CFU/ml. Following incubation at 37°C for 48 to 72 h, preliminary identification of resulting cultures was performed, based on morphological examination of colony types and API20NE biochemical testing. Presumptive BCC organisms identified on the basis of colony phenotype and/or API20NE profile were confirmed as such by the BCC-specific *recA* PCR assay using the BCR1/BCR2 primer pair (15). Subsequent HaeIII RFLP analysis of the *recA* PCR product was followed by the relevant species-specific PCR.

FISH analysis of sputum. The 1:1 mixture of sputum-Sputolysin prepared previously (see "Microbiological analysis of sputum") was further diluted in Sputolysin to an optical density at 600 nm (OD_{600}) of approximately 0.6 in a final volume of 1 ml. Typically this OD_{600} equated to a final dilution factor ranging from 1:10 to 1:40, depending on the viscosity of the original sputum sample. This 1-ml sputum mixture was vortexed thoroughly and split into two equal aliquots. Both aliquots were centrifuged at $10,000 \times g$ for 5 min, and the supernatant was removed. One cell pellet was retained for subsequent DNA extraction (see "Extraction of genomic DNA"), while the second cell pellet was resuspended in 1 ml sterile saline. Ten microliters of this saline suspension was applied per field on an eight-field microscope slide. FISH analysis was performed using the seaFAST Cystic Fibrosis I kit (IZINTA, Hungary; formerly SeaPro Theranostics International, The Netherlands) according to the manufacturer's instructions. The kit enables identification of four CF pathogens (*B. cepacia*, *Haemophilus influenzae*, *P. aeruginosa*, and *S. maltophilia*) through the use of two tandem probe pairs, the sequences of which are the same as the Burcep, Haeinf, PseaerA/PseaerB, and Stemal probes previously employed by Hogardt and colleagues (10). In brief, following application of samples to the slide, slides were dried at 55°C, fixed for 3 min in a SeaWAVE programmed microwave, and immersed in methanol for 10 min. After the slides were dried, 10 μl of reconstituted probe mixture was added to each field and slides were placed in a hybridization chamber containing filter paper soaked in 3 ml hybridization buffer. Hybridization at 46 to 48°C was performed in the SeaWAVE programmed microwave for 12 min according to the manufacturer's protocol, following which slides were washed for 16 min (46 to 48°C). Slides were rinsed briefly in methanol and allowed to dry prior to applying mounting medium and a coverslip. Fluorescence was read immediately using a Leica DM LB2 fluorescence microscope fitted with a Hamamatsu ORCA-ER digital camera.

Extraction of genomic DNA. To assess the specificity of the diagnostic PCR assay, crude genomic DNA was prepared from all isolates within the strain panel (see "Bacterial strains and culture conditions"). In brief, two bacterial colonies were resuspended in 20 μl lysis buffer (0.25% sodium dodecyl sulfate, 0.05 M NaOH) and incubated at 95°C for 15 min. After brief centrifugation, 180 μl sterile water was added and the mixture was centrifuged at $13,000 \times g$ for 5 min. The supernatant containing genomic DNA was used directly in PCR assays.

For application of PCR assays to sputum, three alternative DNA extraction methods were compared to assess the impact on sensitivity of the PCR assays. Starting material for DNA extractions was the cell pellets obtained following centrifugation of the sputum-Sputolysin mixture (see "FISH analysis of sputum"). These cell pellets were either resuspended in 100 μl lysis buffer (described above) or 100 μl Chelex-100 (5%, wt/vol; Sigma) or processed using the QIAamp DNA kit (QIAGEN) according to the manufacturer's instructions (100-μl final elution). Pellet suspensions in lysis buffer were incubated at 95°C for 15 min prior to centrifugation at $13,000 \times g$ for 5 min. Supernatant was diluted 1:10 prior to use in PCRs. Pellet suspensions in Chelex-100 were subjected to two cycles of heating (95°C, 5 min) and chilling on ice (5 min), prior to centrifugation at $13,000 \times g$ (5 min). Supernatant from Chelex-100 preparations was used directly in PCRs.

PCR detection of BCC from sputum. Representative 16S rRNA sequences were aligned for all available BCC species, generating a consensus sequence which was then compared with alignments of relevant non-BCC 16S rRNA sequences. On the basis of these alignments, a BCC-specific 16S rRNA gene-based PCR assay was designed (forward, 5′-TCC GGA AAG AAA WCC TTG GY; reverse, 5′-AAT GCA GTT CCC AGG TTG AG). Additionally, a human 16S rRNA gene-based PCR assay was designed to act as a positive control in PCRs directly from sputum (forward, 5′-GCT CAG GGA GGA CAG AAA CC; reverse, 5′-AGT GGG TGA ACA ATC CAA CG). BCC PCRs were performed in a 50-μl volume containing 600 nM forward primer, 200 nM reverse primer, 3 mM $MgCl_2$, 260 μM of each deoxynucleoside triphosphate (dNTP), 4% (vol/vol) dimethyl sulfoxide, 1 U *Taq* polymerase (Invitrogen), and appropriate manufacturer's reaction buffer. For multiplex PCR assays with both human- and BCC-specific primers, 100 nM of each human primer was used, $MgCl_2$ was increased to 4 mM, dNTPs were increased to 400 μM, and *Taq* polymerase was increased to 1.5 U. Thermal cycling was performed on a GeneAmp PCR System 9700 (Applied Biosystems) with the following parameters: 95°C for 2 min; 40 cycles of 95°C (30 s), 55°C (20 s), and 72°C (30 s); and 72°C for 10 min. PCR products were electrophoresed on a 4% E-Gel (Invitrogen) and visualized by UV illumination.

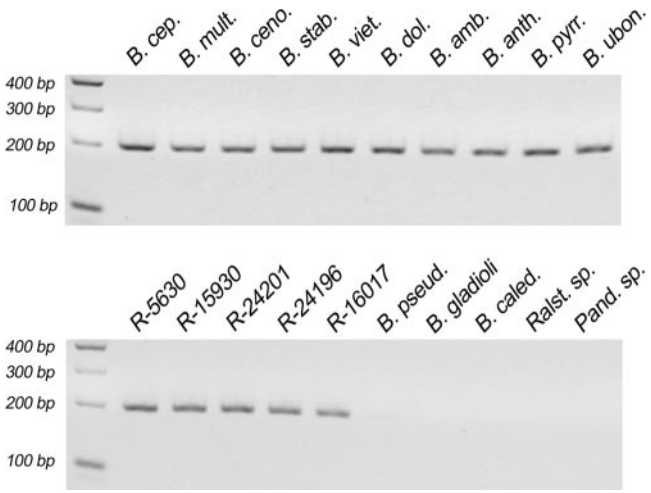

FIG. 1. Application of the BCC 16S rRNA gene-based PCR assay to BCC and closely related non-BCC species. A 196-bp product was obtained from all BCC species examined to date. B. cep., *B. cepacia*; B. mult., *B. multivorans*; B. ceno., *B. cenocepacia*; B. stab, *B. stabilis*; B. viet., *B. vietnamiensis*; B. dol., *B. dolosa*; B. amb., *B. ambifaria*; B. anth., *B. anthina*; B. pyrr., *B. pyrrocinia*; B. ubon., *B. ubonensis*; B. pseud., *B. pseudomallei*; B. caled., *B. caledonica*; Ralst., *Ralstonia*; Pand., *Pandoraea*. R-5630, R-15930, R-24201, R-24196, and R-16017 represent five presumptive novel species of the BCC (24a). Results shown are representatives of each species tested. Refer to Materials and Methods ("Bacterial strains and culture conditions") for full details of the number of isolates of each species tested.

Assessment of assay sensitivity using spiked sputum. To assess assay sensitivity, a known BCC-negative sputum sample was diluted with Sputolysin to an approximate OD_{600} of 0.6. BCC organisms were grown overnight in Luria-Bertani broth or BM2-glucose minimal media (9) and viable counts performed. Aliquots of overnight culture were spun and cells resuspended in 1 ml of the sputum-Sputolysin mixture. Tenfold serial dilutions of this suspension were prepared using the sputum-Sputolysin mixture as the diluent. These suspensions were then processed for FISH and PCR as described above. Viable counts of the overnight culture enabled calculation of equivalent CFU/ml in the spiked sputum samples.

RESULTS

Specificity of BCC diagnostic assays applied to the strain panel. The BCC rRNA gene PCR assay exhibited 100% sensitivity and specificity for the identification of BCC when applied to the strains described above, correctly identifying all 88 BCC isolates tested. No reactivity was observed with any of the 67 non-BCC isolates tested, comprising pseudomonads (n = 40), non-BCC *Burkholderia* species (n = 6), *S. maltophilia* (n = 4), *Ralstonia* spp. (n = 6), *Pandoraea* spp. (n = 4), *Achromobacter xylosoxidans* (n = 5), and *Acinetobacter baumannii* (n = 2). Representative PCR results from BCC and non-BCC isolates are shown in Fig. 1. Inefficient PCR amplification was observed when the BCC PCR assay was applied to *B. pseudomallei* DNA, which occasionally resulted in a weak PCR band that could readily be distinguished from a true-positive result from BCC (Fig. 1).

In contrast to the PCR assay, the *B. cepacia*-specific probe within the seaFAST Cystic Fibrosis I FISH kit failed to identify all members of the BCC when applied to the strain panel (Fig. 2). No reactivity was observed with four of four *B. stabilis* strains, two of four *B. pyrrocinia* strains, and one of three *B. ambifaria* strains. Examination of available 16S rRNA sequences revealed a 3-nucleotide mismatch between the *B. cepacia* probe sequence and the published gene sequence for all *B. stabilis* strains sequenced and one-half of the *B. pyrrocinia* strains sequenced, thus explaining the observed lack of reactivity. The reason for the lack of reactivity observed with one *B. ambifaria* strain is unclear. The *B. cepacia* FISH probe did not cross-react with other closely related non-BCC species studied. Thus the specificity and sensitivity of the FISH kit for BCC when applied to the strain panel were 100% and 83%, respectively. Subsequent to the analysis of the strain panel, the FISH assay was shown to successfully identify *B. ubonensis* LMG20358^T (BCCM/LMG Bacteria Collection, Ghent, Belgium) (Fig. 2J) and five presumptive novel species of the BCC (24a) (data not shown).

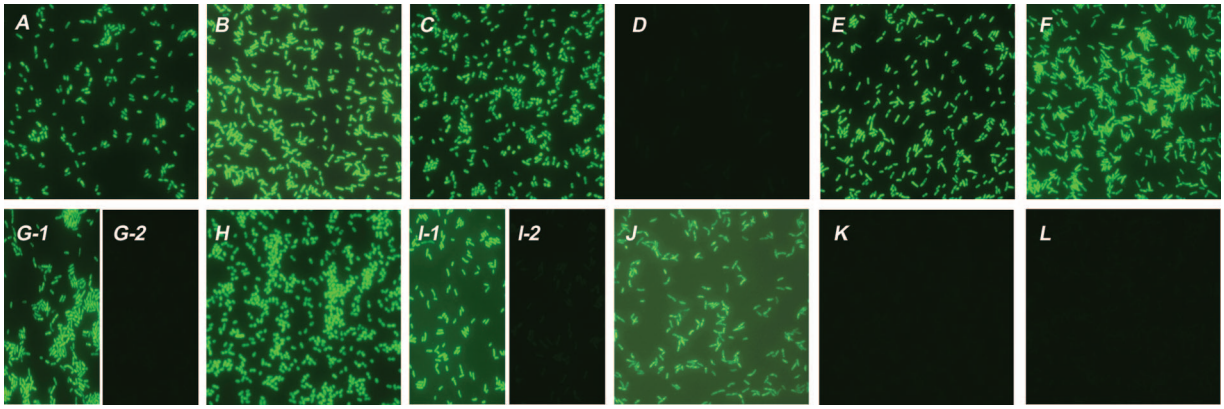

FIG. 2. Application of the seaFAST Cystic Fibrosis I FISH kit to representative BCC and non-BCC strains. (A) *B. cepacia*; (B) *B. multivorans*; (C) *B. cenocepacia*; (D) *B. stabilis*; (E) *B. vietnamiensis*; (F) *B. dolosa*; (G-1 and G-2) *B. ambifaria*; (H) *B. anthina*; (I-1 and I-2) *B. pyrrocinia*; (J) *B. ubonensis*; (K) *B. gladioli*; (L) *Ralstonia* sp. The *B. cepacia* probe failed to identify four of four *B. stabilis* strains, one of three *B. ambifaria* strains, and two of four *B. pyrrocinia* strains tested. All other BCC species were reliably detected. No reactivity was observed with closely related species, including *B. gladioli* (K) and *Ralstonia* species (L). Results shown are representative of each species tested. Refer to Materials and Methods ("Bacterial strains and culture conditions") for full details of the number of isolates of each species tested.

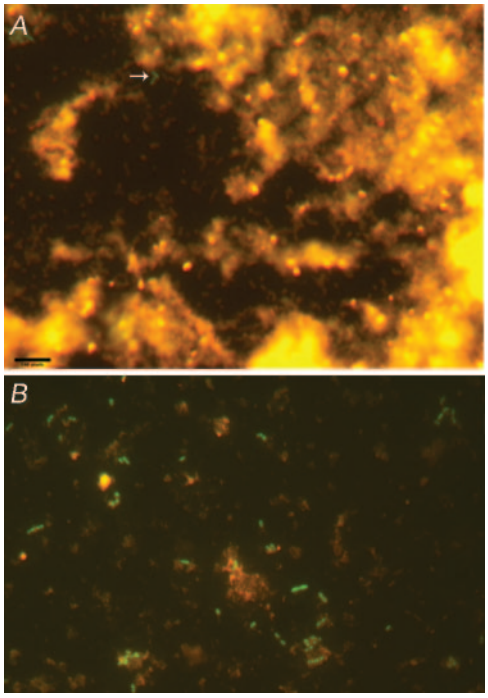

FIG. 3. Application of the seaFAST Cystic Fibrosis I kit to sputa prior to optimization (A) and following optimization (B). (A) Sputum sample spiked with BCC organisms to an equivalent of $>10^{10}$ CFU/ml. The arrow highlights two BCC cells showing weak fluorescence. (B) Clinical sputum sample containing 10^7 CFU/ml BCC organisms, with strongly fluorescent BCC organisms clearly visible.

Direct detection of BCC in sputum by FISH. Initially, FISH analysis was attempted on sputum that had been diluted 1:2 with Sputolysin, similar to the protocol described by Hogardt et al. (10), or 1:10 with Sputolysin (according to the manufacturer's instructions). These sputum-Sputolysin dilutions were applied directly to the microscope slide for FISH analysis. However in our hands, neither approach yielded satisfactory results. When sputum was diluted 1:2, a high level of nonspecific fluorescence prevented reliable identification of BCC organisms (Fig. 3A), even when present in high numbers ($>10^{10}$ CFU/ml), while 1:10 dilutions with Sputolysin were associated with inefficient fixing of the sample to the slide. Optimization of the sputum preparation protocol focused on reducing the nonspecific fluorescence associated with the 1:2 Sputolysin dilution, thus enabling ready detection of the specific fluorescence attributable to BCC organisms (Fig. 3B). The optimized methodology (as given in Materials and Methods) essentially eradicated the background fluorescence by (i) performing dilution in Sputolysin (the extent of which varied according to sample viscosity), (ii) recovering the sputum cell pellet by centrifugation, and (iii) resuspending the pellet in sterile saline. When applied to serial dilutions of sputum spiked with *B. cenocepacia* or *B. multivorans*, this optimized methodology exhibited a lower limit of detection of 8×10^5 CFU/ml (data not shown). The same sensitivity was obtained irrespective of whether the spike organism was grown in Luria-Bertani broth or BM2-glucose minimal media, despite the fact that the fluorescence signal was considerably weaker from cells grown in minimal media, presumably due to lower metabolic activity. When the seaFAST Cystic Fibrosis I FISH kit was used, a comparable lower limit of detection ($\geq 7 \times 10^5$ CFU/ml) was observed for the identification of *P. aeruginosa* within sputum (data not shown).

To reduce the nonspecific fluorescence and thus aid the rapid identification of BCC organisms within sputum, the sputum cell pellet was resuspended in a final volume of 1 ml saline, with only 10 μl of this being applied to the slide for FISH analysis. We anticipated that, while this dilution factor results in low background fluorescence, it would adversely affect the sensitivity of the assay. We assessed this by resuspending a sputum cell pellet in various volumes of saline (0.1, 0.5, and 1.0 ml) and applying 10 μl of each suspension to a slide for subsequent FISH analysis. Despite the sputum harboring 10^7 CFU/ml BCC organisms (as determined by culture), no BCC-positive signal was detected when FISH was applied to the 0.1-ml pellet suspension due to high background fluorescence (Fig. 4A). Weak BCC-associated fluorescence was observed when FISH was applied to the 0.5-ml pellet suspension (Fig. 4B). However, strong fluorescence enabling reliable identification of BCC was observed only when the sputum cell pellet was resuspended in 1 ml with a resulting decrease in background fluorescence (Fig. 4C). Thus while the dilution factor results in fewer BCC organisms per field, the resulting low background effectively

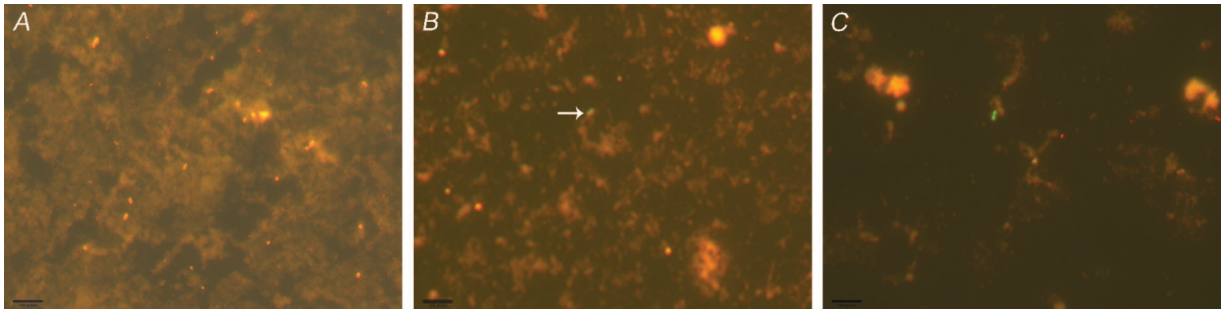

FIG. 4. Assessing the impact of sputum dilution on the sensitivity of FISH analysis. Shown is a representative FISH analysis a clinical BCC-positive sputum sample (10^7 CFU/ml) prepared according to the protocol in Materials and Methods. The sputum cell pellet was resuspended in either 0.1 ml (A), 0.5 ml (B), or 1 ml (C) saline. No BCC fluorescence was detectable in the 0.1-ml sputum suspension owing to the high background. While very weak fluorescence attributable to BCC organisms was occasionally evident when the pellet was resuspended in 0.5 ml saline (B, arrow), reliable detection of the BCC organism was achieved only when the pellet was resuspended in 1 ml.

increases the sensitivity of the assay. Additionally, the low background fluorescence significantly reduces the time required for microscopy, thus increasing sample throughput. Attempts to improve sensitivity further by differential centrifugation of liquefied sputum (thus concentrating bacterial cells in the absence of material responsible for nonspecific fluorescence) were unsuccessful (data not shown).

The FISH results obtained using spiked sputum correlated fully with the results obtained when applied to 69 clinical sputum samples (see Table S1 in the supplemental material). With one exception, all BCC-positive sputum samples examined harbored at least 10^6 CFU/ml BCC organisms (higher than the limit of detection by FISH) and thus were correctly identified as BCC positive by FISH analysis. FISH did not identify a *B. vietnamiensis*-positive sputum sample containing 5×10^4 CFU/ml. No BCC-positive sputa examined in the course of this study harbored *B. stabilis*, *B. pyrrocinia*, or *B. ambifaria*, species which are associated with a false-negative FISH result (see above; Fig. 2). No FISH false positives were observed in any BCC culture-negative sputum samples studied.

Direct detection of BCC in sputum by PCR. Three different sputum DNA extraction protocols were tested in the present study to assess the impact that the quality and cleanliness of the DNA preparation has on PCR sensitivity. All three methods were successfully applied to the direct detection of BCC in sputum. However, the crude DNA extraction method (cell pellet resuspended in lysis buffer and heated at 95°C for 15 min) was associated with occasional PCR failures, as determined by the failure of the human 16S rRNA gene-based positive control. These PCR failures were presumed to arise from PCR inhibitors in the sputum that were not removed by the DNA extraction process. Consequently, the crude DNA extraction protocol was not investigated further. No PCR failures were observed with either of the other DNA extraction protocols tested (Chelex-100 supernatant or QIAamp DNA kit [QIAGEN]).

When the BCC-specific 16S rRNA gene-based PCR assay was applied to DNA prepared from serial dilutions of sputum spiked with *B. cenocepacia*, both the Chelex-100 and QIAamp DNA extraction protocols enabled reliable detection of 10^4 CFU/ml when 10 μl of template DNA was used per 50-μl reaction mixture (Fig. 5A). A comparable limit of detection (between 10^4 and 10^5 CFU/ml) was observed for all other BCC species examined in the same way (genomovars I to X). In contrast, the *recA*-based PCR assay for the identification of BCC (15) was 100- to 1,000-fold less sensitive (limit of detection, 10^7 CFU/ml) when applied directly to sputum (Fig. 5B), irrespective of the DNA extraction protocol.

When applied to 69 clinical sputum samples, the 16S rRNA gene-based PCR assay (applied to Chelex-100 supernatants) exhibited 100% sensitivity and specificity for detection of BCC-positive sputa compared with culture results (see Table S1 in the supplemental material). All BCC-positive sputum samples studied had bacterial counts in excess of the PCR limit of detection previously established using BCC-spiked sputum. No PCR false positives were observed in any BCC culture-negative sputum samples.

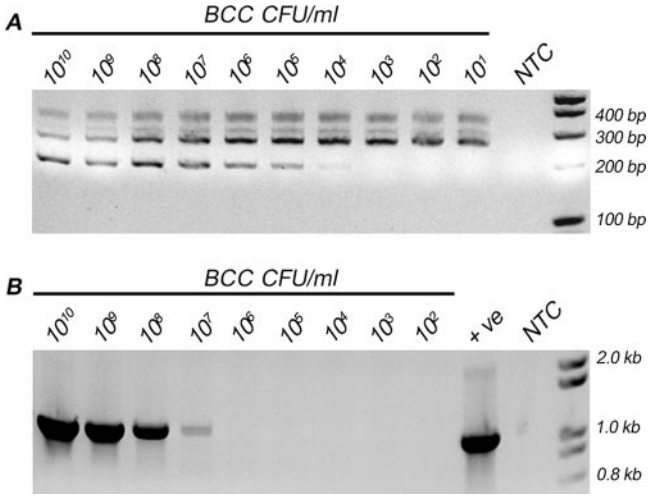

FIG. 5. Application of the 16S rRNA gene-based PCR assay (A) and the *recA*-based PCR assay (B) to DNA prepared from 10-fold serial dilutions of sputum spiked with known numbers of *B. cenocepacia*. (A) The rRNA gene-based PCR assay (196-bp product) detected the *B. cenocepacia* spike at 10^4 CFU/ml. The assay is multiplexed with a human rRNA gene PCR assay to provide a positive control (270-bp product). Additional PCR bands evident between 300 and 400 bp are an artifact of multiplexing the BCC and human PCR primers and applying to a sputum DNA template. These bands are not evident when either primer pair is applied individually to sputum DNA or when primers are multiplexed and applied to bacterial DNA (data not shown). (B) The *recA* PCR (1,043-bp product) detected the *B. cenocepacia* spike at 10^7 CFU/ml. The positive control (+ ve) is genomic DNA prepared from *B. cenocepacia* J2315. NTC, no-template control.

DISCUSSION

The emergence of BCC as a CF pathogen and the complex and rapidly evolving BCC taxonomy have placed considerable demands on laboratories responsible for the diagnosis of lung infections in CF. While the identification of BCC species has undoubtedly improved since their emergence within the CF population in the 1980s (20), misidentification remains a significant cause for concern (18, 26). Conventional biochemical systems are unreliable in the identification of BCC (26); thus, attention has invariably turned to PCR-based methodologies in the quest for BCC-specific diagnostic methods. Generally, there are two main options when considering a BCC diagnostic assay: either a single assay that encompasses all BCC species but does not discriminate between species or individual assays that are specific for each of the BCC species. Currently, the *recA*-based PCR assay (15) is the only assay that achieves both aims, as the initial PCR identifies all BCC species and subsequent HaeIII RFLP analysis informs on the species. However, the *recA*-HaeIII RFLP assay is itself challenged by the expanding taxonomy of the BCC, as different BCC species can now be associated with similar HaeIII RFLP profiles that cannot be adequately resolved by conventional agarose gel electrophoresis (authors' unpublished observations). While these patterns can be resolved by polyacrylamide gel electrophoresis (25), this approach is not routinely undertaken. Species level identification of BCC is essential for epidemiological surveillance and for enabling the clinical significance of less common species to be established. However, the current BCC infection control

policy is not species dependent. Thus, there is also a need for rapid and sensitive assays that can be applied directly to sputum for the identification of BCC-positive samples, avoiding the inherent delays (48 to 72 h) in culture-based methods and enabling rapid implementation of appropriate infection control measures. Accordingly, we assessed a commercially available FISH assay and a novel 16S rRNA gene-based PCR assay for the rapid identification of BCC in CF patient sputum.

FISH is a well-established method for the accurate identification of pathogens within a clinical sample without the need for culture. While the seaFAST Cystic Fibrosis I FISH assay assessed in the present study utilizes the same probe sequences as those employed by Hogardt and colleagues (10), BCC taxonomy has evolved considerably since their study in 2000. Thus, we assessed the specificity of the *B. cepacia* probe with the commercially available kit for all members of the BCC and demonstrated that, while the probe fails to detect all BCC species, it correctly identifies the majority, including the two most clinically relevant species, *B. cenocepacia* and *B. multivorans*. Accordingly, based on current BCC epidemiology, the seaFAST Cystic Fibrosis I FISH kit can be expected to correctly identify the causative organism in greater than 90% of BCC infections of CF patient lungs.

When applied to sputum spiked with either *B. multivorans* or *B. cenocepacia*, the FISH assay exhibited a sensitivity of approximately 8×10^5 CFU/ml. The *B. cepacia* FISH probe sequence appears to be conserved across the majority of BCC species; thus, the same level of sensitivity can be assumed for the less common species that have 100% identity to the probe sequence. To put this level of sensitivity in context, approximately 10% of recent BCC-positive sputum samples received by the Edinburgh CF Microbiology Laboratory had BCC counts less than 8×10^5 CFU/ml. Considerable optimization of the sputum processing protocol was required in the present study to achieve a comparable level of sensitivity to that reported previously (10), despite the use of the same probe sequences. When considering this, it is important to highlight that, since the study by Hogardt and colleagues, considerable changes in the FISH methodology have been implemented with the commercialization of the assay. For example, while Hogardt et al. employed a 30-min period of prehybridization with unlabeled oligonucleotides to reduce nonspecific binding of labeled probes, followed by a 1.5-h hybridization (10), the current commercial assay using the same probe sequences has no prehybridization step and a 12-min hybridization. The optimized sputum processing protocol described herein circumvents the problems associated with nonspecific binding of labeled probes that was reduced by the prehybridization step used by Hogardt and colleagues. Elimination of nonspecific background fluorescence is of particular importance for the detection of BCC, as the intensity of fluorescence signal from hybridized BCC bacteria is typically lower than that observed for other bacterial species. Generally, this low fluorescence signal in FISH analyses correlates with a low ribosome content, and in the case of *B. cepacia* infection of CF patient lungs it has been proposed that it reflects a low rate of in situ metabolic activity during chronic infection (10). In the present study we spiked sputum with BCC organisms grown in either complex or minimal media. Despite the BCC signal intensity being significantly lower when organisms were grown in minimal media, the low background fluorescence ensured that the sensitivity of detection was unaffected. It is worth noting that the intensity of BCC fluorescence we observed in clinical samples was comparable to that observed in sputum spiked with BCC organisms grown on complex media. Additionally, we have not observed the extremely low signal intensity associated with growth on minimal media in any clinical samples studied. Thus, while the basal level of metabolic activity of BCC may be lower than that of other bacterial species, we see no evidence that the metabolic level is specifically reduced as a consequence of chronic infection of CF patient lungs.

Numerous PCR-based assays for the identification of BCC have been described previously, for application both to cultures and directly to clinical samples. We aimed to improve on existing methods for the rapid identification of BCC by developing an rRNA gene-based PCR assay that (i) is capable of identifying all species within the BCC and (ii) offers a higher sensitivity, when applied to sputum, than the existing *recA* PCR assay. Multiple copies of the rRNA gene exist throughout the bacterial genome; thus, rRNA gene-based PCR assays typically offer higher sensitivity than PCR assays targeted against a single-copy gene, such as *recA*. This is the case in the present study, with a sensitivity of approximately 10^4 CFU/ml for the rRNA gene-based assay versus 10^7 CFU/ml for the *recA*-based assay. Over 30% of recent BCC-positive sputum samples referred to the Edinburgh CF Microbiology Laboratory had less than 10^7 CFU/ml BCC, while none had less than 10^4 CFU/ml. The *recA* PCR sensitivity of 10^7 CFU/ml was obtained by increasing the number of cycles from 30 to 40 in the absence of any PCR additives. It has been reported that inclusion of dimethyl sulfoxide enhances amplification of the *recA* gene from sputum, enabling the detection of 10^6 CFU/ml (17). Even with this enhancement, the rRNA gene-based assay described in the present study offers a 10- to 100-fold increase in sensitivity. However, the rRNA gene assay described herein is not as sensitive as similar BCC rRNA gene assays described previously (29). This is likely to be a consequence of the assay design, as the inclusion of "wobble" nucleotides within the forward primer to ensure that all BCC species are identified appears to result in lower PCR amplification efficiency. Removal of one wobble nucleotide increased sensitivity 10-fold when applied to sputum, but the resulting assay lacked 100% sensitivity and specificity for the BCC when applied to the strain panel (data not shown). Thus while the PCR assay reported herein is less sensitive than other rRNA gene-based BCC assays, to the authors' knowledge it is the only rRNA gene assay that encompasses all members of the BCC. As such, this novel assay is currently the most sensitive BCC-specific PCR assay for the detection of all BCC species direct from sputum.

In conclusion, the BCC diagnostic assays assessed in the present study have potential application for diagnostic and research purposes. Both the FISH and PCR assays assessed in the present study are valuable tools to aid in the identification of atypical BCC cultures. Upon application directly to sputum, and based on current BCC epidemiology within the CF patient population and typical bacterial counts within BCC-infected CF patient lungs, the seaFAST Cystic Fibrosis I FISH kit is capable of identifying BCC-positive sputa in the majority (80 to 90%) of cases. However, the FISH assay has two major

weaknesses. First, the sensitivity of FISH analysis applied to sputum is ultimately limited by the inability to isolate bacterial cells from the sputum sample. Second, current FISH methodologies are not suited to high-throughput analysis, primarily due to the need for traditional fluorescence microscopy. Optimized sputum processing protocols, as presented herein, reduce microscopy time, but the throughput of samples remains limited. In contrast, PCR-based methodologies are well suited for high-throughput assays, in both conventional 96-well formats and real-time PCR systems, which are increasingly available. The novel BCC-specific rRNA gene-based PCR assay presented herein identifies all BCC species with a level of sensitivity that is capable of identifying the vast majority of BCC-infected clinical samples (indeed, 100% of those received by our laboratory in the course of this study) irrespective of the BCC species. While this PCR assay does not enable species level identification, which remains important for epidemiological purposes, it is a valuable addition to existing diagnostics for the rapid identification of BCC-positive sputum.

ACKNOWLEDGMENTS

This work was undertaken under the framework of the UK Cystic Fibrosis Microbiology Consortium, an initiative funded by the Big Lottery Fund in association with the Cystic Fibrosis Trust.

We thank C. Doherty, F. Pike, V. Barcus, and L. O'Gorman of the Edinburgh CF Microbiology Laboratory for their assistance with sputum microbiology; H. Malnick and T. Pitt of the Health Protection Agency for the provision of *B. pseudomallei* genomic DNA; A. King (Microgen Bioproducts) and I. Thrippleton (formerly of SeaPro Theranostics International) for helpful discussion regarding FISH methodology; and E. Mahenthiralingam (Cardiff University) for helpful discussion regarding sputum DNA extraction and PCR methodologies.

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