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Fetal tissues tested for microbial sterility by culture- and PCR-based methods can be safely used in clinics

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ABSTRACT

Cell preparations to be used in clinical practice must be free of infectious agents. Safety concerns are especially elevated upon the use of human fetal tissues which are otherwise highly advantageous in cell therapy. We demonstrate that treating fetal samples with antibiotic, extensive washing and homogenization prior to cryoconservation efficiently removes microbes in general. Screening a large collection by an automatic culture system showed that 89% fetal tissue samples were sterile while contamination was detected in 10.8% samples. Liver and chorion were contaminated more than brain, kidney, lung and soft tissues. Broad-range PCR from the bacterial 16s rRNA gene was adopted as a confirmatory assay; however, the concordance between the culture-based and PCR assays was weak. Taxonomic identification was done for contaminated samples by bacteriological methods and sequencing 16s rRNA PCR products. The two approaches revealed different spectra of taxonomic groups sharing only *Lactobacillus*, the most frequently found genus. In addition, other representatives of vaginal microbiota were detected by culture-based identification while PCR product sequencing has also revealed a subset of nosocomial microorganisms. Importantly, species known to cause sepsis were identified by both techniques arguing for their indispensability and mutual complementarity. We suggest that most contamination is uptaken during collection of fetal material rather than originate from an *in utero* infection. In conclusion, a rigorous microbiological control by culture and PCR is a pre-requisite for safe clinical use of fetal tissue suspensions.

Keywords: fetal tissues, broad-range PCR, 16s rRNA sequencing, BacT/ALERT, microbial contamination

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INTRODUCTION

The therapeutic potential of fetal cell preparations is widely acclaimed. An extensive success record exists for treatment of conditions that result from regeneration defects at the cellular level[1,2]. Particularly, fetal cell therapy was suggested to be efficient in autism[3], Parkinson's disease[4], heart failure[5], diabetes[6], lateral sclerosis[7], etc. The advantage of fetal cells could be explained by their high proliferative potential, availability in high quantities and low antigenicity. Fetal preparations contain a subpopulation of stem cells considered to be analogous to embryonic stem cells in terms of pluripotency and primitiveness, yet no tumorigenicity of fetal stem cells has been reported[8].

Safety issues arise when considering fetal cell transplantation for therapy because fetal material originates from an environment highly populated by microorganisms. In most cases, fetal material is harvested upon voluntarily elected termination of pregnancy and does not insure complete sterility. We and others[9,10] use stringent washing in antibiotic-containing medium upon processing crude fetal material. A study[10] to specifically assess the efficacy of washing for microbial decontamination was carried out earlier showing a complete removal of cultivable microorganisms in 21 fetal samples.

Meanwhile, questions remain to be answered: how to detect fastidious uncultivable microorganisms? What are the species that occasionally make it through the processing procedure? How dangerous are they in terms of sepsis, abscess, and other post-transplantation complications? Additional methods are needed to approach these questions and to expand the safety control toolbox. Polymerase chain reaction (PCR) targeting conservative regions of the bacterial 16s rRNA (and fungal 18s rRNA) gene has recently gained a wide spread in many areas where fast and sensitive detection of microbial contamination is required[11–14]. This broad-range PCR showed a good concordance with culture-

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based techniques in many[12] but not all[14] experimental settings depending on the constitution of the material tested. As a culture-independent method, PCR allows detecting a much wider range of species. The extreme sensitivity of PCR is definitely a plus yet sometimes exceeding the necessary level of clinical significance. In addition, DNA from dead microorganisms could be detected leading to overdiagnosis and rejection of therapeutically valuable preparations. Proper quantification of PCR results would help to rightly place this method among others. The next dimension of microbiological analysis opens with the implementation of sequencing 16s rRNA PCR products allowing for taxonomic identification[15,16].

Here we assayed the efficiency of washing for microbial decontamination by fluorescent microscopy and broad-range PCR thus expanding the culture-based approach used earlier for this purpose by Piroth et al[10]. We summarized the data accumulated after two years of the routine use of the BacT/ALERT automatic system to screen for contaminated samples in our collection. We identified the taxonomic groups predominant in contaminated samples by means of bacteriology and sequencing 16s rRNA PCR products. As a result, we adopted more stringent criteria for the release of a sample for clinical use thus eliminating the risk of sepsis after transplantation of fetal tissues.

MATERIALS AND METHODS

Fetal tissue samples

Human fetal samples were collected after elective termination of pregnancy. Each woman donating fetal material gave informed consent and signed an appropriate form. The research and clinical practice at our Center are performed according to the Law of Ukraine "On transplantation of organs and other anatomical materials to the person", the Law of Ukraine “On licensing types of economic activity”, under the License of Health of

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Ukraine for Medical practice, the License for the activities of the bank of umbilical cord blood, other human tissues and cells issued by the State Service of Ukraine on prevention of HIV-infection/AIDS and other publicly dangerous diseases (No. 222-VIII, 02.03.2105). According to the licensing conditions, an ethical approval is required and has been granted. The full list of regulatory documents and original patents covering the activity of our Center could be found at www.emcell.com.

Processing fetal tissues was performed as described earlier[3]. 215 aborted fetuses aged 6-12 weeks were harvested at the site of operation in accordance with aseptic surgical protocol. Fetal material was placed in a sterile transport medium made of Dulbecco-modified Eagle's medium (DMEM) without L-glutamine with gentamicin (100 mg/ml). Whole fetal organs were placed in Hank's balanced salt solution (HBSS) without calcium and magnesium (Sigma-Aldrich, St. Louis, MO, USA), then dissociated and homogenized mechanically. This produced a collection of 938 tissue samples used in this study. Cell suspension was filtered using individual 100-µm filters (Becton-Dickinson, Franklin Lakes, NJ, USA) and isolated cells were cryopreserved using 5% dimethyl sulfoxide (DMSO) in HBSS using a computer-controlled rate freezer ICE Cube 14 (Sy-LAB Geräte GmbH, Neupurkersdorf, Austria). Tissues samples were transferred to liquid nitrogen for long term storage.

Assaying bacterial contamination by fluorescence in situ

Samples were assayed with Molecular Probes Cell Culture Contamination Detection Kit (C-7028; Thermo Fisher Scientific, Waltham, MA). The following dyes were used: SYTO-9 (staining nucleic acids and emitting green fluorescence) and *N*-acetylglucosamine-specific Texas Red (claimed to bind stronger to the cell wall of gram-positive bacteria). Briefly, 20 µl sample (or supernatant after centrifugation at 800 g, 5 min for pre-clearing) was placed on the slide, dried for 10 min at room temperature, flame-

fixed, washed with BSA-saline solution (0.25% bovine serum albumin in 0.15 M NaCl). Then solutions of SYTO-9 (10 µl) or Texas Red (50 µl) were applied and slides were incubated for 5 min. Texas-red slides were washed once again with BSA-saline. Visualization was done on an Axio Vert.A1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). A *Lactobacillus rhamnosus* culture were used as a positive control (Probiotical SpA, Novara, Italy).

Semi-automatic microbial culture and taxonomic identification

A portion of cryoconserved cell suspension was transferred to BacT/ALERT® Pediatric FAN® assay bottles (containing peptone-enriched trypton-soy broth supplemented with brain heart infusion solids and activated charcoal) and incubated on a BacT/ALERT® 3D instrument (bio-Mérieux, Marcy-l'Etoile, France). In this system, the automatic monitoring of growth is based on the colorimetric detection of CO_2 emitted by metabolically active microorganisms. If no growth was detected during seven days of incubation the sample was deemed to be sterile otherwise the incubation was stopped at the day of detection.

Taxonomic identification was done at the Laboratory for Microbiology, Kyiv Military Hospital (Kyiv, Ukraine). Culture from BacT/ALERT positive bottles was transferred to rich solid media, Gram-stained, pre-analyzed by light microscopy and MIKROLATEST® ID kits (Erba-Lachema, Brno, Czech Republic). Pure cultures were than analyzed on a VITEK® 2 microbial identification system (bio-Mérieux, Marcy-l'Etoile, France). Cultures were normalized to the appropriate turbidity measured by a DensiChek Nephelometer (bio-Mérieux, Marcy-l'Etoile, France). Suspensions were transferred to test cards containing regents for identification in 30 wells. The following cards were used: GNI+ (Gram-negative and non-fermenting bacteria), GPI (Gram-positive bacteria), YBC

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(fungi), BAC (bacilli), ANI (anaerobes and coryneform bacteria), NHI (haemophilus, neisseria). Results were processed by VITEK 2 software and the taxonomic group was determined along with the estimate of confidence.

DNA extraction, PCR and sequencing

DNA was extracted using Molysis Complete 5 (Molysis, Bremen, Germany) specifically tailored for broad-range 16s rRNA PCR. The procedure consists of DNAse pretreatment to reduce human DNA content, enzymatic lysis of bacterial cell walls and spin-column purification. Indeed, we noted that the presence of human DNA interfered with 16s rRNA PCR. Moreover, another spin column kit (non-disclosed) added a detectable amount of 16s rRNA templates to mock samples which was not observed with Molysis Complete 5. In addition, 16s rRNA PCR was cleaner from DNA prepared by Molysis Complete 5.

Broad-range real-time PCR was performed with the following primer/probe sets: BactQuant (forward primer, 5′-CCTACGGGDGGCWGCA-3′; reverse primer, 5′-GGACTACHVGGGTMTCTAATC-3′; probe, 6-FAM-5′-CAGCAGCCGCGGTAATACGGAGG-3′-BHQ1 which is an extended version of the probe in the original paper[13]) and Jordan (forward primer, 5'-AACTGGAGGAAGGTGGGGAT-3'; reverse primer, 5'-AGGAGGTGATCCAACCGCA-3'; probe, 5'-TACAAGGCCCGGGAACGTATTCACCG-3'-BHQ2)[11]. For human DNA, PCR was done using primers to the b-Globin gene: 5'-GGCAGGTTGGTATCAAGGTTAC-3', 5'-CCTAAGGGTGGGAAAATAGACC-3', and probe HEX-5'-ACTGGGCATGTGGAGACAGA-3'-BHQ2. The reaction mix for these reactions was: 1x Tris-KCl *Taq* buffer (Thermo Fisher Scientific, Waltham, MA, USA), 0.2 mM dNTP, 4

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mM $MgCl_2$, 2.5 U *Taq* polymerase (Gene and Cell Technologies, Vallejo, CA, USA), total volume 25 µl. Real-time PCR for *Lactobacillus* 16s rRNA was done with the forward 5'-TGGAAACAGATGCTAATACCG-3' and reverse 5'-GTCCATTGTGGAAGATTCCC-3' primers published earlier[17]. The reaction was done in the M-427 SYBR-green reaction mix (Syntol, Moscow, Russia) with the addition of $MgCl_2$ to 4 mM. All the PCRs were run using the following touchdown program: pre-denaturation, 95^0C for 5 min; touchdown cycling (10 cycles): 95^0C for 10 sec, 69-64^0C (-2^0C/cycle) for 15 sec, 72^0C for 30 sec; cycling with constant annealing t^{0}C (30 cycles): 95^0C for 10 sec, 63^0C for 15 sec, 72^0C for 30 sec. PCR products were sequenced at commercial facilities using Jordan reverse primer. The readable part of sequence (260-320 bp) was used for searching the bacterial database by BLASTN algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For taxonomic identification, only BLAST hits with the Identity >80% were taken into account and ranged by the Identity. In cases, when this parameter was not sufficient for the identification, hits leading to lower taxon were given preference.

RESULTS

Microbial decontamination of fetal tissues

Fetal cell suspensions passed through the processing procedure that consists of incubation with antibiotic, washing, homogenizing and freezing, are expected to be essentially free of microorganisms[10]. To screen for microbes that could still remain in our preparations we examined more than 40 nitrogen-frozen tissue samples by fluorescent microscopy using the Texas Red dye that targets bacterial cell wall components (see Material and Methods). We found entities resembling bacilli (Fig. 1A and B) only in rare cases (2 samples). We then prepared pre-cleared samples of transportation medium in which the fetal material had been collected right after the surgery ('entry'). The pre-cleared tissue samples

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and 'entry' were stained with the DNA-binding dye SYTO-9 (Fig. 1C-F). Round-shaped entities resembling bacterial cells (Fig. 1E) were always found in 'entry' (Fig. 1C) and, rarely, in tissue samples (Fig. 2D). Most samples did not have such entities (Fig. 2F) suggesting an efficient elimination of bacteria by the processing. It should, however, be noted that non-specific fluorescent dyes are of limited usefulness for fetal samples which contain high amounts of human cells and cell clumps. In addition, the precise quantitative analysis is hampered because of subjective image perception.

To better demonstrate the efficiency of microbe removal we used real-time PCR, offering relative quantification of bacterial DNA load. We used two previously published primer/probe sets, BactQuant[13] and Jordan[11], that target different conservative regions of the bacterial 16s rRNA gene, thus covering a broad range of bacterial species. First, we analyzed the 'entry' samples. A strong real-time PCR signal was registered (*Ct* = 23; Fig. 2A and B) pointing to a high bacterial DNA content. At the same time, the 'mock' sample showed a much weaker signal (*Ct* around 30). This background amplification was likely templated by traces of bacterial DNA (i) inevitably picked up during DNA isolation and/or (ii) always present in the reaction as an admixture to *Taq* polymerase. Fetal tissues (liver, brain, soft tissues) prepared from 'entry' produced results close to 'mock' (*Ct* = 30).

The major component of the normal vaginal microbiome is *Lactobacillus*. Thus it could be used as an indicator genus for monitoring the efficiency of decontamination of fetal tissues. Now we used primers specific to *Lactobacillus* 16s rRNA[17] and registered results mirroring those of the broad-range PCR: much less *Lactobacillus* DNA in processed samples than in 'entry' (Fig. 2C). Noteworthy, the content of fetal material estimated by PCR for human DNA was, actually, higher in the processed tissues than in 'entry' (Fig. 2D). This rebukes the argument that the observed loss of bacterial DNA could have been

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simply due to diluting the 'entry' sample. Together, the PCR results further argue that our processing procedure is efficient for microbe removal.

Routine screening for contaminated fetal samples

To screen for samples that might remain contaminated after processing, we utilized the BacT/ALERT automated microbial detection system. Of 932 samples tested, microbial growth was detected in 10.8% (Fig. 3A). A various number of tissues could be prepared from one fetus that is why the *n* values of tissues are so different. In most cases, four tissues were available: liver, brain, soft tissues and chorion. Liver and chorion samples were more contaminated whereas the brain and lung appear to be the least contaminated. If a tissue sample turns out to be non-sterile it does not mean that other tissues from the same fetus are so as well. Rather, one fetus might provide both clean and contaminated samples (Fig. 3B) arguing against a systemic fetal infection. In most of such fetuses, only one of the four harvested tissues has produced growth in BactT/ALERT falling in the category '25-40%'. Two non-sterile samples of the four available would place the fetus in the category '50-80%'. There were only five of the 47 non-sterile fetuses in which all tissues (100%) were contaminated.

Classification by the age of gestation (932 samples from Fig. 3A plus six with mixed tissues) showed that tissues originating from older fetuses have a tendency to be more contaminated (Fig. 3C). Thus, among 11- and 12-week fetuses the percentage of contaminated samples reached 20% and above, while the average in all fetuses was only 10.8%. In contrast, "younger" samples (5-7 weeks) produced BacT/ALERT growth only in less than 10% cases. The BacT/ALERT system allows for the rough estimation of the microbial load by the time at which growth becomes detectable. Most samples grew within 1-

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2 days of incubation (Fig. 3D) with few rare exceptions producing growth after up to six days. No significant difference in the microbial load was found between tissues.

To detect microorganisms for whatever reason unable to grow in BacT/ALERT conditions, we recently adopted broad-range PCR with the BactQuant and Jordan primer/probes. The dynamic range of these sets is wide enough to ensure the proper detection of bacterial DNA in samples (Fig. 4A and B, grey PCR curves from ten-fold dilutions of purified *Escherichia coli* DNA). The sensitivity was as low as five genome-equivalents per reaction. The PCR system appears to be oversensitive as it detects trace amounts of DNA even in the 'mock' sample (Fig. 4 inserts). This argues that only quantitative real-time PCR could be used to estimate the bacterial DNA content. We established an arbitrary cut-off criterion: to call a sample "contaminated with bacterial DNA", Ct_{sample} must be at least 2 cycles less than Ct_{mock} in at least one of the two PCRs (BactQuant or Jordan). The plateau RFU value in contaminated samples may sometimes be lower than in non-contaminated ones. This could be due to (i) an incomplete match of our primers to the bacterial DNA predominant in the given 'contaminated' sample and (ii) large amounts of human DNA introduced to reaction with the tissue sample (despite the DNAse treatment step during DNA purification; see Material and Methods). Therefore we do not take the plateau height in consideration when deeming a sample contaminated.

Another kind of broad-range PCR that we adopted for the safety control is aimed to detect the fungal 18s rRNA using the previously published FungiQuant system[18]. We optimized this primer/probes set to fetal material, however fungi-positive samples occur extremely rarely and hence insufficient data has been accumulated.

Of 85 BactT/ALERT culture-positive samples, PCR confirmed the presence of bacterial DNA in 54 samples. On the other hand, we detected a positive PCR signal in two

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of 24 BactT/ALERT culture-negative samples. Thus, for fetal tissue samples, the concordance between BacT/ALERT and PCR is not that remarkable as reported earlier for blood samples[12]. Interestingly, when the two assays were used for platelet concentrates (which are also rich in human cells) the analytic sensitivity of PCR was not high enough either[14]. Yet the advantages offered by BacT/ALERT and PCR encourage using both techniques which increases the accuracy of contamination screening.

Microbial identification in contaminated samples

This study could not bypass two questions: (i) where does microbial contamination originate from (*in utero* fetus infection, female reproductive tract, handling procedure, etc.), (ii) how dangerous are the bacteria found in tissue samples in terms of the risk of sepsis? We set out to identify the prevalence of taxonomic groups and species of microorganisms that most frequently make it through the processing procedure. It is crucial to stress that we did not aim to reveal the composition of the entire microbiome but rather outline the most abundant groups and categorize them by the clinical importance.

First, we analyzed microbial cultures grown in BacT/ALERT using a series of differentiating and chromogenic media and stainings (see Materials and methods). Eleven groups were identified among 56 samples at the level of genus and species (Table). The most prevalent geni were *Staphylococcus* (represented by three species), *Streptococcus* (two species), *Corynebacterium* (no special identification). Next follow *Lactobacillus sp*, *Enterococcus sp*, *Escherichia coli.* Rare samples showed the presence of *Micrococcus* and the fungus *Candida albicans*. The revealed set of microorganisms suggests a strong connection between fetal tissue contamination and the female urogenital tract[19].

Analysis of 16s rRNA sequence is another method of bacterial identification adoptable in clinical setting[20]. We sequenced Jordan PCR products (Fig. 4B, insert) by

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Sanger method and confirmed that the PCR products indeed correspond to 16s rRNA. Despite the good quality of the analyzed DNA there was quite a lot of ambiguous bases in the sequence reads suggesting that the the target band on the agarose gel might represent amplicons from more than one species. Nevertheless, we were able to assign sequences to the species, genus, or family in 42 samples (Table). Interestingly, the reads from two 'mock' samples were too mixed and did not produce any conclusive results in BLAST analysis. At the same time, 16s rRNA in 'entry' samples (Fig. 2) belonged to *Lactobacillus sp* in two cases (not included in Table) and one was unrecognizable. Most tissues samples contained *Lactobacillus sp* as well. Other species normally occurring in the female reproductive tract were detected too, such as *Haemaphilus sp, Gardnerella vaginalis* and *Lachnospiracea* (similar to studies of the vaginal microbiome[15,21]). *Pseudomonadales* and *Burkholderiales* were represented by soil/water inhabitants, some of which are still poorly investigated (*Comamonas*) while the others are known as ventilation-born/hospital infections (*Pseudomonas, Acinetobacter*[22]).

With the exception of *Lactobacillus*, there was no overlap between the culture-based and sequencing identification, even in samples analyzed by both techniques. Tissues originating from the same fetus might often show the prevalence of more than one group. More often this would be *Lactobacillus* in one tissue and another species in the other tissue. Other interesting combinations found were *Pseudomonas* + *Acinetobacter* (by sequencing), *Streptococcus mitis* + *Staphylococcus haemolyticus* + *Escherichia coli* + *Candida albicans* + *Enterococcus sp* (by culture). Fewer fetuses gave more than one tissue with the same taxonomic group (e.g. *Streptococcus mitis* in one case assayed by culture; *Acinetobacter*, *Lachnospiraceae*, *Haemophilus*, one case per each, assayed by sequencing; and, of course, *Lactobacillus* in several cases). Although we did not have a goal of studying the penetrance and

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tissue distribution of microorganisms, the obvious conclusion rises that, in most cases, the contamination is picked up upon the surgery rather than carried over with fetal material infected *in utero*.

Summarizing the results of microbial identification we attempted to assess the risk of sepsis should a contaminated sample be, nevertheless, erroneously used for therapy (Table, the rightmost column; Fig. 5). We assigned a risk to each group of microorganisms after a literature search for the reports on bacteremia and sepsis. One can hardly define strict criteria for this matter yet we followed the major rule: if a species or a group was suggested to cause sepsis in some systematic multicenter survey with a strong statistics we assigned the risk as high. If, however, a microorganism appears only in case reports and/or negative clinical consequences of the bacteremia have not been convincingly shown we ranked the risk as low. The use of this criteria and, of course, numerous discussions with specialists in the relevant medical fields, prompted us to assign the high risk to *Escherichia coli*, *Enteroccoccus sp*, *Candida albicans*, *Acinetobacter*, *Pseudomonas*[23], *Haemophilus influenza*[24], *Burkholderia cepacia*[25], *Staphylococcus haemolyticus* and *epidermidis*[26], *Streptococcus intermedicus* (known to belong to the so called *milleri* group[27]). We would like to reiterate that the discrimination of microorganisms by the risk has been undertaken here solely for the purpose of research and analysis, not to give low-risk samples a chance to pass the contamination control.

DISCUSSION

In this work, we analyzed the extent to which fetal suspensions are safe in terms of microbial sterility. While our processing procedure is efficient for the elimination of microorganisms (Fig. 1 and 2), traces of contamination remained in about 10.8% samples (Fig. 3A). Different, largely non-overlapping subsets of taxonomic groups were found with the use

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of two methods of identification: automatic culture followed by bacteriological analysis and PCR followed by sequencing (Fig. 5). To explain this disconcordance, let us remind first that both methods have an amplification stage ensuring their high sensitivity. Indeed, microbial growth must occur in the BacT/ALERT bottle and many cycles of target DNA synthesis must proceed in PCR before the respective signals become detectable. In both cases, the increase of indicator parameter follows an exponential kinetics. The fetal tissue sample is a highly heterogeneous system containing many species, and each of them may serve as a template for the signal amplification. Inevitably, the amplification processes from these templates compete to a certain extent so that only one of them will likely prevail by the moment of signal registration. The initial quantity of template is the major determinant of the success in this competition. Therefore, the final result will provide the information on the most abundant species only. Thus, in the majority of contaminated samples, we revealed *Lactobacillus* (Fig. 5), the most abundant component of the normal vaginal microbiota. At the same time, both culture and PCR could have missed other species, some of which might be quite dangerous. Indeed, within the other groups, we found subsets featuring different potency for bacteremia upon intravenous administration (e.g., low- and high-risk species of *Staphylococcus, Streptococcus, Haemophilus*). In view of this, we stay rather conservative in our routine screening discarding all samples found to be contaminated by either method, regardless of the identification results.

Further, there is a plethora of fastidious non-cultivated microorganisms, yet clinically important, which would not have a chance to be detected by BacT/ALERT. There are reports of both success and failure of automatic culture even for the same species[28], some of which were also revealed here by sequencing, for example, *Burholderia sp*[29],

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Pseudomonas sp[30], *Haemophilus influenza*[31] (see also BacT/ALERT manual). Inconsistencies may arise due to differences at the pre-analytical step: sometimes the presence of an antibiotic in the inoculum would affect growth[32], sometimes pre-incubation of samples at room temperature or 37^0C would be beneficial[30]. This might be relevant to our practice because we inoculate the BacT/ALERT medium with an aliquote of a cryoconserved sample.

Thus, we ultimately needed a supplementary technique to the automatic culture and we hence added broad-range PCR as a relative quantitative assay for the bacterial (Fig. 4) and fungal (not shown) DNA load.We did not analyze the correlation between PCR and BacT/ALERT thoroughly enough, yet our data from the routine co-usage of the two techniques so far point to a weak concordance. This could be due to failure to grow in BacT/ALERT, PCR inhibition, primer/template mismatch, DNA loss, pre-analytical contamination, etc. Yet the results of taxonomic identification by culture and PCR/sequencing (Fig. 5 and Table) strongly suggest that the two techniques complement each other and have a unique value for the contamination control.

As for the origin of contamination, we tend to think that most part of it is uptaken during the surgery and handling fetal tissues. Indeed, the incomplete penetrance of contamination (Fig. 3B) together with the detection of low-risk bacteraemic vaginal (Lactobaciaceae, Corynebacteriaceae and others, Table) and nosocomial ventilation-born microorganisms (*Pseudomonas, Acinetobacter*) supports this hypothesis. On the other hand, we observed a non-uniform distribution of the culture-positive samples by tissue (more in chorion and liver, less in brain, Fig. 3A) and the stage of gestation (Fig. 3C). These two pieces of data may suggest an infection *in utero* which penetrates differently and is more likely to establish itself in older samples. Nevertheless, we believe that the share of operation-related contamination is much larger.

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During two years after the introduction of the automatic culture system at our Center, 790 patients received fetal tissue transplants following the safety control procedures approved by the license-issuing agencies (Materials and Methods). No case of bacteremia has been registered. However, we believe that a clinic must use as many approaches as possible to protect the patient from transplant-associated infections even if the reciprocal validation is incomplete. Having adopted broad-range PCR as a complementary test, we further strengthened the sample release criteria whereby the detection of contamination either by culture or PCR absolutely excludes the sample from any kind of clinical use.

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All the authors are Emcell employees. The authors declare no other conflicts of interest.

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FIGURE LEGENDS

Figure 1: Microbial contamination in fetal tissue samples assayed by fluorescence *in situ.* Fluorescent staining with Texas-red (A, B) and SYTO-9 (C-E). A: *Lactobacillus rhamnosus* culture (positive control). B: processed fetal tissue. Rod-shaped bacterial cells (arrows) occur among human cells and cell clumps in rare samples. C: *'*entry*'* sample (transportation medium used for harvesting the fetal material at the operation site). D: a rare contaminated sample. Note round shaped bacterial cells with the brighter area in the center likely representing the DNA. E: the magnified portion of panel *D* outlined by dashed square to show an individual bacterial cell. F: a typical non-contaminated sample. Scale bar, 10 µm.

Figure 2: PCR showing an efficient removal of bacterial contamination by the processing procedure Real-time PCR against 16s rRNA regions conservative for the entire bacterial kingdom (targeted by the BactQuant (A) and Jordan (B) primers), regions specific for *Lactobacillus* (C) and human DNA (D). Abscissa: PCR cycle. Ordinate: relative fluorescence units. Samples: *ENTRY,* transportation medium used for harvesting the fetal material at the operation site. *liver, brain, soft tissues,* samples prepared from the entry material. *mock,* an H_2O sample passed through the DNA isolation procedure to serve as a negative control. Note the much earlier rise of 16s rRNA PCR kinetics for "entry" in comparison to the prepared samples witnessing a dramatic reduction of bacterial DNA content after processing. The experiment has been repeated more than three times.

Figure 3: BactAlert automatic culture from fetal samples A: Percentage of culture-positive samples of fetal liver (*n*=200), brain (*n*=193), soft tissues (*n*=205), kidney (*n*=32), lung (*n*=36), chorion (*n*=215), spleen (*n*=51); all samples (*n*=932). B: Tissue samples were first grouped by fetuses they were obtained from. Only fetuses providing 4-8 tissues were considered. Then fetuses were ranged by the percentage of contaminated tissues as shown un-

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der the abscissa. Number of fetuses per each range is at the ordinate. C: Percentage of culture-positive samples by the week of gestation: 5, (*n*=110), 6 (*n*=111), 7 (*n*=184), 8 (*n*=241), 9 (*n*=184), 10 (*n*=45), 11 (*n*=33), 12 (*n*=30) and all samples with an identifiable age (*n*=938). D: The day at which the microbial growth was detected in BactAlert culture bottles containing samples from liver (*mean*=1.34, *n*=62), brain (*mean* =1.57, *n*=16), soft tissues (*mean*=1.08, *n*=25), chorion (*mean*=1.26, *n*=59). The mean is indicated by the dash.

Figure 4: Detection of bacterial DNA in processed fetal preparations by PCR

PCR against 16s rRNA regions conservative for the entire bacterial kingdom (targeted by the BactQuant (A) and Jordan (B) primers). Abscissa: PCR cycle. Ordinate: relative fluorescence units. *Grey curves*, dilutions of *E.coli* DNA were used as standards: *1, 2, 3, 4* log genome-equivalents per reaction. *Dark curves*, PCR from clinical samples labeled as 'contaminated' and 'clean' following the criteria described in the text. *mock*, an H_2O sample passed through the DNA isolation procedure to serve as a negative control. Inserts: gel electrophoresis with Bactiquant (447 bp) and Jordan PCR (371 bp) products from mock and clinical samples. Molecular weight is given in kilobases.

Figure 5: Microbial identification in fetal samples A: BactAlert bottle culture was analyzed by bacteriological techniques. B: Sequencing of PCR product from 16s rRNA gene (Fig. 4b, insert) followed by BLAST nucleotide database search. *Dark* portions of histograms correspond to the number of samples containing microorganisms known to pose a high risk of sepsis. Taxonomic grouping was done unevenly for a better representation of clinical importance.

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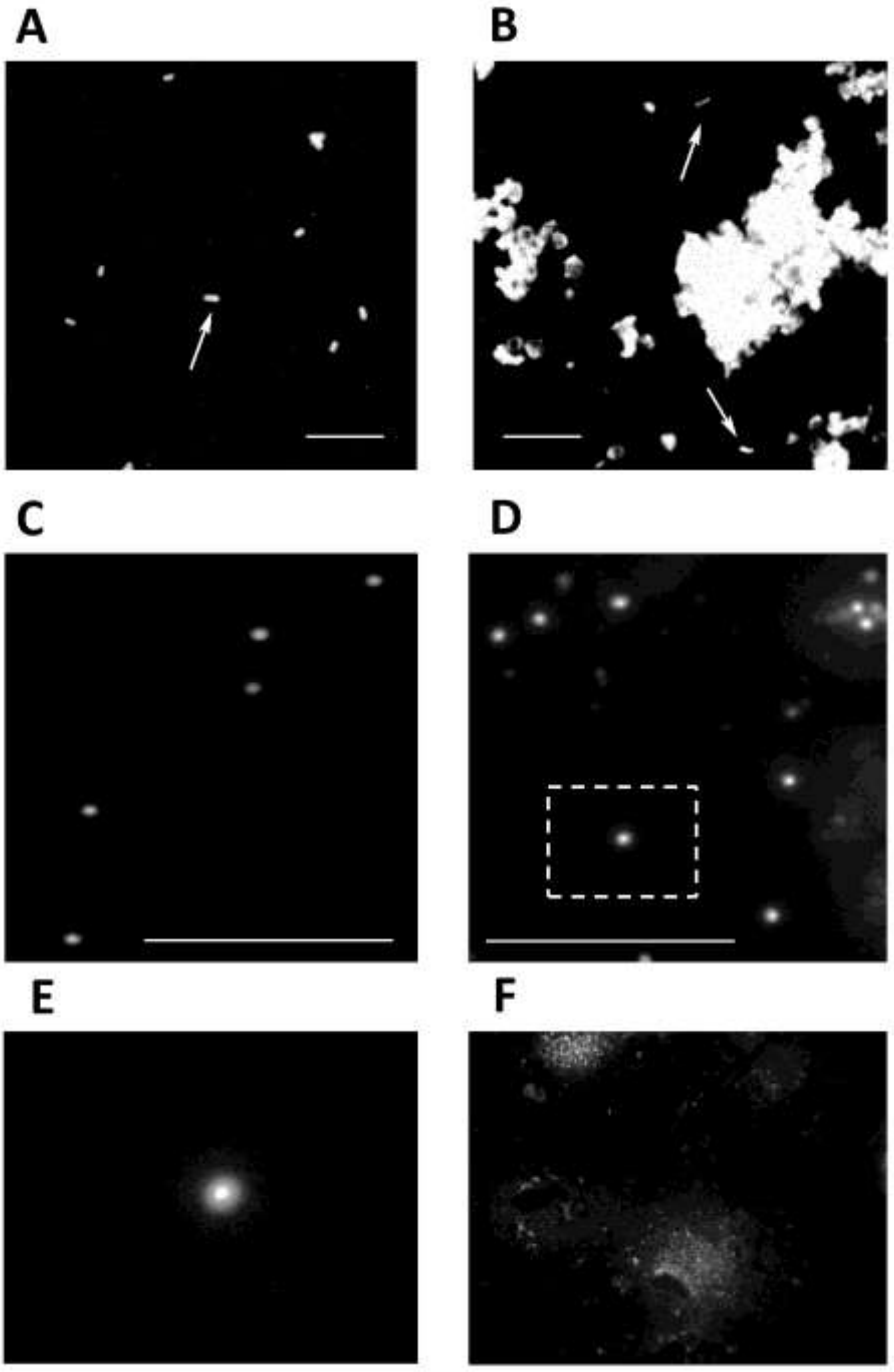

Figure 1

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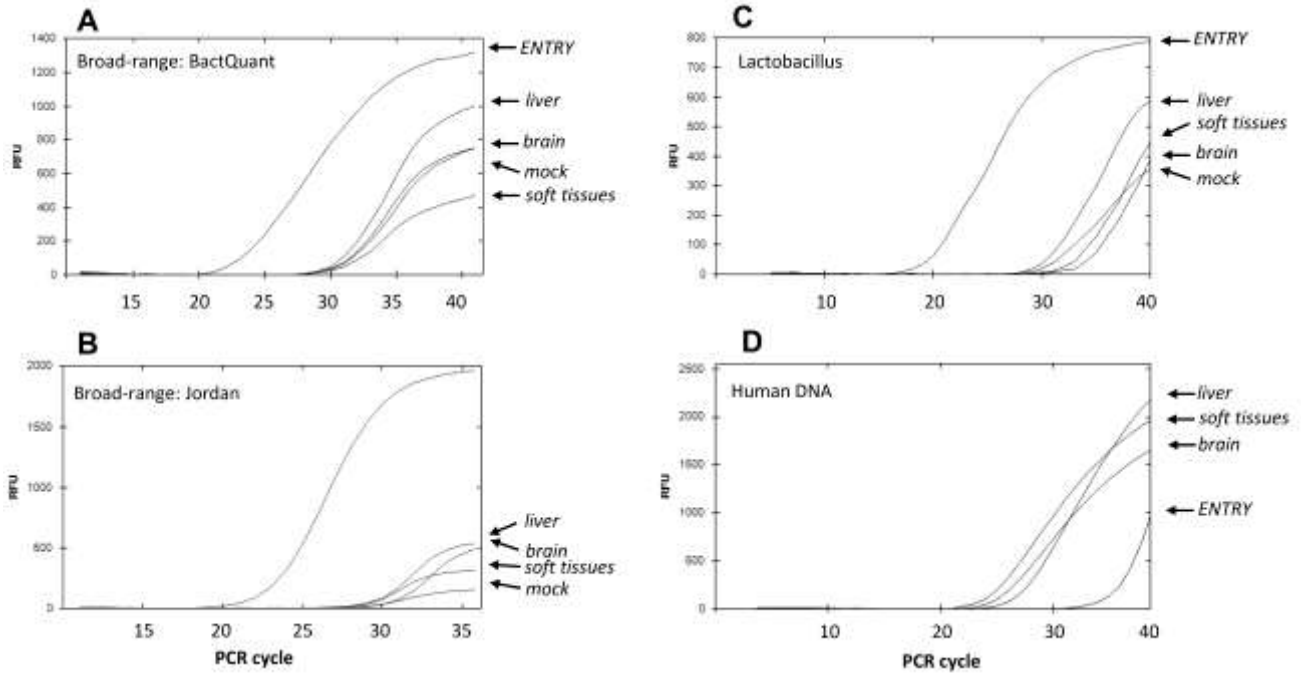

Figure 2

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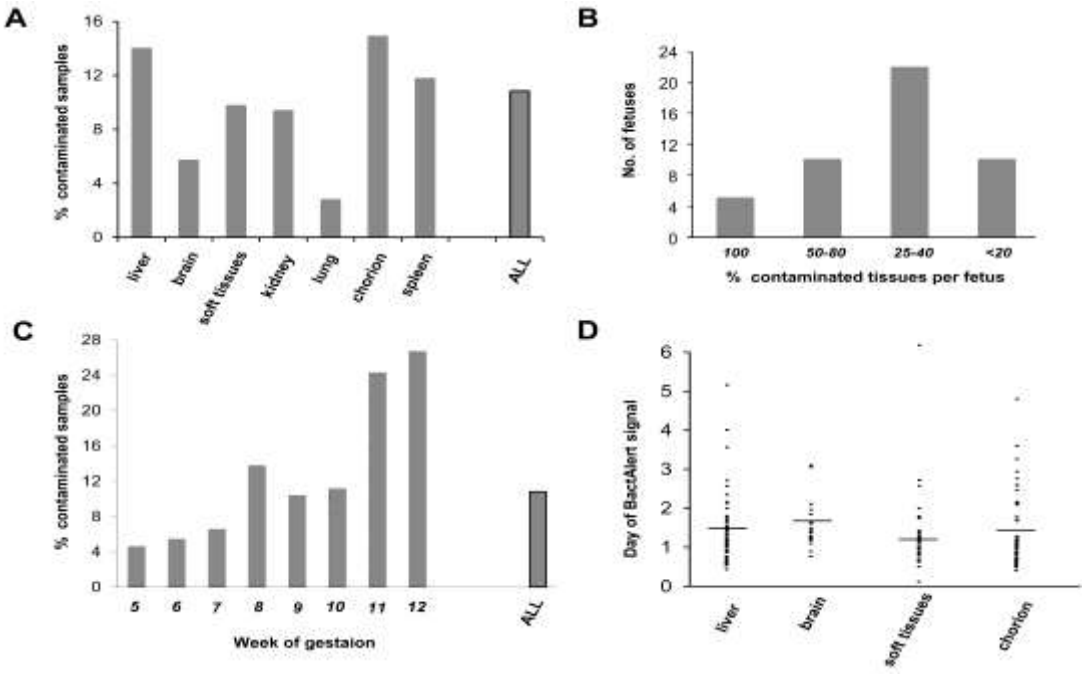

Figure 3

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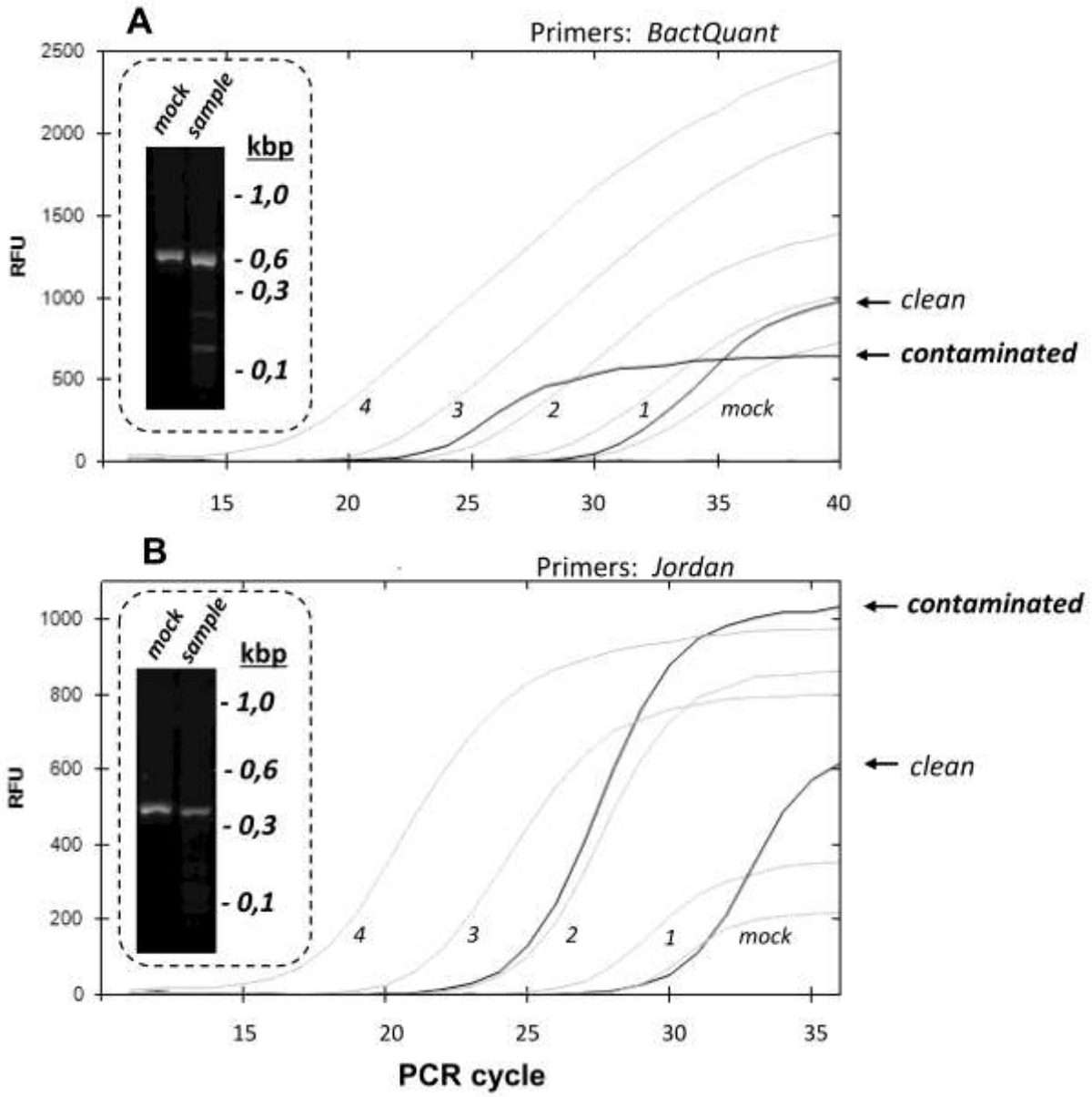

Figure 4

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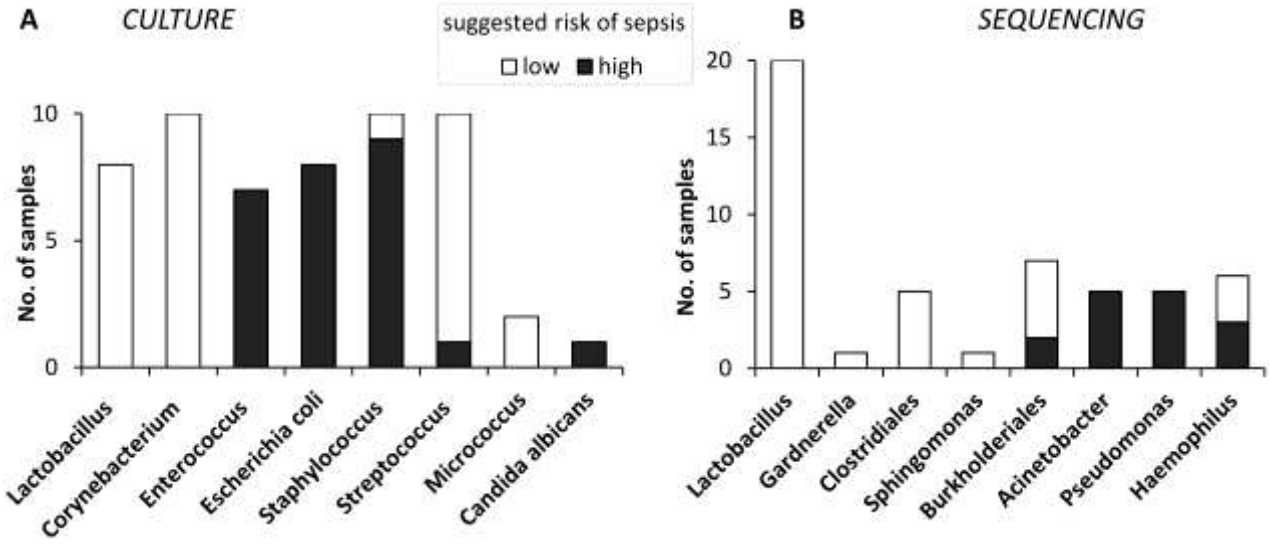

Figure 5

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Table 1. Microbial identification by culture-based methods and 16s rRNA sequencing

Method	Order	Family	Genus	Species	No. samples	Assessed risk of sepsis
Culture	Saccharomycetales	Saccharomycetaceae	*Candida*	*albicans*	1	high
	Actinomycetales	Corynebacteriaceae	*Corynebacterium*	ND	10	low
	Actinomycetales	Corynebacteriaceae	*Micrococcus*	ND	2	low
	Bacillales	Staphylococcaceae	*Staphylococcus*	*haemolyticus*	7	high
	Bacillales	Staphylococcaceae	*Staphylococcus*	*epidermidis*	2	high
	Bacillales	Staphylococcaceae	*Staphylococcus*	*saprophyticus*	1	low
	Lactobacillales	Enterococcaceae	*Enterococcus*	ND	7	high
	Lactobacillales	Lactobacillaceae	*Lactobacillus*	ND	8	low
	Lactobacillales	Streptococcaceae	*Streptococcus*	*mitis*	10	low
	Lactobacillales	Streptococcaceae	*Streptococcus*	*intermedicus*	1	high
	Enterobacteriales	Enterobacteriaceae	*Escherichia*	*coli*	7	high
16s rRNA sequencing	Bifidobacteriales	Bifidobacteriaceae	*Gardnarella*	*vaginalis*	1	low
	Clostridiales	Syntrophomonadaceae	*Syntrophomonas*	ND	1	low
	Clostridiales	Lachnospiraceae	ND	ND	4	low
	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*crispatus*	7	low
	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*iners or gasseri*	3	low
	Lactobacillales	Lactobacillaceae	*Lactobacillus*	ND	9	low
	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	*echinoides*	1	low
	Burkholderiales	Alcaligenaceae	*Alcaligenes*	ND	1	low
	Burkholderiales	Burkholderiaceae	*Burkholderia*	*cepacia*	1	high
	Burkholderiales	Burkholderiaceae	*Burkholderia*	ND	1	high
	Burkholderiales	Comamonadaceae	*Comamonas*	ND	1	low
	Burkholderiales	Comamonadaceae	*Comamonas*	*testosteroni*	1	low
	Burkholderiales	Comamonadaceae	*Delftia*	ND	2	low
	Pasteurellales	Pasteurellaceae	*Haemophilus*	*haemolyticus*	3	low
	Pasteurellales	Pasteurellaceae	*Haemophilus*	*influenzae*	3	high
	Pseudomonadales	Moraxellaceae	*Acinetobacter*	*bereziniae*	1	high
	Pseudomonadales	Moraxellaceae	*Acinetobacter*	*calcoaceticus*	1	high
	Pseudomonadales	Moraxellaceae	*Acinetobacter*	ND	3	high
	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	ND	5	high

Risk assessment was done on the basis of literature search. When the lower taxon identification was not possible (ND), the risk was assigned as ‘high’ if the higher taxon known to have dangerous species.

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