Use of blood-free enrichment broth in the development of a rapid protocol to detect Campylobacter in twenty-five grams of chicken meat

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ARTICLE INFO

Article history:
Received 10 February 2012
Received in revised form 30 January 2013
Accepted 12 February 2013
Available online 18 February 2013

Keywords:
Enrichment culture
Campylobacter jejuni
Food pathogen
PCR

ABSTRACT

A Food Pathogen Enrichment (FPE) broth, which supports the growth of Campylobacter without lysed blood and CO₂, was developed. The FPE broth supports the growth of Campylobacter to the same degree as Bolton and Preston broths. Using the FPE broth, we developed a novel rapid protocol to detect small numbers of Campylobacter in 25 g of food. The sensitivity of FPE enrichment and PCR to detect Campylobacter spp. from spiked chicken meat was determined. The detection sensitivities for non-stressed C. jejuni and C. coli from fresh meat ranged from 5.8 to 1.1 × 10⁴ CFU per 25 g of chicken meat, and those for freeze-stressed C. jejuni and C. coli from frozen meat ranged from 9.9 × 10¹ to 2.0 × 10² CFU. The FPE broth enrichment culture (24 h) of chicken meat, followed by PCR, resulted in a significantly higher detection score (80% positive) than conventional Bolton enrichment and subsequent colony isolation using mCCDA agar plates (18% positive). Differences between our new protocol and the Bolton enrichment method were due to the overgrowth of many resistant bacteria, especially extended-spectrum beta-lactamase-producing bacteria in the Bolton enrichment broth.

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1. Introduction

Campylobacter is the most frequent causative agent of food poisoning in the world and the increase in antimicrobial-resistant bacteria is becoming a noteworthy concern for its selective isolation from food and feces (Humphrey et al., 2007). Detection of Campylobacter from food, fecal and environmental samples is performed by either direct plating onto selective agars or by selective enrichment culture. However, Campylobacter numbers in foods are typically very low, growth is slow, and detection is further hindered by the difficulty in culturing damaged cells from food samples (Garenaux et al., 2008; Verhoeff-Bakkenes et al., 2008; Yamasaki et al., 2004). The detection of Campylobacter after enrichment is recommended for the recovery of sublethally damaged cells from food and environmental samples. The formulations of Campylobacter enrichment broth media have been extensively modified over the last two decades, primarily to avoid the inhibitory effects on Campylobacter spp. of components in the broth formulae (Corry et al., 1995; Williams et al., 2009). The standard detection protocol requires 1- or 2-day selective enrichment in Bolton or Preston broth. It is recommended that enrichment procedures be conducted at 37 °C for 4 to 6 h followed by 44 h at 41.5 °C. After enrichment, plating is carried out on modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) (ISO, 2006a). However, the standard methods require large volumes of blood and a microaerobic atmosphere, both of which are costly. Eliminating the need for blood and a microaerobic atmosphere would simplify the overall enrichment method for Campylobacter spp. from chicken meat samples.

Several methods based on the polymerase chain reaction (PCR) have been developed to detect pathogens in foods after enrichment culture (Melero et al., 2011; Rantsiou et al., 2010; Swaminathan and Feng, 1994). However, the presence of lysed blood and meat in these conventional enrichment cultures can contribute to PCR amplification inhibition (Al-Soud and Rådström, 2001; Josefson et al., 2004; Mercier et al., 1990; Rantsiou et al., 2010), thereby necessitating several purification steps.

In the present study, we formulated a novel enrichment broth that supports Campylobacter growth without CO₂ and blood supplement, and named it Food Pathogen Enrichment (FPE) broth. To detect small numbers of Campylobacter in 25 g of food sample, we developed a rapid protocol that combines short FPE enrichment culture and rapid PCR detection.

The purpose of the present study was to establish a simple and rapid detection method for Campylobacter spp. in chicken meat and chicken feces. To confirm the utility of our protocol, we compared the growth capacity of Campylobacter in a newly developed enrichment broth (FPE), Bolton broth, or Preston broth. The sensitivity of the enrichment broth and PCR for the detection of Campylobacter in chicken meat spiked with non-stressed and freeze-stressed Campylobacter cells was...
tested, and lastly, detection of *Campylobacter* from naturally contaminated samples was attempted using the FPE broth. Here, we report the efficacy of the combined FPE enrichment and PCR.

2. Materials and methods

2.1. Bacterial strains

The type strains *Campylobacter jejuni* GTC 259, derived from ATCC 33560, and *Campylobacter coli* GTC14978, from ATCC 33559, and the wild-type strains *C. jejuni* GTC 14977 and *C. coli* GTC15058, freshly isolated from chicken meat, were supplied from the Microbial Genetic Resource Stock Center, Gifu University Graduate School of Medicine (Gifu, Japan). All strains were cultured on blood agar at 37 °C under a microaerobic atmosphere for 2 days, and fresh cultures were used for each experiment.

2.2. Preparation of FPE broth

A number of preliminarily formulae were prepared, and the final FPE broth formula was composed of: 30 g of soybean-casein digest, 10 g of Lab-Lemco Meat Extract, 0.6 g of l-Cystine-HCl, 1.0 g of Na-Pyruvate, 0.5 g of NaHSO3, 30 mg of hemin dissolved in 10% of Na2CO3, and 0.8 g of K2HPO4 per 1 l of distilled water. FPE broth (225 ml) was autoclaved in 500-ml screw capped bottles. After autoclaving, the following selective agents were added to the enrichment broth before use: 5 IU/ml of polymyxin B, 10 μg/ml of trimethoprim, 100 μg/ml of cycloheximide, and 10 μg/ml of rifampicin.

2.3. Media

Bolton broth (CM0983 with supplement SR183, Oxoid, Cambridge, UK), which is recommended in the current International Organisation for Standardization (ISO) standard method, and Preston broth (with supplement SR117, Oxoid), which is recommended in the former version of the ISO standard method, were prepared with 5% lysed horse blood for comparison to the FPE broth. In addition, modified mCCDA (Oxoid), which is also recommended in the current ISO standard method, was used in this study.

2.4. Evaluation of enrichment broth

Different enrichment broths were evaluated in this study. One milliliter of a 1×106-CFU/ml bacterial suspension in phosphate-buffered saline was transferred into 225 ml of each of the enrichment broths. Bolton and Preston enrichment broths were prepared in 500-ml loosely closed screw-capped bottles and incubated under a microaerobic atmosphere. FPE broth was incubated in 500-ml tightly closed screw-capped bottles with shaking in a gyratory shaker (Innova 4230, New Brunswick Scientific, New Jersey, NJ) under an aerobic atmosphere. After 0, 1, 2, 3, 4, 5, 6, 24 and 48 h, 0.1 ml of each enrichment culture was collected, inoculated onto agar media (Eddy Jet, IUL Instruments, Barcelona, Spain), and incubated at 41 °C for 48 h under a microaerobic atmosphere. *Campylobacter jejuni* multiplication was used as an index of the growth capacity in the enrichment media.

2.5. Detection sensitivity for spiked chicken meat samples

The sensitivity of the FPE broth enrichment and PCR method (Fig. 1; Protocol 1) to detect non- and freeze-stressed *Campylobacter* spp. from spiked chicken meat was determined. Four strains (*C. jejuni* GTC 259, *C. jejuni* GTC 14977, *C. coli* GTC 14978 and *C. coli* GTC 15058) were used for the spike experiments. The bacterial strains were cultured on blood agar plates at 37 °C for 48 h under a microaerobic atmosphere, and then suspended in PBS. Two-fold dilutions of each bacterial suspension were prepared in PBS, and 0.1 ml of each dilution was spiked into a 25-g portion of chicken meat, which had been confirmed as *Campylobacter* culture negative. Identical non-spiked chicken meat samples were used as negative controls. The spiked and non-spiked samples were immediately tested using both Protocols 1 and 2 (Fig. 1). To assess the effect of freeze stress, portions of spiked meat were stored at −20 °C for 10 days and then tested using both Protocols 1 and 2 (Fig. 1). To measure spiked *Campylobacter* numbers, 0.1 ml of each serial dilution was plated onto blood agar and mCCDA in triplicate and incubated at 41 °C for 48 h under a microaerobic atmosphere.

2.6. Microbiological testing

The growth capacity of the FPE broth was assessed using 50 fresh chilled chicken meats, 89 frozen chicken meats, 34 frozen beef, and 40 chicken feces samples. All samples were purchased from August to October 2011 from supermarkets and poultry processing plants located in the city of Gifu. Specifically, the 50 fresh chilled chicken meats were tested by the two protocols described below and the other samples were only tested by our FPE protocol. Fig. 1 shows a working scheme of the FPE broth enrichment protocol (Protocol 1) and the conventional Bolton broth enrichment (Protocol 2) used in the study. For *Campylobacter* detection using Protocol 1, 25 g of samples were homogenized in Stomacher bags (Eiken Chemical Co., Tokyo, Japan) containing 225 ml of FPE broth. The entire homogenate was transferred to a culture bottle and incubated with shaking at 37 °C for 6 to 7 h, then at 41 °C until 24 h. After incubation, 1 ml of the supernatant was collected, and DNA was extracted using the physical disruption method described below. Subsequently, an aliquot of extracted DNA was analyzed by real-time PCR using SYBR premix Ex Taq (Takara Bio, Shiga, Japan) according to the manufacturer’s instructions. After completion of the analysis, PCR-positive samples were confirmed by the culture method. Briefly, 0.1-ml aliquots of the FPE culture supernatant of PCR-positive samples were inoculated onto mCCDA plates and subsequently incubated at 41 °C for 48 h under a microaerobic atmosphere.

Similarly, following the conventional culture method (Protocol 2), 25-g samples were homogenized in Stomacher bags (Eiken Chemical Co.) containing 225 ml of Bolton broth and the entire homogenate was transferred to a culture bottle and incubated at 37 °C for 6 to 7 h, then at 41 °C until 48 h under a microaerobic atmosphere. One milliliter of culture from the 24- and 48-h Bolton broths was also collected for PCR. After 48 h of incubation, 0.1 ml of the culture supernatant was inoculated onto mCCDA plate and subsequently incubated at 41 °C for 48 h under a microaerobic atmosphere.

After 48 h, all inoculated mCCDA plates from Protocols 1 and 2 were assessed for presumptive colonies as well as contaminating bacteria using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

2.7. MALDI-TOF MS identification

MALDI-TOF MS was carried out to identify unknown bacteria isolated from chicken meat samples. Measurements were performed on a microflex™ MALDI-TOF Mass Spectrometer (Bruker Daltonics, Leipzig, Germany). The spectra were calibrated externally using a standard calibration mixture (Bruker Daltonics). Preparation of samples included sterilization in 75% ethanol, with the mixture subsequently centrifuged at 15 000 rpm for 2 min. The resulting supernatant was discarded and the pellet was air dried. Subsequently, 70% formic acid was added to the pellet and mixed thoroughly before the addition of acetonitrile. The mixture was centrifuged again at 15 000 rpm for 2 min. One micro-liter of the supernatant was spotted on the steel target plate and air dried at room temperature. Each sample was covered with 1 μl of matrix solution and air dried. Automated data acquisition was performed with FlexControl software (Bruker Daltonics). The raw spectra obtained for each isolate were imported into MALDI BioTyper™ version 3.0 software.
(Bruker Daltonics), and analyzed by standard pattern matching (using the default parameter settings) against reference libraries using the integrated pattern-matching algorithm of the MALDI BioTyper™ database, an integrated part of the software. The unknown spectra were compared with a reference spectra library based on a pattern recognition algorithm using peak positions, peak intensity distributions and peak frequencies. Once spectra had been generated and captured by the software, the complete identification process was performed automatically, without user intervention.

2.8. Extended-spectrum beta-lactamase (ESBL)-producing Escherichia coli

Strains identified as E. coli were tested for ESBL production using the double-disk synergy test (DDST), conducted according to the method described by Tzelepi et al. (2000). DDST employs a disk diffusion method, with disks of amoxicillin plus clavulanate, cefpodoxime, cefotaxime, ceftazidime, ceftriaxone, and aztreonam in addition to cefepime. ESBL production was considered positive when the clavulanate-mediated enhancement of the activity of these antibiotic disks increased towards the disk containing clavulanate.

2.9. DNA extraction

DNA was extracted using a physical disruption method (MORA-EXTRACT, AMR, Gifu, Japan) according to the manufacturer’s instructions, with a final DNA elution volume of 100 µl. The DNA extracts from cultures were stored at −20 °C for further Campylobacter species confirmation.
2.10. Real-time PCR analysis

Further confirmation of C. jejuni and C. coli in the samples was based on amplification of part of the dnaJ gene (Hung et al., 2011) using primers for C. jejuni (Cjejuf: 5’-AGCAATTGACAGAAGTCTACG-3’, Cjejur: 5’-GAAAGCAAGTACGTGAGGC-3’) and C. coli (CcoliF: 5’-ATTTACATTAC AAGTTACCTT-3’, CcoliR: 5’-TGCGTTGATAGCAGGCGAG -3’) that were designed by multiple sequence alignment (DNASIS Pro, Hitachi Software Engineering Co., Ltd., Tokyo, Japan.). The real-time PCR conditions used in the present study are described below.

Real-time PCR amplification was performed in 20-μl reaction mixtures containing 10 μl of 2× SYBR premix Ex Taq (Takara Bio, Shiga, Japan), 0.4 μM of primer, distilled water, and 5 μl of DNA template. PCR was carried out using the StepOne™ System (Life Technologies, Tokyo, Japan) under the following conditions: 94 °C for 3 min, 40 cycles of 45 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s, with a final melting analysis rising from 65 °C to 95 °C by 0.1 °C/5 s.

3. Results

Different enrichment broths were used in this study to evaluate the growth of Campylobacter. After enrichment culture in FPE, Bolton, and Preston broths, the culture broths were inoculated onto mCCDA and colonies were enumerated. As a result, FPE broth showed the same Campylobacter growth performance to Bolton and Preston broths. FPE broth was also found to be positive by the culture method with mCCDA. Colonies appearing on each medium were analyzed by MALDI-TOF MS.

Table 3 shows a list of the contaminating bacteria, excluding Campylobacter jejuni and Arcobacter, on the mCCDA plates used in this study. In the 50 fresh chilled chicken samples, E. coli (1 sample), Proteus mirabilis (5 samples) and Pseudomonas aeruginosa (1 sample) were detected from FPE broth. From Bolton broth, E. coli (32 samples), Myroides odoratimimus (1 sample), Ochrobacter intermedium (1 sample), P. mirabilis (1 sample) and Pseudomonas putida (1 sample) were isolated. In the DDST, 24 (75%) of the total 32 isolated E. coli were found to be ESBL producers (data not shown).

Campylobacter jejuni detection from frozen and fresh meat, employing FPE broth enrichment followed by PCR and mCCDA detection, is shown in Table 2. Protocol 1 (FPE-PCR method) provided a significantly higher detection rate compared to Protocol 2; 40 (80%) vs. 9 (18%) of 50 samples were positive for C. jejuni, respectively. Among the 40 samples screened positive by PCR, 39 specimens were also found to be positive by the culture method with mCCDA. On the other hand, using Protocol 2 (Bolton-mCCDA method) 9 samples were positive by culture method, whereas 22 were detected by PCR in samples from 48-h cultures in Bolton broth. The difference in performance between the two methods was mainly due to the overgrowth of contaminating bacteria in the Bolton enrichment broth; the mCCDA agar plate was covered with contaminating bacteria. Colonies appearing on each medium were analyzed by MALDI-TOF MS.

Fig. 2. Growth of C. jejuni GTC 259 in three different enrichment broths.
Table 1
Detection of C. jejuni and C. coli in spiked chicken-meat samples using Protocol 1 (FPE-PCR) and Protocol 2 (Bolton-mCCDA).

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>No. of spiked cells (CFU/25 g of chicken meat)</th>
<th>Mean FPE</th>
<th>SD FPE</th>
<th>Mean Bolton-mCCDA</th>
<th>SD Bolton-mCCDA</th>
<th>Mean FPE-PCR</th>
<th>SD FPE-PCR</th>
<th>Mean Bolton-mCCDA</th>
<th>SD Bolton-mCCDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. jejuni</td>
<td>1.7 × 10² 01.96 + + + +</td>
<td>0.5</td>
<td>1.2</td>
<td>1.7 × 10² 01.96 + + + +</td>
<td>0.5</td>
<td>1.2</td>
<td>1.7 × 10² 01.96 + + + +</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>GTC 259</td>
<td>8.8 × 10¹ 16.8 + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
<td>8.8 × 10¹ 16.8 + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
<td>8.8 × 10¹ 16.8 + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Type strain</td>
<td>4.7 × 10¹ 9.0 + + + + + + + + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
<td>4.7 × 10¹ 9.0 + + + + + + + + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
<td>4.7 × 10¹ 9.0 + + + + + + + + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>C. jejuni</td>
<td>2.0 × 10² 10.1 + + + + + + + + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
<td>2.0 × 10² 10.1 + + + + + + + + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
<td>2.0 × 10² 10.1 + + + + + + + + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>GTC14977</td>
<td>9.2 × 10¹ 5.1 + + + + + + + + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
<td>9.2 × 10¹ 5.1 + + + + + + + + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
<td>9.2 × 10¹ 5.1 + + + + + + + + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Clinical isolate</td>
<td>4.2 × 10¹ 4.8 + + + + + + + + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
<td>4.2 × 10¹ 4.8 + + + + + + + + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
<td>4.2 × 10¹ 4.8 + + + + + + + + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>C. coli</td>
<td>3.3 × 10² 13.9 + + + + + + + + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
<td>3.3 × 10² 13.9 + + + + + + + + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
<td>3.3 × 10² 13.9 + + + + + + + + + + + + + + + + +</td>
<td>0.5</td>
<td>1.2</td>
</tr>
</tbody>
</table>

The above food pathogens grew to a number of several thousands to 10 thousand after 7-h enrichment culture (data not shown); however, Listeria (data not shown) and Campylobacter required 24 h of incubation to reach a thousand-fold increase (Fig. 2). In establishing the protocol, we selected 24-h enrichment culture for the FPE broth enrichment-based PCR protocol (Protocol 1 in Table 2 and Fig. 1) to detect small numbers of Campylobacter in 25 g of chicken meat.

The effect of freeze stress on Campylobacter largely depends on the duration of frozen storage (Habib et al., 2010). The number of Campylobacter was reduced by approximately one log immediately after freezing, and remained relatively constant during the frozen storage (Georgsson et al., 2006).

Our results revealed that samples stored at −20 °C for 10 days showed decreased sensitivity (decreased detection limit). In addition, we showed that the recovery from freeze stress was identical between the Bolton broth and FPE broth. Sensitivity was reduced by further long-term freezing (Chan et al., 2001; Eideh and Al-Qadiri, 2011; Georgsson et al., 2006).

In conventional enrichment culture, Bolton broth has been widely used for the detection of Campylobacter from meat samples (Baylis et al., 2000) and the procedure is recommended by the ISO (ISO, 2006b). In our study, the Bolton-based conventional method failed to isolate Campylobacter from a number of samples, with only 9 of 50 samples (18%) positively cultured due to the heavy overgrowth of contaminating bacteria (Table 2). In contrast, among the 40 C. jejuni-positive specimens detected by Protocol 1 (FPE-PCR method), 39 specimens were also found to be positive by the culture method with mCCDA. These findings confirm the accuracy of the results yielded by our combination method, and suggest that the detection ability of our protocol is equal to that of plating onto selective agars. Additionally, these findings demonstrate that our protocol is more suitable for enriching Campylobacter spp. from chicken meat samples than standard enrichment protocols, even though it is conducted in an aerobic atmosphere.

Jasson et al. (2009) demonstrated that the growth of ESBL-producing E. coli is not suppressed in Bolton broth. Since the same antibiotic, cefoperazone, is used in mCCDA, these E. coli are able to outgrow Campylobacter on these plates. On the other hand, FPE medium contains polymyxin B and rifampicin at concentrations that inhibit the growth of ESBL-producing E. coli. Consequently, transfer of these bacteria from FPE medium to mCCDA is limited and does not lead to an overgrowth of the ESBL-producing E. coli on mCCDA plates.

Although 80% (40/50) of the fresh chicken meat samples were contaminated with Campylobacter, the isolation frequency from frozen chicken was only 3.4% (3/89). This is an important observation in efforts to reduce Campylobacter food poisoning, as Campylobacter is sensitive to freeze-thawing (Chan et al., 2001; Eideh and Al-Qadiri, 2011; Georgsson et al., 2006). However, another important pathogen, A. butzleri, was detected in 43.8% (39/89) of frozen chicken samples. This organism is a notable source of food poisoning from frozen chicken, as many cases of diarrhea and systemic infection due to this pathogen have been reported (Vandenberg et al., 2004).

In conclusion, the performance of our newly developed protocol for Campylobacter detection in chicken meat, employing FPE broth enrichment and PCR, is rapid and superior to the conventional Bolton-based enrichment culture method.

Acknowledgments

This work was partially funded by the Regional Innovation Strategy Support Program of the Ministry of Education, Culture, Sports, Science and Technology, and Grant for Health and Labour Sciences Research, Research on Global Health Issues supported by the United States–Japan Cooperative Medical Science Program.

Table 2
Campylobacter detection in fresh chilled chicken meat using Protocol 1 and Protocol 2.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Fresh chicken (CFU/25 g)</th>
<th>Frozen chicken (CFU/25 g)</th>
<th>Chicken stool (CFU/25 g)</th>
<th>Frozen beef (CFU/25 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campylobacter jejuni</td>
<td>39/50</td>
<td>3/89</td>
<td>29/40</td>
<td>0/34</td>
</tr>
<tr>
<td>Campylobacter coli</td>
<td>0/50</td>
<td>0/89</td>
<td>0/40</td>
<td>0/34</td>
</tr>
<tr>
<td>Arcobacter butzleri</td>
<td>0/50</td>
<td>39/89</td>
<td>0/40</td>
<td>0/34</td>
</tr>
</tbody>
</table>

Table 3
List of contaminating bacteria on mCCDA inoculated with FPE and Bolton broths.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Enrichment media</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>Bolton</td>
</tr>
<tr>
<td>Mycobacterium smegmatis</td>
<td>Bolton</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>Bolton</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>Bolton</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Bolton</td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>Bolton</td>
</tr>
</tbody>
</table>
References


