Rapid Molecular Characterization of *Acinetobacter baumannii* Clones with rep-PCR and Evaluation of Carbapenemase Genes by New Multiplex PCR in Hospital District of Helsinki and Uusimaa

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**Abstract**

Multidrug-resistant *Acinetobacter baumannii* (MDRAB) is an increasing problem worldwide. Prevalence of carbapenem resistance in *Acinetobacter* spp. due to acquired carbapenemase genes is not known in Finland. The purpose of this study was to examine prevalence and clonal spread of multiresistant *A. baumannii* group species, and their carbapenemase genes. A total of 55 *Acinetobacter* isolates were evaluated with repetitive PCR (DiversiLab) to analyse clonality of isolates, in conjunction with antimicrobial susceptibility profile for ampicillin/sulbactam, colistin, imipenem, meropenem, rifampicin and tigecycline. In addition, a new real-time PCR assay, detecting most clinically important carbapenemase genes just in two multiplex reactions, was developed. The assay detects genes for KPC, VIM, IMP, GES-1/-10, OXA-48, NDM, GIM-1, SPM-1, IMI/NMC-A, SME, CMY-10, SFC-1, SIM-1, OXA-23-like, OXA-24/40-like, OXA-58 and ISAb1-1-OXA-51-like junction, and allows confident detection of isolates harbouring acquired carbapenemase genes. There was a time-dependent, clonal spread of multiresistant *A. baumannii* strongly correlating with carbapenemase gene profile, at least in this geographically restricted study material. The new carbapenemase screening assay was able to detect all the genes correctly suggesting it might be suitable for epidemiologic screening purposes in clinical laboratories.

**Introduction**

*A. baumannii* is a hospital-acquired pathogen which commonly causes pneumonia, bloodstream infections, meningitis, wound infections and urinary tract infections, especially in patients with impaired host defences. *A. baumannii* isolates are resistant to many antimicrobial classes: fluoroquinolones, tetracyclines, cephalosporines and aminoglycosides [1]. However, today carbapenem resistance is more frequently encountered [1–3]. In *A. baumannii* carbapenem resistance is usually conferred by carbapenem-hydrolyzing class D oxacillinas (CHDLs), including OXA-23-like (*bla*OXA-23-like), OXA-40-like (*bla*OXA-40-like), OXA-58-like (*bla*OXA-58-like), and OXA-143-like (*bla*OXA-143-like) oxacillinas. Additionally *A. baumannii* has the intrinsic OXA-51-like (*bla*OXA-51-like) oxacilinase [4,5]. Although CHDLs exhibit weak carbapenem hydrolysis, they can confer resistance when overexpressed. This resistance is mediated through a combination of naturally low permeability to β-lactams, efflux pumps and IS**Aba** elements located upstream of the gene, providing a strong promotor activity [6]. In addition, *A. baumannii* may harbour many other carbapenemases more commonly found among *Enterobacteriaceae* and *Pseudomonas* species [7].

To determine genetic and epidemiological relatedness, genomic fingerprinting of clinical isolates is required. One of the most effective method is the repetitive extragenic palindromic sequence-based polymerase chain reaction (rep-PCR), which is commercially available known as the DiversiLab microbial typing system (bioMérieux, Marcy l’Etoile, France) [8]. This system has been proven useful in the typing of *A. baumannii* and has demonstrated good discriminatory ability, comparable with pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) [9,10]. Recently this rep-PCR typing system, DiversiLab, has identified eight carbapenem-resistant *A. baumannii* clonal lineages (WW1 to WW8) that are distributed worldwide [4]. DiversiLab fingerprinting has been requested by laboratories more recently tested and clustering was found to be conserved [11].

The carbapenemase resistance has recently attracted new interest as a subset among tens of gene families has spread to *Enterobacteriaceae* [12–14], despite a much longer history among *Pseudomonas* and *Acinetobacter* species. *A. baumannii* may harbour...
most of the acquired carbapenemase genes within Enterobacteriaceae, and Pseudomonas in addition to their characteristics CDHL genes [7].

Recently, new molecular assays have been described to detect most prevalent carbapenemase genes [15], or a subset of A. baumannii selective carbapenemase genes. Due to limited gene set, or technical limitations, most new tests are not suitable for clinical routine monitoring in low prevalence settings [16]. In addition, combinations of other resistance mechanisms, such as reduced permeability due porin mutations, or defect, and efflux pumps in conjunction with ampC β-lactamases are the most common cause of carbapenem resistance in low prevalence areas [14]. Therefore, an imipenem hydrolysis test or dedicated MALDI-TOF [17] and more extensive screening of resistance mechanisms in a reference laboratory are often needed to reliably exclude carbapenemase genes.

The aim of this study was to investigate the carbapenemase genes of A. baumannii and the correlation between these genes and clonal lineages. The feasibility of a new real-time PCR assay was tested for screening of most important carbapenemase genes detected among A. baumannii, Enterobacteriaceae, and Pseudomonas species.

**Materials and Methods**

**Bacterial strains and culture conditions**

A total of 55 Acinetobacter isolates from 44 patients were detected. 51 isolates with reduced susceptibility to carbapenem from HUSLAB (Laboratory of Helsinki University Central Hospital) between Jun 19th 1993 and Jan 18th 2008 were collected and four Acinetobacter isolates susceptible to carbapenem were included as controls. Helsinki University Hospital is responsible for the secondary and tertiary care of app. 1.5 million people. The culture samples from this area received by HUSLAB are both from these hospitals as well as from outpatients of this geographical area, the Helsinki and Uusimaa district in southern Finland. The culture samples in this study were from patients treated in nine different hospitals (Table S1).

Acinetobacter isolates were cultured in aerobic atmosphere on chocolate and cystine lactose electrolyte deficient (CLED) agar and incubated at 35°C for 18 h. Colonies with typical morphology and biochemistry were identified as A. baumannii complex. Identification with the VITEK 2 (bioMérieux, Marcy L’Etoile, France) system with GN card was performed, as well. 16S rRNA gene sequencing was performed when biochemical identification was equivocal. In addition a house-keeping OXA-51-like gene was detected separately within all the clinical isolates susceptible to carbapenem, whereas carbapenem susceptible control strains did not harbour OXA-51-like genes.

Antimicrobial susceptibility testing was performed by the disk diffusion method according to the CLSI guidelines [http://www.clsi.org]. MICs for ampicillin/subbactam, colistin, imipenem, meropenem, rifampicin and tigecycline by E-test (AB BIODISC, Solna, Sweden) were determined on Mueller-Hinton agar according to manufacturer’s instructions.

**Design of multiplex Real-Time carbapenemase gene screening assay**

The assay was designed to detect most clinically relevant carbapenemase genes described within A. baumannii, Pseudomonas aeruginosa, and Enterobacteriaceae species. The design was performed using AlleleID software [http://www.premierbiosoft.com], taking into account all the globally known sub-variants in NCBI data base. For practical purposes, the assay was divided in two multiplex reactions consisting of nine and eight gene families, respectively. The assay was validated in vitro using 43 positive

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**Table 1. Description of validation isolates.**

| Target     | Species  | Isolation site | Travel history |
|------------|----------|----------------|----------------|
| GES-1      | P. aeruginosa | wound          | no             |
| GES-14     | K. pneumoniae | trachea        | n/a            |
| GES-5      | P. aeruginosa | incision wound | no             |
| GES-5      | P. aeruginosa | incision wound | no             |
| IMI-1      | E. cloacae | stool           | Thailand       |
| IMI-2      | E. cloacae | wound           | no             |
| IMP-15     | P. aeruginosa | blood          | no             |
| IMP-15     | P. aeruginosa | incision wound | no             |
| IMP-15     | P. aeruginosa | urine          | no             |
| IMP-15     | P. aeruginosa | urine          | n/a            |
| IMP-15     | P. aeruginosa | urine          | n/a            |
| ISAbaI-OXA-51 | A. baumannii | stool         | Spain          |
| ISAbaI-OXA-51 | A. baumannii | stool        | no             |
| ISAbaI-OXA-51 | A. baumannii | trachea      | no             |
| KPC        | K. pneumoniae | stool          | US             |
| KPC-2      | K. pneumoniae | stool          | Greece         |
| KPC-2      | K. pneumoniae | urine          | no             |
| KPC-2      | K. pneumoniae | blood          | Mexico/US      |
| KPC-2      | K. pneumoniae | urine          | no             |
| NDM-1      | K. pneumoniae | stool          | n/a            |
| OXA-23     | A. baumannii | blood          | n/a            |
| OXA-23     | A. baumannii | wound          | no             |
| OXA-23     | A. baumannii | trachea        | Thailand       |
| OXA-48     | E. coli    | stool          | Syria          |
| OXA-48     | K. pneumoniae | stool         | Turkey         |
| OXA-48     | A. baumannii | stool          | n/a            |
| OXA-58     | A. baumannii | stool          | Tunis          |
| OXA-58     | A. baumannii | wound          | no             |
| OXA-58     | A. baumannii | wound          | no             |
| OXA-58     | A. baumannii | stool          | Greece         |
| OXA-58     | A. baumannii | incision wound | n/a            |
| OXA-58     | A. baumannii | urine          | no             |
| SFC-1      | S. fonticola | control strain | Portugal       |
| SIM-1      | A. baumannii | control strain | South-Korea    |
| SME        | S. marcescens | control strain | n/a            |
| VIM        | P. aeruginosa | stool          | Thailand       |
| VIM        | K. pneumoniae | stool          | Spain          |
| VIM        | K. pneumoniae | stool          | Greece         |
| VIM        | K. pneumoniae | CV catheter    | n/a            |
| VIM-1      | K. pneumoniae | blood          | Greece         |
| VIM-2      | P. aeruginosa | trachea        | Russia         |
| VIM-2      | P. aeruginosa | trachea        | Russia         |

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control strains (Table 1), which were confirmed at National Institute for Health and Welfare, Turku, Finland [14]. Since the target primer regions were fully conserved in silico, it was considered adequate to demonstrate PCR performance with one or more control species representing all the gene variants. In addition, synthetic gene constructs for SFC, CMY-1/10, SIM, SME, OXA-25, and OXA-58 genes containing a partial, non-functional resistance gene in *E. coli* plasmid (pIDTsmart), including the amplicon and app. 20 bp upstream and downstream sequence (Integrated DNA Technologies Inc, CA, USA). The plasmid was then transfected into the TOP10 strain according to manufacturer’s instructions. The construct was ordered from IDT using pSMART plasmid, blunt-ended, containing a kanamycin resistance gene. The SFC, and SIM the control strains were obtained later (as a kind gift from Dr. Correia and Dr. Yunsop Chong and Kyungwon Lee, consequently). All the gene products were confirmed by sequencing with reference primers, or the gene

| Table 2. Primers used for amplification of resistance genes by polymerase chain reaction (PCR). |
| Prime | Sequence 5' - 3' | Reference | Oligomix |
|-------|-----------------|-----------|----------|
| F_ges_001 | ACACCTGGGCACCTTCAAGATAC | This study | 1 |
| R_ges_001 | ACTTGACGACCTATCAATTGACACTCC | This study | 1 |
| F_gim_001 | CGAATGGTTGTTAGTCTGGATAAATTC | This study | 1 |
| R_gim_001 | ATGGTATGAGGAAATTGACTTTGATTTAGC | This study | 1 |
| F_imm_001 | CGAATGGGTTGGTAGTCTGGATAAATTC | This study | 1 |
| R_imm_001 | ATGGTATGAGGAAATTGACTTTGATTTAGC | This study | 1 |
| F_kpc_001 | CAGCGGCAGCAGTTTGTTGATTG | This study | 1 |
| R_kpc_001 | CCAGACGACGGCATAGTCATTTG | This study | 1 |
| F_oxa48_003 | TTACTGAACATAATCACAGGGCGTAG | This study | 1 |
| R_oxa48_003 | ATTATTCGTAAATCCTTGCTGCTTATTCTC | This study | 1 |
| F_sme_006 | CAGATGAGCGGTTCCCTTTATGC | This study | 1 |
| R_sme_006 | CAGAAGCCATACTACCTAATGTCATACC | This study | 1 |
| F_vim_03 | GTGTTTGGTGCAGAATATGAGG | This study | 1 |
| R_vim_03 | GCTGTATCAATCAAAAGCAACTCATC | This study | 1 |
| F_cmy_01 | CAGGTGCTCTTCAACAAG | This study | 1 |
| R_cmy_01 | CGGCTCTTTTCTCAAC | This study | 1 |
| F_is5_01 | GTCATAGTATTCGTCGGTAGA | This study | 1 |
| R_is5_01 | GTAAGAGTGGCTTATAATGTTTACAT | This study | 1 |
| F_oxa24_02 | ACCTTTAGTGAGGCAATG | This study | 1 |
| R_oxa24_02 | TAACTCTCTGTACTGGTGTTATAA | This study | 1 |
| F_sfc_01 | ATATTTACTTGGTATATGTTTCTGCTTC | This study | 1 |
| R_sfc_01 | ATAATCGTGGCGGTGTACCC | This study | 1 |

| Table 2. Cont. |
| Prime | Sequence 5' - 3' | Reference | Oligomix |
|-------|-----------------|-----------|----------|
| F_oxa58_02 | GACAAATTACACCTATCAAAGAAG | This study | 2 |
| R_oxa58_02 | CCCTCTCATACAAATCTTTC | This study | 2 |
| F_sfc_01 | CCTCTTGAGTATAGAGGAAATG | This study | 2 |
| R_sfc_01 | ATAATCGTGGCGGTGTACC | This study | 2 |
| F_sim_01 | CAGGTGCTCTTCAACAAG | This study | 2 |

* = gene construct containing the partial, non-functional resistance gene in *E. coli* plasmid.

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| Table 3. Control strains. |
| Gene | Bacterium | Ct (50 ng/ul) | T(m) | PCR reaction |
|------|----------|--------------|------|--------------|
| GES-1 | *K. pneumoniae* | 23 | 84 | PCR1 |
| GIM-1 | *P. aeruginosa* | 16 | 80 | PCR1 |
| IMI-2 | *E. cloacae* | 14 | 78 | PCR1 |
| IMP-15 | *P. aeruginosa* | 15 | 77 | PCR1 |
| KPC-2 | *K. pneumoniae* | 17 | 87 | PCR1 |
| OXA-48 | *E. coli* | 15 | 75 | PCR1 |
| SME | *S. marcescens* | 11 | 77 | PCR1 |
| SPM | *P. aeruginosa* | 16 | 80 | PCR1 |
| VIM-1 | *K. pneumoniae* | 17 | 81 | PCR1 |
| CMY-1/10 | *E. coli* | 16 | 88 | PCR2 |
| Saab1-OXA-51- family | Acinetob. spp | 19 | 72 | PCR2 |
| NDM-1 | *K. pneumoniae* | 18 | 87 | PCR2 |
| OXA-23- family | Acinetob. spp | 22 | 78 | PCR2 |
| OXA-24/40- family | Acinetob. spp | 17 | 79 | PCR2 |
| OXA-58 | *E. coli* | 15 | 76 | PCR2 |
| SFC-1 | *E. coli* | 16 | 81 | PCR2 |
| SIM-1 | Acinetob. spp | 21 | 80 | PCR2 |

* = gene construct containing the partial, non-functional resistance gene in *E. coli* plasmid.

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specific primers alone, when published reference primers were not available. For additional species identification, OXA-51 gene (blaOXA-51-like), with or without IS
AbaI, was detected separately, using F_oxa51_001 AATTTATTTAACGAAGCACACATACGG, and R_oxa51_001 GCACGAGCAAGATCATTACCATAGC primers and the PCR program shown below.

The specificity was tested with 58 carbapenem susceptible Enterobacteriaceae isolates (Table S2) [18], and 710 isolates with putative reduced susceptibility A. baumannii, P. aeruginosa and Enterobacteriaceae isolated from clinical samples during 2008–2011. These isolates were selected among samples growing on CHROMagar ESBL, or CHROMagar KPC plates (bioMérieux, Marcy L’Etoile, France), or from other culture isolates with disk diffusion diameter <25 mm for ertapenem, or <22 mm for meropenem, or MIC>0,5 mg/l for ertapenem and meropenem.

Validation of multiplex Real-Time PCR assay

Template DNA was extracted from a single colony on CLED plate grown overnight, and re-suspended in 100 µl TE-buffer (0,5 McF) and boiled 15 min. Each 20 µl real time PCR-reaction included 10 µl Maxima SYBR Green qPCR Master Mix (2X) (Scientific Fermentas, Schwerte, Germany), 6 µl Oligomix 1 or 2 (Table 2), IDT (Integrated DNA Technologies, Inc.), 3 µl H2O, and 1 µl DNA template. Amplification was performed as follows: 95°C 10 min initial denaturation, 30 cycles with 95°C 20 sec denaturation, 58°C 30 sec annealing and extension, final extension 56°C 1 min and final denaturation 95°C 30 sec (MxPro.

Table 4. Primers used for sequencing of resistance genes by polymerase chain reaction (PCR).

| Gene       | Primer        | Sequence (5'–3')       | Size (bp) | T (m) | Reference               |
|------------|---------------|------------------------|-----------|-------|-------------------------|
| CMY        | F_cmy_s1      | TAAAGATACTTCGGATGAGAG   | 695       |       |                         |
|            | R_cmy_s1      | GCACTTCTCGGATGAGATC    |           |       |                         |
| GES-C      |               | GCTTCATAGCAATAGGCTTAG  | 371       | 60    |                         |
| GES-D      |               | GGAGAGCAAATGCGACTTG    |           |       |                         |
| GIM        | F_gim_1       | AATCAGAATTCCTGCTCAG    | 748       | 60    |                         |
| GIM-1R     |               | GTCAGAGTACGTTATGAG     |           |       |                         |
| IMI-A      |               | ATATCGATCCCTGGTTGAT    | 818       | 55    |                         |
| IMI-B      |               | TCTCGGATTACTTTATACCT   |           |       |                         |
| IMP        | R_imp_1       | TGATGGATGCTTCAGTTAT    | 740       | 55    |                         |
| IMP-2      |               | GCGATGCGTCAAAACAA      |           |       |                         |
| ISaba1/OXA-S1 | F_is51_01   | GTCAATGATTCGTGTTAGA    | 301       | 60    |                         |
|            | R_oxa51_001   | GCACGAGCAAGATCATTAC    |           |       |                         |
| KPC        | R_kpc         | ATGTCAGCTATCGGCTCT     | 893       | 55    |                         |
| NDM        | F_ndm_s1      | GACAACGGAATGCGATAA     | 447       |       |                         |
|            | R_ndm_s1      | AAGGAAAATCTGTAGGATG    |           |       |                         |
| OXA-23 family | F_oxa23_s1   | GTGCTACTATCTGTTGTT    | 592       | 60    |                         |
|            | R_oxa23_s1    | TATCAACTCTGCTGTTCAAT  |           |       |                         |
| OXA-24 family | F_oxa24_s1   | ATTAGGGCTTGAGGAAA      | 521       | 60    |                         |
|            | R_oxa24_s1    | TTGATATGTTGCAATCTAGT   |           |       |                         |
| OXA-48     | OXA-48B       | GACCACCTTTTGTGAGTGC    | 744       | 62    |                         |
| SFC        | F_sfc_s1      | CTATGTTCTCGTGATCTGA    | 351       | 60    |                         |
|            | R_sfc_s1      | GTGCTCTCTGTTGTTAAT     |           |       |                         |
| SIM-1      | F_sim_s1      | TACAAAGGATTCGCGATCG    | 571       | 60    |                         |
| SIM-2      | R_sim_2       | TAATGGGGTTTCTCCATGTG   |           |       |                         |
| SME        | R_sme_s1      | GGCATATATGCGAGCATTA    | 410       | 60    |                         |
| SPM        | F_spm_1       | CCTAACATCTAGCAGCGA     | 650       | 55    |                         |
|            | R_spm_2       | CGCGCGTTCGAGGATGAT     |           |       |                         |
| VIM        | F_vim_1       | TTGGGACGACAGCAAGATG    | 920       | 60    |                         |
|            | R_vim_2       | AAAAGTGGTGGCCAGCCTAG   |           |       |                         |

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3005P, Stratagene, La Jolla, CA, USA). Melting curve was determined between temperatures 58–95°C. Control strains are presented in Table 3.

The PCR was run as a preformed oligonucleotide mixture with master mixture and template to avoid quality variations between the runs. A new oligonucleotide mixture was always tested with all the panel targets with set expected 19–25 Cq range in qPCR depending on the target (Table 3). The oligonucleotide mixture was stored in stock concentrations in small aliquots, and a working dilution was formed for short term usage only. In addition, each PCR run including a representative negative and positive control for the given multiplex: KPC for multiplex 1 and NDM for multiplex 2. An acceptance range for positive controls (target +/−3 Cq) was implemented to accept test series.

All positive isolates were confirmed by further analysing by an independent, conventional PCR and by sequencing the carbapenemase gene. Primers used in sequencing are presented in Table 4. Reaction included 2.5 mM dNTP 1.6 μl, HotStarTaq polymerase (Qiagen, Helsinki, Finland), 0.1 μl, Polymerase Buffer 10 ×2 μl, primer F and R 1 μl each, H2O 13.3 μl and 1 μl template making a total of 20 μl reaction volume. Amplification was performed as follows: initial denaturation 95°C 15 min, 35 cycles with denaturation 94°C 30 sec, variable annealing temperature 55/60/62°C 30 sec depending on the carbapenemase gene to be amplified, extension 72°C 10 min, final extension 72°C 10 min (DNA Engine Tetrad 2, Peltier Thermal Cycler, BioRad, CA, USA).

Rep-PCR

DNA was extracted from colonies on CLED plates using the UltraClean microbial DNA isolation kit (Mo Bio Laboratories, Solona Beach, CA, USA) and diluted to 35 ng/μl. The DNA was amplified using the DiversiLab Acinetobacter kit (Bacterial Barcodes, Inc. cat no DL-AB01, Athens, GA, USA) for DNA fingerprinting following the manufacturer’s instructions. PCR was run on preheated thermal cycler (DNA Engine Tetrad 2, Peltier Thermal Cycler BioRad, Hercules, CA, USA) using the parameters according to manufacturer’s recommendations. The kit specific positive and negative controls were run with each reaction set for the validation of amplification. The rep-PCR products were detected and the amplicons were separated using microfluidics lab-on-a-chip technology and analysed using the DiversiLab system (Bacterial Barcodes, Inc.). Further analysis was performed with the web-based DiversiLab software (version 3.4) using the band-based modified Kullback-Leibler distance for the calculation of percent similarities. The manufacturer provides guidelines for strain-level discrimination; similarity more than 97% is considered as indistinguishable (no differences in fingerprints), similarity more than 95% as similar (1-2 band difference in fingerprints) and similarity less than 95% as different. In this study optimal cut-off for clustering was 95%.

Ethics statement

The bacterial isolates analyzed in this study belong to the microbiological collections of HUSLAB (Laboratory of Helsinki University Central Hospital) and were obtained as part of routine clinical care in the past. Furthermore, all patient identifiers had been previously removed and data were analyzed anonymously. As the isolates were not clinical samples in the legal sense, no written or verbal consent was needed.

Figure 1. DiversiLab analysis. Dendogram and computer-generated image of rep-PCR banding patterns showing clustering between oxacillin genes; OXA-23-like, OXA-24-like and OXA-58. doi:10.1371/journal.pone.0085854.g001
Results

Characterization of carbapenemase genes with *A. baumannii*

All the strains were analysed for 17 carbapenemase gene groups using the new assay. Among these *A. baumannii* isolates the most prevalent gene was OXA-23-like ( bla\textsubscript{OXA-23-like} ). In addition we also found eight OXA 58 ( bla\textsubscript{OXA-58} ) genes and one OXA-24-like ( bla\textsubscript{OXA-24-like} ) gene (Figure 1). No other carbapenemase genes, including genes for KPC, VIM, IMP, GES-1/-10, OXA-48, NDM, GIM-1, SPM-1, IMI/NMC-A, SME, CMY-10, SFC-1, and SIM-1, were detected. The IS\textsubscript{Abi}-OXA-51-like junction PCR was negative in all strains, as well (data not shown).

Temporal variation of prevalent, endemic *A. baumannii* clones

A time dependent clonal variation among the analysed *A. baumannii* was observed. A predominant clone was detected during the follow-up period, typically lasting a few years, which was then substituted by a new clone (Figure 2). Briefly, first a few isolates, harbouring a mobile element with OXA-58 gene, appeared 1993–1996 and 2003–2006 (Clone 1, Figure 1), which was not detected in the following years, followed by a clone harbouring a mobile OXA-23-like gene (Clone 2, Figure 1). The results were consistent with DiversiLab typing, and characteristic antibiotic susceptibility profile associated with the OXA clones analyzed. Only five out of 55 species having OXA-23/-58 gene displayed a different rep-PCR profile. Based on rep-PCR analysis, two predominant clones were detected. One isolate having OXA-24-like gene was unique in DiversiLab analysis, as well. As expected, all the control isolates from patient with no known connection were unique in their rep-PCR profiles.

Association of antibiotic susceptibility with clonality and carbapenemase gene profile

In our study, OXA-58 isolates had lower MIC-values for to meropenem than OXA-23-like positive isolates that systematically had higher MIC-values (Table 5). The isolates with non-acquired OXA-gene, displayed a marked variation and they included also some carbapenem resistant isolates. The control isolates (Figure 1) consisted of *Acinetobacter* spp not harbouring any of the OXA genes analyzed. These isolates were all carbapenem susceptible (Table 5).

Discussion

The carbapenemase producing multi-resistant gram negative rods are probably the most important challenge for hospital hygiene at the moment [13,19]. The great variety of underlying mechanisms, in contrast to simple *mecA* or *mecC* in MRSA, possesses a significant challenge to clinical screening process. Phenotypes are highly variable and many overlapping other resistance mechanisms complicate any simple screening approach. A straight-forward, economical method suitable for routine clinical diagnostics has not been available yet. In this paper we demonstrate the good performance of a new multiplex real-time PCR assay, detecting most important carbapenemases based on melting curve analysis, by applying it to an epidemiologically important set of clinical *A. baumannii* isolates. In a striking contrast to carbapenemase producing *Enterobacteriaceae*, which were first detected in Finland 2008 [14], the carbapenem resistant *A. baumannii* were detected in Finland already three decades ago. This study highlights the emergence of carbapenem-resistant *A. baumannii* isolates carrying the bla\textsubscript{OXA-23-like} gene (Clone 1), which replaced the bla\textsubscript{OXA-51} gene (Clone 2) in three years (Figure 2). These major clones might have been endemic.

The new carbapenemase detection assay was initially developed to detect carbapenemase producing *Enterobacteriaceae* isolates, but it also appeared to be a useful tool for *P. aeruginosa* and *A. baumannii*. After three years of clinical use, it has been proved to be sensitive and highly specific screening assay among more than 700 hundred
isolates with reduced carbapenem susceptibility analysed to date [14]. One of the major problems related to molecular detection of many antibiotic resistance genes is the appearance of new genomic variants. For example, the variable regions of \textit{bla}\textsubscript{OXA-181} are up to 9% different from \textit{bla}\textsubscript{OXA-48} [20]. The new variants may not be detectable with the existing systems. To minimize the risk for false negative results, the primers were designed at conserved gene regions to achieve optimal amplification of all the current and forthcoming sub-variants. The SYBR Green chemistry was preferred to avoid false negative results due to minor mutations in the probe sequence. The probe based assays are often sensitive to just 1–2 mutations in probe sequence, whereas primers are usually less sensitive to minor target mutations. These design features were considered relevant to achieve a high exclusion power of clinically relevant, acquired carbapenemase genes among carbapenem resistant strains.

\textit{A. baumannii} is a nosocomial pathogen, and epidemiological tools are important to develop effective strategies for better monitoring of MDRAB clinical isolates [21]. In this study we used rep-PCR because the method is suitable for comparison of isolate genetic profiles using standardized and automated format [22]. This method has previously demonstrated good discrimination ability of \textit{A. baumannii} isolates [23,24]. We found two major clones with DiversiLab (Clone 1 and 2, Figure 1.) harbouring most of the isolates with \textit{bla}\textsubscript{OXA-23-like} and \textit{bla}\textsubscript{OXA-58} genes. There were only few exceptions. The cases were mostly from departments of treating patients with severe burn trauma, or intensive care units. In this study, a good correlation between the carbapenemase gene and DiversiLab typing suggested that they both could be effectively applied for epidemiological screening of \textit{A. baumannii} species. The new carbapenemase gene screening assay has been in clinical use for more than three years, and it has been a highly suitable method for rapid unequivocal identification of isolates harbouring acquired carbapenemase genes among \textit{Acinetobacter}, \textit{Pseudomonas aeruginosa}, and \textit{Enterobacteriaceae} species. This study suggests that the new molecular methods could be successfully applied in clinical diagnostics to monitor acquired carbapenemase genes, provided that they are user-friendly and cost-effective as well.

### Table 5. MIC distributions for 55 \textit{Acinetobacter} isolates.

| Drug | \(\leq0.5\) | \(\leq1\) | \(\leq2\) | \(\leq4\) | \(\leq8\) | \(\leq16\) | \(\leq32\) | \(\leq64\) | \(\leq128\) | \(\leq256\) | Isolate |
|------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|---------|
| MP   | 0,0      | 0,0      | 12,5     | 75,0     | 75,0     | 87,5     | 100,0    |          |          |          | OXA-58  |
| IP   | 0,0      | 0,0      | 0,0      | 0,0      | 12,5     | 37,5     | 100,0    |          |          |          | OXA-58  |
| RI   | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 3,0      | 100,0    |          |          |          | OXA-23  |
| AB   | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 100,0    | OXA-23  |
| TGC  | 0,0      | 6,0      | 12,0     | 94,0     | 97,0     | 100,0    |          |          |          |          | OXA-23  |
| CO   | 100,0    |          |          |          |          |          |          |          |          |          |         |
| MP   | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 100,0    |          |          |          | OXA-23  |
| IP   | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 100,0    | OXA-24  |
| RI   | 0,0      | 0,0      | 0,0      | 100,0    |          |          |          |          |          |          |         |
| AB   | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 0,0      | 100,0   |
| TGC  | 0,0      | 0,0      | 100,0    |          |          |          |          |          |          |          |         |
| CO   | 100,0    |          |          |          |          |          |          |          |          |          |         |
| MP   | 20,0     | 40,0     | 50,0     | 50,0     | 50,0     | 80,0     | 100,0    |          |          |          | non OXA |
| IP   | 10,0     | 50,0     | 50,0     | 50,0     | 50,0     | 50,0     | 100,0    |          |          |          | non OXA |
| RI   | 0,0      | 0,0      | 0,0      | 30,0     | 90,0     | 90,0     | 100,0    |          |          |          | non OXA |
| AB   | 0,0      | 0,0      | 0,0      | 0,0      | 20,0     | 90,0     | 100,0    |          |          |          | non OXA |
| TGC  | 0,0      | 0,0      | 70,0     | 100,0    |          |          |          |          |          |          |         |
| CO   | 100,0    |          |          |          |          |          |          |          |          |          |         |
| MP   | 75,0     | 100,0    |          |          |          |          |          |          |          |          | Control |
| IP   | 100,0    |          |          |          |          |          |          |          |          |          | Control |
| RI   | 0,0      | 25,0     | 50,0     | 50,0     | 75,0     | 100,0    |          |          |          |          | Control |
| AB   | 0,0      | 75,0     | 75,0     | 75,0     | 75,0     | 75,0     | 75,0     | 100,0    |          |          | Control |
| TGC  | 50,0     | 100,0    |          |          |          |          |          |          |          |          | Control |
| CO   | 100,0    |          |          |          |          |          |          |          |          |          | Control |

MP, meropenem; IP, imipenem; RI, rifampicin; AB, ampicillin+sulbactam; TGC, tigecycline; CO, colistin.
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Rapid Molecular Identification of A. baumannii

Supporting Information

Table S1  Acinetobacter isolate description.

Table S2  Species included in analytical specificity testing.

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Author Contributions

Conceived and designed the experiments: TP PT JK. Performed the experiments: TP SK SM JK. Analyzed the data: TP JK. Contributed reagents/materials/analysis tools: TP SK SM ET PT MV JK. Wrote the paper: TP SK SM ET PT MV JK.