2. インフラストラクチャー要件
  a. CLABSI を有する患者を特定するための，感染予防管理プログラム
  b. 発生率算出の分母となるカテーテル・日（catheter-day）および CVC 利用率を算出するための患者・日（patient-day）を収集，算出する
  c. 教育および訓練の実施
  d. 適切な臨床検査施設の支援
3. 実際に行うこと
  a. 医師，看護師に対し，CLABSI 予防ガイドラインについて教育する
  b. カテーテル挿入チェックリストを作成し実践する
  c. 医療従事者にカテーテルの挿入および維持について教育する
  d. カテーテル挿入キット／カートを用意する

セクション 4：感染防止・モニタリング戦略

Ⅰ．CLABSI 防止・モニタリングのための基本実践事項

A. 挿入前

1. 感染防止について教育する A－Ⅱ

B. 挿入時

1. カテーテルチェックリストを利用して，感染防止実践事項を遵守する B－Ⅱ
  a. 無菌的手技実践の遵守を確認する
  b. 手技における無菌性の破たんが認められた際に挿入を中止させる
2. カテーテルの挿入前または操作前に手指衛生を行う B－Ⅱ
  a. アルコール製剤または抗菌石鹸と水を使う
  b. 手袋を使用する場合でも手指衛生は必要である
3. 成人患者では大腿静脈の利用を避ける A－Ⅰ
  a. 小児では，全身麻酔なしに大腿静脈カテーテル挿入が可能で，感染リスク増加を伴わない
  b. 鎖骨下静脈への挿入に伴う CLABSI リスクは内頸静脈よりも低いが，危険性と有益性を個別に考慮して挿入部位を決める
  c. 末梢静脈からの挿入 CVC の利用は，ICU 患者では，感染リスクが鎖骨下静脈または内頸静脈挿入 CVC に伴う感染リスクに迫る
4. カテーテルセットまたはキットを利用する B－Ⅱ
5. CVC 挿入中は，マキシマルバリアプリコーションをする A－Ⅰ
  a. マスク，キャップ，滅菌ガウンおよび滅菌手袋を着用する。カテーテル挿入中は，患者を滅菌覆布で覆う
6. 生後 2 ヵ月超の患者では，皮膚消毒にクロルヘキシジン製剤を使用する A－Ⅰ

カテーテル挿入前に，0.5%以上のクロルヘキシジンアルコールにて皮膚消毒をする。この消毒薬が乾くのを待って皮膚穿刺を行う。米国 FDA は，生後 2 ヵ月未満の小児へはクロルヘキシジン製剤の使用を承認していない。この場合には，ポビドンヨードを使用する。

C. 挿入後

1. カテーテルを利用する前に，カテーテルハブ，ニードルレスコネクタ，および注入ポートを消毒する B－Ⅱ。
  a. クロルヘキシジンアルコール製剤または 70% アルコールで消毒する
2. 不必要なカテーテルを抜去する A－Ⅱ
  a. 患者ケアに不必要なカテーテルを抜去する
3. カテーテル皮膚刺入部は，透明なドレッシングを 5～7 日おきに交換し，汚れ，弛みまたは湿り気があるときはもっと頻繁に交換してクロルヘキシジン製剤で挿入部を消毒する。ガーゼ包帯の場合は 2 日おきに交換し，汚れ，弛みまたは湿り気があるときはもっと頻繁に交換する A－Ⅰ
4. 血液，血液製剤または脂肪乳剤に使用しなかった投与セットは，96 時間以内の間隔で交換する A－Ⅱ
5. CLABSI についてのサーベイランスを実施する B－Ⅱ
  a. 病棟特異的な CLABSI 発生率（1,000 カテーテル・日あたりの CLABSI 発生件数）を調べ，定期的にデータを報告する
  b. CLABSI 発生率を公式なデータ（すなわち NHSN のデータ）と比較する
6. 血液透析カテーテル挿入部位には抗微生物軟膏を使用する A－Ⅰ
  a. 再発性 *Staphylococcus aureus* CLABSI の既往がある患者には，血液透析カテーテル挿入部位にポビドンヨード軟膏などを塗布する
  b. 耐性およびポリウレタンカテーテル素材の損傷のリスクがあるため，カテーテル挿入部位にムピロシン軟膏を塗布すべきでない

D. 説明責任（省略）

Ⅱ．CLABSI 予防のための特殊なアプローチ

CLABSI リスクアセスメントを実施し，CLABSI 発生率が特に高い領域では，以下に示す特殊な対応を行うことが推奨される。

1. 生後2ヵ月超のICU患者については，毎日クロルヘキシジン製剤で挿入部位を処理する B－Ⅱ
   a. FDAは，生後2ヵ月未満の小児に対するクロルヘキシジン製剤の使用を承認していない
   b. 生後2ヵ月未満の小児，特に出生時低体重の新生児におけるCVC挿入部位の清浄化にはポビドンヨード製剤を使用すべきである
2. 成人患者には，抗菌薬または抗微生物薬充填CVCを使用する A－Ⅰ
   a. 現在市販されている一部の抗菌薬（クロルヘキシジン－スルファジアジン銀など）または抗微生物薬（ミノサイクリン－リファンピンなど）充填カテーテルではCLABSIリスクが低下するため，こうしたカテーテルの利用を考慮する
   b. FDAは，小児に対する抗菌薬充填カテーテルの使用を承認していない
3. 生後2ヵ月超の患者のCVCにはクロルヘキシジン含浸スポンジ包帯を使用する B－Ⅰ
   出生時低体重の新生児にクロルヘキシジン含浸スポンジ包帯を使用しない
4. 抗微生物薬によるCVCロックを用いる A－Ⅰ
   a. カテーテルハブを次に利用する時まで，超生理学的濃度の抗微生物薬溶液をカテーテル内腔に満たしたままにすることによって，抗生物質ロックを行う。耐性発現の可能性およびロック液が血流に入り込むことによる全身毒性の可能性が懸念されるため，抗微生物薬ロックは以下の場合に限る
      ⅰ．静脈アクセスが限定的で，再発性CLABSIの既往がある患者のための予防
      ⅱ．CLABSI重度続発症リスクが高い患者（人工弁や大動脈グラフトなどの血管内デバイスが最近移植された患者など）

Ⅲ．ルチーンのCLABSI予防対策とみなすべきでないアプローチ

1. 抗微生物薬投与によるカテーテル感染予防を行わない A－Ⅰ
   a. 抗微生物薬の予防的な全身投与は推奨されない
2. CVCまたは動脈カテーテルをルチーンで交換しない A－Ⅰ
3. 機械弁を備えた陽圧ニードルレスコネクタをルチーンで使用しない B－Ⅱ

Ⅳ．未解決事項

1. 患者に対する看護師の人数比およびICUにおけるヘルプ看護師（float nurses）
   a. CVC挿入患者を管理するICUでは看護師対患者の比を少なくとも2：1にすべきである。ICUで働くヘルプ看護師の数は最小限にすべきであることを示唆している。正式な勧告は，介入試験の結果待ちである
2. CLABSI発生率抑制のための静脈内療法チーム
   a. 末梢静脈カテーテルの挿入および維持において静脈内療法チームの設置は血流感染リスクを減少させるが，静脈内療法チームがCLABSI発生率に及ぼす影響についての研究はほとんど行われていない
3. 末梢動脈カテーテルなどについてのサーベイランス
   a. これからは血流感染サーベイランスシステムにおいて，末梢動脈カテーテルを含める必要があるかもしれない
4. CLABSIのカテーテル･日を推定することにより，サーベイランスを促進することができる可能性がある

セクション5：状況評価

Ⅰ．内部報告

病院の責任者，看護師責任者およびCLABSIリスクのある患者のケアにあたる臨床医に，プロセスおよび成果の数値を報告する。

A. プロセス測定値（優先順位の高いものから順に提示）

1. CVC挿入ガイドラインの遵守率
   a. CVC挿入が行われるICU，救急部，手術室，放射線科，一般病棟などにおけるチェックリストの遵守率を評価する
   b. 手指衛生，マキシマルバリアプリコーションの実施，挿入部位へのクロルヘキシジン製剤の使用の遵守率を求める
      ⅰ．分子：CVC挿入時に3つの介入（手指衛生，マキシマルバリアプリコーション，皮膚消毒へのクロルヘキシジン製剤の使

用）すべてが実行されたことが記録されたCVC挿入の件数
ii．分母：CVC挿入の総実施件数
iii．商に100を乗じて測定値をパーセンテージで表す

2. CVCアクセスの必要性に関する毎日の評価の記録の遵守率
a．毎日の評価が記録されているCVC留置患者のパーセンテージを調べる
i．分子：毎日の評価が記録されているCVC留置患者の数
ii．分母：CVC留置患者の総数
iii．商に100を乗じて測定値をパーセンテージで表す

3. カテーテルハブおよび注入ポートの利用前清浄化の遵守率
a．実践の観察を通じて遵守率を評価する
i．分子：カテーテルハブまたはポートの利用前清浄化が観察された回数
ii．分母：カテーテルハブまたはポートの利用が観察された回数
iii．商に100を乗じて測定値をパーセンテージで表す

4. 成人患者における大腿静脈からのCVC挿入回避の遵守率
a．CVCが鎖骨下静脈または内頸静脈ではなく大腿静脈に挿入された患者のパーセンテージを求める
b．大腿静脈カテーテル挿入患者のパーセンテージを算出する
i．分子：CVCが大腿静脈に挿入された患者の数
ii．分母：評価したユニットにおいてCVCが挿入された患者の総数
iii．商に100を乗じて測定値をパーセンテージで表す

B. 成果測定値

1. CLABSI発生率
a．NHSNの定義を使用する
i．分子：評価した各ユニットにおけるCLABSI発生件数（NHSNの定義を使用）
ii．分母：評価した各ユニットにおけるカテーテル・日の総数（NHSNの定義を使用）
iii．商に1,000を乗じて，測定値を1,000カテーテル・日あたりのCLABSI発生件数で表す
iv．リスク調整：患者ケアユニットのタイプでCLABSI発生率を層別化する

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In vitro antimicrobial effects of aztreonam, colistin, and the 3-drug combination of aztreonam, ceftazidime and amikacin on metallo- -lactamase-producing *Pseudomonas aeruginosa*

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Abstract

Background: There are limited choice of antimicrobial agents to treat infection with metallo- -lactamase-producing *Pseudomonas aeruginosa*. We evaluate the antimicrobial effects of aztreonam alone, colistin alone and the 3-drug combination of aztreonam, ceftazidime and amikacin on 23 strains of metallo- -lactamase-producing *P. aeruginosa* by time-killing tests.

Methods: Strains used were from different hospitals in Japan and had different pulse-field gel electrophoresis patterns by restriction with *Spe*I. The minimum inhibitory concentrations of 11 antimicrobial agents (piperacillin, piperacillin/tazobactam, imipenem, meropenem, aztreonam, ceftazidime, amikacin, tobramycin, arbekacin, ciprofloxacin and colistin) were determined using the agar dilution test. The effects of aztreonam, colistin and the combination of aztreonam, ceftazidime and amikacin were determined by time-killing studies.

Results: Bacteriostatic effects after 6 hours of drug exposure were observed in 12 strains (52.2%) of 23 strains of metallo- -lactamase-producing *P. aeruginosa* with 48 mg/l aztreonam, in 19 strains (82.6%) with the 3-drug combination of 16 mg/l aztreonam, 16 mg/l ceftazidime, and 4 mg/l amikacin, and in 23 strains (100%) with 2 mg/l colistin. Bactericidal effects after 6 h drug exposure were observed in 1 strain (4.3%) with 48 mg/l aztreonam, in 8 strains (30.4%) with the 3-drug combination and in all 23 strains (100%) with 2 mg/l colistin.

Conclusion: Evaluation of *in vitro* antimicrobial effects on metallo- -lactamase-producing *P. aeruginosa* revealed relatively good effects of the 3-drug combination of aztreonam, ceftazidime and amikacin and marked effects of colistin.

Background

Pseudomonas aeruginosa is a major bacterium causing nosocomial infection, and the development of multidrug resistance has become a problem [1-7]. Since metallo- -lactamase (MBL)-producing *P. aeruginosa* is often resistant not only to all -lactams, but also aminoglycosides, and fluoroquinolones, there are often no drugs to treat infection with this bacterium [8-10]. In addition, no extended

survey involving a series of human infections with MBL-positive isolates has been performed to determine the optimal treatment. Thus, appropriate therapy for those infections remains unknown [11].

We previously reported the effects of the 3-drug combinations of aztreonam, ceftazidime and amikacin or aztreonam, piperacillin and amikacin on 7 strains of multidrug-resistant *P. aeruginosa* [8-10]. In this study, to confirm the effectiveness of the 3-drug combinations, we evaluated the effects on a total of 23 strains of MBL-producing *P. aeruginosa* isolated in 23 hospitals in Japan in comparison with the effects of aztreonam or colistin alone.

Methods

Bacterial strains

Among *P. aeruginosa* strains sent from hospitals in Japan to the Japanese National Institute of Infectious Disease for detailed examination between January 2007 and July 2008, MBL-producing *P. aeruginosa* strains were screened, and MBL typing was performed according to the method of Shibata *et al* [12]. All 23 strains (one strain/hospital) of MBL-producing *P. aeruginosa* isolated during this period were donated by the Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Disease, and used for the experiments.

Pulsed-field gel electrophoresis

The high-molecular-weight chromosomal DNA was prepared according to the method of Murray *et al* [13], and the DNA sample in a small slice of an agarose plug in 200 μl of reaction buffer was digested with 30 U *Spe*I (New England Bio Labs, USA). Pulsed-field gel electrophoresis was carried out with the Bio-Rad Gene Path system (Bio-Rad, USA) in a 1% agarose gel in 0.5 × TBE buffer at 14°C with a linear ramp time of 1 to 23 s over a period of 18.5 h. Thereafter, the gels were stained with ethidium bromide and photographed.

Susceptibility tests using agar dilution methods

The minimum inhibitory concentrations (MICs) were determined after 18 h of incubation at 37°C by dilution on Sensitivity Disc Agar-N (Nissui Pharmaceuticals, Tokyo, Japan). The following antimicrobial agents were tested: piperacillin, piperacillin/tazobactam (Toyama Chemicals, Tokyo, Japan), imipenem, amikacin (Banyu Pharmaceuticals, Tokyo, Japan), meropenem (Dainippon-Sumitomo Pharmaceuticals, Tokyo, Japan), aztreonam (Eisai Co., Tokyo, Japan), ceftazidime (Glaxo Japan Co., Tokyo, Japan), tobramycin (Shionogi Pharmaceuticals, Tokyo, Japan), arbekacin (Meiji Seika Co., Tokyo, Japan), ciprofloxacin (Bayer Japan Co., Tokyo, Japan) and colistin (Wako Junyaku Co., Osaka, Japan). These antibiotics except for ciprofloxacin were provided in the form of a freeze-dried amorphous powder. The inocula (10^4 colony-forming units [cfu]/spot) were plated using a multipoint inoculator (Sakuma Co., Tokyo, Japan). The MIC was defined as the lowest drug concentration that inhibited visible growth. *P. aeruginosa* IFO 3919 was used as the reference strain. The drug concentrations (breakpoints) were set as follows: piperacillin, 64 mg/l; imipenem and meropenem, 8 mg/l; aztreonam and ceftazidime, 16 mg/l; amikacin, tobramycin and arbekacin, 4 mg/l; ciprofloxacin and colistin, 2 mg/l. Breakpoints used for all agents (except for amikacin, tobramycin, arbekacin and colistin) were according to the National Committee for Clinical Laboratory Standards (NCCLS) criteria [14]. The concentration of amikacin and tobramycin was 4 mg/l, which is lower than the criteria of the NCCLS. This was because in Japan, the routine dose of these agents is lower (ex. in the case of amikacin, 200–400 mg/day in one to two divided doses) than that in Western countries. The concentration of colistin used was according to a report by Soussy *et al* [15].

Drug effects in killing tests

Killing experiments were carried out to evaluate the bactericidal activities of 48 mg/l aztreonam, 2 mg/l colistin, and the 3-drug combination of 16 mg/l aztreonam, 16 mg/l ceftazidime and 4 mg/l amikacin. The final concentration of the log-phase inocula was approximately 10^5–10^7 cfu/ml [16-19]. Viability was determined based on bacterial counts at 2, 4, 6 and 24 h after incubation with drugs at 37°C by plating 500 μl of serial dilutions from each tube onto trypticase soy agar plates followed by incubation of the plates at 37°C for 24 to 48 h. In a preliminary experiment, drug carryover was ruled out by plating samples of a bacterial suspension containing 2×10^2– 4×10^2 cfu/ml in the presence or absence of antimicrobial agents alone or in combination. We also carried out preliminary killing tests with the 3-drug combination of 16 mg/l aztreonam, 16 mg/l ceftazidime and 4 mg/l amikacin, of 16 mg/l aztreonam, 64 mg/l piperacillin and 4 mg/l amikacin, and of 16 mg/l aztreonam, 64 mg/l piperacillin/4 mg/l tazobactam and 4 mg/l amikacin on 23 strains of MBL-producing *P. aeruginosa*. As a result, the viable cell count at 4 h after drug addition decreased to 1/100 or less of the initial count in 12 strains with aztreonam, ceftazidime and amikacin, 6 strains with aztreonam, piperacillin and amikacin and 4 strains with aztreonam, piperacillin/tazobactam and amikacin. Thus, the combination of aztreonam, ceftazidime and amikacin was the most effective, and therefore, the *in vitro* antimicrobial effects of this drug combination were evaluated.

Bactericidal activity was defined as a ≤ 3 $\log_{10}$ cfu/ml decrease in the starting inoculum. A bacteriostatic effect was defined as any decrease in the viable count from the starting inoculum [17].

Data analysis

In the killing tests, the effects on the 23 strains of MBL-producing *P. aeruginosa* were compared among aztreonam alone, colistin alone and the three-drug combination of aztreonam, ceftazidime and amikacin using the Kruskal-Wallis test based on the decrease in the viable count from the initial count at 2, 4, 6 and 24 h after drug addition.

Results

Of the 23 MBL-producing *P. aeruginosa* strains, 1 strain (strain no. 7) was bla_{VIM-2}, and the other 22 strains were bla_{IPM-1} by MBL typing. All 23 strains tested were confirmed to differ in their DNA pattern by pulsed-field gel electrophoresis. Concerning differences in the PFGE pattern, a one band difference was observed in 10 strains of 5 groups, 2–3 band differences in 5 strains of 2 groups, and more than 3 band differences in the other strains. Table S1 [additional file 1] shows the MICs of the 11 drugs against the 23 strains of MBL-producing *P. aeruginosa*. The MIC of piperacillin was ≤ 64 mg/l in 16 (69.6%) of the 23 strains. The MIC of piperacillin/tazobactam was ≤ 64 mg/l in 20 (87.0%), that of aztreonam was ≤ 16 mg/l in 13 (56.5%) and that of colistin was ≤ 2 mg/l in all 23 strains. However, the MICs of the other antimicrobial agents were high in most strains.

Aztreonam (48 mg/l) had bacteriostatic effects on 43.5–56.5% of the strains but bactericidal effects on only 0–4.3% at 2–24 h after its addition (Table 1, Figure 1). The 3-drug combination of aztreonam (16 mg/l), ceftazidime (16 mg/l) and amikacin (4 mg/l) had bacteriostatic effects on 69.6–82.6% of the strains and bactericidal effects on 8.7–39.1% at 2–24 h after their addition (Table 2, Figure 2). On the other hand, colistin (2 mg/l) exhibited bactericidal effects on all strains (100%) at 2–24 h after its addition (Table 3, Figure 3). Kruskal-Wallis tests showed significant decreases in the viable cell count at 2, 4, 6 and 24 h after the addition of colistin (Figure 3) compared with aztreonam alone (Figure 1) or the 3-drug combination of aztreonam, ceftazidime and amikacin (Figure 2).

Table 1: Antimicrobial effects of aztreonam (48 mg/l) against 23 strains of metallo- -lactamase-producing *P. aeruginosa*

	No. of strains (%) showing effects[a]	
Time of exposure (h)	Bacteriostatic effects	Bactericidal effects
2	13 (56.5)	0 (0)
4	10 (43.5)	0 (0)
6	12 (52.2)	1 (4.3)
24	11 (47.8)	1 (4.3)

[a]Bacteriostatic effects, any decrease in the viable count from the starting inoculum; bactericidal effects, ≥ 3 log cfu/ml decrease in the starting inoculum.

Table 2: Antimicrobial effects of the 3-drug combination of aztreonam (16 mg/l), ceftazidime (16 mg/l) and amikacin (4 mg/l) against 23 strains of metallo- -lactamase-producing *P. aeruginosa*

	No. of strains (%) showing effects[a]	
Time of exposure (h)	Bacteriostatic effects	Bactericidal effects
2	18 (78.3)	2 (8.7)
4	16 (69.6)	5 (21.7)
6	19 (82.6)	7 (30.4)
24	16 (69.6)	9 (39.1)

[a]Bacteriostatic effects, any decrease in the viable count from the starting inoculum; bactericidal effects, ≥ 3 log cfu/ml decrease in the starting inoculum.

Discussion

No hydrolysis of aztreonam by MBL has been reported, and studies using an animal model of pneumonia infection with a VIM-2-positive *P. aeruginosa* isolate showed that a high-dose of aztreonam reduced the bacterial load and may be a useful drug [20,21]. Aztreonam is negligibly toxic and can be administered at high doses. After high-dose administration, a blood concentration three times the breakpoint can be achieved [22].

A 2-drug combination of a -lactam antibiotic and an aminoglycoside antibiotic was reported to be effective against *P. aeruginosa* [16,19,23-27]. However, for multidrug-resistant *P. aeruginosa*, 3-drug combinations such as that of aztreonam, ceftazidime and amikacin rather than 2-drug combinations were shown to exhibit more marked *in vitro* antimicrobial effects [8,9]. Such observation is based on experiments in one country. In addition, colistin is effective *in vitro* against multidrug-resistant *P. aeruginosa* [28-30]. Therefore, we evaluated the bacteriostatic and bactericidal effects of aztreonam, a 3-drug combination (aztreonam, ceftazidime and amikacin) and colistin against 23 strains of MBL-producing *P. aeruginosa*. We found that although aztreonam had relatively low MIC (≤

Table 3: Antimicrobial effects of colistin (2 mg/l) against 23 strains of metallo- -lactamase-producing *P. aeruginosa*

	No. of strains (%) showing effects[a]	
Time of exposure (h)	Bacteriostatic effects	Bactericidal effects
2	23 (100)	23 (100)
4	23 (100)	23 (100)
6	23 (100)	23 (100)
24	23 (100)	23 (100)

[a]Bacteriostatic effects, any decrease in the viable count from the starting inoculum; bactericidal effects, ≥ 3 log cfu/ml decrease in the starting inoculum.

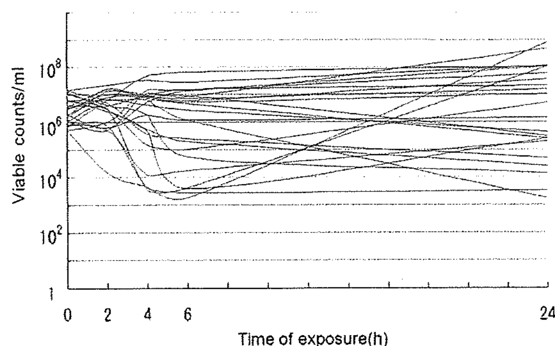

Figure 1
Bactericidal effects of 48 mg/l aztreonam against 23 strains of metallo- -lactamase-producing *P. aeruginosa* (37°C).

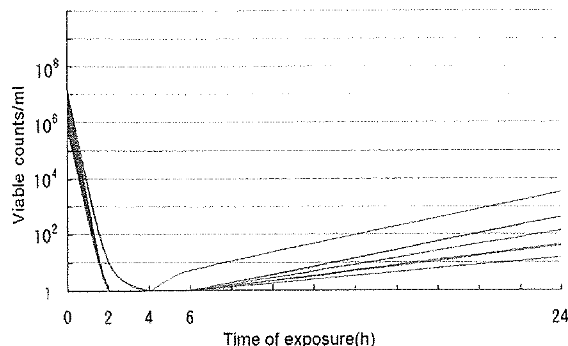

Figure 3
Bactericidal effects of 2 mg/L colistin against 23 strains of metallo- -lactamase-producing *P. aeruginosa* (37°C).

64 mg/l) among -lactam antimicrobial agents against 21 of the 23 strains of MBL-producing *P. aeruginosa* [additional file 1] the drug at 3-fold the breakpoint concentration, 48 mg/l, had bactericidal effects only on 1 of the 23 strains when used alone (Table 1).

On the other hand, the 3-drug combination of aztreonam, ceftazidime and amikacin showed bacteriostatic effects against 19 (82.6%) of the 23 MBL-producing strains and bactericidal effects against 7 (30.4%) of the 23 strains at 6 h after drug addition, indicating relatively good *in vitro* antimicrobial effects. Therefore, the combination of the 3 drugs should be considered as a treatment method for infection with MBL-producing *P. aeruginosa*.

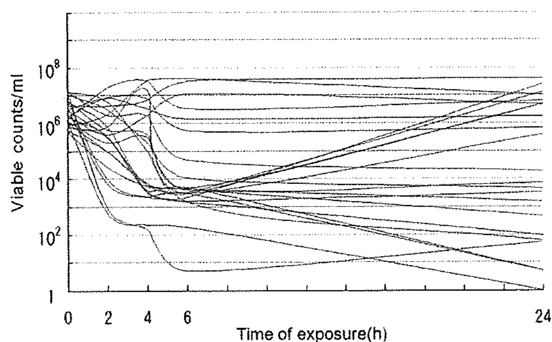

Figure 2
Bactericidal effects of the 3-drug combination of 16 mg/l aztreonam, 16 mg/l ceftazidime and 4 mg/l amikacin against 23 strains of metallo- -lactamase-producing *P. aeruginosa* (37°C).

Colistin had more marked *in vitro* antimicrobial effects than that of the 3-drug combination against MBL-producing *P. aeruginosa*, showing bactericidal effects against all 23 strains at 2–24 h after drug addition. Although colistin has severe side effects such as renal damage [31,32], some studies showed the clinical effectiveness of colistin against multidrug-resistant *P. aeruginosa* [28,29].

Conclusion

Evaluation of *in vitro* antimicrobial effects on metallo- -lactamase-producing *P. aeruginosa* revealed relatively good effects of the 3-drug combination of aztreonam, ceftazidime and amikacin and marked effects of colistin.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SO and KA conceived the idea for the study; YF, MY, and YM collected the data; SO and KA drafted the manuscript. All authors contributed in the writing and preparation of the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1
Table S1 MICs (mg/l) of 11 drugs against 23 strains of metallo-β-lactamase-producing Pseudomonas aeruginosa. *The data provided the MICs of the 11 drugs against the 23 strains of MBL-producing* P. aeruginosa.
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Short Communication

Microbial Contamination of Suction Tubes Attached to Suction Instruments and Preventive Methods

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SUMMARY: We investigated the microbial contamination of suction tubes attached to wall-type suction instruments. Microbial contamination of suction tubes used for endoscopy or sputum suction in hospital wards was examined before and after their disinfection. In addition, disinfection and washing methods for suction tubes were evaluated. Suction tubes ($n = 33$) before disinfection were contaminated with 10^2–10^8 colony-forming units (cfu)/tube. The main contaminants were *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Stenotrophomonas maltophilia*. The suction tubes were disinfected with sodium hypochlorite ($n = 11$) or hot water ($n = 11$), or by an automatic tube cleaner ($n = 11$). After 2-h immersion in 0.1% (1,000 ppm) sodium hypochlorite, 10^3–10^7 cfu/tube of bacteria were detected in all 11 tubes examined. After washing in hot running water (65°C), 10^3–10^7 cfu/tube were detected in 3 of the 11 examined tubes. The bacteria detected in the suction tubes after disinfection with sodium hypochlorite or hot water were *P. aeruginosa*, *A. baumannii*, and *S. maltophilia*. On the other hand, after washing with warm water (40°C) using the automatic tube cleaner, contamination was found to be <20 cfu/tube (lower detection limit, 20 cfu/tube) in all 11 tubes examined. These results suggest the usefulness of washing with automatic tube cleaners.

In hospitals in Japan, the suction of body fluids such as sputum or blood is performed daily using wall-type suction instruments in wards and outpatient clinics such as endoscopy rooms. These wall-mounted suction instruments are connected to a suction tube. The suction instruments are used for procedures such as sputum suction, endoscopy using a suction tube connected to a gastrofiberscope, and bronchoalveolar lavage (BAL) using a suction tube connected to a bronchofiberscope. In sputum suction and suction in gastrofiberscopy, the sucked body fluid (such as sputum and saliva) flows from the patient's side toward the suction tube (suction instruments). However, in BAL, regurgitation from the suction tube side toward the bronchofiberscope or bronchoalveolar lavage fluid (BALF) sometimes occurs (1); indeed, we experienced such regurgitation several times during BAL. BAL using suction tubes that are contaminated or that have not been disinfected therefore risks contaminating the patient and/or BALF, which may induce nosocomial infection (2,3). Additionally, when suction tubes are washed or disinfected in a sink in the ward or outpatient clinic, water drops containing patients' body fluids and microorganisms may splash on health care workers, who then run the risk of exposure and infection (4–6). Thus, it is essential to use disposable (single-use) suction tubes or to wash or disinfect suction tubes for each patient. However, to the best of our knowledge, there are currently no guidelines (or recommendations) regarding washing/disinfection methods for suction tubes as non-critical instruments, nor are there clinical data on the relationship between the microbial contamination of suction tubes and their disinfection methods. The purpose of the present study was to evaluate microbial contamination and methods of disinfection of suction tubes.

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We investigated the microbial contamination of suction tubes connected to wall-type suction instruments (Central Uni Co., Tokyo, Japan), and evaluated their disinfection/washing methods. Microbial contamination in a total of 33 suction tubes used for endoscopy or sputum suction in wards was compared before and after disinfection/washing. The tubes were disinfected with sodium hypochlorite ($n = 11$) or hot water ($n = 11$), or washed using an automatic tube cleaner ($n = 11$). We used one suction tube per patient. All suction tubes were 3 m in length and 4 mm in internal diameter, and were made of high-purity latex (Deluxe type latex tubing; Central Uni).

For disinfection with sodium hypochlorite, the suction tubes were washed after use under running water, immersed in 0.1% (1,000 ppm) sodium hypochlorite for 2 h (Fig. 1A), and air-dried in the ward or endoscopy room.

For disinfection with hot water, the suction tubes were washed under running water and then immersed in an enzyme detergent (Biotect®55; Sakura Seiki Co., Tokyo, Japan) at 40°C for 30 min. Subsequently, hot water (65°C) was run through the suction tubes for 5 min (Fig. 1B). In addition, the tubes were flushed with 20 mL of 80% (v/v) ethanol for disinfection (Yoshida Pharmaceutical Co., Tokyo, Japan) using a syringe, and air-dried in the ward.

For washing with the automatic tube cleaner, the suction tubes were washed using the cleaner in the central supply room, flushed with 20 mL of 80% (v/v) ethanol for disinfection, and dried using an automatic drier at 70°C for 2 h. This automatic tube cleaner automatically performs a cleaning process consisting of washing with an enzyme detergent, washing without a detergent, rinsing, and drying (Fig. 1C; Automatic Tube Cleaner MU-72 K; Sharp System Product Co., Tokyo, Japan). Warm water at 40°C, the temperature at which the optimal effects of the enzyme detergent can be expected, was used in the automatic tube cleaner.

Microorganisms in suction tubes after use and after disin-

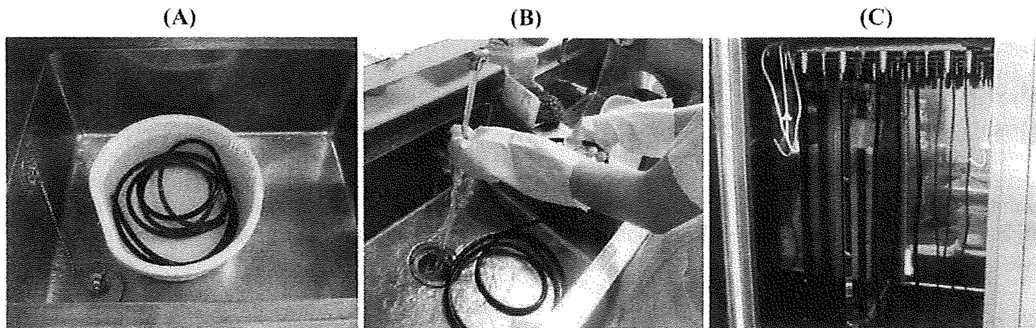

Fig. 1. Immersion in sodium hypochlorite (A), washing under running hot water (B), and washing with an automatic tube cleaner (C). (A) Disinfection by sodium hypochlorite solution. Suction tubes were washed after use under running tap water and immersed in 0.1% (1,000 ppm) sodium hypochlorite for 2 h. (B) Disinfection with hot water. Suction tubes were washed under running tap water and immersed in an enzyme detergent at 40°C for 30 min. Subsequently, hot water (65°C) was run through the suction tubes for 5 min. (C) Washing with an automatic tube cleaner. This automatic tube cleaner automatically performs a cleaning process consisting of washing with an enzyme detergent, washing without a detergent, rinsing, and drying.

Table 1. Microbial contamination inside suction tubes before disinfection with sodium hypochlorite solution, disinfection with hot water, or washing with automatic tube cleaner

Before disinfection with sodium hypochlorite		
Sample no.	Colony (cfu/tube)	Contaminant
1	2.4×10^2	*Escherichia coli*
2	2.7×10^7	*Klebsiella oxytoca*
	2.2×10^4	*Acinetobacter baumannii*
	2.0×10^7	*Stenotrophomonas maltophilia*
3	8.0×10^4	*Pseudomonas aeruginosa*
	3.5×10^6	*Acinetobacter baumannii*
	8.4×10^5	*Sphingobacterium multivorum*
4	2.8×10^5	*Acinetobacter baumannii*
	7.2×10^5	*Sphingobacterium multivorum*
	5.5×10^6	*Stenotrophomonas maltophilia*
5	3.5×10^6	*Acinetobacter baumannii*
	1.4×10^6	*Sphingobacterium multivorum*
6	1.3×10^5	*Acinetobacter baumannii*
	1.0×10^7	*Pseudomonas pertucinogena*
	3.2×10^6	*Escherichia coli*
7	4.2×10^2	*Pseudomonas pertucinogena*
	1.5×10^3	*Acinetobacter baumannii*
	6.0×10^3	*Escherichia coli*
8	2.3×10^8	*Acinetobacter baumannii*
	1.2×10^7	*Pseudomonas pertucinogena*
9	6.5×10^5	*Stenotrophomonas maltophilia*
	4.5×10^7	*Chryseobacterium meningosepticum*
	2.0×10^5	*Pseudomonas aeruginosa*
10	3.0×10^6	*Pseudomonas oryzihabitans*
11	6.4×10^4	*Stenotrophomonas maltophilia*
	2.6×10^5	*Chryseobacterium meningosepticum*

Before disinfection with hot water		
Sample no.	Colony (cfu/tube)	Contaminant
1	5.5×10^5	*Acinetobacter baumannii*
	3.6×10^4	*Pseudomonas aeruginosa*
2	3.0×10^3	*Stenotrophomonas maltophilia*
	4.4×10^7	*Pseudomonas aeruginosa*
3	2.5×10^6	*Acinetobacter baumannii*
4	3.4×10^5	*Acinetobacter lwoffii*
	3.0×10^5	*Chyseobacterium meningosepticum*
5	4.5×10^6	*Acinetobacter baumannii*
	5.0×10^3	*Pseudomonas aeruginosa*
	3.0×10^5	*Stenotrophomonas maltophilia*
6	3.0×10^4	*Acinetobacter lwoffii*
	6.0×10^5	*Stenotrophomonas maltophilia*
7	4.2×10^6	*Pseudomonas aeruginosa*
	2.7×10^3	*Acinetobacter baumannii*
8	7.0×10^6	*Pseudomonas aeruginosa*
	8.0×10^7	*Sphingomonas paucimobilis*
	3.5×10^6	*Acinetobacter lwoffii*
9	5.0×10^4	*Stenotrophomonas maltophilia*
10	2.1×10^7	*Chyseobacterium meningosepticum*
	4.8×10^6	*Pseudomonas aeruginosa*
11	5.3×10^5	*Pseudomonas aeruginosa*
	2.0×10^7	*Acinetobacter calcoaceticus*

Before washing with automatic tube cleaner		
Sample no.	Colony (cfu/tube)	Contaminant
1	3.0×10^6	*Acinetobacter baumannii*
2	3.0×10^5	*Stenotrophomonas maltophilia*
	4.4×10^5	*Pseudomonas aeruginosa*
3	2.6×10^7	*Acinetobacter lwoffii*
4	2.0×10^4	*Acinetobacter baumannii*
	2.4×10^6	*Pseudomonas aeruginosa*
	5.8×10^5	*Sphingobacterium multivorum*
5	3.0×10^5	*Acinetobacter baumannii*
	5.0×10^3	*Pseudomonas aeruginosa*
6	1.0×10^5	*Sphingomonas paucimobilis*
	1.0×10^7	*Stenotrophomonas maltophilia*
7	4.8×10^5	*Pseudomonas aeruginosa*
	5.0×10^4	*Acinetobacter haemolyticus*
	6.0×10^2	*Acinetobacter baumannii*
8	2.3×10^7	*Pseudomonas aeruginosa*
	8.0×10^7	*Sphingomonas paucimobilis*
9	5.8×10^5	*Stenotrophomonas maltophilia*
	6.6×10^6	*Acinetobacter baumannii*
	7.8×10^4	*Pseudomonas aeruginosa*
10	2.8×10^5	*Stenotrophomonas maltophilia*
	3.6×10^6	*Acinetobacter lwoffii*
	4.4×10^7	*Pseudomonas aeruginosa*
11	6.4×10^4	*Stenotrophomonas maltophilia*
	3.8×10^6	*Acinetobacter baumannii*

fection were quantified and identified by the following method. The tubes were flushed with sterile physiological saline (20 mL) using a syringe, and 20 mL of the saline that flowed out of each tube were used as a sample. The microorganisms in each saline sample were counted to determine the number of contaminants per suction tube. To count microorganisms, 10-fold serial dilutions of the samples with sterile saline were incubated in Trypticase® Soy Agar II with 5% sheep blood (Nippon Becton Dickinson, Co., Tokyo, Japan) at 37°C for 24–48 h. Microorganisms were identified by gram staining, morphological examination, the oxidation-fermentation (OF) test, the cytochrome-oxidase test, a test using a kit for the identification of glucose non-fermentative rods (ID Test・ENF-18®; Nissui Pharmaceutical, Co., Tokyo, Japan),

or a test using a kit for the identification of glucose fermentative rods (ID Test • ENF-20®; Nissui Pharmaceutical).

An interview survey was performed regarding suction tubes used in 18 institutions (including our hospital) in Yamaguchi Prefecture regarding the use or nonuse of disposable tubes, disinfection and automatic cleaners. Three institutions reported that disposable tubes were used, in 2 (including our hospital), disinfection was performed by immersion in sodium hypochlorite; and in 2, automatic tube cleaners were used. In the other remaining 11 institutions, tubes were reused without disinfection.

Table 1 shows the results of microbial contamination in suction tubes before disinfection by any of the 3 method under consideration. Suction tubes before disinfection with sodium hypochlorite solution or hot water were contaminated with 10^2–10^8 colony-forming units (cfu)/tube, and the main contaminants were *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Stenotrophomonas maltophilia*. Table 2 shows the results of microbial contamination in suction tubes after disinfection by any of the 3 methods under consideration. Bacteria were detected in all 11 examined tubes after 2-h immersion in 0.1% (1,000 ppm) sodium hypochlorite solution, and in 3 of 11 tubes after washing in hot running water. Contamination after disinfection was 10^3–10^7 cfu/tube, and the contaminants detected in the tubes were glucose non-fermentative Gram-negative rods such as *P. aeruginosa*, *A. baumannii*, *Sphingomonas paucimobilis*, and *S. maltophilia*. Contamination was <20 cfu/tube (lower detection limit, 20 cfu/tube) in all 11 examined tubes after washing using the automatic tube cleaner.

After disinfection by immersion in sodium hypochlorite solution or washing in hot running water, 14 (63.6%) of the 22 tubes examined were contaminated with 10^3–10^7 cfu/tube. In the case of immersion in sodium hypochlorite solution, this inadequate disinfection may be due to the insides of the tubes not being fully immersed in the solution because of the long thin tube structure (≥3 m), which inhabits the removal or dilution of organic matter and microorganisms in the tubes. Indeed, in one suction tube after disinfection by immersion in sodium hypochlorite solution, a mass of body fluid was discovered (Fig. 2). On the other hand, all 11 tubes disinfected by an automatic tube cleaner were contaminated with

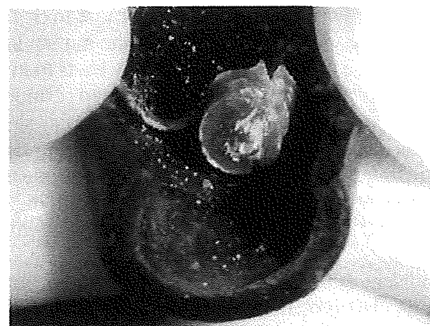

Fig. 2. A mass of body fluid discovered in a suction tube after disinfection with sodium hypochlorite solution.

Table 2. Microbial contamination inside suction tubes after disinfection with sodium hypochlorite solution, disinfection with hot water, or washing using automatic tube cleaner

After disinfection with sodium hypochlorite			After disinfection with hot water			After washing with automatic tube cleaner		
Sample no.	Colony (cfu/tube)	Contaminant	Sample no.	Colony (cfu/tube)*	Contaminant	Sample no.	Colony (cfu/tube)*	Contaminant
1	4.2×10^5	*Pseudomonas aeruginosa*	1	<20	–	1	<20	–
2	2.0×10^4	*Pseudomonas aeruginosa*	2	<20	–	2	<20	–
	2.0×10^4	*Acinetobacter baumannii*	3	7.2×10^3	*Acinetobacter baumannii*	3	<20	–
	7.4×10^5	*Stenotrophomonas maltophilia*	4	<20	–	4	<20	–
	1.2×10^6	*Sphingomonas paucimobilis*	5	1.6×10^7	*Pseudomonas aeruginosa*	5	<20	–
3	1.2×10^4	*Pseudomonas aeruginosa*		4.4×10^6	*Stenotrophomonas maltophilia*	6	<20	–
	3.6×10^4	*Acinetobacter baumannii*	6	<20	–	7	<20	–
	3.4×10^5	*Sphingobacterium multivorum*	7	<20	–	8	<20	–
	7.2×10^5	*Sphingomonas paucimobilis*	8	1.0×10^6	*Pseudomonas aeruginosa*	9	<20	–
4	6.0×10^5	*Acinetobacter baumannii*		3.2×10^5	*Acinetobacter lwoffii*	10	<20	–
	4.0×10^5	*Pseudomonas aeruginosa*	9	<20	–	11	<20	–
	1.1×10^7	*Sphingobacterium multivorum*	10	<20	–			
5	1.2×10^4	*Acinetobacter baumannii*	11	<20	–			
	1.6×10^3	*Pseudomonas aeruginosa*						
	6.6×10^4	*Sphingobacterium multivorum*						
6	8.4×10^6	*Pseudomonas aeruginosa*						
	2.8×10^6	*Stenotrophomonas maltophilia*						
7	8.0×10^4	*Pseudomonas aeruginosa*						
	6.4×10^5	*Sphingomonas paucimobilis*						
	4.8×10^5	*Stenotrophomonas maltophilia*						
8	8.0×10^3	*Pseudomonas aeruginosa*						
	1.6×10^4	*Acinetobacter baumannii*						
	1.5×10^5	*Stenotrophomonas maltophilia*						
	5.1×10^5	*Sphingomonas paucimobilis*						
9	3.2×10^6	*Stenotrophomonas maltophilia*						
	6.8×10^6	*Chryseobacterium meningosepticum*						
10	4.0×10^3	*Pseudomonas oryzihabitans*						
	2.0×10^2	*Empedobacter brevis*						
11	4.8×10^6	*Stenotrophomonas maltophilia*						
	4.0×10^6	*Chryseobacterium meningosepticum*						

*Lower detection limit, 20 cfu/tube.

<20 cfu/tube, showing accurate disinfection effects. Automatic cleaners can reduce microorganisms and organic matter inside suction tubes by a mean of 4 log (99.9%) (7). Therefore, the observed disinfection effects may be due to the effective removal of microorganisms and organic matter attached to suction tubes by the automatic cleaner. The disinfection and sterilization of medical equipment are indispensable as anti-infection measures in hospitals. To ensure appropriate disinfection/sterilization, the removal of contaminants from medical instruments is essential (8). In England and the United States, disinfection using automatic cleaners is widely performed. However, in Japan, disinfection is generally performed with disinfectants rather than automatic cleaners (9,10). Disinfectants are much more toxic than antibiotics and have various influences on the human body. The inappropriate use of disinfectants not only leads to inadequate effects but may also cause side effects due to residues. There have been reports of cases of chemical burns, shock, and protocolitis due to residual disinfectants resulting from inadequate rinsing (11–14). The use of automatic cleaners is a useful disinfection method that has marked disinfection effects without causing side effects due to residual toxicity (15).

The present status survey of 18 institutions revealed 3 institutions (16%) using disposable tubes and 2 (11%) (including our hospital) where disinfection is performed by immersion in sodium hypochlorite in the ward or outpatient clinic. When moist/respiratory tract medical instruments such as suction tubes are disinfected in the ward or outpatient clinic, medial workers or sinks may be contaminated by water droplets from suction tubes, which may cause occupational infection (16–18). On the other hand, washing with automatic tube cleaners not only provides superior decontamination/washing effects than disinfection methods performed in the ward or outpatient clinic, but is also desirable in terms of the prevention of occupational contamination of medical workers (19,20). Therefore, we strongly recommend the use of disposable suction tubes or disinfection using automatic tube cleaners.

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Particulate and Microbial Contamination in In-Use Admixed Intravenous Infusions

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We compared particulate and microbial contamination in residual solutions of peripheral intravenous admixtures after the termination of drip infusion between intravenous fluids admixed with glass ampoule drugs and those admixed with pre-filled syringe drugs. The mean number of particles ≥1.3 μm in diameter per 1 ml of residual solution was 758.4 for fluids (n=60) admixed with potassium chloride in a glass ampoule (20 ml volume), 158.6 for fluids (n=63) admixed with potassium chloride in a pre-filled syringe (20 ml volume), 736.5 for fluids (n=66) admixed with sodium chloride in a glass ampoule (20 ml volume), 179.2 for fluids (n=15) admixed with sodium chloride in a pre-filled syringe (20 ml volume), 1884.5 in fluids (n=30) admixed with dobutamine hydrochloride in 3 glass ampoules (5 ml volume), and 178.9 (n=10) in diluted dobutamine hydrochloride in pre-filled syringes (50 ml volume: For these samples alone, particulate and microbial contamination were evaluated in sealed products.) Thus, for potassium chloride or sodium chloride for injection, the number of particles ≥1.3 μm in diameter in the residual intravenous solution was significantly higher for fluids admixed with glass ampoule drugs than for those admixed with pre-filled syringe drugs (p<0.0001). For dobutamine hydrochloride for injection, the number of particles ≥1.3 μm in diameter in the residual intravenous solution was estimated to be higher for fluids admixed with its glass ampoule drug than for those admixed with its pre-filled syringe drug. Observation of the residual solutions of fluids admixed with potassium chloride, sodium chloride, or dobutamine hydrochloride in glass ampoules using an electron microscope with an X-ray analyzer showed glass fragments in each residual solution. Therefore, for the prevention of glass particle contamination in peripheral intravenous admixtures, the use of pre-filled syringe drugs may a useful method. No microbial contamination was observed in any of the residual solutions of 5 types of admixture.

Key words intravenous infusion; admixture; glass ampoule; pre-filled syringe; contamination; glass

Glass particles from glass ampoules not only causes phlebitis but also gives damage to the lungs, brain, kidneys, liver, and spleen.[1—7] Therefore, in western countries, the use of glass ampoule drugs is minimized, and vial drugs are used. However, in Asian countries such as Japan, glass ampoule drugs are still frequently used.

In recent years, pre-filled syringe drugs, which are expected to be free of glass fragments, have become commercially available in Japan. However, there have been no clinical data on the mixture of particles in pre-filled syringe drugs. Therefore, we collected residual solutions of peripheral infusions administered to patients, and compared the number of particles in the residual solutions between fluids admixed with glass ampoule drugs and those admixed with pre-filled syringe drugs. In addition, microbial contamination in these residual solutions was also examined.

MATERIALS AND METHODS

We evaluated particulate and microbial contamination in a total of 300 samples of residual solutions of peripheral infusions administered at the intensive care unit (6 beds) of Saiseikai Yamaguchi General Hospital (310 bed) in Yamaguchi Prefecture, Japan. The following 6 types of peripheral infusions were examined: electrolyte solution (500 ml volume: n=60) admixed with potassium chloride in a glass ampoule (K.C.L.®, 20 ml volume), electrolyte solution (500 ml volume: n=63) admixed with potassium chloride in a pre-filled syringe (Mediject®K, 20 ml volume), electrolyte solution (500 ml volume: n=66) admixed with sodium chloride in a glass ampoule (Conclyte®-Na, 20 ml volume), electrolyte solution (500 ml volume: n=15) admixed with sodium chloride in a pre-filled syringe (Mediject®Na 10%, 20 ml volume), physiological saline (Physisalz®-PL, 100 ml volume) admixed with dobutamine hydrochloride in 3 ampoules (Dobutrex®, 5 ml volume: n=30), and diluted dobutamine hydrochloride in pre-filled syringes (Dobupum®Injection 0.3% syringe, 50 ml volume: n=10). Since dobutamine hydrochloride in pre-filled syringes is administered using a syringe pump, the recovery of residual solutions of administration was difficult, and sealed products were examined. Electrolyte solutions used for admixture were Otsuka Glucose Injection 5%, Potacol®-R, Solita®-T No. 3, Physio®35, and Veen-®D. As controls, particulate and microbial contamination of these electrolyte solutions (500 ml volume: n=56) and physiological solution (Physisalz®-PL, 100 ml volume: n=10) were also examined.

The size and number of particles were measured using a light blockage particle counter KL-04 (Rion K.K., Tokyo, Japan). In addition, the volume of the residual solution in the bag was measured. For 2 samples each of the 5 types of intravenous admixture and diluted dobutamine hydrochloride in pre-filled syringes (total, 6 types), each residual solution (5 ml) was passed through 0.22 μm membrane filters, 5 cm in diameter (Nippon Becton Dickinson Co., Tokyo, Japan), and the particles on this filter were observed and identified using a scanning electron microscope JSM-5600LV coupled to an energy dispersion spectroscope JEO-2200 (JSM, Tokyo, Japan).

Microorganisms were quantified by the filter filtration

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method. The residual solution (5 ml) in the bag was passed through 0.22 μm membrane filters (5 cm in diameter), and 100 ml sterile physiological solution was passed through the filters to eliminate carry-overs such as sugar and amino acid on the filter. The filters were placed on Trypticase soy agar II with 5% sheep blood (Nippon Becton Dickinson Co., Tokyo, Japan) and incubated for 1—7 d at 30 °C.

The number of particles in the residual solution was compared according to the particle size ($\geq$1.3 μm, $\geq$5 μm, $\geq$10 μm, $\geq$50 μm) between fluids admixed with potassium chloride in a glass ampoule and those admixed with potassium chloride in a pre-filled syringe and between fluids admixed with sodium hydrochloride in a glass ampoule and those admixed with sodium hydrochloride in a pre-filled syringe by the Wilcoxon rank sum test, and p values were obtained.

RESULTS

The mean volume of residual solution samples of intravenous infusions was 22 ml for electrolyte solution (500 ml volume) admixed with drugs and 10 ml for physiological solution (100 ml volume) admixed with drugs. Table 1 shows the diameter and number of particles in 244 samples. The number of particles $\geq$1.3 μm in diameter was significantly higher in fluids admixed with potassium chloride in a glass ampoule than those admixed with this drug in a pre-filled syringe (p<0.0001). The number of particles $\geq$1.3 μm in diameter was significantly higher in fluids admixed with sodium chloride in a glass ampoule than those admixed with this drug in a pre-filled syringe (p<0.0001). The number of particles $\geq$1.3 μm in diameter was estimated to be higher in fluids admixed with dobutamine hydrochloride in glass ampoules than in those admixed with this drug in pre-filled syringes. The mean number (range) of particles $\geq$1.3 μm in electrolyte solution (500 ml volume) and physiological solution (100 ml volume) as controls was 95.9 (58—130)/ml for Otsuka Glucose Injection 5% (n=13), 109.1 (86—160)/ml for Potacol®-R (n=10), 112.5 (61—160)/ml for Solita®-T

Table 1. Mean (Range) of Particles/ml in In-Use Various Intravenous Infusion

Infusion	Particles greater than			
	1.3 μm	5 μm	10 μm	50 μm
Fluids admixed with potassium chloride in a glass ampoule (n=60)	758.4* (388—1560)	20.9* (5—44)	9.9* (2—43)	2.9* (0—9)
Fluids admixed with potassium chloride in a pre-filled syringe (n=63)	158.6 (98—299)	6.5 (1—22)	1.8 (0—11)	0
Fluids admixed with sodium chloride in a glass ampoule (n=66)	736.1* (379—1009)	23.9* (9—65)	10.6* (2—28)	2.5* (0—6)
Fluids admixed with sodium chloride in a pre-filled syringe (n=15)	179.2 (128—245)	7.6 (3—16)	3.2 (0—7)	0
Fluids admixed with dobutamine hydrochloride in glass ampoules (n=30)	1884.5 (1001—3890)	29.4 (15—56)	11.1 (2—30)	3.2 (1—8)
Dobutamine hydrochloride in pre-filled syringes (n=10)	178.9 (160—199)	2.6 (0—6)	0	0

* p<0.0001.

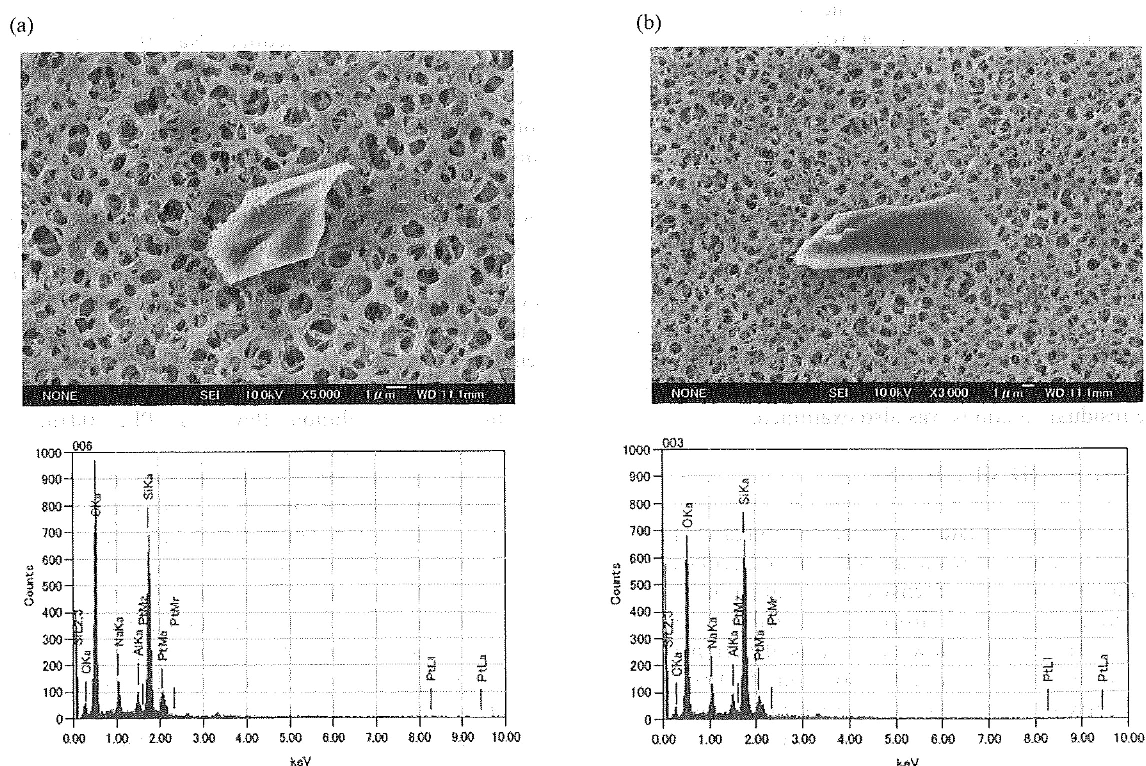

Fig. 1. Identification of Two Types of Particles by Scanning Electron Microscopy Coupled to Energy Dispersion Spectroscopy
(a) and (b) suggest glass particles.

No. 3 (n=12), 117.0 (68—166)/ml for Physio®35 (n=11), 107.0 (81—165)/ml for Veen-®D (n=10), and 97.2 (65—121)/ml for Physisalz®-PL (n=10).

Figure 1 shows a scanning electron micrograph of two types of particles on the filter after filtration of 5 ml residual solution of physiological solution admixed with dobutamine hydrochloride in glass ampoules and results of the identification of these particles by energy dispersion spectroscopy. Both types of particles were glass fragments. Glass ampoule fragments were also observed in infusion solutions admixed with potassium chloride in a glass ampoule and those admixed with sodium chloride in a glass ampoule. However, no particles such as glass fragments were observed in solutions admixed with potassium chloride in a pre-filled syringe, those admixed with sodium chloride in a pre-filled syringe, or diluted dobutamine hydrochloride in pre-filled syringes.

No microbial contamination was observed in any of the 244 samples and the 66 control samples examined in this study.

DISCUSSION

The risk of the mixture of particles such as glass fragments in injection fluid at ampoule cutting has long been suggested.[1—7] In the U.S., England, and Canada, the use of glass ampoules is minimized. For example, our previous survey in Calgary University Hospital in Canada showed the use of glass ampoules only for digoxin and mesna, and predominant use of vial drugs. In Japan, though plastic ampoules for distilled water and glucose solution for injection became commercially available,[8] there have been few measures against particles in injection fluids. However, pre-filled syringes have introduced into the market, showing advantages such as saving the trouble of preparing injections, absence of the risk of injury during handling because ampoule cutting is not necessary, and a reduction in the risk of mixture of particles and microorganisms. Therefore, we compared the number of particles between infusion fluids admixed with glass ampoule drugs and those admixed with pre-filled syringe drugs, and found a significantly lower number of particles in the latter. The particles in fluids admixed with glass ampoule drugs were identified as glass fragments. These results were consistent with the presence of a large amount of glass fragments in hyperalimentation fluids admixed with glass ampoule drugs observed in our previous study.[9] Therefore, for the prevention of mixture of particles such as glass fragments, pre-filled syringe drugs may be useful.

The sequelae of glass particle contamination require a considerable time to develop.[5] Therefore, the adverse effects of glass fragments in drugs for injection tend to be overlooked. However, glass fragments in drugs for injection have been shown to induce severe conditions such as adult distress syndrome and pulmonary artery granuloma in immature infants.[6,7] Therefore, the use of glass ampoule drugs should be also minimized in Japan. Since 12 types of drug in pre-filled syringes are commercially available at present, the use of pre-filled syringes instead of glass ampoules is desirable for these drugs. In the future, changes from glass ampoule drugs to vial drugs may be necessary as in western countries. When glass ampoule drugs are used because there is no other choice, admixtures should be prepared using membrane filters at pharmacies whenever possible.[10] In the present survey, 98—299 particles (diameter, $\geq$1.3 μm)/ml were detected even in residual solutions of intravenous fluids admixed with pre-filled syringe drugs. However, no particles such as glass fragments were observed in solutions admixed with drugs in pre-filled syringes. Therefore, we speculate that these particles are dust that was originally present in intravenous fluids or drugs in pre-filled syringes. These results also exclude the possibility that glass particulates come to be mixed into the solutions under experimental process to measure particulates.

The Pharmacopoeia of Japan describes the Foreign Insoluble Matter Test for Injection and Insoluble Particulate Matter Test for Injections. In the Foreign Insoluble Matter Test for Injection, drugs for injection meet the standard when no foreign matters are macroscopically observed under 8000—10000 luxes. In other words, drugs meet the standard when the diameter of contained foreign matters is less than about 40 μm. In the Insoluble Particulate Matter Test for Injections, the criteria are 25 or less particles with a diameter of $\geq$10 μm and 3 or less particles with a diameter of $\geq$25 μm per ml. Thus, these standards refer to foreign matters and particles with a diameter of $\geq$10 μm. However, foreign matters and particles with a diameter of $\leq$10 μm may also be harmful to the body.[4] Therefore, in the future, the contents of the Foreign Insoluble Matter Test for Injection and Insoluble Particulate Matter Test for Injections in the Pharmacopoeia of Japan should be revised. In addition, these criteria in the Pharmacopoeia are used for drug products for injection, and there are no criteria for foreign matters and particles in drugs for injection after ampoule cutting. In the future, the criteria of the counts of foreign matters and particles in drugs for injection after ampoule cutting should be established for the promotion of changes in the drug form from glass ampoules to plastic ampoules or vials.

Concerning microbial contamination of peripheral intravenous admixtures, a previous study in Mexico showed microbial contamination in 16 (2.13%) of 751 samples.[11] In this study, none of the 234 samples showed microbial contamination. Therefore, the microbial contamination rate after admixture is estimated to be low, but further investigation is necessary.

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ORIGINAL ARTICLE

Prevalence of *Helicobacter* and *Acanthamoeba* in natural environment

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Keywords
acanthamoeba, environment, *Heliocbacter pylori*, PCR, prevalence, river water, soil.

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Abstract

Aims: We examined whether the presence of *Helicobacter* is related to that of *Acanthamoeba* in river and soil environments.
Methods and Results: The samples (river n = 51, soil n = 75) were collected in Sapporo City, Japan. PCR with primers for *Helicobacter* genus-specific and standard culture techniques were used to detect helicobacter. Prevalence of acanthamoeba was also evaluated by genus-specific PCR. The prevalence of *Helicobacter* genus-specific DNA in river water samples and in soil samples was 88% and 0%, respectively. No successful culture of helicobacter was achieved. The prevalence of *Acanthamoeba* genus-specific DNA in river samples and in soil samples was 61% and 96%, respectively. No statistical correlation between the prevalence of helicobacter and either that of acanthamoeba or water quality parameters (pH, turbidity and coliform group) except for temperature was found.
Conclusions: We revealed the presence of helicobacter in river water and non-existence of helicobacter in soil. However, the distribution of helicobacter did not overlap with that of acanthamoeba in rivers.
Significance for Impact of the Study: The role of acanthamoeba on the survival of helicobacter might be limited as the both are coincidentally present in the environment.

Introduction

Helicobacter pylori can colonize the human stomach and become a causative agent of chronic gastritis, peptic and duodenal ulcers, and be an aetiologic agent in gastric cancer (Parsonnet *et al.* 1991, 1994; Peterson 1991). *Helicobacter pylori* is believed to be one of the world's commonest pathogens, with over half of the world's population being infected (Westblom *et al.* 1991; Parsonnet 1998). Despite many studies, the transmission route of *H. pylori* remains to be determined, however, previous reports suggest that humans are the only significant reservoir of *H. pylori*, indicating that this infection may spread by the faecal–oral route (Mégraud 1995; Brown 2000; England 2001; Bellack *et al.* 2006). Indeed, recent papers have reported the detection of *H. pylori* DNA in water samples, suggesting extensive distribution of *H. pylori* in the aquatic environment (Hultén *et al.* 1998; Sasaki *et al.* 1999; Voytek *et al.* 2005). Moreover, recent studies have described several other *Helicobacter* species that infect not only human but also other hosts, including cheetah, dog, cattle, pig, ferret, pigtailed macaque and rhesus macaque (Solnick and Schauer 2001; Kusters *et al.* 2006). Thus, these findings indicate that a broad range of river and soil environments may be contaminated by helicobacter, possibly spreading in the natural environment through animal faeces. However, the survival of helicobacter and its way in the natural environment remain unknown.

Free-living amoebae, which are morphologically classified into several genera, such as *Acanthamoeba*, *Filamoeba*, *Hartmannella*, *Saccamoeba*, *Vahlkampfia* and *Naegleria*, inhabit a wide rage of natural ecological niches, including ponds, river water, soil and air (Khan 2006). Acanthamoeba is more abundant than the other amoeba

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genera (Khan 2006). Interestingly, a Swedish group has reported that, under experimental conditions, *H. pylori* is capable of propagation and remains viable for several weeks in the presence of acanthamoeba (Winiecka-Krusnell *et al.* 2002). *In vitro* studies have also shown that acanthamoeba can tolerate survival and multiplication of human pathogens, such as pseudomonas, mycobacterium, listeria, vibrio and cryptococcus, as well as legionella (Greub and Raoult 2004). These findings suggested that helicobacter, as well as other pathogens, could survive through 'hiding' in amoebae, thereby sheltering from environmental stress and that amoebae could be a natural reservoir for helicobacter. However, the overlap between the distribution of helicobacter and that of amoebae in the field remains unknown, despite its importance in understanding the transmission route of *H. pylori*. Therefore, we examined whether the prevalence of helicobacter would overlap with that of acanthamoeba in river and soil environments of Sapporo City, Japan.

Materials and methods

Bacterial and amoebal strains

Helicobacter pylori clinical isolates (TK1021, TK1023, TK1029) separately obtained from three patients with gastritis and *Acanthamoeba castellanii* C3 purchased from the American Type Culture Collection were used as positive controls for PCR analysis. Other bacteria from our stock collections (*Bacillus subtilis*, *Chlamydophila pneumoniae*, *Enterococcus faecalis*, *Escherichia coli*, *Legionella pneumophila*, *Mycobacterium avium*, *Parachlamydia acanthamoebae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*) were also used.

Samples

River water samples from 51 different sites were collected from the rivers that pass through Sapporo City from June to October, 2007. The samples (300–1000 ml) were put in disposable polypropylene bottles. Collections were made as surface grab samples from near to the shore to reflect local inputs. Temperature was measured on site at the time of collection. Collected river water samples were kept at 4°C and immediately taken to our laboratory. As indicators for water quality, turbidity, pH and the amount of faecal indicator bacteria (coliform group) were measured. The detection of helicobacter was performed by the culture method as follows: The river sample (100 μl) was spread on plates containing a selective agar medium (Eiken, Tokyo, Japan) and incubated under microaerophilic conditions for 7 days. Water turbidity was determined using a standard turbidimetrical assay, as described previously (Ravichandran 2003). Water pH was measured by a standard pH meter. Faecal indicator bacteria were quantified using a standard membrane filtration technique and Petri film assay (3M, St. Paul, MN, USA), according to the manufacturer's instructions.

Seventy-five soil samples were collected from public parks located in the central area of Sapporo City from June, 2006 to August, 2007. The samples (10–20 g) were taken at a depth of 5–10 cm using a disposable centrifuge tube. Soil samples were put in individual sterile bags, immediately taken to our laboratory, and kept in the dark at 25°C until use.

PCR detection limit for helicobacter

To determine the detection limit of PCR for helicobacter from river water and soil samples, spike experiments were performed. Several sets of Page's amoeba saline (Buck and Rosenthal 1999) (500 ml) or soil (500 mg), which were confirmed as helicobacter negative, were prepared. The sets were spiked with serial dilutions of *H. pylori* TK1029 strain from 2·4 to $2 \cdot 4 \times 10^5$ CFU (river water) or 0·7 to $0 \cdot 7 \times 10^5$ CFU (soil) per sample. The DNA extraction of spiked samples, as well as from environmental samples, was performed by the method described below.

DNA extraction

Each river water sample was passed through a membrane filter (pore size; 0·2 μm). The filter was then intensely vortexed for 60 s in 20 ml sterilized Page's amoeba saline containing 0·05% (v/v) Tween 80, and the suspension then centrifuged at 1600 *g* for 30 min. Pellets were then used for DNA extraction. The DNA extraction was performed using a DNA mini kit (QIAmp; Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The DNA was eluted in 50 μl of the elution buffer supplied with the kit, and stored at −20°C until use. Five hundred mg of soil per sample was used for DNA extraction using an UltraClean Soil DNA isolation Kit (MBL, Carlsbad, CA, USA), according to manufacturer's instructions.

PCR

Table 1 shows primer sets for PCR amplification of *Helicobacter* spp. (Lu *et al.* 2002), *Acanthamoeba* spp. (Horn *et al.* 1999) and bacteria (excluding order *Chlamydiales*) (Schroeder *et al.* 2001). For relief of the inhibition of PCR amplification by humic acid, BSA was added to each reaction according to the method described previously (Kreader 1996; Wilson 1997). The quality of extracted DNA was confirmed by PCR amplification using universal

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Table 1 Primer sequences used for this study

Micro-organism	Target gene	Primer	Sequence	Expected fragment size	Reference
Helicobacter spp.	*16S rRNA*	HP1	5′-GCA ATC AGC GTC AGT AAT GTT C-3′	521 bp	Lu *et al.* 2002
		HP2	5′-GCT AAG AGA TCA GCC TAT GTC C-3′		
Acanthamoeba spp.	*18S rRNA*	JDP1	5′-GGC CCA GAT CGT TTA CCG TGA A-3′	423–551 bp	Schroeder *et al.* 2001
		JDP2	5′-TCT CAC AAG CTG CTA GGG AGT CA-3′		
Bacteria	*16S rRNA*	Forward	5′-AGA GTT TGA TYM TGG CTC AG-3′*	Nearly full-length	Horn *et al.* 1999
		Reverse	5′-CAK AAA GGA GGT CC-3′†		

*Y, C or T; M, A or C.
†K, G or T.

primers that target bacterial *16S rRNA*, which is conserved across a broad spectrum of bacteria. DNA samples that resulted in negative PCR for bacterial *16S rRNA* were omitted. The samples (soil, $n = 71$; river water, $n = 51$) that yielded PCR products of the expected size were used for PCR amplification with *Helicobacter* genus-specific, and *Acanthamoeba* genus-specific primers. Primers for genus *Helicobacter* was targeted to *16S rRNA* and to the *16S rRNA* hypervariable 5′ flanking region, respectively. Primers targeting *Acanthamoeba* spp. *18S rRNA* were also used. The results of search with the BLAST program showed that the primers used for each PCR were specific for genus *Helicobacter* and genus *Acanthamoeba* detection. Template DNA of 2 μl (for soil) or 5 μl (for river) was used for each PCR reaction. Reactions were carried out in 25 μl reaction buffer (each dNTP, 200 μmol l^{-1}; BSA, 10 μmol l^{-1}; 1× commercial reaction buffer) containing *Taq* DNA polymerase [0·625 U *Taq* DNA polymerase (New England Biolabs, Herts, UK)]. The PCR cycle consisted of 10 min denaturation at 94°C followed by 35 cycles, each of 30 s denaturation at 94°C; 30 s of annealing at 55°C for *Helicobacter* genus-specific *16S rRNA*, 60°C for *Acanthamoeba* genus-specific *18S rRNA*, 52°C for bacterial *16S rRNA*; and 45 s of extension at 72°C. The amplified products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The presence of amplified target genes in randomly selected five positive specimens was confirmed by direct oligonucleotide sequencing of the PCR products (Macrogen, Seoul, Korea) (Fig. 1). As a quality control for each PCR, diluted DNA extracted from *H. pylori* TK1029 or *A. castellanii* C3 strains were used in each amplification. To prevent contamination, the preparation of the PCR mixture was performed in a separate room.

Statistical analysis

Comparison between the frequency of genus *Helicobacter* and that of genus *Acanthamoebae* was analysed by Fisher's exact test. Correlation between the prevalence of *Helicobacter* spp. and water qualities including pH, temperature, turbidity and amount of faecal indicator bacteria (coliform group) was assessed by an unpaired *t*-test. A $P < 0·05$ was considered significant.

Results

Specificity of PCR for genus *Helicobacter 16S rRNA* region

The specificity of PCR primers targeting the region of genus *Helicobacter 16S rRNA* was assessed. Figure 2 shows the PCR results generated from *H. pylori* (TK1021, TK1023, TK1029) and other bacteria. The PCR primers amplified from *H. pylori* but not from templates extracted from other bacteria.

PCR detection limits of genus *Helicobacter* from environmental samples

The detection limit of the PCR for *Helicobacter* genus-specific *16S rRNA* was examined by using DNAs extracted from the river or soil samples that had been spiked with defined numbers of *H. pylori*. The detection limit of the PCR in spiked river water was $2·4 \times 10^2$ CFU (for both targets) and in soil samples was $0·7 \times 10^3$ CFU (for *16S rRNA*) (Fig. 3).

Water quality of the rivers and prevalence of genus *Helicobacter* and genus *Acanthamoeba* in river and soil samples

Fifty-one river water samples were assessed. Water quality parameters, including pH, temperature, turbidity and amount of faecal indicator bacteria (coliform group) were determined as follows: pH, 7·22 ± 0·75; temperature, 20·31 ± 4·27°C; turbidity, 5·28 ± 6·28 NTU; coliform group, 2109 ± 2979 CFU l^{-1} (each value is an average ± SD). *Helicobacter* genus-specific DNA was detected in 45 of 51 river samples (75%). No successful culture for helicobacter was achieved. The prevalence of *Acanthamoeba* genus-specific DNA in the rivers was 30 of 51 river

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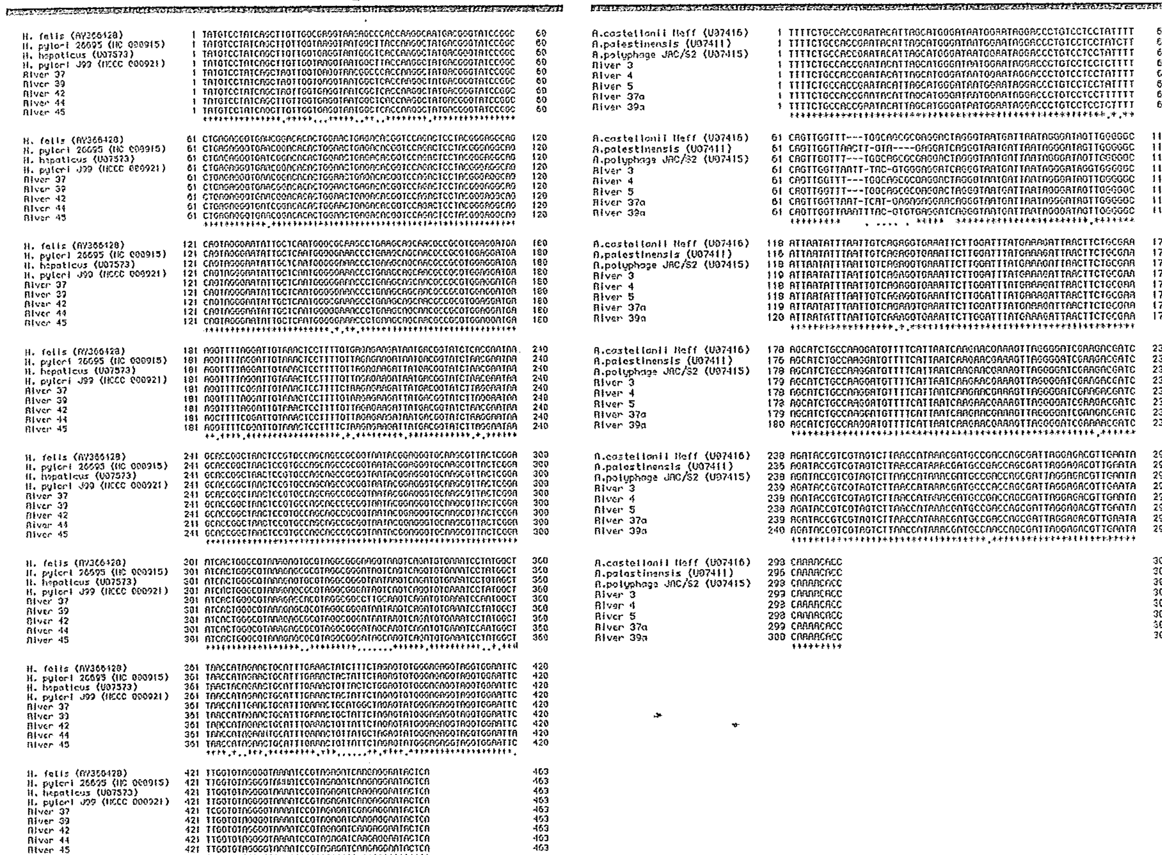

Figure 1 Alignment analysis for *Helicobacter* and *Acanthamoeba* amplicons with previously reported sequences. The alignment analysis was performed using Genetyx-Mac (ver. 10·1, Genetyx, Tokyo, Japan). The asterisk represents conserved sequences in amplicons. Dots and unmarked positions represent a base mismatch and an additional base on the alignment, respectively. *Helicobacter* amplicons amplified from environmental specimens: River, 37, 39, 42, 44, 45. *Acanthamoeba* amplicons amplified from environmental specimens: River 3, 4, 5, 37a, 39a.

water samples (61%). No statistical correlation between the distribution of helicobacter and either that of acanthamoeba or water qualities including pH, turbidity and amount of faecal indicator bacteria was found (Table 2). The temperature of rivers that were genus *Helicobacter* positive differed significantly from the temperature of rivers that were genus *Helicobacter* negative ($P < 0{\cdot}05$).

The presence of helicobacter in the 71 soil samples was also assessed. However, genus *Helicobacter* was not detected in any soil sample. In contrast, *Acanthamoeba* genus-specific DNA was detected in 69 of 71 soil samples (97%).

Discussion

The use of standard culture techniques to detect micro-organisms in complex biological samples obtained from natural environments, such as river water and soil, is limited when the target population is in a minority and because most micro-organisms in the environment sift to a non-culturable stage. Therefore, the molecular technique of PCR, which can be applied to analyse the entire micro-organism community, was used for this study. Since it is known that humic acid, which is ubiquitous to environments such as rivers and soil, inhibits PCR amplification (Wilson 1997), BSA was added to each reaction according to the method described previously (Kreader 1996). No amplification of bacterial *16S rRNA* was seen from soil samples without the addition of BSA (data not shown).

Acanthamoeba was detected in both river water and soil, in agreement with other studies (Khan 2006). The genus *Acanthamoeba* has been classified into 15 different genotypes (T1–T15) (Khan 2006). Ninety percent of acan-

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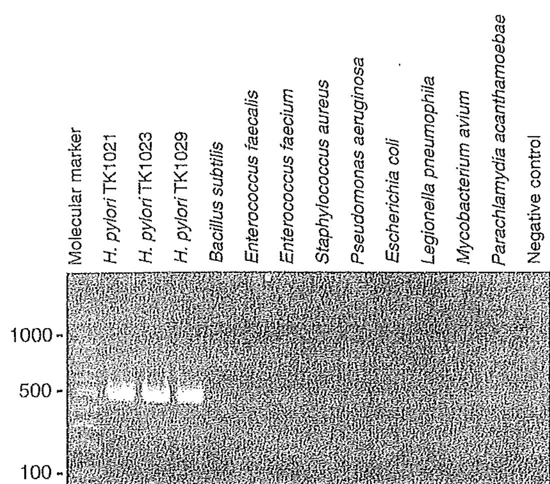

Figure 2 Specificity of PCR primers targeting to *Helicobacter* genus-specific *16S rRNA* region.

Table 2 Comparisons between prevalence of *Helicobacter* spp. and either that of *Acanthamoeba* spp. or water quality in the rivers

	Prevalence of *Helicobacter* spp. DNA		
	Positive (n = 45)	Negative (n = 6)	P value
Prevalence of *Acanthamoba* spp. DNA (n = 30)	28 (90%)	2 (6%)	0·181*(NS)
Water qualities (unpaired t-test)			
pH	7·17 ± 0·73†	7·7 ± 0·78	0·166 (NS)
Temperature (Åé)	19·68 ± 3·94	25·08 ± 3·8	0·013 (S)
Turbidity (NTU)	5·43 ± 6·64	4·23 ± 2·46	0·405 (NS)
Coliform group (CFU l^{-1})	2160 ± 3149	1733 ± 1129	0·52 (NS)

*Comparison between the frequency of genus *Helicobacter* and that of genus *Acantamoebae* was analysed by Fisher's exact test.
†Average ± SD.
NS, not significant; S, significant.

thamoeba isolates belong to the T4 genotype, which is the most likely genotype to express a strong virulence against human infection (Khan 2006). We have also confirmed that the T4 genotype rather than other genotypes was also frequently amplified (data not shown).

Several previous studies, using PCR methods for the detection of environmental helicobacter and assuming waterborne infection, have already been reported. Voytek *et al.* detected *Helicobacter* spp. and *H. pylori* in 10/33 (55%) and 11/33 (33%) of the rivers of North America, respectively, indicating a broad range of distribution of *H. pylori* as well as of other *Helicobacter* spp. in this area (Voytek *et al.* 2005). Hulten *et al.* also detected *Helicobacter* spp. in many Swedish water samples: 9/24 (38%) of household wells, 3/25 (25%) of municipal tap-water samples and 3/25 (12%) of waste-water samples (Hultén *et al.* 1998). Sasaki *et al.* reported that *H. pylori* is widely distributed in the domestic environment of Japan, such as in rivers and ponds (Sasaki *et al.* 1999). Thus, recent published data indicated that helicobacter including *H. pylori* can be detected in different water samples obtained from environments such as river and well water and that this bacterium is widely distributed in the natural environment. In the present study, *Helicobacter* spp. were frequently detected in river water samples, but not in soil samples. Our results support previous reports showing a wide range of distribution of helicobacter in water type environment, and also indicate the PCR conditions for helicobacter detection was optimized as well as that of other papers. The reason for the uneven distribution of helicobacter remains unknown, but may be explained thus: it is easy for water environment such as river to be contaminated by helicobacter that spread through animal faeces.

Among the river water samples, there was a significant correlation between the temperature values and the prevalence of genus *Helicobacter*, showing that the presence of helicobacter was affected by temperature values. The results support the hypothesis of previous reports that the

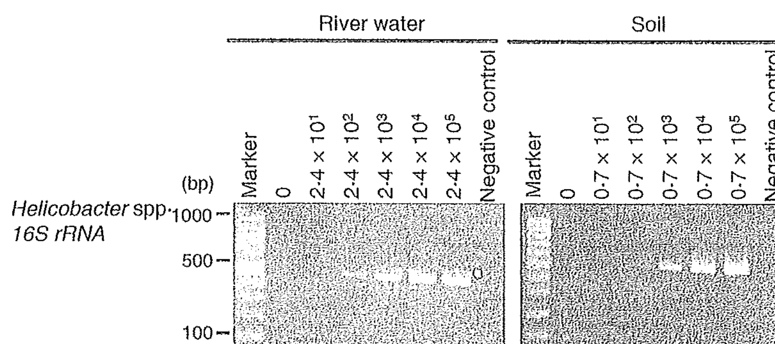

Figure 3 Detection limits of primers specific for *Helicobacter 16S rRNA*. N, negative control. P, positive control.

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