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Full Length Research Paper

Detection of *Pseudomonas aeruginosa* in sputum samples by modified fluorescent *in situ* hybridization

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Pseudomonas aeruginosa **is the most common and dominant infectious agent that causes chronic pneumonia in patients with cystic fibrosis (CF). Fluorescent in situ hybridization (FISH) is a powerful molecular method for the specific and rapid diagnosis of bacteria, including the detection of *P. aeruginosa* in sputum samples from CF patients. High background fluorescence of viscous sputum samples obtained from CF patients may impede detection of microorganisms by FISH. The aim of this study was to test the application of biotin during FISH technique to reduce unspecific background fluorescence in sputum samples to facilitate and improve detection of *P. aeruginosa*. Sixty-three sputum samples from CF patients were tested by FISH to detect *P. aeruginosa*. All the 63 samples were also examined by a modified FISH procedure including biotin treatment. The FISH results were compared with those of conventional culture method. The specificity of FISH was 100%. The sensitivity of FISH for detection of *P. aeruginosa* from samples without biotin treatment was 83.3%, whereas in biotin-treated samples was 88.1%. Biotin reduced background fluorescence of 12 sputum samples of CF patients and it did not show any adverse effect on FISH results of the remaining sputum samples. Therefore, using of biotin in FISH procedure seems to facilitate and improve the detection of respiratory tract infections by *P. aeruginosa* in this population.**

Key words: *Pseudomonas aeruginosa*, fluorescent in situ hybridization, FISH, biotin.

INTRODUCTION

Pseudomonas aeruginosa is a common nosocomial pathogen that can cause serious human infections and is the most prevalent respiratory pathogen among patients suffering from cystic fibrosis (CF) (Forbes et al., 2002). Cystic fibrosis is a genetic disorder associated with the secretion of a sticky mucus and impaired mucocilliary clearance that initially leads to intermittent and then persistent microbial infections (Hogardt et al., 2000; Knowles et al., 2000). *P. aeruginosa* has a predilection for infecting the respiratory tract of these patients which is life-threatening (Knowles et al., 2000). Therefore, accurate and rapid diagnosis of infections is essential for initiation of appropriate patient treatment and hygienic measurements. Isolation and identification of *P. aeruginosa* by conventional bacteriological methods requires at least 2 days for incubation (Forbes et al., 2002). Furthermore, antibiotic therapy before clinical sampling may impede cultivation of bacteria (Hogardt et al., 2000).

At present, fluorescent *in situ* hybridization (FISH) is considered in the field of clinical microbiology as a rapid and specific detection method. The FISH technique is based on fluorescently-labeled oligonucleotide probes that specifically target and hybridize to ribosomal RNA (rRNA), so that the whole microbial cell can be visualizied directly by fluorescence microscopy (Moter et al., 1998; Trebesious et al., 2000). FISH has already been successfully applied for the detection of several bacteria such as *Salmonella* (Nordentoft et al., 1997), *Legionella* (Hayden et al., 2001), *Chlamydiae* (Poppert et al., 2002),

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Helicobacter pylori (Russmann et al., 2001; Samarbaf-Zadeh et al., 2006; Moosavian et al., 2007), *Neisseria meningitidis* (Poppert et al., 2005), *Staphylococci* (Peters et al., 2006; Kempf et al., 2000) and *Brucella* spp. (Wellinghausen et al., 2006). Moreover, FISH has been used for definite species identification of bacteria, including *P. aeruginosa* in sputum samples obtained from CF patients (Hogardt et al., 2000). High background fluorescence of CF sputum may interfere with the detection of microorganisms by FISH (Hogardt et al., 2000). Therefore, the objective of this study was to test the application of biotin in the FISH procedure to reduce background fluorescence in sputum samples in order to facilitate the detection of *P. aeruginosa*.

MATERIALS AND METHODS

Cultivation of *P. aeruginosa* from CF sputum samples

Sixty-three sputum samples from CF patients with documented lung infection were collected. For liquefaction, each sample was treated with an equal volume of dithiothreitol (DTT; Sigma, Germany) solution (1 mg/ml) (Hogardt et al., 2000). DTT-treated sputum samples were then plated on tryptone soy agar and MacConkey agar. Identification of cultivated *P. aeruginosa* was carried out by standard bacteriological methods.

Fixation of sputum samples

An aliquot of each DTT-treated specimen was fixed for FISH as follows: 200 µl of sputum was mixed with 3 volumes of 4% paraformaldehyde and incubated at 4ºC for 4 h (Amann et al., 1990; Samarbaf-Zadeh et al., 2006). After incubation, the mixture was centrifuged at 8000 rpm for 5 min, the pellet was resuspended in PBS and recentrifuged (PBS washing). The pellet was resuspended in 100 µl PBS and an equal volume of absolute ethanol (Merck, Germany) was added (Perry-O'keefe et al., 2001; Samarbaf-Zadeh et al., 2006).

Bacterial reference strains

P. aeruginosa (ATCC 27853) was used as positive control, whereas *Escherichia coli* (ATCC 25922) and *Haemophilus influenzae* (DSM 46901) were used as negative controls for FISH. The strains were fixed by 4% paraformaldehyde similar to that described above. Fixed bacterial cells were stored at -20ºC (Kempf et al., 2000; Perry-O'keefe et al., 2001; Samarbaf-Zadeh et al., 2006).

FISH

Oligonucleotide probes Pseaer and EUB338 that synthesized and labeled by Metabion (Germany), were used in this study. Probe Pseaer (5'-TCT CGG CCT TGA AAC CCC- 3') that specifically targets and hybridizes to 23S rRNA of *P. aeruginosa*, was used for specific detection of this bacterium (Hogardt et al., 2000; Kempf et al., 2000). The 5' end of Pseaer was labeled with fluorochrome Cy3 (red signal). Probe EUB338 (5'-GCT GCC TCC CGT AGG AGT- 3') is complementary to the sequence of a region of 16S rRNA that is unique for all bacteria (Manz et al., 1995; Hogardt et al., 2000; Kempf et al., 2000). The 5' end of EUB338 was labeled with FLOUS [5(6)-carboxyfluorescein- N- hydroxy succinimide-ester] which emits green signal (Trebesius et al., 1998; Hogardt et al., 2000).

FISH was performed on six-field glass slides (Marienfeld, Bad Mergentheim, Germany). 10 µl of each fixed sputum sample or fixed bacterial reference strain were put on each field of glass slides which were left at room temperature to air-dry (Hogardt et al., 2000). The dehydration steps were carried out by putting the slides in a serial dilution of ethanol (50, 80, and 96% ethanol) for 3 min each (Hogardt et al., 2000; Samarbaf-Zadeh et al 2006). For hybridization, each field of slides was covered with 10 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 8], 0.01% SDS, 20% formamide) (Kempf et al., 2000; Samarbaf-Zadeh et al., 2006) containing a mixture of probes EUB338 and Pseaer. The concentration of each probe in hybridization buffer was 5 ng/µl (Samarbaf-Zadeh et al., 2006). Then, the slides were put separately in a moisture chamber and incubated at 46ºC for 90 min for hybridization step (Hogardt et al., 2000; Trebesius et al., 2000; Samarbaf-Zadeh et al., 2006).

An aliquot of all the 63 sputum samples were also tested with biotin on separate glass slides. The specimens were put on glass slides, air-dried and dehydrated as described above. Before hybridization, the samples were covered with 10 µl hybridization buffer containing 80 µM biotin (Sigma) and incubated for 30 min. Afterwards, the specimens were covered with hybridization buffer containing 80 µM biotin and the mixture of probes EUB338 and Pseaer, so that the concentration of each probe was 5 ng/µl. The slides were then incubated for hybridization as aforementioned.

After hybridization, stringent washing was performed in a washing buffer (20 mM Tris-HCl [pH 8], 0.01%SDS, 225 mM NaCl] at 48ºC for 15 min (Hogardt et al., 2000; Samarbaf-Zadeh et al 2006). The slides were then stained with 1µg/ml DAPI (4',6-diamidine-2'-phenylindole dihydrochloride) for 5 min (Neef et al., 1998; Samarbaf-Zadeh et al., 2006), which stains both eukaryotic and prokaryotic DNA (Kempf et al., 2000). Finally, the slides were washed with PBS, air-dried, mounted in Citifluor (Citifluor Ltd, United Kingdom), and analyzed with a Leica epi-fluorescence microscope equipped with a standard filter set (Leica, Germany).

RESULTS

Sixty-three sputum samples were examined by microbiological culture and FISH to detect *P. aeruginosa* (Table 1). By conventional culture, 42 of 63 specimens were culture-positive and 21 specimens were culture-negative for *P. aeruginosa*. Without biotin-treatment, *P. aeruginosa* was detected by FISH in 35 of 42 culture-positive samples, whereas 37 biotin-treated samples were FISH-positive. All FISH-positive specimens were culture-positive. In seven samples that were not treated with biotin, the culture was positive for *P. aeruginosa* but negative according to FISH. Strikingly, five biotin-treated samples were culture-positive but FISH-negative. According to this study, the specificity of FISH in comparison with culture methods was 100%. The sensitivity of FISH for the detection of *P. aeruginosa* in samples without biotin treatment was 83.3%, whereas in biotin-treated samples was 88.1%.

In 12 of 63 biotin-treated samples the background fluorescence was reduced (Figure 1). Out of these 12 samples, eight were both culture-positive and FISH-positive. In these eight samples, two samples were FISH-negative without biotin-treatment but FISH-positive after biotin treatment. In other six samples, the specific fluorescence signal intensity of *P. aeruginosa* increased

Table 1. Examination of 63 sputum samples by FISH (without and with biotin) and culture method for the detection of *P. aeruginosa.*

Method and result	Number of specimens
Culture:	
Positive	42
Negative	21
Culture positive:	
FISH positive (without biotin)	35
FISH positive (with biotin)	37
FISH negative (without biotin)	7
FISH negative (with biotin)	5
Culture negative:	
FISH positive (without biotin)	0
FISH positive (with biotin)	0
FISH negative (without biotin)	21
FISH negative (with biotin)	21

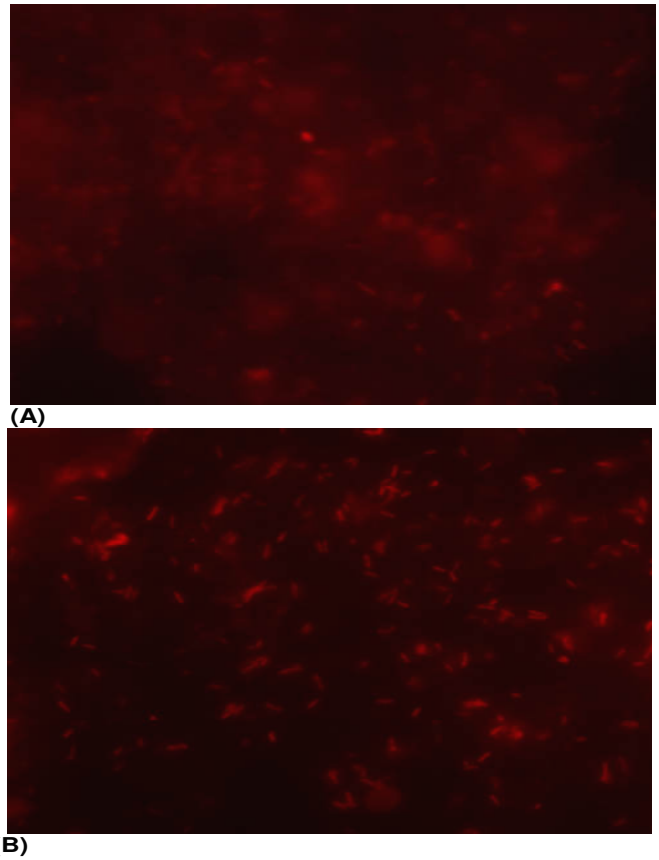

Figure 1. Detection of *P. aeruginosa* in a sputum sample by means of FISH with probe Pseaer-Cy3. Panel (A) shows the sample without biotin treatment in which the specific signal of the bacterium was decreased by background fluorescence. Panel (B) shows the same sample after biotin treatment in which reducing background fluorescence led to increase the intensity of specific fluorescence signal of *P. aeruginosa*, so that the identification of the bacterium was facilitated.

because of reducing the background fluorescence, so that the identification of *P. aeruginosa* was facilitated. Although both FISH and culture were negative for *P. aeruginosa* in 4 of 12 aforementioned samples, but background fluorescence was clearly reduced by biotin treatment. Fifteen of 63 specimens had an inherent low background fluorescence even without biotin treatment. Finally, biotin did not show any effect on remaining 36 sputum samples.

DISCUSSION

P. aeruginosa is a life-threatening infectious agent in respiratory tract of cystic fibrosis patients. Conventional bacteriological methods require at least 48 h for definite diagnosis of *P. aeruginosa.* Thus in severe infections and exacerbations empirical, antimicrobial therapy frequently starts without knowledge of the causative microorganism. Specific and rapid detection of infectious agents is essential for administration of appropriate antibiotics and prevention of rapid deterioration of pulmonary functions (Hogardt et al., 2000; Knowles et al., 2000).

FISH has been proven to be a rapid, precise and cost-effective technique for the identification of microbial pathogens within the clinical samples without the need for culture. Background fluorescence of sputum samples obtained from CF patients is often high probably due to abundant DNA, mucin content etc., that may impede detection of infectious agents by FISH (Hogardt et al., 2000; Tajbakhsh et al., 2004). In this study, treatment with biotin was applied in FISH procedure to reduce the background fluorescence of sputum. Biotin is a small molecule that is a cofactor in many enzymes and thus, probably easily bind to many proteins in general (Dakshinamurti and Chauhan, 1990) which may lead to a reduction of unspecific background fluorescence. We chose *P. aeruginosa,* the most common and most important pathogen in patients with CF (Hogardt et al., 2000; Knowles et al., 2000; Forbes et al., 2002), and designed our experiments to diagnose this organism.

As shown in Table 1, seven sputum samples were culture-positive but FISH-negative (without biotin). High background fluorescence was a limitation and led to false negative results in these 7 specimens. However after treatment with biotin, 2 out of 7 mentioned samples were found to be positive by FISH suggesting that reduction of the background fluorescence could be the reason. Therefore, in this study, higher sensitivity for FISH was achieved by using biotin (88.1%) in comparison with the FISH procedure without using biotin (83.3%).

In 12 sputum samples, reduced background fluorescence was observed by biotin treatment, whereas biotin was not effective on 36 samples. This probably is due to differences in nature, viscosity, and materials of different sputum samples. Although biotin was effective only in 12 sputum samples, but it did not have any adverse effect

on FISH results of the remaining sputum samples.

The specificity of FISH was 100%, since there was not any false positive result. Thus, the FISH-positive results are highly reliable and administration of choice drugs can promptly start in severe cases of infections.

In conclusion, biotin could be effective to reduce background fluorescence and to facilitate detection of *P. aeruginosa* at least in some of sputum samples. It should be also emphasized that biotin did not show any adverse effect on FISH results. Therefore, using of biotin in FISH procedure can be recommended to improve diagnosis of infections in sputum samples obtained from cystic fibrosis patients.

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