Rapid Identification of Bacteria and Candida Pathogens in Peritoneal Dialysis Effluent from Patients with Peritoneal Dialysis-Related Peritonitis Using Multilocus PCR Coupled with Electrospray Ionization Mass Spectrometry

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Running title: PCR/ESI-MS for pathogen detection in PD peritonitis

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ABSTRACT

Polymerase chain reaction coupled with electrospray ionization mass spectrometry (PCR/ESI-MS) was compared with culture for pathogen detection in peritoneal dialysis (PD)-related peritonitis. Of 21 samples of PD effluent, PCR/ESI-MS identified microorganisms in 18 (86%) samples, including Mycobacterium tuberculosis in 1 culture-negative sample. Of 15 double-positive samples, PCR/ESI-MS and culture reached levels of agreement of 100% (15/15) and 87.5% (7/8) at the genus and species levels, respectively. PCR/ESI-MS can be used for rapid pathogen detection in PD-related peritonitis.

Key words: Peritoneal dialysis (PD)-related peritonitis, Polymerase chain reaction coupled with electrospray ionization mass spectrometry (PCR/ESI-MS), Mycobacterium tuberculosis, Rapid diagnosis.
Peritonitis is one of the major complications of peritoneal dialysis (PD). The majority of PD-related peritonitis cases are caused by pathogenic bacteria, with only a small number of cases being caused by fungi, primarily *Candida* species, and *Mycobacterium* species. Currently, the novel diagnostic techniques that have been explored for the early diagnosis of PD-related peritonitis are primarily limited to the identification of bacterial pathogens (1-3). In this study, we aimed to determine the feasibility of and potential application of the BAC Detection assay (Ibis Bioscience) in multilocus polymerase chain reaction coupled with electro spray ionization mass spectrometry (PCR/ESI-MS) (PLEX-ID, Abbott Laboratories, Illinois, U.S.) for the rapid detection, identification and information of drug resistance of bacteria and *Candida* species in PD effluent from patients with PD-related peritonitis.

Twenty-one episodes of PD-related peritonitis, including 19 episodes from 17 patients at National Cheng Kung University Hospital (NCKUH) (2 patients experienced the second episodes) and 2 episodes at Chi-Mei Medical Center (CMMC) (patients 12 and 17), were enrolled after informed consent was obtained between July 2012 and April 2013 (Table 1). The diagnosis of PD peritonitis followed the diagnostic criteria recommended by the International Society for Peritoneal Dialysis (3). Three samples of PD effluent from patients without peritonitis were used as control samples. For pathogen detection, PD effluent, obtained prior to antimicrobial treatments, was subjected to both traditional culture and PCR/ESI-MS analyses. For culture analyses, 20 ml of centrifugation sediment from 50 ml and 20 ml samples of PD effluent obtained from patients at NCKUH and CMMC, respectively, was inoculated into BACTEC aerobic/anaerobic blood culture bottles (10 ml per bottle) and MYCO/F lytic bottles where indicated and cultivated using the BACTEC 9240 blood culture system (BD Diagnostics, U.S.). Microorganisms were identified based on standard phenotypic tests used in clinical microbiology laboratories (4), and antimicrobial susceptibility was determined using the disk diffusion method (5).
PCR/ESI-MS analyses, 50 ml of PD effluent was centrifuged at 14,000 rpm for 10 minutes. DNA was then extracted and purified from the resulting cell pellets using the UMD-Universal extraction kit according to the protocol supplied by the manufacturer (Molzym, Bremen, Germany), and elutes were stored at -20°C until testing. The DNA extraction kit includes a protocol for the lysis of human cells, degradation of the human DNA by a DNase, and lysis of the cell walls of any bacteria and yeasts by treating with BugLysis and β-mercaptoethanol. On PCR/ESI-MS analyses, extracted DNA was added to a BAC Detection assay plate and amplified and analyzed using PCR/ESI-MS. The BAC Detection assay plate contains a set of 18 different primer pairs for the detection and identification of bacterial and Candida species and the mecA, vanA, vanB and Klebsiella pneumoniae carbapenemase antibiotic resistance genes (6). The procedures for PCR/ESI-MS analysis followed the manufacturer’s instructions as previously described, with a test turnaround time within 6-8 hours (6, 7). Organism identification was based on the total mass and base compositions of the PCR amplicons compared to those in the molecular signature database established by the PLEX-ID manufacturer. The genome level, indicating the amount of amplified DNA present in the sample reported as genome equivalents/well, was calculated as previously described method (8). The limit of detection determined by PCR/ESI-MS was $10^2$ cfu/ml for Staphylococcus aureus ATCC25923 and Escherichia coli ATCC25922 and $10^1$ cfu/ml for Candida albicans ATCC14053 using three independent tenfold-dilution panels ($10^1$–$10^3$ cfu/ml) of 3-ml whole blood samples.

Of the 21 episodes, culture and PCR/ESI-MS identified microorganisms in 16 (76%) and 18 (86%) samples, respectively, and overall, 19 (90%) samples were positive either by culture or PCR/ESI-MS (Table 1). Notably, PCR/ESI-MS failed to identify a second pathogen in two samples (patients 12 and 18). No pathogen was detected by either method in three control samples. Among the 15 organisms identified by both methods, PCR/ESI-MS
and culture reached an agreement of 100% (15/15) at the genus level, and among 8 isolates with species-level identification by the conventional phenotypic method, an agreement of 87.5% (7/8) was reached at the species level. 16S rRNA gene sequence analyses of the PD effluent samples further confirmed the PCR/ESI-MS results at the species level in 6 isolates without species-level identification, *Mycobacterium tuberculosis* and *Legionella pneumophila* (Table 1). Target-specific PCR analyses confirmed the PCR/ESI-MS result of *Escherichia coli* in patient 12, whereas no PCR product was found for detection of *Klebsiella pneumoniae* in patient 12 and for *Streptococcus salivarius* in patient 18. In patient 3, the pathological finding of the peritoneal biopsy in the second episode of peritonitis showed suppurative granulomatous inflammation composed of multinucleated giant cells and visible acid-fast bacilli, suggestive of *M. tuberculosis* infection, and anti-tuberculosis therapy were initiated. Retrospectively, PCR/ESI-MS analysis of PD effluent obtained 80 days earlier in an episode of cloudy PD effluent (dialysate leukocyte count 233 per μL, neutrophils 33%) also detected *M. tuberculosis*. Mycobacterial cultures of PD effluent samples from both occasions were negative. Although patient 7 recovered with piperacillin/tazobactam treatment, the clinical significance of detection of nucleic acid of *L. pneumophila* was not clear. Antimicrobial susceptibility testing of the culture isolates did not demonstrate carbapenem resistance in Gram-negative bacilli, vancomycin resistance in gram-positive cocci, or oxacillin resistance in *Staphylococci*, consistent with the finding that antimicrobial resistance genes were not detected in any of the samples by PCR/ESI-MS. PCR/ESI-MS herein demonstrated its capability to rapidly identify a broad spectrum of pathogens in a single examination, including uncommonly encountered organisms such as *Mycobacterium* and *Candida* species, without foreknowledge of the microorganisms potentially present. This is of particular importance for patients with tuberculous peritonitis because the presentation of tuberculous peritonitis in this population could be insidious,
making the diagnosis challenging. The potential of PCR/ESI-MS for the early diagnosis of
tuberculous peritonitis is promising because earlier anti-tuberculosis treatment would aid the
preservation of the peritoneal membrane ultrafiltration capacity (9). Furthermore, both our
study and previous reports indicate that at the genus and species levels, PCR/ESI-MS
identification can achieve high levels of concordance with standard techniques used in
clinical microbiology laboratories (6, 7). In addition, the presence of antibiotic resistance
genes can be examined. Rapid and accurate identification of pathogens and their associated
antibiotic resistance genes directly from PD effluent could guide clinicians toward a more
rapid and earlier shift from the use of broad-spectrum empirical antimicrobials to targeted
antibiotics.

However, PCR/ESI-MS does have its limitations. Although PCR/ESI-MS has the ability
to resolve mixtures, the technology failed to detect mixed pathogens in two of our samples.
Additionally, in a previous report, it had a relatively high false-negative error rate of 24% in
detecting mixtures in blood culture bottles, most likely due to preferential amplification, as
the primers were quickly saturated with DNA from the most abundant organism, leaving
microorganisms present in lower titers undetected (6). Because polymicrobial peritonitis
represented approximately 10% of all PD-related peritonitis episodes (10), the possibility of
a false-negative PCR/ESI-MS result should be considered. Second, PCR/ESI-MS cannot
provide detailed antimicrobial susceptibility information for the causative organisms, unlike
culturing, which was used to guide post-empirical antibiotic treatment. Therefore, although
sensitive and rapid in pathogen detection, PCR/ESI-MS cannot replace, but may
complement, culture methods in the diagnosis of PD-related peritonitis.

In conclusion, this study supports the use of PCR/ESI-MS as a potential tool for rapid
and broad-range pathogen detection in PD-related peritonitis.
ACKNOWLEDGMENTS. This study was supported by grants from the National Health Research Institutes (IV-101-SP-18). We thank Miss Ming-I Hsieh and Miss Yu-Chun Wang for performing laboratory studies and Mrs. Wei-Yin Hsu, Mrs. Yu-Wei Wang, Mrs. Hsueh-Chi Chou, Mrs. Pei-Jung Wu, Mrs. Mei-Hsien Wu and Miss Wang-Ping Chen collecting peritoneal dialysis effluent from the patients.

CONFLICT of INTEREST. None to declare.
REFERENCES


### TABLE 1. Patient data and bacteria and Candida species detected in peritoneal dialysis (PD) effluent collected from patients with PD-related peritonitis using three independent methods

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Effluent WBC count /sex</th>
<th>Gram stain</th>
<th>Culture identification</th>
<th>PCR/ESI-MS Culture identification</th>
<th>16S rRNA gene sequence analyses^e</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70/F</td>
<td>1360 (93%)</td>
<td>GPC</td>
<td>Group D Streptococcus</td>
<td>S. salivarius (240)</td>
<td>S. salivarius (no. CP002888, 1413/1413)</td>
</tr>
<tr>
<td>2</td>
<td>51/M</td>
<td>8270 (97%)</td>
<td>GPC</td>
<td>Streptococcus pneumoniae</td>
<td>S. pneumoniae (156)</td>
<td>NP</td>
</tr>
<tr>
<td>3</td>
<td>52/M</td>
<td>670 (92%)</td>
<td>GPC</td>
<td>Streptococcus, viridans group</td>
<td>S. salivarius (173)</td>
<td>S. salivarius (no. NR_102816, 1422/1422)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>830 (87%)</td>
<td>NP</td>
<td>no growth</td>
<td>M. tuberculosis (426)</td>
<td>M. tuberculosis (no. CP006578, 1404/1404)</td>
</tr>
<tr>
<td>4</td>
<td>55/F</td>
<td>1250 (78%)</td>
<td>NF</td>
<td>no growth</td>
<td>not detected</td>
<td>NP</td>
</tr>
<tr>
<td>5</td>
<td>54/M</td>
<td>7310 (98%)</td>
<td>GNB</td>
<td>K. pneumoniae</td>
<td>K. pneumoniae (129)</td>
<td>NP</td>
</tr>
<tr>
<td>6</td>
<td>71/F</td>
<td>1980 (97%)</td>
<td>NF</td>
<td>Fusobacterium spp.</td>
<td>Fusobacterium nucleatum (98)</td>
<td>F. nucleatum (no. AE009951, 1377/1378)</td>
</tr>
<tr>
<td>7</td>
<td>62/M</td>
<td>142 (94%)</td>
<td>NF</td>
<td>no growth</td>
<td>L. pneumophila (380)</td>
<td>L. pneumophila (no. JX827099, 417/419)</td>
</tr>
<tr>
<td>No.</td>
<td>Age/Sex</td>
<td>Score</td>
<td>Site</td>
<td>Isolation</td>
<td>Organism</td>
<td>Genotype</td>
</tr>
<tr>
<td>-----</td>
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</tr>
<tr>
<td>8</td>
<td>57/M</td>
<td>1650 (95%)</td>
<td>NF</td>
<td>Streptococcus, viridans group</td>
<td>Streptococcus mitis (211)</td>
<td>S. mitis (no. AY281077, 1385/1395 [99.3%])^f</td>
</tr>
<tr>
<td>9</td>
<td>52/M</td>
<td>5120 (89%)</td>
<td>GPC</td>
<td>Streptococcus, viridans group</td>
<td>S. salivarius (251)</td>
<td>S. salivarius (no. CP002888, 1417/1417 [100%])^f</td>
</tr>
<tr>
<td>10</td>
<td>52/F</td>
<td>620 (80%)</td>
<td>NF</td>
<td>Candida parapsilosis^z</td>
<td>C. parapsilosis (89)</td>
<td>NP</td>
</tr>
<tr>
<td>11</td>
<td>49/M</td>
<td>580 (78%)</td>
<td>NP</td>
<td>E. coli</td>
<td>not detected</td>
<td>not detected</td>
</tr>
<tr>
<td>12</td>
<td>57/M</td>
<td>2640 (92%)</td>
<td>NF</td>
<td>E. coli; K. pneumoniae</td>
<td>E. coli (96)</td>
<td>E. coli (no. HG738867, 130/130 [100%])^f</td>
</tr>
<tr>
<td>13</td>
<td>21/M</td>
<td>3820 (86%)</td>
<td>NF</td>
<td>Pseudomonas stutzeri^z</td>
<td>P. stutzeri (206)</td>
<td>NP</td>
</tr>
<tr>
<td>14</td>
<td>57/M</td>
<td>3040 (93%)</td>
<td>GPC</td>
<td>no growth</td>
<td>Streptococcus vestibularis (274)</td>
<td>S. salivarius (no. NR_042776, 1399/1399 [100%])^f, or S. vestibularis (no. HM596286, 1397/1399[99.9%])^f</td>
</tr>
<tr>
<td>15</td>
<td>31/M</td>
<td>82 (75%)</td>
<td>NF</td>
<td>Staphylococcus warneri^z</td>
<td>S. warneri (127)</td>
<td>NP</td>
</tr>
<tr>
<td>16</td>
<td>10/F^d</td>
<td>800 (91%)</td>
<td>GPC</td>
<td>Streptococcus agalactiae</td>
<td>S. agalactiae (161)</td>
<td>NP</td>
</tr>
<tr>
<td>17</td>
<td>56/M</td>
<td>1192 (89%)</td>
<td>NF</td>
<td>Streptococcus, viridans group</td>
<td>S. salivarius (177)</td>
<td>S. salivarius (no. CP002888, 1402/1402 [100%])^f</td>
</tr>
<tr>
<td>18</td>
<td>75/M</td>
<td>4980 (80%)</td>
<td>GPC</td>
<td>Streptococcus, viridans group; GPB</td>
<td>S. salivarius (9)</td>
<td>not detected</td>
</tr>
<tr>
<td>19</td>
<td>53/F</td>
<td>8960 (89%)</td>
<td>NP</td>
<td>S. warneri^z</td>
<td>Staphylococcus haemolyticus (244)</td>
<td>S. haemolyticus (no. NR_074994, 1422/1423 [99.9%])^f</td>
</tr>
</tbody>
</table>
Abbreviations not defined in text: F, female; GNB: Gram-negative bacilli; GPB: gram-positive bacilli; GPC: gram-positive cocci; M, male; NF: not found; NP: not performed; WBC, white blood cell count; PMN, polymorphonuclear neutrophils.

Two separate episodes occurred 5 months apart.

Isolate for which genus identification was further confirmed using the Rapid ID 32A system (bioMérieux, Marcy-l’Etoile, France).

Isolates for which species identification was further confirmed using the Vitek system (Biomerieux Vitek, Hazelwood, MO).

Two separate episodes occurred 1 month apart.

PD effluent was analyzed by 16S rRNA gene sequence analysis. The primers for 16S rRNA gene sequence analyses included: Fd1/Rp2 for *M. tuberculosis* (11); UMD-Universal kit (Molzym, Bremen, Germany) for *L. pneumophila*; 8F/1492R for the other isolates (8).

PD effluent was analyzed by target-specific PCR. The primers for target-specific PCR included: UAL1936b/UAL2105b for *E. coli*; K16SF/K16SR for *K. pneumoniae*; MKK-F/MKK-R and 13BF/6R for *S. salivarius* (12-15).

The 16S rRNA gene sequences from both PD effluent and the isolate were only 98.3% (1401/1425) identical to that of *S. warneri* (no. NR_025922).