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1 **Bacillus cereus** biofilm formation on central venous catheters of hospitalised
2 cardiac patients

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Abstract

Formation of bacterial biofilms is a risk with many *in situ* medical devices. Biofilm-forming *Bacillus* species are associated with potentially life-threatening catheter-related blood stream infections in immunocompromised patients. Here, bacteria were isolated from biofilm-like structures within the lumen of central venous catheters (CVCs) from two patients admitted to cardiac hospital wards. Isolates belonged to the *Bacillus cereus* group, exhibited strong biofilm formation propensity, and mapped phylogenetically close to the *B. cereus* emetic cluster. Together, whole genome sequencing and quantitative PCR confirmed that the isolates constituted the same strain and possessed a range of genes important for and up-regulated during biofilm formation. Antimicrobial susceptibility testing demonstrated resistance to trimethoprim-sulphamethoxazole, clindamycin, penicillin and ampicillin. Inspection of the genome revealed several chromosomal β -lactamase genes and a sulphonamide resistant variant of *folP*. This study clearly shows that *B. cereus* persisting in hospital ward environments may constitute a risk factor from repeated contamination of CVCs.

Keywords: Biofilm, central venous catheters, cardiac patients, *Bacillus cereus* group, whole genome sequencing, comparative genomics

39 **Introduction**

40 In clinical settings, development of biofilms can promote infection by the persistence
41 of bacteria on medical devices, thereby acting as reservoirs for disease (Bridier et al.
42 2012). *Bacillus cereus* is a Gram-positive, facultatively anaerobic, spore-forming
43 bacterium, which is ubiquitous in natural environments and a notorious contaminant of
44 fomites as well as food. Biofilm-forming strains of *B. cereus* have been reported as
45 causing nosocomial bacteraemia following catheter-related infections (Kuroki et al.
46 2009), and have the potential to cause catheter related blood stream infections (CRBSIs)
47 even in immunocompetent patients (Hernaiz et al. 2003). Known to produce a variety
48 of toxins, *B. cereus* also has the ability to cause two forms of food poisoning, as well
49 as severe conditions such as septicaemia, central nervous system infection and
50 endocarditis in immunocompromised individuals (Bottone 2010, Drobniewski 1993,
51 Lede et al. 2011). The capability of *B. cereus* to act as a pathogen is largely attributable
52 to the synergistic effects of several virulence factors that cause tissue degradation and
53 resistance to the immune system of the host (Visiello et al. 2016). Among the secreted
54 virulence factors are several haemolysins, three phospholipases (including PI-PLC and
55 PC-PLC), an emesis-inducing toxin (emetic toxin), and three pore-forming
56 enterotoxins; haemolysin BL (HBL), non-haemolytic enterotoxin (NHE), and cytotoxin
57 K (reviewed by (Bottone 2010, Ehling-Schulz et al. 2015, Gohar et al. 2008, Kotiranta
58 et al. 2000, Schoeni and Wong 2005, Stenfors Arnesen et al. 2008). Many key genes
59 involved in *B. cereus* group biofilm formation and biofilm regulatory networks have
60 also recently been elucidated (Fagerlund et al. 2014, Fagerlund et al. 2016, Okshevsky
61 et al. 2018, Pflughoeft et al. 2011, Yan et al. 2017).

62 As an emerging potential nosocomial pathogen, more thorough characterisation
63 of *B. cereus* strains from hospital environments is warranted. Here a *B. cereus* strain

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3 64 was isolated twice from central venous catheters (CVCs) of two different cardiac
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5 65 patients admitted to different hospital wards during overlapping time intervals, and an
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7 66 extensive phenotypic and genotypic characterisation of the isolate was carried out.
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9 67 Direct staining of catheters indicated that the isolates were able to produce biofilm *in*
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11 68 *situ*, which was confirmed using scanning electron microscopy and a microtitre plate *in*
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13 69 *vitro* biofilm formation assay. Whole genome shotgun sequencing (WGS) confirmed
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15 70 that both isolates constituted the same strain, placed the strain in close proximity of the
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17 71 *B. cereus* emetic strain cluster, and allowed a comparative analysis of genes related to
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19 72 biofilm formation. The characterisation approach in this study represents a
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21 73 comprehensive strategy for studying biofilm forming bacterial strains associated with
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Methods

Staining of bacteria within central venous catheter (CVC) lumen with Alcian Blue

CVCs used in this study were made from polyurethane (Model No. CVC-3175, Royal Fornia Medical, China) and adherence of bacteria to the CVC lumen walls was visualised using Alcian Blue stain, a cationic dye that stains acidic polysaccharides such as those found in the bacterial cell wall (Nermut and Svobodová 1965). Catheter lumens were locked with the stain solution (0.1%) for 10 minutes and then washed three times with phosphate buffer saline (PBS) (Qurashi and Sabri 2012).

Isolation, characterisation and identification of bacteria from central venous catheters of cardiac patients

Bacterial isolates S1 and S12 were isolated by sampling from CVCs of two cardiac patients, removed on post-operative days 6 and 10, respectively. Bacteria were grown on blood agar (haemolysis) at 37°C for 24 hours, followed by biochemical tests (API-20E kit, bioMerieux) and 16S rRNA sequencing (PCR amplification using primers 27F: AGAGTTTGATCMTGGCTCAG and 1492R: TACGGYTACCTTGTTACGACTT, and Sanger sequencing using primers 785F: GGATTAGATACCCTGGTA and 907R: CCGTCAATTCMTTTRAGTTT, through Macrogen Inc., Korea).

Microtitre plate assay for biofilm formation determination

Biofilm formation was studied with a microtiter plate assay essentially as described by

Fagerlund *et al.* (Fagerlund et al. 2016), using 96-well round-bottomed polystyrene plates (Nest Biotechnology Co., LTD, China). Culture medium containing planktonic bacterial cells was removed after 24, 48 and 72 hours of static incubation at 37°C and wells were carefully washed twice with PBS. Biofilm staining was performed with crystal violet (0.1%) for 10 minutes at room temperature. After rinsing with water three times, 200 µL of 33% (v/v) glacial acetic acid was added to extract biofilm-bound dye, and absorbance was measured at 550 nm using a microplate reader (CLARIOstar®, BMG LABTECH). Strains were tested in technical duplicates for each experiment and statistical significance determined by one-way ANOVA (Dunn's multiple comparisons test), using GraphPad Prism v7.

Scanning electron microscopy analysis of biofilm structure

Scanning electron microscopy (SEM) was used to analyse cells in *B. cereus* biofilm. For growth of the biofilm, glass slides (8mm) were placed in culture medium and incubated at 37 °C for 48 hours. Slides were then fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) and kept over night at 4 °C. Samples were washed twice in 0.1M sodium cacodylate buffer (pH 7.4) for 10 minutes and treated with osmium tetroxide 1.0% in 0.1M cacodylate buffer for 1 hour at room temperature post fixation. Washing was repeated three times for 10 minutes in the same buffer. Dehydration was carried out in an ethanol gradient (50, 70, 80, 90, 96, 100%) for 10 minutes and four times each in 100% ethanol for 15 minutes. Dehydrated samples were subsequently critical-point dried using carbon dioxide in a CPD 030 critical-point dryer (Bal-Tec, CA, USA), and mounted on aluminium stubs with 9.0mm carbon adhesive tabs (Electron Microscopy Sciences) prior to sputter coating with 3.5nm platinum in a

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3 123 Cressington coating system 308R (Cressington Scientific Instruments, UK). A Hitachi
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5 124 S-4800 field emission gun scanning electron microscope was used for the analysis of
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8 125 samples (Hitachi, Japan).
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12 126 ***Antibiotic resistance profiling***
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15 127 The antimicrobial susceptibility of the isolates was elucidated using a standard broth
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17 128 microdilution method performed on Sensititre™ Gram Positive plates GPALL1F
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19 129 (ThermoFisher Scientific). The broth microdilution method was executed according to
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22 130 CLSI guidelines with minor modifications (CLSI 2015). Sensititre™ plates were
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24 131 inoculated according to instructions given by the manufacturer, with 50 µL of 4-6 x 10⁵
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26 132 CFU ml⁻¹ bacterial suspension per well, and the plates were incubated for 24 hours at
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28 133 37°C. The CFU ml⁻¹ of the inoculum was confirmed by serial dilutions on agar plates.
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35 135 ***Whole genome shotgun sequencing (WGS), genome assembly and***
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37 136 ***bioinformatics analysis***
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40 137 Genomic DNA for WGS was prepared from overnight cultures using the MO BIO
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42 138 DNeasy UltraClean Microbial kit (Qiagen, USA) and sequenced using 150 bp paired-
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44 139 end reads on the Illumina HiSeq platform (Illumina Inc., Hayward, CA). To facilitate
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46 140 *de novo* assembly by reducing computational requirements, 1.0 million reads were
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48 141 randomly sampled from both forward and reverse reads using Seqtk (Shen et al. 2016)
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50 142 *De novo* assembly was performed using A5-MiSeq (Coil et al. 2015) and annotation
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52 143 was performed using the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et
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54 144 al. 2016). Draft genomes of *Bacillus cereus* isolates S1 and S12 were submitted to
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56 145 GenBank under BioProject PRJNA419487 with accession numbers PHHT000000000
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3 146 and PHHU000000000, respectively. Average nucleotide identity between the two
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5 147 isolates was calculated using the ANI: Average Nucleotide Identity calculator
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7 148 (Rodriguez-R and Konstantinidis 2016). MegaBLAST searches were performed within
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9 149 Geneious version 11.0.5 (Kearse et al. 2012). For comparative genomics analyses, the
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11 150 complete genome of *Bacillus thuringiensis* strain 407 cry- (accession number
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13 151 NC_018877) (Sheppard et al. 2013) was used.
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152 ***Multilocus sequence typing (MLST) and phylogenetic tree construction***

153 A phylogenetic tree of the *B. cereus* group was constructed as reported earlier
154 (Fagerlund et al. 2016) by applying the distance based Neighbour-Joining method
155 BioNJ (Gascuel 1997) on MLST data from seven housekeeping genes using gene
156 sequences extracted from the WGS data. Analyses were performed according to the
157 Tourasse-Helgason MLST scheme (Tourasse et al. 2006) (<http://mlstoslo.uio.no>). Tree
158 construction was executed using Seaview 4 (Gouy et al. 2010) and tree visualisation
159 was done with Tree Graph 2 (Stover and Muller 2010). Six of the seven major
160 phylogenetic clusters of the *B. cereus* group (Guinebretiere et al. 2008, Tourasse et al.
161 2011) were represented in the analysis.
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163 **RNA isolation and reverse transcription quantitative PCR (qPCR)**

164 To harvest RNA from biofilm cells, *B. cereus* cells were cultured in 10 mL of LB
165 medium (Falcon tubes) in three biological replicates (technical duplicates) at 37°C in a
166 static incubator. Biofilms were harvested after 48 hours of incubation. Planktonic cells
167 were removed from the culture vial by removing the liquid broth (pipetted out)
168 underneath the floating surface pellicle biofilm. Fresh LB broth was added and the
169 surface pellicle biofilm along with the ring biofilm on the walls of the tube (liquid-air
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170 interface, scraped off aseptically) were resuspended in the fresh broth, before harvesting
171 by centrifugation. To harvest RNA from planktonic cells, bacterial isolates were grown
172 to exponential phase (2.5 hours, $OD_{600}=1.3$) in 5 mL LB medium (Falcon tubes) at
173 37°C, 220 rpm, and then added ice-cold methanol (1:1 ratio) prior to harvesting of cells
174 by centrifugation. For extraction of RNA from the planktonic and biofilm cultures, a
175 Precellys 24 Tissue Homogeniser (Bertin) was used for cell lysis, followed by RNA
176 isolation using the RNeasy Mini Kit (Qiagen).

177 Prior to qPCR, RNA was treated with TURBO DNase (Ambion) and then
178 cleaned using the RNeasy Mini Kit (Qiagen; RNA Cleanup protocol). cDNA synthesis
179 was performed in technical duplicates for each sample, using the High-Capacity cDNA
180 Reverse Transcription Kit (Thermo Fisher Scientific) and random hexamers (Applied
181 Biosystems). Appropriate negative control reactions in which reverse transcriptase
182 enzyme was not added, were included for all RNA samples.

183 qPCR was performed essentially as previously described (Fagerlund et al.
184 2016). Briefly, qPCR was performed using the LightCycler 480 Real-Time PCR
185 System (Roche) in 96-well microtiter plates with a final reaction volume of 10 μ L per
186 well, using 0.1 μ L cDNA, 0.5 μ M of each primer, and 5 μ L of Light Cycle 480 SYBR
187 Green I Master (Roche). The primers used are listed in Supplementary table 1. Three
188 housekeeping genes, *gatB/yqeY*, *rpsU*, and *udp*, were used as reference genes, and were
189 included for each cDNA sample. These genes have previously been shown to be stably
190 expressed throughout the *B. cereus* life cycle, and to be well suited as reference genes
191 in *B. cereus* qPCR (Reiter et al. 2011). Cycling conditions were as follows: 95°C for 5
192 min followed by 45 cycles at 95°C for 10 s, 58°C for 10 s, and 72°C for 8 s. Following
193 the completion of cycling, a melting curve analysis was performed, resulting in a single
194 product specific melting temperature for all samples (single peak). The lack of

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3 195 amplification in all negative control reactions (no reverse transcriptase added) was used
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5 196 to confirm the absence of amplification of contaminating DNA. The quantification
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7 197 cycle (C_q) value for each qPCR reaction was determined using the second derivative
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9 198 maximum method, employing LightCycler 480 software (Roche).

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12 199 For gene expression analysis, the average of the C_q values for the two technical
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14 200 replicates for each sample was transformed into a linear scale expression quantity using
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16 201 the formula 2^{C_q} (Pfaffl 2001). For each target gene in each of the biological replicates,
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18 202 the gene expression was then normalised to the geometric mean of the 2^{C_q} values
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20 203 obtained for the three reference genes (ΔC_q -method). Averages and standard deviations
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22 204 were then calculated from the normalised expression values in the biological triplicates.
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Results

Isolation of bacteria contaminating CVCs of hospitalised patients

Bacteria were isolated from the CVCs of two cardiac patients who had undergone heart surgery, and had been treated with inotropes, anticoagulants (post-operative only) and antibiotics (pre-operative and post-operative) (Supplementary table 2). Whilst inserted in patients, catheters had been flushed with 0.9% saline each time after administration of intravenous drugs or after drawing blood, and patients did not have symptoms of infection at the time of catheter removal. Staining of CVCs with Alcian Blue clearly revealed the presence of a bacterial biofilm throughout the CVC (Fig. 1), with a total bacterial load described in Supplementary table 3. Altogether nineteen morphologically different colonies were isolated by sampling of the CVCs (Supplementary table 4). Two isolates (S1 and S12) were found to produce large, circular, grey coloured colonies with a zone of haemolysis when grown on blood agar plates.

[Figure 1 near here]

Identification of bacterial isolates by biochemical characterisation and 16S rRNA gene sequencing

Using 16S rRNA sequencing, the nineteen morphologically different isolates were found to belong to *Bacillus* species, both within and outside of the *B. cereus* group, as well as to *Staphylococcus haemolyticus* (two isolates) (Supplementary table 4). Isolates S1 and S12 were both characterised biochemically as being citrate-positive, indole-negative, and positive for gelatin liquification and acid production from D-glucose (Supplementary table 5) (Logan and Berkeley 1984). Furthermore, nucleotide BLAST

searches (Altschul et al. 1990, Altschul et al. 1997) of their respective 16S rDNA sequence toward the NCBI nr/nt database (<https://blast.ncbi.nlm.nih.gov/>) showed that both isolates belonged to the *B. cereus* group.

Genome sequencing of *B. cereus* isolates S1 and S12

Draft genome assembly of the S1 and S12 isolates produced a total of 131 scaffolds (N50 = 3682392) for S1 and 46 scaffolds (N50 = 2913153) for S12. Total draft genome sizes were approximately 5.28 Mbp and 5.23 Mbp for S1 and S12, respectively, both with a mean coverage >50, and a %GC of 35.5. Two-way average nucleotide identity (Goris et al. 2007) was >99.9% (S.D. 0.1%) indicating that both isolates were the same strain. Mapping of reads from S12 on to the S1 draft genome assembly produced only four single nucleotide polymorphisms, three of which exerted no protein effect, and one which resulted in a substitution in sphingomyelin phosphodiesterase. An insertion (tandem repeat) was the only other variation between the two isolates identified at 20X coverage or greater, further illustrating the extremely high similarity between the two genomes, and essentially confirming that the two isolates are indeed the same strain. Inspection of the S1 and S12 genomes revealed annotation of CDSs (in both isolates) for a class A β -lactamase Bla1, a BcII family subclass B1 metallo- β -lactamase and the *folP* gene encoding sulphonamide resistance, providing a genetic basis for the antibiotic resistance observed in Table 1.

Phylogenetic mapping of *B. cereus* S1 and S12

MLST genes were extracted from the whole genome sequence data set of *B. cereus*

isolates S1 and S12, employing the modified Tourasse-Helgason scheme (Tourasse et al. 2006), and used to build a distance-based tree, showing that S1 and S12 mapped close to the *B. cereus* emetic cluster, most closely matching the *B. cereus* m1293 strain (Fig. 2). Phylogenetic position and the absence of any *cyt*, *cry*, *vip*, *py* or emetic toxin genes indicated that the isolates were most likely non-emetic *B. cereus*. Phylogenetic mapping of S1 and S12 strongly suggested that these organisms are two separate isolates of the same strain. Based on the MLST data and the extremely high similarity between the genome sequences of the two isolates (practically identical) it was concluded that S1 and S12 were indeed the same strain, isolated on two separate occasions (hereafter named *B. cereus* strain CVC_S1) and chose to focus on CVC_S1 for further phenotypic characterisation.

[Figure 2 near here]

***B. cereus* CVC_S1 biofilm formation and structure**

A microtitre plate assay for biofilm formation revealed that *B. cereus* strain CVC_S1 had the ability to form biofilm after 24 hours incubation time, with a maximum after 48 hours (Fig. 3). SEM analysis clearly showed that the isolate formed aggregated communities (Fig. 4), further demonstrating that biofilm formation is an integral characteristic of strains that colonise semi-invasive medical devices.

[Figures 3 and 4 near here]

Antimicrobial susceptibility of B. cereus CVC_S1

Antimicrobial susceptibility testing using the SensiTitre™ microbroth dilution method

showed that the CVC_S1 strain was resistant to trimethoprim/sulfamethoxazole, oxacillin, ampicillin and penicillin, and intermediate to clindamycin, employing CLSI guideline M45 (CLSI 2016) (Table 1).

[Table 1 near here]

Comparative genomics of B. cereus CVC_S1 with other B. cereus group strains

The size of *B. cereus* CVC_S1 (5.28 Mb; 5,702 CDS) and CVC_S12 (5.23 Mbp; 5,607 CDS) genomes was in good agreement with previously sequenced *B. cereus* group reference strains. Although being closely related to emetic strains, the isolates did not map inside the emetic cluster (Fig. 2), and accordingly did not carry the emetic toxin plasmid (pXO1 family plasmid) nor genes specifying enzymes known to produce this toxin. Also, sequence similarity searches (BLAST) did not detect an ortholog to the pXO1 plasmid family replication gene, *repX*.

The proficiency with which *B. cereus* CVC_S1 formed biofilms, both *in situ* on CVCs and in the *in vitro* microplate assays, led us to investigate the presence / absence of genes known to be important in *Bacillus* sp. biofilm formation (Caro-Astorga et al. 2014, Fagerlund et al. 2014, Fagerlund et al. 2016, Gao et al. 2015, Gelis-Jeanvoine et al. 2017, Okshevsky et al. 2018, Rasko et al. 2004). Coding sequences for genes linked to biofilm formation were extracted from the complete genomes of reference strains *Bacillus thuringiensis* 407 cry- (*B. cereus* group biofilm reference strain; accession number NC_018877), *B. cereus* ATCC 14579 (*B. cereus* type strain, accession numbers AE016877 and AE016878) or *B. cereus* ATCC 10987 (accession numbers AE017194 and AE017195), and a megaBLAST search was performed against the *B. cereus*

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3 299 CVC_S1 draft genome. Orthologs showing 75 – 95% pairwise identity (96 – 100%
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5 300 query coverage) were identified for almost all key biofilm-formation genes
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7 301 investigated, including those encoding key biofilm transcriptional regulators, matrix
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9 302 structural genes (matrix protein, putative matrix polysaccharide), potential extracellular
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11 303 DNA synthesis genes, and cyclic-di-GMP metabolism genes (Table 2), in line with the
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13 304 strong biofilm-forming properties of the isolates. However, the genome sequence of
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15 305 neither isolate (S1, S12) carried genes for the lipopeptide kurstakin (Gelis-Jeanvoine et
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17 306 al. 2017) nor the cyclic-di-GMP responsive effector protein BspA (Sudarsan et al. 2008,
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19 307 Tang et al. 2016). Interestingly, delineation of the *B. cereus* CVC_S1 accessory genome
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21 308 relative to its closest neighbours (*B. cereus* m1293 and the *B. cereus* emetic reference
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23 309 strain F4810/72 [AH187]) as well as to the *B. thuringiensis* Bt407 cry- reference strain,
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25 310 revealed that the CVC_S1 isolate contained the following unique CDS with predicted
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27 311 functions that may indicate a potential involvement in biofilm formation; a unique
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29 312 DegU-like transcriptional regulatory protein, a putative antitoxin HigA2, and a second
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31 313 copy of: i) a putative Autoinducer 2 sensor kinase/phosphatase LuxQ (*luxQ_2*) and ii)
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33 314 a biofilm operon *icaADBC* HTH-type negative transcriptional regulator IcaR.
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45 317 ***Expression analysis of conserved biofilm-related genes***

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47 318 In order to investigate the functional role of conserved biofilm-related genes in the *B.*
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49 319 *cereus* CVC strain S1, a qPCR analysis was performed to compare gene expression in
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51 320 biofilms of *B. cereus* S1 relative to planktonic growing cells of the same strain.
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53 321 Amongst the genes analysed, *sinI*, encoding an antagonist of the SinR biofilm repressor,
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55 322 was the most strongly upregulated gene in CVC_S1 biofilm (~7x upregulation), while
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57 323 the *sinR* transcriptional repressor (~3.6x) itself, as well as the *tasA* amyloid-like protein
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324 (~2.2x) were both induced in biofilm relative to planktonic growth (Fig. 5).

325 Furthermore, *galU*, encoding a putative polysaccharide synthesis protein, was down-

326 regulated in biofilm, while for genes encoding cyclic-di-GMP metabolising proteins,

327 *cdgF* and *cdgE* were both down-regulated in biofilm, while *cdgD* was up-regulated.

328 **[Figure 5 near here]**

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329 Discussion

330 In addition to its role as a common food-borne pathogen, *B. cereus* has the potential to
331 cause serious infections in immunocompromised individuals (reviewed in (Bottone
332 2010, Drobniewski 1993)). In immunocompetent patients, catheter-related blood
333 stream infections have also been described. Typically patients recover upon catheter
334 removal and antibiotic therapy, however fatal outcomes have been reported (Lede et al.
335 2011). Key members of the *B. cereus* group, including *B. cereus*, *B. thuringiensis* and
336 *B. anthracis*, have been shown to be efficient biofilm formers, in lab models (Lebeaux
337 et al. 2013), *ex vivo* on dental equipment (Majed et al. 2016) and on invasive medical
338 devices (Wright 2016). In this study, the same strain of *Bacillus cereus* was isolated
339 twice (CVC_S1 and CVC_S12), from central venous catheters of two cardiac patients
340 admitted to two adjacent wards within a large surgical intensive care unit of a cardiac
341 hospital. *B. cereus* CVC_S1 (and CVC_S12) mapped within the phylogenetic cluster
342 harbouring most of the *B. cereus* group clinical isolates (Cluster III), and close to the
343 cluster of emetic strains known to have the potential to cause critical human infections
344 (Dierick et al. 2005, Naranjo et al. 2011). However, similar to other non-emetic near-
345 neighbours to the emetic cluster, such as *B. cereus* Q1 (Xiong et al. 2009), *B. cereus*
346 CVC_S1 (and CVC_S12) did not carry the emetic gene cluster, nor the emetic plasmid
347 pCER270, and thus does not constitute an emetic strain. Within the *B. cereus* group
348 (*B. cereus sensu lato*), adaptation to various environments and hosts results in the
349 formation of ecotypes within major phylogenetic lineages (Guinebretiere et al. 2008,
350 Fiedoruk et al. 2017). Although the CVC_S1 and CVC_S12 isolates map close to the
351 Q1 strain and the clonal emetic strains (ST26), these strains are however isolated from
352 widely diverse sources, emetic strains frequently isolated from cases of food poisoning,

353 and Q1 being an environmental strain isolated from a deep sea oil reservoir (Xiong et
354 al. 2009).

355 The CVC_S1 strain showed resistance to β -lactam antibiotics (ampicillin,
356 penicillin and oxacillin), related to the presence of several β -lactamases carried on the
357 chromosome (Luna et al. 2007, Torkar and Bedenic 2018), a diagnostic feature used to
358 separate *B. cereus* from *B. anthracis* (Bottone 2010, Chen et al. 2004, Turnbull et al.
359 2004). Moreover, resistance to trimetophrim-sulphametoxazole may be explained by
360 the presence of the *folP* gene encoding a conserved dihydropteroate synthase, which
361 provides intrinsic sulfonamide resistance in *B. anthracis* (Valderas et al. 2007). Genes
362 encoding the lipopeptide kurstakin have been shown to be important for biofilm
363 structure (Gelis-Jeanvoin et al. 2017), and the cyclic-di-GMP responsive effector
364 protein BspA (an MSCRAMM family adhesion protein), which is regulated through a
365 c-di-GMP responsive riboswitch, has been suggested to affect biofilm formation
366 (Sudarsan et al. 2016). Neither kurstakin nor BspA are however encoded in the
367 CVC_S1 (or CVC_S12) isolate, showing that these factors are not essential for catheter-
368 related *in situ* *B. cereus* biofilm formation in the hospital environment. qPCR analysis
369 did however show that key biofilm genes such as *sinI*, *sinR*, and *tasA* (Fagerlund et al.
370 2014), which are conserved among different *B. cereus* group strains, including the
371 CVC_S1 and CVC_S12 isolates described here, follow the same
372 transcriptional regulation pattern during biofilm growth in the *B. cereus* CVC isolate
373 (CVC_S1 strain) as in the model laboratory strain of *B. thuringiensis* (strain 407 cry-,
374 originally isolated from insect larvae). The qPCR analyses also confirmed that *cdgF*,
375 which is known to encode a diguanylate cyclase (c-di-GMP synthesising enzyme)
376 essential for formation of biofilms in oxygenated environments, similar to what was
377 previously shown in *B. thuringiensis* (Fagerlund et al. 2016), is transcriptionally down-

regulated in biofilm also in *B. cereus*. This would again be in line with a model for c-di-GMP metabolising enzymes being mainly post-transcriptionally controlled and/or controlled through spatial localisation in the cell, as previously suggested (Fagerlund et al. 2016). Importantly, trends in relative expression during biofilm versus planktonic growth for all of the three *cdg* genes included here (*cdgD*, *cdgE*, *cdgF*) were in accordance with the previous analyses in the reference strain *B. thuringiensis* 407 cry- (Fagerlund et al. 2016).

Recent publications, such as that from Tamburini and co-workers (Tamburini et al. 2018), highlight the importance of using powerful genomic tools such as WGS for strain tracking and more precise identification of the origins of BSIs, in order for hospitals and health care providers to most effectively work to prevent infections. Here WGS was used successfully as a predictive tool for the antimicrobial resistance profile of the *B. cereus* CVC_S1 strain. Also, it is anticipated that the findings presented here will facilitate future investigations into the identification and dissemination of *Bacillus* spp. as nosocomial pathogens. The isolation of the same *B. cereus* strain, from CVCs of two patients in two different wards within an overlapping time period, points to *B. cereus* successfully colonising CVCs used in critically ill patients, constituting a potential source of serious bloodstream infections. Herein, the CVC colonising strain has been extensively characterised by biochemical profiling, phylogenetic mapping, biofilm structure and formation propensity, antibiotic resistance profiling, and whole genome sequencing – allowing this isolate to constitute a reference strain for further work on *B. cereus* group isolates contaminating surfaces in hospital environments and thereby comprising a considerable risk factor for severe nosocomial infections. Previously, *B. cereus* has often been regarded as an environmental contaminant in clinical samples. However, the repeated isolation of *B. cereus* from the CVCs of cardiac

patients in this study, together with its known biofilm- and spore-formation capabilities, as well as the results from a recent longitudinal study of the epidemiology of *B. cereus* infections in French hospitals (Glasset et al. 2018), all point towards *B. cereus* constituting a true nosocomial pathogen of importance to human health.

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Table 1. Susceptibility of *B. cereus* CVC_S1 to antimicrobials using the Sensititre™ microbroth dilution method. For interpretation of MIC values, breakpoints from the CLSI guideline M45 (CLSI 2016) were used. S: susceptible, I: intermediate, R: resistant, NS: non-susceptible. ^aAccording to interpretative criteria for *Staphylococcus* spp. from the CLSI guideline M100 (CLSI 2018). ^bInterpretation was determined as non-susceptible since there only is a breakpoint set for susceptible. ^cAccording to interpretative criteria for *Staphylococcus* spp. from the (EUCAST 2018). ^dNo interpretation available.

	MIC (mg L ⁻¹)	Interpretation
Chloramphenicol	8	S
Daptomycin	> 4	NS ^{ab}
Gentamicin	≤ 2	S
Linezolid	2	S ^a
Rifampin	≤ 0.5	S
Trimethoprim/sulfamethoxazole	> 4/76	R
Quinupristin/dalfopristin	1	S ^a
Tetracycline	≤ 2	S
Erythromycin	≤ 0.25	S
Oxacillin	2	R ^a
Ampicillin	1	R
Penicillin	2	R
Vancomycin	2	S
Levofloxacin	0.5	S
Tigecycline	0.06	S ^c
Moxifloxacin	≤ 0.25	S ^a
Clindamycin	1	I
Streptomycin	≤ 1000	^d
Ciprofloxacin	≤ 1	S
Nitrofurantoin	≤ 32	S ^a

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Table 2. MegaBLAST analyses identifying genes in *B. cereus* CVC_S1 with the highest sequence identity to experimentally identified biofilm-related genes from the *B. thuringiensis* 407 cry- and *B. cereus* ATCC 10987, both fully sequenced biofilm model strains from the *B. cereus* group. For the rightmost column, a star indicates the reference is the same as the one immediately above in the table. *Bt 407* - *B. thuringiensis* 407; *Bc 14579* - *B. cereus* ATCC 14579; *Bc 10987* - *B. cereus* ATCC 10987.

Gene/function	Locus tag	Organism	Protein product	Identity (%)	Coverage (%)	BcS1 locus tag	Reference
Regulators							
<i>spoOA</i>	BTB_RS21200	<i>Bt 407</i>	Sporulation transcription factor	93	100	CV717_17110	(Fagerlund et al. 2014)
<i>sinI</i>	BTB_RS06545	<i>Bt 407</i>	DNA-binding anti-repressor	92	100	CV717_02990	*
<i>sinR</i>	BTB_RS06540	<i>Bt 407</i>	Transcriptional regulator	95	100	CV717_02985	*
<i>codY</i>	BTB_RS19405	<i>Bt 407</i>	GTP-sensing pleiotropic transcriptional regulator	95	100	CV717_15285	(Lindback et al. 2012)
<i>plcR</i>	BTB_RS27305	<i>Bt 407</i>	XRE family transcriptional regulator	90	99	CV717_20790	(Hsueh et al. 2006)
<i>nprR</i>	BTB_RS03040	<i>Bt 407</i>	Transcriptional regulator	77	93	CV717_23020	(Verplaetse et al. 2015)
<i>abrB</i>	BTB_RS00215	<i>Bt 407</i>	AbrB/MazE/SpoVT family DNA-binding domain-containing protein	97	100	CV717_27350	(Fagerlund et al. 2014)
Matrix protein encoding genes							
<i>calY</i>	BTB_RS06535	<i>Bt 407</i>	Biofilm extracellular matrix fibre protein and cell surface adhesin	95	100	CV717_02980	(Caro-Astorga et al. 2014)

<i>tasA</i>	BTB_RS06525	<i>Bt</i> 407	Amyloid-like structural fibre element dedicated to formation of the extracellular biofilm matrix	90	100	CV717_02970	*
<i>sipW</i>	BTB_RS06520	<i>Bt</i> 407	Signal peptidase I	91	100	CV717_02965	*
<i>bc_1280</i>	BTB_RS06530	<i>Bt</i> 407	DUF4047 domain-containing protein	73	98	CV717_02975	*
Putative matrix polysaccharide synthesis genes							
<i>lytR</i>	BC5265	<i>Bc</i> 14579	LytR family transcriptional regulator	84	100	CV717_21245	(Ivanova et al. 2003)
<i>galU</i>	BTB_RS26920	<i>Bt</i> 407	UTP--glucose-1-phosphate uridylyltransferase	88	99	CV717_21200	(Sheppard et al. 2013)
<i>gtaB2</i>	BC_5275	<i>Bc</i> 14579	UTP--glucose-1-phosphate uridylyltransferase	88	97	CV717_21200	(Ivanova et al. 2003)
glycosyl transferase	BCE_5356	<i>Bc</i> 10987	Glycosyl transferase domain protein, putative	98	100	CV717_21395	(Rasko et al. 2004)
<i>ugd</i>	BCE_5385	<i>Bc</i> 10987	UDP-glucose 6-dehydrogenase	67	57	CV717_21230	*
polysaccharide transport	BCE_5386	<i>Bc</i> 10987	Polysaccharide transport protein, putative	81	3	CV717_21810	*
capsular exopolysaccharide	BCE_5398	<i>Bc</i> 10987	Capsular exopolysaccharide family protein	89	99	CV717_21185	*
eDNA synthesis genes							
<i>purA</i>	BC_5468	<i>Bc</i> 14579	Adenylosuccinate synthetase	96	100	CV717_20155	(Vilain et al. 2009)
<i>purC</i>	BC_0326	<i>Bc</i> 14579	Phosphoribosylaminoimidazole succinocarboxamide synthetase	99	100	CV717_23470	*
<i>purL</i>	BC_0329	<i>Bc</i> 14579	Phosphoribosylformylglycinamidine synthetase	94	100	CV717_23485	*

Kurstakin synthesis genes							
<i>krsE</i>	BC_2450	<i>Bc 14579</i>	Macrolide-efflux protein	82	3	CV717_26600	(Gelis-Jeanvoine et al. 2017)
<i>krsA</i>	BC_2452	<i>Bc 14579</i>	Peptide synthetase	65	27	CV717_08300	*
<i>krsB</i>	BC_2453	<i>Bc 14579</i>	Peptide synthetase	68	6	CV717_08300	*
<i>krsC</i>	BC_2456	<i>Bc 14579</i>	Peptide synthetase	65	4	CV717_08300	*
c-di-GMP metabolism genes							
<i>cdgF</i>	BTB_RS03185	<i>Bt 407</i>	GGDEF domain-containing protein	91	100	CV717_22875	(Fagerlund et al. 2016)
<i>cdgD</i>	BTB_RS27060	<i>Bt 407</i>	diguanylate cyclase	88	100	CV717_21055	*
<i>cdgE</i>	BTB_RS18755	<i>Bt 407</i>	sensor domain-containing phosphodiesterase	93	100	CV717_14835	*
<i>cdgB</i>	BTB_RS20530	<i>Bt 407</i>	GGDEF domain-containing protein	89	100	CV717_16430	*
<i>cdgG</i>	BTB_RS17620	<i>Bt 407</i>	phosphodiesterase	68	93	CV717_14835	*
<i>cdgJ</i>	BTB_RS20255	<i>Bt 407</i>	diguanylate phosphodiesterase	94	100	CV717_16120	*
c-di-GMP effectors							
<i>bspA (cap)</i>	BC_1060	<i>Bc 14579</i>	MSCRAMM family adhesion protein	63	4	CV717_00935	(Tang et al. 2016)

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99x60mm (300 x 300 DPI)

Figure captions

Figure 1: Alcian Blue stained proximal lumen of CVC from patient 1. (A) Near port. (B) Mid lumen. (C) Near manifold. (D) Control (CVC, not used).

Figure 2. Positioning of *B. cereus* isolates S1 and S12 (marked with arrow) within a phylogenetic tree representative of the *B. cereus* group, by MLST analysis using seven housekeeping genes. The tree was constructed as described (Methods section). Six of the main phylogenetic clusters (I, III to VII) within the *B. cereus* group are indicated (grey). The S1 and S12 isolates mapped close to the emetic cluster, represented by the *B. cereus* emetic type strain (F4810/72), *B. cereus* BDRD-ST26 and *B. cereus* NC7401.

Figure 3. Biofilm formation of *B. cereus* CVC_S1 in a microtiter plate assay after 24 hours, 48 hours and 72 hours of incubation. Bars constitute the mean of background-subtracted OD₅₅₀ values. The asterisk indicates adjusted p-value = 0.0341 (one-way ANOVA).

Figure 4. Scanning electron micrograph of *B. cereus* CVC_S1 biofilm, showing extracellular matrix production at 48 hours of incubation. Date of imaging: Oct 17, 2017 (Supplementary text file 1).

Figure 5. Differentially regulated genes in biofilm growth relative to planktonic growth of *B. cereus* CVC_S1. The bar plots show log₂ ratios of gene expression in biofilm cells relative to planktonic cells, as determined by quantitative PCR. Bars extending to the right: genes upregulated in biofilm cells, bars extending to the left: genes upregulated in planktonic cells.

Supplementary table 1. Sequences of primers used in qPCR analyses.

Gene	Primer sequence (F, forward; R, reverse)
<i>Spo0A</i>	F- TCCACTGCCATCTTTCCGATC
	R- AGCAGGCACACCAATTTTCATG
<i>calY</i>	F- AATTGGTGGAGGAACATTTCGC
	R- AAGCGTTGTAGGGTCTAATGTAAG
<i>sinI</i>	F- AGACTGACGCATTGGATCAAGA
	R- ATTGAGCCTGGCTTGATGGT
<i>sinR</i>	F- TGCAGCAGTTCTACAAATTCAG
	R- TTGCATCTTTAACGAGTTGTGTCC
<i>tasA</i>	F- GCAGTATTAGGAGCAGCGTTAG
	R- CTAAATCAAGCGTACCAGCCG
<i>cdgD</i>	F- GTAGCAGCCATGAACGAACC
	R- ACGGATCTTCACCTGCTTCAG
<i>cdgE</i>	F- CCGATTGCAGAAGAAACGCTAC
	R- GTGAATATCCGCTTGTGTGCC
<i>cdgF</i>	F- TGTGCTCATATCCGCAAGATG
	R- CACGCTGCATTTCTTCCCTG
<i>galU</i>	F- CGACGAAAGCAATGCCGAAA
	R- ACGCTTTCCTTTCCAGTCA
<i>udp</i>	F- ACTAGAGAACTTGGAAATGATCG
	R- GACGCTTAATTGCACGGAAC
<i>gatB</i>	F- AGCTGGTCGTGAAGACCTTG
	R- CGGCATAACAGCAGTCATCA
<i>rpsU</i>	F- AAGATCGGTTTCTAAACTGGTACA
	R- TTTCTTGCCGCTTCAGATTT

Supplementary Table 2. Patient background.

	Patient 1	Patient 2
Age	57	22
Sex	F	F
Diagnosis	TVD (Tripple vessel disease)	MR (Mitral Regurgitation)
Procedure	CABG (Coronary Artery Bypass Grafting)	MVR (Mitral Valve Repair)
Treatment	Tab; Ascard 75mg q.d, NoClot 75mg q.d, (Anti-coagulants) Losic 20mg IV; Vancomycin 500mg t.i.d (1 shot Pre-Op + Post-Op), Zinacef 750mg t.i.d (Post-Op) Adrenalin, Dopamin, Noradrenalin, Lipofundin, Nephrostril (q.d)	Tab; Ascard 75mg q.d, NoClot 75mg q.d (Anti-coagulants) IV; Zinacef 1.5gm (1 shot Pre- Op) 750mg t.i.d (Post-Op), Targocid 400mg q.d (Post-Op) Adrenalin, Dopamin, Noradrenalin, Lipofundin, Aminovel
Length of catheter insertion	6 days	10 Days
Isolate	CVCS1	CVCS12

Supplementary table 3. Bacterial load, given in colony forming units (CFU) per mL, for each sample (CVC1-CVC6) from central venous catheters of hospital admitted patients, and specification of the bacterial isolate(s) from each respective sample. DOI described the duration of catheter insertion (in days). Bacterial isolates taken from each sample were selected based on colony morphology.

Sample	DOI (Days of Insertion)	CFU mL ⁻¹				Bacterial isolates from sample
		10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	
CVC 1	6	110000	30000	0	0	S1, S2, S3, S4
CVC 2	6	160000	110000	40000	0	S5, S6, S7, S8
CVC 3	3	40000	0	0	0	S9
CVC 4	10	330000	160000	130000	10000	S10, S11, S12, S13, S14, S15
CVC 5	4	60000	10000	0	0	S16, S17
CVC 6	8	190000	80000	30000	0	S18, S19

Supplementary table 4. Bacterial isolate number, identified organism (species name) and source of isolation, for the 19 bacterial strains isolated from central venous catheters in the present study.

Bacterial isolate	Identified organism (species name)	Source
S1	<i>Bacillus cereus</i>	CVC Lumens
S2	<i>Bacillus subtilis</i>	CVC Lumens
S3	<i>Bacillus subtilis</i>	CVC Lumens
S4	<i>Staphylococcus haemolyticus</i>	CVC Tip
S5	<i>Bacillus subtilis</i>	CVC Lumens
S6	<i>Bacillus tequilensis</i>	CVC Lumens
S7	<i>Bacillus tequilensis</i>	CVC Lumens
S8	<i>Staphylococcus haemolyticus</i>	CVC Tip
S9	<i>Bacillus subtilis</i>	CVC Lumens
S10	<i>Bacillus subtilis</i>	CVC Lumens
S11	<i>Bacillus subtilis</i>	CVC Lumens
S12	<i>Bacillus cereus</i>	CVC Lumens
S13	<i>Bacillus sp.</i>	CVC Lumens
S14	<i>Bacillus aryabhattai</i>	CVC Lumens
S15	<i>Bacillus subtilis</i>	CVC Lumens
S16	<i>Bacillus subtilis</i>	CVC Lumens
S17	<i>Bacillus sonorensis</i>	CVC Lumens
S18	<i>Bacillus subtilis</i>	CVC Lumens
S19	<i>Staphylococcus haemolyticus</i>	CVC Tip

Supplementary table 5. Biochemical characterisation of bacterial isolates CVCS1 (S1) and CVCS12 (S12) from contaminated CVCs of admitted cardiac patients.

	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
S1	-	+	+	+	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
S12	-	+	+	+	+	-	+	-	-	-	+	+	-	-	-	-	-	-	-	-

ONPG: test for β -galactosidase enzyme by hydrolysis of the substrate o-nitrophenyl-b-D-galactopyranoside

ADH: decarboxylation of the amino acid arginine by arginine dihydrolase

LDC: decarboxylations of the amino acid lysine by lysine decarboxylase

ODC: decarboxylations of the amino acid ornithine by ornithine decarboxylase

CIT: utilisation of citrate as only carbon source

H2S: production of hydrogen sulfide

URE: test for the enzyme urease

TDA (Tryptophan deaminase): detection of the enzyme tryptophan deaminase: Reagent to put- Ferric Chloride.

IND: Indole Test-production of indole from tryptophan by the enzyme tryptophanase . Reagent- *Indole is detected by addition of Kovac's reagent.*

VP: the Voges-Proskauer test for the detection of acetoin (acetyl methylcarbinol) produced by fermentation of glucose by bacteria utilizing the butylene glycol pathway

GEL: test for the production of the enzyme gelatinase which liquefies gelatin

GLU: fermentation of glucose (hexose sugar)

MAN: fermentation of mannose (hexose sugar)

INO: fermentation of inositol (cyclic polyalcohol)

SOR: fermentation of sorbitol (alcohol sugar)

RHA: fermentation of rhamnose (methyl pentose sugar)

SAC: fermentation of sucrose (disaccharide)

MEL: fermentation of melibiose (disaccharide)

AMY: fermentation of amygdalin (glycoside)

ARA: fermentation of arabinose (pentose sugar)

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