Whitepaper

New Developments in DNA Extraction for Microbiome Analysis

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Summary

The Human Microbiome Project and other approaches are dedicated to the exploration of the vast complexity of microbial communities living in association with the human body, including the skin, oral cavity, upper respiratory tract, stomach, intestine, and genitourinary tract. The aim of these projects is to understand host-microbe interactions with regard to human health and disease. Next Generation Sequencing (NGS) is a powerful analytical tool capable of resolving the microbiota in respect to community structure, dynamics, and activity. This overview discusses new solutions for enrichment and extraction of microbial DNA, removal of irrelevant non-target host DNA and ways to discriminate for DNA from live microbes.

Keywords: Microbiome - Next Gen Sequencing - removal of human DNA - removal of extracellular microbial DNA - lysis of microbes - DNA-free reagents

Introduction

The collectivity of microbial genomes associated with the human body, the microbiome, and changes thereof are a prominent topic of next generation sequencing. Changes in the microbiome structure and dynamics may be responsible for digestive disorders, skin diseases, gum disease and even obesity. Because of their vital importance in human health and disease, microbial communities of the human body are a matter of intensive research.

Various NGS technologies and platforms enable a wide variety of applications to address specific research objectives. Next to whole genome sequencing researchers are focussing on specific regions from genomic DNA. This targeted NGS approach is the method of choice in profiling of microbial communities at high resolution and is typically performed by massive parallel sequencing of individual sequences from bacteria. Usually, variable regions of the 16S rRNA gene are PCR-amplified using primers that target flanking conserved stretches. Analysis of hundreds of amplicon reads leads to assignment of sequences to microbial taxa by comparison with gene libraries [42].

Independant on the NGS platform modelling of microbiota on the grounds of empirical data involves a series of core steps, including sample collection, handling and processing the sample, DNA extraction, library construction, template preparation, sequencing and data analysis [42]. At each of these steps inaccuracies may occur which, if not considered appropriately, in their sum can generate meaningless data [42]. This article is dedicated to pre-analytical factors taking influence on the results obtained. Problems associated with DNA extraction will be discussed with special emphasis on the quality and quantity of reflection of community structure and trade-off between detection limit and contamination. New solutions for DNA extraction will be introduced that enable the lysis of a broad-range of organisms and the enrichment of DNA from live microbes thereby offering clues to analyze structures of live communities and their dynamics.

Negative Effect of Human DNA on Bacterial DNA-Specific Amplification and Sequencing

The scenario of human-microbe associations can be described as communities of microorganisms freely floating in a liquid host environment or attached to surfaces in biofilms. In view of analysis of such associations by DNA-based methods, there are target sequences that need to be separated from non-target (human) sequences and high and low molecular substances that inhibit PCR amplification. PCR inhibiting substances are removed in the course of DNA purification using in-house or commercial systems. Separation of target and non-target sequences usually is obtained by

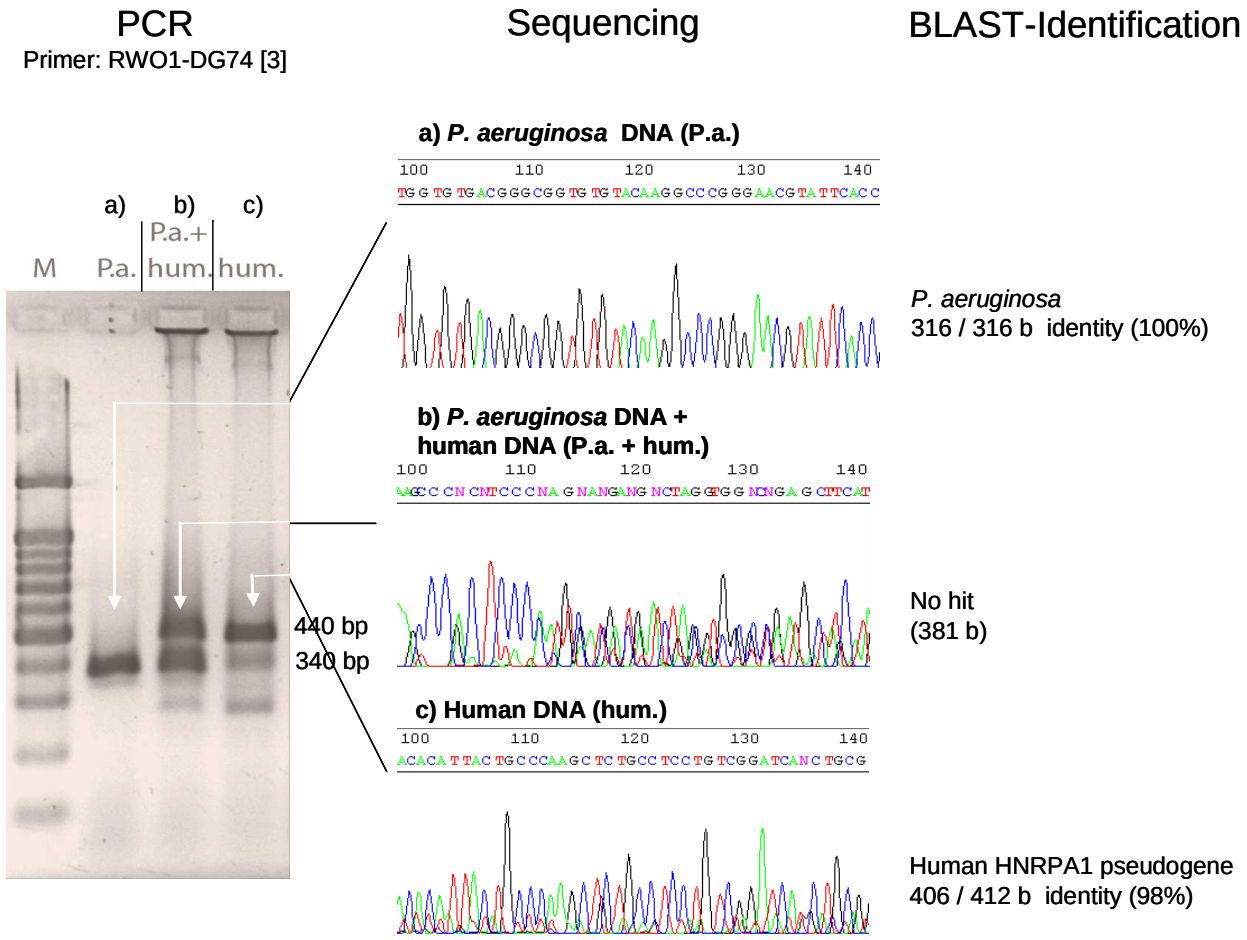

Fig. 1: *Influence of the presence of human DNA on the amplification and Sanger sequencing of 16S rRNA gene sequences. In the assay, primers were used that bind to conserved sites flanking V8/V9 hypervariable regions [19]. a) Pseudomonas aeruginosa DNA as template (13pg; corresponding to approximately 2,000 genome equivalents); b) mixture of P. aeruginosa and human DNA (42ng); c) human DNA only. Data are adapted from [10].*

designing primers and establishing PCR amplification conditions aimed at a high specificity. However, since the development of a variety of broad-range, bacteria-specific primers for diagnostics and community analysis including NGS in the past years, it became obvious that primers can bind to non-target sequences. Unspecific primer binding has two effects, generation of irrelevant information and loss of sensitivity.

Disqué [10] simulated the situation of a high overload of human against pathogen DNA and analyzed the quality of sequence reads obtained after bacteria-specific 16S rRNA gene PCR amplification (V8/V9 regions; Fig. 1). In this experiment human DNA was present in a 3.230-fold mass excess to pathogen DNA like it may occur in a fulminant septic scenario. Apparently, the unspecific amplification of human sequences (Fig. 1c) obscured specific sequence readouts in the Sanger sequencing analysis (Fig. 1b). Cross reactivity was observed with other universal primers for the amplification of the V1-V3 [27] and V3-V4 hypervariable regions [10]. The effect of the presence of human DNA on the amplification efficiency of bacterial sequences is

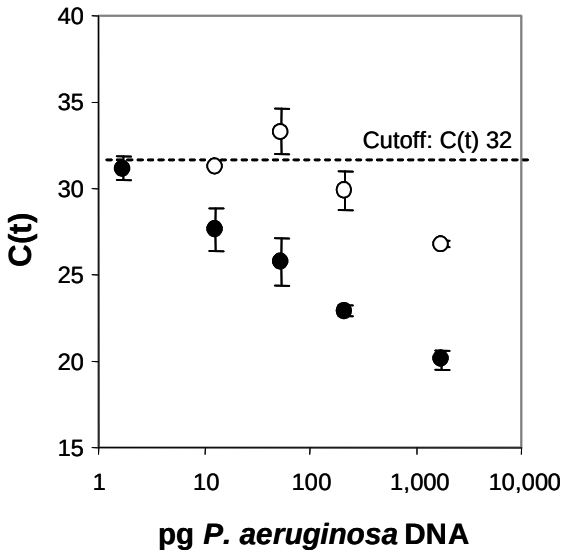

Fig. 2: *Quantitative effect of the presence of human DNA (42ng) on the amplification of P. aeruginosa DNA. The assay was a 16S rRNA gene Real-Time PCR (SYBR Green 1) using primers for amplification of the V3/V4 region. Human DNA present (o) or absent (●). Data are from [10]. At C(t)s below the cutoff bacteria-specific peaks were identified by dissociation analysis.*

Table 1: *Human DNA contamination in microbiome libraries*

Material	Target	Method [a]	Human sequences (%)
Saliva	resistance genes	cloning, Sanger	61 [44]
	microbiome	16S PCR, WGS, Illumina	>99 [28]
CF sputum	virome	WGS, 454	>90 [50]
Dental plaque	microbiome	WGS, 454	0.5-40 [2]
	microbiome	WGS, 454	60.6-89.8 [29]
Tissue	microbiome	cloning, Sanger	47 [9]
Feces	microbiome	WGS, 454	0.3-10.3 [20]

[a] WGS: Whole genome shotgun sequencing

depicted in Fig. 2. The results show that human DNA shifted the crossing points to values 6 to 7 C(t)s higher than when human DNA was absent. As a consequence, with human DNA present, the cutoff level (see dashed line in Fig. 2) was reached at approximately 2 orders of magnitude higher target DNA amount than in the absence of human DNA (Fig. 2). A plausible explanation for this loss of detection sensitivity is competition of unspecific human with specific bacterial binding sites for primers. According to this hypothesis, unspecific primer binding to human sites leads to primer limitation for target sites and hence decreased amplification efficiency of bacterial sequences [10]. Primer binding to and amplification of human sequences may explain the predominance of human reads in microbiome analyses of some sample types (Table 1). It appears that human DNA is an important factor that can obscure microbial signals [16] thereby influencing cutoff thresholds of in-depth analysis of microbial communities. Therefore, development of methods minimizing amplification of human sequences is necessary [25].

New pre-analytical approaches have been made available employing methods to either enrich the bacterial DNA or remove the human DNA from specimens before analysis [40]. Bacterial DNA enrichment technology involves a chromatographical procedure that uses a protein which binds to non-methylated (bacterial) DNA while human DNA is washed out (Pureprove® technology, SIRS-Lab). Human DNA removal technology (MolYsis™, Molzym) takes advantage of the fact that human and bacterial DNAs are present in different cell types (in blood, for instance, white blood cells and bacterial cells). MolYsis™ involves lysis of the fragile human cells while rigid bacterial cells are unaffected. An added nuclease degrades the released human

Table 2: *Detection sensitivities of two bacteria spiked into whole blood (0.2ml) using total DNA (A) and human DNA-depleted extracts (B).* [a]

Kit / dilution [b]	*S. aureus*	*E. coli*
A) QiaAmp® [c]		
10^{-3}	3/3	2/3
10^{-4}	0/3	2/4
10^{-5}	0/3	1/4
10^{-6}	n.d	0/3
B) MolYsis™ Basic + QiaAmp® [c]		
10^{-3}	3/3	n.d.
10^{-4}	4/4	2/2
10^{-5}	4/4	2/2
10^{-6}	4/4	4/4
Factor B/A [d]	**>1,000**	**>1,000**

[a] Assay: 16S rRNA gene PCR (Mastermix 16S Complete, Molzym)

[b] Overnight cultures: S. aureus ($1.6 \cdot 10^9$ cfu/ml), *E. coli* ($6.2 \cdot 10^9$ cfu/ml)

[c] QiaAmp® is a trade name of Qiagen; MolYsis™ is a trade name of Molzym

[d] Highest dilutions with 100% positive results compared

DNA. After sedimentation bacterial cells are lysed and DNA is purified by common manual or automated protocols.

Horz et al. [24] tested both technologies for oral specimens using a PCR assay targeting the β-2-microglobulin gene to quantify human DNA. The authors found a mean reduction of the human DNA load in caries and periodontal specimens by 93.4% and 93.6% with Pureprove® and 86.8% and 99.5% with MolYsis™, respectively. In another study, human DNA was completely removed from blood samples by MolYsis™ as measured in a PCR assay for human ß-globin gene [21]. The effect of human DNA removal technology on the sensitivity of detection of *Staphylococcus aureus* spiked into blood or blood culture was evaluated recently [22,31]. In these studies, methicillin resistance genes were PCR-assayed using DNA extracted by conventional and MolYsis™ method, respectively. The results showed that the analytical sensitivities obtained with conventional, i.e., total DNA extraction were increased 20-fold (blood) and 10 to 100-fold (blood culture) when samples were extracted with MolYsis™. Employing universal 16S rRNA gene PCR, the detection sensitivities of *S. aureus* and *E. coli* spiked into whole blood were more than 1,000-times higher when MolYsis™ was coupled with an extraction kit (Table 2, experiment B) than when the extraction kit was used alone (experiment A). Thus, removal of human DNA increases the sensitivity of PCR detection of bacteria in blood.

Table 3: *Influence of the removal of human DNA on bacterial reads of 16S-targeted resequencing.* [a]

Reads	QiaAmp® [a]	MolYsis™ Basic + QiaAmp® [a]
Human sequences	61.5 %	9.5 %
Bacterial sequences	34.0 %	90.0 %

[a] 0.2ml EDTA-blood spiked with *E. coli* and *S. aureus* at 5,000 cfu in total. Library preparation: amplification (V3/V4 region), Molzym Mastermix 16S Complete; fragmentation and barcoding, Life Technology Ion Xpress® Fragment Library kit; template enrichment and sequencing: Life Technology OneTouch® 300 Template and PGM® Sequencing 300 kits, Ion 316™ Chip; depth of coverage: approx. 300,000X; QiaAmp® and MolYsis™ are brand names of Qiagen and Molzym, respectively

The influence of the removal of human DNA on the 16S rRNA gene-targeted resequencing of blood spiked with bacteria was studied. For this, blood samples were spiked with *S. aureus* and *E. coli* and extracted for total DNA (QiaAmp®) and human DNA-depleted microbial DNA (MolYsis Basic™ combined with QiaAmp®). Table 3 shows that the proportion of human reads (total DNA extracts, 61.5%) was reduced approx. 6.5-fold by the MolYsis™ pre-treatment (9.5%). On the other hand, bacterial reads reached 90% with human DNA-depleted extracts as compared to only 34% with total DNA extracts. This result demonstrates that pre-analytical reduction of the human DNA load can improve the output of target sequence reads in a clinical sample like blood.

Large volumes are one of the important advantages of cultivation over molecular methods determining the unrivaled detection thresholds of culturable bacteria (theoretically 1cfu/ bottle). By testing the influence of volume of blood from pediatric patients on blood culture results, Tenney et al. [46] observed an increase of the detection of pathogens with increasing blood volume. Results of a similar approach employing MolYsis™ and Real-Time PCR analysis for the study of the influence of blood volume on the detection of bacteria is shown in Table 4. Obviously, large volumes of samples greatly enhanced the analytical sensitivities of two strains spiked into whole blood. This result encourages the notion that large volume extraction approaches may be helpful to increase detection sensitivity and in-depth microbiome resolution of microbial communities.

Table 4: *Influence of the blood volume on the detection sensitivity of bacteria.* [a]

Strain	Blood volume (ml)	Titer (cfu/ml)	PCR result
S. aureus	1	60	3/3
	2	30	3/3
	5	12	3/3
	10	6	3/3
E. coli	1	120	2/3
	2	60	3/3
	5	24	3/3
	10	12	3/3

[a] Strains spiked (10µl) into blood (1-10ml) at the final viable counts (cfu)/ml indicated. Extraction: MolYsis™ Complete5 (1ml, 2ml, 5ml) and MolYsis™ Complete10 (10ml). Universal 16S rRNA gene Real-Time PCR (Mastermix 16S, Molzym) and dissociation analysis

Lysis of Microorganisms for DNA Preparation

Microorganisms are very diverse with respect to the chemistry and structure of cell walls and outer layers like capsules. Moreover, microorganisms can be embedded in biofilms consisting of a complex structure. A crucial step in the preparation of samples for analysis is the breakage of cells for nucleic acid extraction and purification. Several studies have shown that differences in the structures of cell walls cause cell lysis to be more or less efficient [16]. In order to reduce bias by the DNA extraction methodology [18] and hence realistically reflect representative members and their abundances in a given community, the ideal procedure would extract DNA from any microbe present in a sample.

To investigate the effect of extraction methodology on the analysis of the human microbiome, Yuan et al. [51] tested 6 common procedures based on physical, enzymatic and chemical treatments and combinations thereof. In this study, the authors statistically evaluated the DNA extraction procedures using 11 human-associated bacterial species in equal numbers in a mock community. The conclusion from the results was that protocols employing bead beating and/or lytic enzymes, in particular mutanolysin, for cell lysis better represented the bacterial mock community than protocols without both of them. Recently, Disqué et al. [11] analyzed 430 clinical specimens, including blood, synovia, swabs from wounds, prostheses, pus and bones, cerebrospinal fluid, heart valves and aortal tissues. For extraction the authors used a commercial kit (*UMD-Universal*, Molzym) that includes a solution, BugLysis, consisting of a blend of hydrolytic enzymes for the degradation of microbial cell walls. The data from PCR and sequence analysis of the 186 positive samples found indicated that the reagent was able to lyse a broad range of microorganisms, including such diverse groups like Firmicutes (staphylococci,

enterococci, *Listeria*, *Dialister*), Gammaproteobacteria (*Pseudomonas*, enterobacteria), Alphaproteobacteria (*Bartonella*), Bacteroidetes, Spirochaetes (*Borrelia*), Actinobacteria (*Corynebacterium*, *Nocardia*, *Tropheryma*), Ascomycota (*Candida*, *Exophiala*), and Alveolata (*Plasmodium*). In a series of studies including different clinical materials, 200 organisms have been listed that are lysed by BugLysis (see appendix, page 9). Because of the high impact of the extraction method on community structure analysis, Rogers and Bruce [42] emphasize careful consideration of the particular characteristics of the sample type and the selection of suitable methods for processing.

Analysis of Live Communities

Extracellular microbial DNA (eDNA) is present in any microbial habitat where it functions as nutrient or carrier of genetic information in the course of natural transformation [32]. The DNA is released spontaneously from live cells or during the decay of cellular entities [12, 32]. eDNA can be included in cellular slime layers of bacteria with a mass ratio of >40%, thereby stabilizing its structure [7]. eDNA also is a component of biofilms constituting of a microcolony of microorganisms embedded in a complex matrix of macromolecules [33]. eDNA plays an important role in host colonization by pathogens, including, e.g., *Pseudomonas aeruginosa, Streptococcus intermedius*, *S. mutans, Enterococcus faecalis* and staphylococci [36]. From environmental studies it is known that eDNA tends to bind to surfaces where it can persist with a half-life of up to several weeks [32, 38]. Also, DNA in heat-killed cells was shown by PCR to persist for up to 55 days in seawater [13]. In the clinical scenario, eDNA can persist for extended periods of time as is well known for cystic fibrosis of the lower respiratory tract [4]. In view of studying living microbial community structures, eDNA and DNA of dead cells generates irrelevant information. More importantly, the persistence of considerable amounts of eDNA in extracellular matrices may mask changes in the community structure [42].

A method for the distinction of live from dead microbes and eDNA is the incubation of samples with ethidium monoazide (EMA) or propidium monoazide (PMA) (15). These dyes intercalate with DNA and form photo-induced crosslinks after exposure to light. The thus modified DNA is not a template for PCR amplification. EMA and PMA are unable to cross the cell membranes of living cells and therefore react only with eDNA and DNA in dead cells having lost their cellular integrity. The effect of PMA treatment of environmental water samples on the community analysis by NGS has been studied recently. For the discrimination between live and dead bacteria, samples were used untreated and heated, respectively. PMA did not substantially change the sequence profiles of non-heated samples, but clearly influenced the relative proportions of certain microbial groups when samples were heat-treated [39]. This experiment suggests that PMA treatment prior to extraction is a suitable procedure to reduce the impact of DNA from dead cells on the analysis of living microbial communities.

The importance of the exclusion of eDNA and DNA from dead cells from DNA-based analysis is stressed by the fact that *Streptococcus pneumoniae* DNA was shown to persist in non-infected heart valve tissue 7 years after a *Streptococcus pneumoniae* endocarditis [5]. Sakka et al. [43] studied the response of a systemic infection by *S. epidermidis* to the administration of antibiotics by blood culture and a quantitative Real-Time PCR test, SepsiTest™ (Molzym). SepsiTest™ includes the MolYsis™ sample pre-treatment procedure which involves a DNase to degrade human and eDNA. Blood culture indicated a decline of the infectious agent by increasing time-to-positivity and, at the end, negative result over a period of 1 day. Analogously, by SepsiTest™ a decrease of the bacterial DNA load was observed. The correlation to the decreased cultivability of *S. epidermidis* suggests that the PCR test monitored the decrease of the viable bacterial load in the blood. The advantage of this method is that it is integrated into the sample preparation pathway for the isolation of microbial DNA from human specimens and does not require further treatments as is necessary with photoactivatable dyes.

DNA Contamination of Reagents

A constraint of NGS analysis is the potential presence of microbial DNA contaminating extraction chemicals, PCR reagents and consumables during the manufacturing process. Table 5 (next page) shows an overview of studies investigating contamination of a variety of extraction and PCR reagents and consumables. The studies were conducted in the context of suitability of the material for molecular diagnosis. Contamination was found in the majority of reagents and consumables studied. The origin of the contamination comprised environmental organisms like *Alcaligenes* spp., *Pseudomonas* spp. and *Ralstonia* spp., skin colonizers like *Propionibacterium* spp., *Serratia*

Table 5: Sources of potential DNA contamination. [a]

Material	% False positives (found/tested)	Origin	Reference
A) Collection of samples			
Blood collection tubes	17 (31/185)	*Aspergillus* spp.	[23]
Blood serum tubes	10 (16/160)	*Aspergillus* spp.	[23]
Urine collection tubes	8 (2/25)	*Aspergillus* spp.	[23]
Forceps for tissue preparation	57 (13/23)	*Escherichia* spp., *Propionibacterium* spp., *Stenotrophomonas* spp. *Pseudomonas* spp.	[26]
Cap of blood culture bottle	n.d.	*Ralstonia pickettii*	[3]
Blood culture medium	n.d.	*Lactococcus lactis*, *Bacillus coagulans*	[34]
B) Nucleic acid extraction and processing			
Zymolase	n.d.	*Saccharomyces cerevisiae*	[30]
DNA extraction	100 (20/20)	*Burkholderia* spp., *Pseudomonas saccharophilia*, *Ralstonia* spp., *Alcaligenes* spp.	[35]
	20 (4/20)	*Legionella* spp., *Aspergillus* spp.	[14, 48]
	n.d.	*Aspergillus* spp., *Candida* spp.	[17]
	n.d.	*Brucella* spp.	[41]
	<3 (0/36)	-	[53]
Nucleic acid precipitation (glycogen)	22 (2/9)	*Acinetobacter lwoffii*	[1]
RNA stabilization reagent	5 (1/20)	*Aspergillus* spp.	[23]
C) PCR reagents			
Taq polymerase	100 (4/4)	bacteria	[8]
	100 (4/4)	*Pseudomonas* spp.	[37]
	8 (2/24)	*Sphingomonas* spp., *Moraxella* spp.	[37]
	2 (1/41)	*Acinetobacter junii*	[37]
	n.d.	*Pseudomonas* spp., *Serratia marcescens*, *Escherichia coli*, *Salmonella* spp., *Shigella* spp.	[45]
	10-30 (n.a.)	*Coxiella burnetii*	[47]
D) Plastic consumables			
Pipette tips	18 (6/32)	bacteria	[52]

[a] Samples of the same or different lots or samples from different manufacturers; signals were observed in negative PCR controls using molecular grade water; species were identified by sequencing of the amplicons and BLASTn search; n.d., not determined.

marcescens and *Sphingomonas* spp. as well as potential pathogens, including *Brucella* spp., *Coxiella burnetii*, *Escherichia coli* and *Legionella* spp. The grade of contamination was heavy (>50%) in some material, including forceps for tissue preparation, DNA extraction reagents and Taq polymerases (Table 5). Further contaminant genera can be found in [43a]. Reagents and consumables used in the molecular laboratory are issued for analyses other than the targeting of microbial sequences. Evidence for low and tolerable loads of contaminating microbial DNA (2 and 8% false positive rate) was given for some Taq polymerases (Table 5). Therefore, it is important to take care to select material specially produced and quality-controlled for molecular microbial analysis.

Conclusions

Nucleic acid extraction is a standard process since long optimized for yield and purity. Originally the procedures were not developed for applications in molecular diagnostics of bacterial pathogens and next generation sequencing of microbial communities. Here the demands are high for further qualities like the preferential supply of microbial DNA, lysis of a broad variety of organisms, extraction of DNA from only live microbes and exclusion of reagent-borne contamination. Therefore, nucleic acid extraction needs to be adapted to NGS. Because there is experience in coping with the majority of above mentioned problems, solutions for nucleic acid

preparation for NGS may come from molecular diagnostics rather than from other applications.

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[52] Own results. Among three manufacturers, one was showing severe contamination of the tips. The other products (PCR tubes, pipette tips) were continuously free of any DNA contamination as analysed by 16S/18S rDNA PCR (n=32 to 320; different lots tested).

[53] Guaranteed results according to the quality control SOP. MolYsis™ buffers, reagents and plastic consumables are quality controlled for the absence of bacterial and fungal DNA (limit of detection, <5cfu *S. aureus*/25µl assay).

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Appendix: *List of species found in clinical evaluations using MolYsis™ microbial DNA isolation procedure which involves broad lysis reagent, BugLysis (Real-Time PCR plus sequencing analysis). PCR Assay: Mastermix 16S Complete (Molzym); identification: sequencing/BLAST.*

Organisms identified					
Gram-negative bacteria		*Pseudomonas spp.*	4	*Listeria monocytogenes*	1
Acinetobacter spp.	7	*Pseudoxanthomonas spadix*	1	*Microbacterium aurum*	1
Actinomyces sp.	1	*Ralstonia pickettii*	1	*Micrococcus spp.*	2
Aeromonas veronii	1	*Raoultella planticola*	1	*Mycetocola sp.*	1
Bacteroides fragilis	1	*Schlegelella aquatica*	1	*Mycobacterium spp.*	3
Bartonella quintana	1	*Serratia spp.*	2	*Mycoplasma sp.*	1
Bifidobacterium spp.	2	*Sphingomonas sp.*	1	*Nocardia sp.*	1
Bordetella petri	1	*Spirosoma rigui*	1	*Paenibacillus sp.*	1
Borrelia garinii	1	*Shigella flexneri*	1	*Parvimonas micra*	1
Bradyrhizobium sp.	1	*Stenotrophomonas maltophilia*	1	*Peptoniphilus harei*	1
Brevibacterium spp.	2	*Tepidimonas thermarum*	1	*Peptostreptococcus stomatis*	1
Burkholderia fungorum	1	*Variovorax sp.*	1	*Planomicrobium okeanokoites*	1
Campylobacter coli	1	*Veillonella sp.*	1	*Propionibacterium acnes*	1
Candidatus Neoehrlichia	1	*Weeksella sp.*	1	*Rothia spp.*	3
Citrobacter freundii	1	*Zoogloea sp.*	1	*Ruminococcus productus*	1
Cloacibacterium normanense	1	**Sum**	**75**	*Staphylococcus spp.*	8
Comamonas testosteroni	1	***Gram-positive bacteria***		*Streptococcus spp.*	19
Coxiella burnetii	1	*Actinomyces sp.*	1	*Tropheryma whippleii*	1
Dialister invisus	1	*Aerococcus urinaeequi*	1	*Vagococcus carniphilus*	1
Edwardsiella tarda	1	*Anaerococcus spp.*	2	**Sum**	**101**
Enhydrobacter aerosaccus	1	*Bacillus spp.*	2	***Fungi***	
Enterobacter spp.	3	*Bifidobacterium spp.*	2	*Aspergillus spp.*	2
Escherichia spp.	2	*Brevibacterium spp.*	2	*Candida spp.*	7
Fusobacterium nucleatum	1	*Carnobacterium viridans*	1	*Cladosporium cladosporioides*	1
Haemophilus spp.	2	*Clostridium spp.*	3	*Cryptococcus spp.*	3
Helicobacter pylori	1	*Corynebacterium spp.*	8	*Didymella exitialis*	1
Hyphomicrobium facile	1	*Dolosigranulum pigrum*	1	*Davidiella tassiana*	1
Janthinobacterium lividum	1	*Enterococcus spp.*	8	*Malassezia spp.*	2
Klebsiella spp.	3	*Eremococcus coleocola*	1	*Peniophora nuda*	1
Lautropia mirabilis	1	*Exiguobacterium sp.*	1	*Saccharomyces cerevisiae*	1
Leptotrichia sp.	1	*Facklamia spp.*	2	*Schizophyllum radiatum*	1
Methylobacterium sp.	1	*Finegoldia magna*	1	*Sistotrema brinkmannii*	1
Moraxella spp.	2	*Gemella spp.*	2	*Sporobolomyces sp.*	1
Morganella morganii	1	*Granulicatella adiacens*	1	*Udeniomyces pannonicus*	1
Neisseria spp.	2	*Janibacter sp.*	1	**Sum**	**23**
Parabacteroides distasonis	1	*Jeotgalicoccus pinnipedialis*	1	***Protist***	
Paracoccus aminovorans	1	*Kocuria spp.*	3	*Plasmodium falciparum*	1
Petrobacter sp.	1	*Lactobacillus spp.*	6		
Proteus spp.	2	*Lactococcus lactis*	1		
Providencia stuartii	1	*Leifsonia sp.*	1	**Sum species:**	**200**