# Detection of potentially pathogenic bacteria in the drinking water distribution system of a hospital in Hungary

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## Abstract

The drinking water distribution system of a hospital was investigated using standard cultivation techniques, taxon-specific PCRs targeting pathogenic bacteria, denaturing gradient gel electrophoresis, cloning and sequencing. The results obtained verify the higher sensitivity of PCR compared to cultivation for detecting *Legionella* and *Pseudomonas aeruginosa*. Moreover, several other opportunistic pathogenic bacteria, such as *Escherichia albertii*, *Acinetobacter lwoffi* and *Corynebacterium tuberculostrearicum*, were detected, emphasizing that drinking water systems, especially those with stagnant water sections, could be the source of nosocomial infections.

**Keywords:** Cloning and sequencing, *Legionella*, PCR detection, *Pseudomonas aeruginosa,* waterborne nosocomial infections

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Hospital drinking (potable) water systems are the most important and controllable, as well as the most overlooked source, of nosocomial pathogens. Conventional culture-based microbiological water quality monitoring techniques take a long time (several days) and usually a small volume of water is sampled (typically 100 mL), which gives rise to inadequate detection limits with regard to drinking water safety. Furthermore, the presence of some important waterborne pathogens (such as *Pseudomonas aeruginosa* or legionellae) shows no correlation with conventional indicator organism counts. Water-related pathogens can also find niches in water systems (i.e. an association with biofilms or free-living amoebae), rendering their observation with conventional techniques more difficult. Molecular techniques provide new and rapid facilities for the detection of pathogens involved in nosocomial infections.

Five representative end-points (taps) in the drinking water system of a hospital (Budapest, Hungary) were sampled in October 2005.

The hospital is located in two adjacent buildings, which share the main inlet pipe, but have separate hot water heating systems, storage facilities and water disribution systems. Samples were taken from a building for adult patients: from a hot water tap in the intensive care unit (TW1) and from an unused tap in a store-room (TW2, lukewarm water); from a different building (children's intensive care unit): from a hot water tap used for hand-washing (TW3) and from a hot water tap used for the washing of surgical instruments (TW4); and from a cold water tap in the garden (GT) close to the main cold water supply.

Samples were taken to simulate every-day usage: with previous flushing, at usage temperature and without previous tap disinfection. One hundred-millilitre samples were used for cultivation purposes and 1-L samples were filtered for DNA extraction from aquatic microbes (47 mm in diameter, 0.45 $\mu$m mixed cellulose ester filter). For selective cultivation of *Legionella*, GVPC agar plates (Hevck, Darmstadt, Germany) (with acidic treatment) were used and *P. aeruginosa* was cultivated on cetrimide agar plates. GVPC plates were incubated at 37°C for 5 days in aerobic conditions; cetrimide plates were incubated at 37°C for 2 days. DNA was extracted with the Aqua Screen® kit (Minerva Biolabs, Berlin, Germany). Each PCR was carried out using a nested protocol: the first PCR reaction was performed with primers 27F and 1492R, specific to 16S rDNA [1]. The nested, taxon-specific PCR [2–4], denaturing gradient gel electrophoresis (DGGE) [5] and cloning and sequencing [1] were performed as described previously.

Higher numbers of positive samples for legionellae and *Pseudomonas* were detected using taxon-specific PCR compared to cultivation on selective media (Fig. 1). This is in good agreement with previous studies [6,7] demonstrating the higher sensitivity of the PCR method (in addition, as we used only one hundredth of the total extracted genomic DNA for PCR, which corresponds to 10 mL of sample water, a ten-fold lower start-up volume could have been used compared to the cultivation method). Other possible explanations for the different sensitivities are: (i) bacterial

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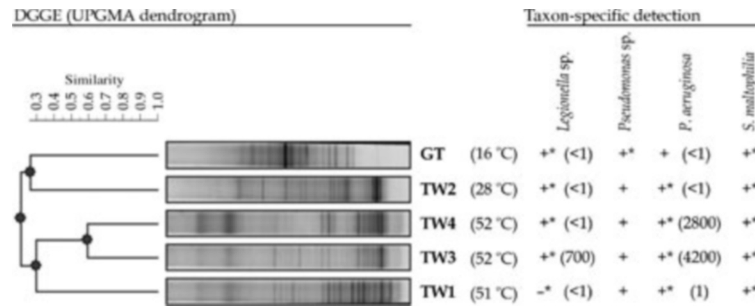


**FIG. 1.** Unweighted pair group method with arithmetic mean (UPGMA) dendrogram obtained from the denaturing gradient gel electrophoresis (DGGE) fingerprints of drinking water samples and the results of taxon-specific detection of selected pathogenic bacteria. −/+, absence/presence of PCR product with the expected size; *PCR product sequenced (Genbank accession numbers: EU445497–EU445510); if cultivation was performed, the results obtained are shown in parentheses (CFU/100 mL). GT, garden tap; TW1, tap in an intensive care unit; TW2, tap in a store-room; TW3, tap used for hand-washing (other building); TW4, tap used for the washing of surgical instruments (other building).

cells in a viable but non-culturable state that cannot be detected by conventional culture [8,9]; (ii) a significant amount of culturable bacteria could be damaged during filtration, resulting in dead cells detectable only by PCR [6]; (iii) both *Legionella* and *Pseudomonas* species may survive in free-living protists [10] and the large number of intracellular and small numbers of planktonic bacteria in the water may account for the variance between low colony counts and higher PCR results; and (iv) growth inhibition of pathogenic bacteria by other organisms present in the water [11,12]. On the other hand, released naked DNA or DNA from dead cells could also be amplified by PCR, which may lead to an overestimation of real infectious risk.

Based on DGGE patterns from the five sampling points, significant differences were found in the drinking water microbial communities in the hospital under investigation

**TABLE 1. Phylogenetic relationships of clones detected in water samples from a hospital distribution system in Budapest, Hungary**

| Number of clones | Sequence similarity, % | Closest relative | Associated human disease |
|---|---|---|---|
| Clone library TW3 (hot water sample) (total 159 clones, 34 sequenced) | | | |
| 20 | 98.5 | *Thermus scotoductus* | Not reported |
| 12 | 97.3 | *Dechloromonas agitata* | Not reported |
| 6 | 100 | *Sphingomonas ursincola/natatoria* | (Bacteraemia, endophthalmitis, osteomyelitis and arthritis) |
| 5 | 95.4 | *Hydrogenophaga intermedia* | Not reported |
| 2 | 98.1 | *Nitrospira moscoviensis* | Not reported |
| 2 | 99.0 | *Sphingomonas yanoikuyae* | (Bacteraemia, endophthalmitis, osteomyelitis and arthritis) |
| 1 | 98.9 | *Hydrogenophilus thermoluteolus* | Not reported |
| 1 | 97.1 | *Pseudomonas aeruginosa* | Mainly skin, urogenital and respiratory tract infections |
| 110 | <95 | Unidentifiable clone | – |
| Clone library GT (cold water sample) (total 140 clones, 44 sequenced) | | | |
| 55 | 95.4 | *Methylocella palustris* | Not reported |
| 11 | 99.8 | *Legionella pneumophila* ssp. *pneumophila* | Pneumonia, Pontiac fever |
| 8 | 99.4 | *Hydrogenophilus thermoluteolus/hirschii* | Not reported |
| 7 | 99.1 | *Cellulosimicrobium funkei* (previously strains of *Oerskovia turbata*) | Bacteraemia, endocarditis |
| 5 | 96.3 | *Dechloromonas agitata* | Not reported |
| 5 | 96.5 | *Methyloversatilis universalis* | Not reported |
| 5 | 99.1 | *Oerskovia turbata* | Strains involved in human infections are reclassified as *C. funkei* |
| 4 | 97.7 | *Streptococcus parasanguinis* | Endocarditis, bacteraemia |
| 3 | 99.8 | *Acidovorax defluvii* | (Sepsis) |
| 3 | 98.8 | *Acinetobacter lwoffii* | Gastritis, cellulitis, endocarditis, endophthalmitis |
| 3 | 98.8 | *Pseudomonas (Stenotrophomonas) hibiscicola*[a] | (Bacteraemia, pneumonia, skin and urinary tract infections[a]) |
| 2 | 96.1 | *Ferribacterium limneticum* | Not reported |
| 2 | 98.8 | *Halomonas marisflavi* | (Bacteraemia, wound infection) |
| 2 | 97.3 | *Propionivibrio limicola* | Not reported |
| 2 | 99.3 | *Thermopolyspora flexuosa* | (Farmer's lung disease) |
| 1 | 99.8 | *Corynebacterium tuberculostrearicum* | Skin lesions, probably mastitis |
| 1 | 99.2 | *Escherichia albertii* | Diarrhoea |
| 1 | 99.6 | *Streptococcus infantis* | Not reported |
| 20 | <95 | Unidentifiable clone | – |

[a]Anzai *et al.* [14] suggested the transfer of *P. hibiscicola* to the genus *Stenotrophomonas* and therefore human diseases associated with *S. maltophilia* are shown.
In cases of OTUs where more than one clone was sequenced, the highest similarity value to the type strain [15] is shown. Sequences with similarity values higher than 95% were considered as the same genus, and those with values higher than 97.5% were considered as the same species [16]. Clones with similarities below 95% were considered unidentifiable. (If an identified clone belonged to a species without any reported human desease, the infections associated with other members of the same bacterial genus are shown in parentheses.) GenBank accession numbers: EU494594–EU494627 (TW3 clones) and EU544249–EU544292 (GT clones).

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(Fig. 1). This could be attributed to the distance of taps from the inlet, types and frequency of water usage, as well as differences in water temperature. Besides the obvious explanations, the similarities and differences in the community profiles can be explained by several other factors that were not investigated in this study (e.g. the two buildings were built some 20 years apart and there were differences in the construction of the pipelines). The condition and set-up of the distribution system could have effects on the chemical composition of the water, on biofilm formation and on the microbial community profiles. Therefore, the detected species composition in drinking water could also be very variable when comparing different hospitals [13].

The species composition divergences were also detectable by cloning and sequencing (Table 1), although the contribution of potentially pathogenic bacteria to the total bacterial community was different in water collected from different sources (0.6% of the hot water TW3 clones and 22.4% of the cold water GT clones).

One explanation for this unexpected phenomenon is most likely the fact that the garden tap is seldom used, and stagnating water provides favourable conditions for the proliferation of microorganisms. Most of the detected taxa, however, are opportunistic pathogenic environmental bacteria known to survive in aquatic habitats at relatively low temperatures. Indoor taps (except for TW2) were in regular use; therefore, biofilm formation may be slower under these conditions. However, the results obtained for the taxon-specific PCR (especially the extremely high *Pseudomonas* counts in case of TW3 and TW4) suggest that mechanical cleaning and disinfection of taps would also be necessary to hinder their colonization by potentially pathogenic bacteria (the phenomenon of some taxa being detected by taxon-specific investigations, and not by cloning, could be explained by the moderate number of analysed clones compared to the complexity of the microbial community).

The results obtained in the present study suggest that hospital residents may be in contact with a wide range of pathogenic bacteria associated with drinking water. Standard methods based on defined selective/differential media or heterotrophic plate counts may lead to an inaccurate estimation of real infectious risk. The hospital under investigation complies with the requirements of current regulations in Hungary (i.e. hospital drinking water systems are monitored yearly by the public health authorities), although the tested microbiological parameters only include heterotrophic plate count and faecal indicator bacteria (*Escherichia coli* and intestinal enterococci). However, as indicated by the results obtained in the study, these indicators fail to predict the presence of opportunistic bacteria of non-faecal origin. The above mentioned biases associated with cultivation methods, insufficient detection limits associated with the conventional use of small sample volumes and the questionable correlation between indicator bacteria and the microbiological quality of water highlight the need for changes in drinking water monitoring.

## Transparency Declaration

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# Molecular typing of methicillin-susceptible *Staphylococcus aureus* isolates collected in the Yogyakarta area in Indonesia, 2006

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## Abstract

The characterization of 62 community-associated methicillin-sensitive *Staphylococcus aureus* (MSSA) isolates from 440 individuals in the Yogyakarta area of Indonesia in 2006 showed that: (i) almost half of the isolates were associated with methicillin-resistant *S. aureus* lineages [clonal complex (CC)1, CC8 and CC45] and (ii) ten Panton–Valentine leukocidin-positive isolates were associated with CC1 ($n$ = 7), CC30 ($n$ = 1) and CC51 ($n$ = 2). The high Panton–Valentine leukocidin prevalence (16%) among *S. aureus* is of concern because these strains can cause severe infections and the introduction of staphylococcal cassette chromosome *mec* into virulent and epidemic MSSA could pose a serious public health threat.

**Keywords:** Antibiotic resistance, community, Indonesia, PVL, *S. aureus*, *spa* typing

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*Staphylococcus aureus* is an important pathogen causing various infectious diseases [1]. *S. aureus* nasal carriers are at increased risk of developing *S. aureus* infection, and *S. aureus* of any genotype can become a life-threatening pathogen [2,3]. Since the 1990s, community-associated methicillin-resistant *S. aureus* (CA-MRSA) has emerged worldwide. CA-MRSA clones are genetically characterized by staphylococcal cassette chromosome *mec* (SCC*mec*) IV or V; some harbour Panton–Valentine leukocidin (PVL) genes and have different geographic distributions, as is the case of clonal complexes (CC)1, CC30, CC59 and CC80 in Asia [4]. The presence of PVL is associated with skin infections and necrotizing pneumonia [4]. However, the role of PVL has recently been questioned, and a possible role of other virulence factors, such as the arginine catabolic mobile element, has been postulated [5]. CA-MRSA could emerge through the transfer of the genes encoding PVL from PVL-positive methicillin-sensitive *S. aureus* (MSSA) into PVL-negative CA-MRSA, because these genes are carried on phages [4,6], or via SCC*mec* transfer into PVL-positive MSSA with a CA-MRSA genetic background. A high PVL prevalence among MSSA supports the hypothesis that SCC*mec* transfer has an important role in the increased prevalence of CA-MRSA [7–9]. For this reason, studies are needed to investigate PVL prevalence among MSSA. The present study aimed to investigate the population structure of *S. aureus* from nasal carriers in the Yogyakarta area of Indonesia and to investigate PVL prevalence.

During September and October 2006, nasal swabs were taken from 220 outpatients and their 220 companions visiting the ear, nose and throat outpatient department at Dr Sardjito Academic Hospital, Yogyakarta, Java, Indonesia. The 220 companions were healthy relatives or household members of patients. Written informed consent was obtained from all participants. *Staphylococcus aureus* isolation and identification was performed as described previously [10]. The susceptibility pattern of the isolates was determined according to CLSI guidelines using the micro-broth dilution method with the antibiotics described previously [10,11]. The isolates were tested for the MRSA-specific *mecA* gene and the *S. aureus*-specific *femA* gene [10].

The genetic background of the isolates was determined using *spa* typing, followed by clustering of the *spa* types into *spa*-clonal complexes (*spa*-CCs) [12]. The associated multilocus sequence typing (MLST) CCs were allocated using the Ridom SpaServer [12–14]. The presence of the genes encoding PVL was determined [15].

*Staphylococcus aureus* was isolated from 62 individuals (14%), including 39 outpatients (18%) and 23 companions (11%). All isolates harbored the *femA* gene. The outpatients (59% female, 41% male) had a median age of 21 years; their

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