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REMIS+

Culture-independent targeted next generation sequencing of the 16S-23S rRNA region for the identification of bacterial species directly from clinical samples: opportunities and challenges

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Disclosure

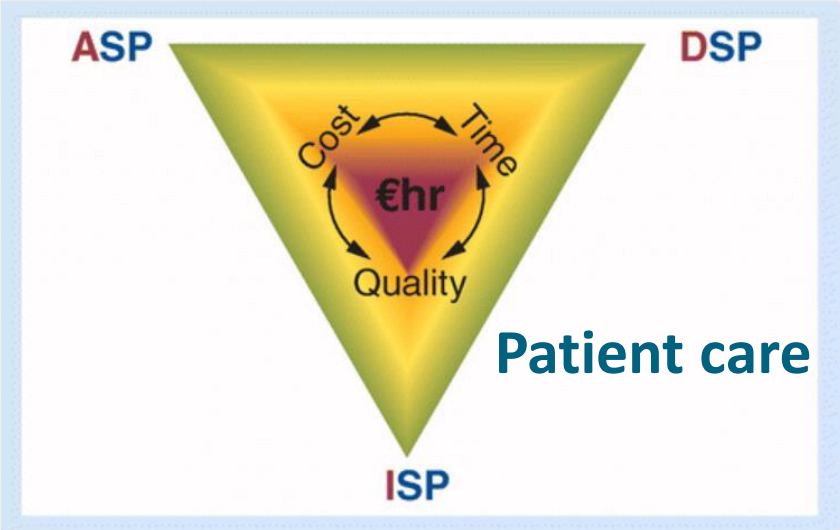
(potential) conflict of interest	None
For this meeting possibly relevant relationships with companies	None
• Sponsoring or research funding • Fee or other (financial) compensation • Shareholder • Other relationship, namely …	None

Diagnostic Stewardship

Modern and rapid diagnostics: focus on individual patient care

Accurate diagnostics for detection and identification of bacterial species

AID stewardship model



Culture methods

Molecular tests

- PCR
- 16S rDNA Sanger sequencing

ASP: Antimicrobial Stewardship Program
DSP: Diagnostic Stewardship Program
ISP: Infection Prevention Stewardship Program

Dik *et. al.* Future Microbiol. 2016

Limitations

Culture methods

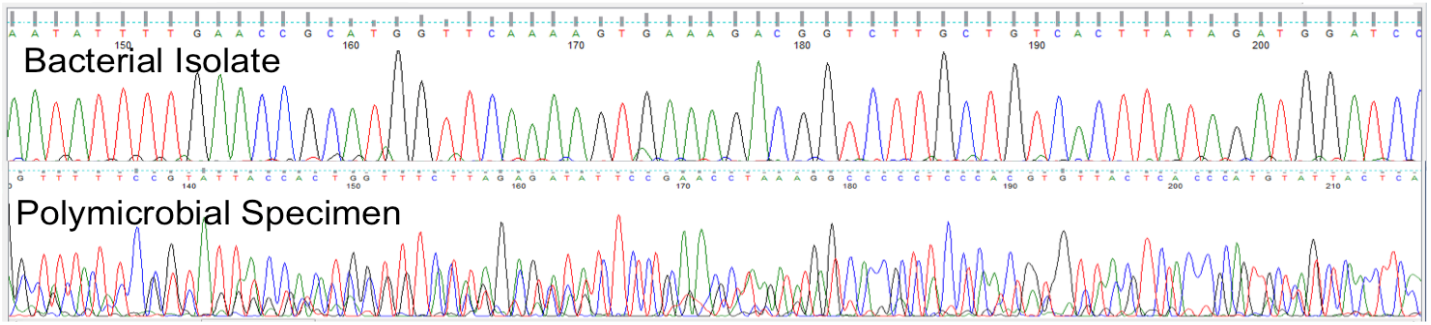
- slow-growing or fastidious bacteria
- antibiotic treatment
- Maldi-TOF

Molecular tests

PCR a priori knowledge of likely pathogenic species

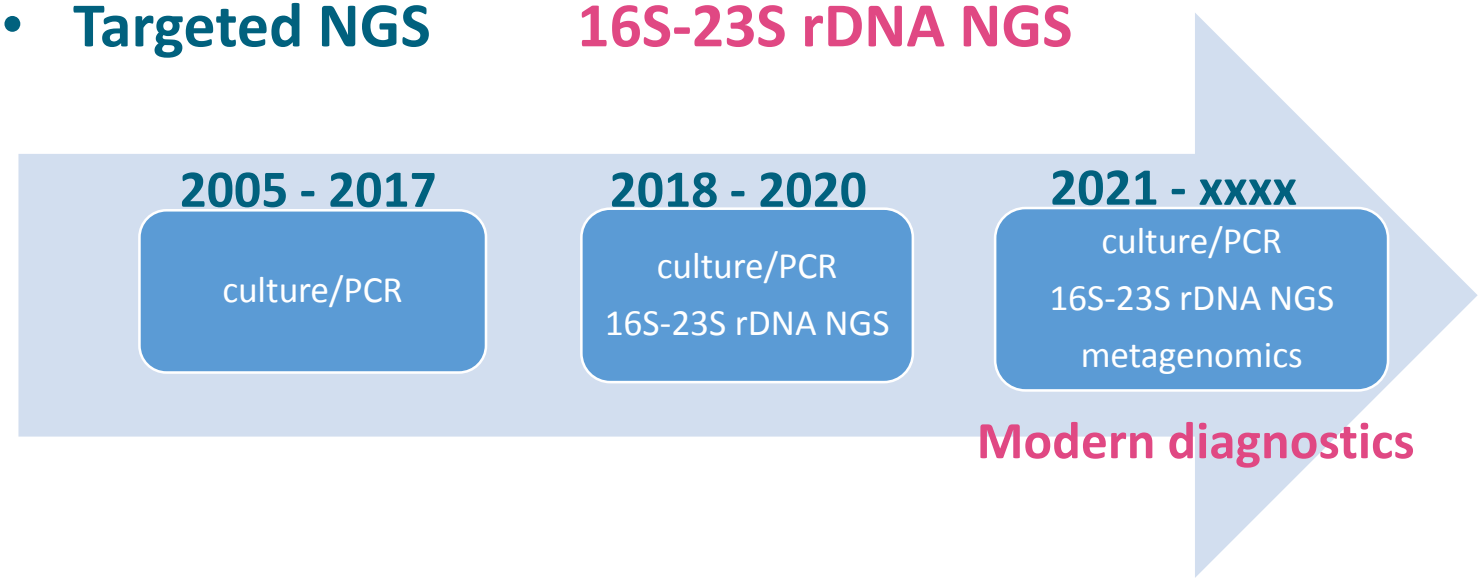
16S rDNA Sanger sequencing

- polymicrobial samples
- high sequence similarities in some species



Culture independent diagnostic method for detection and identification of bacterial species in clinical materials

- Next Generation Sequencing (NGS)
- Benchtop sequencers — Diagnostic tool
- Metagenomics
- Targeted NGS — 16S-23S rDNA NGS



16S rRNA gene (appr. 1,5 kb)

- highly useful in regards to bacterial classification
- poor discriminatory power for some genera

Patel *et. al. Mol. Diag*. 2001

ITS region

- highly variable in size and/or sequence composition

E. coli: García-Martínez *et. al. J Bacteriol. 1996*
Campylobacter spp: Man et. al. *Appl Environ Microbiol*. 2010

23S rRNA gene (appr. 2,9 kb)

- highly useful in regards to bacterial classification
- high sequence variation

Hunt *et. al*. *Appl. Environ. Microbiol*. 2006

Challenge: sequencing whole 16S-23S rRNA region

Advantages of Next Generation 16S-23S rDNA sequencing for culture-free infectious disease diagnostics

Culture-independent assay enables detection of organisms which are:

- fastidious
- slow-growing
- viable not culturable

Identification bacterial species within complex polymicrobial samples

Community DNA

PCR amplification of 16S-23S rRNA region

NGS of amplicons on MiSeq (Illumina)

Aims

1. To develop an easy-to use culture free diagnostic method based on 16S-23S rDNA NGS to improve the resolution of bacterial species identification.

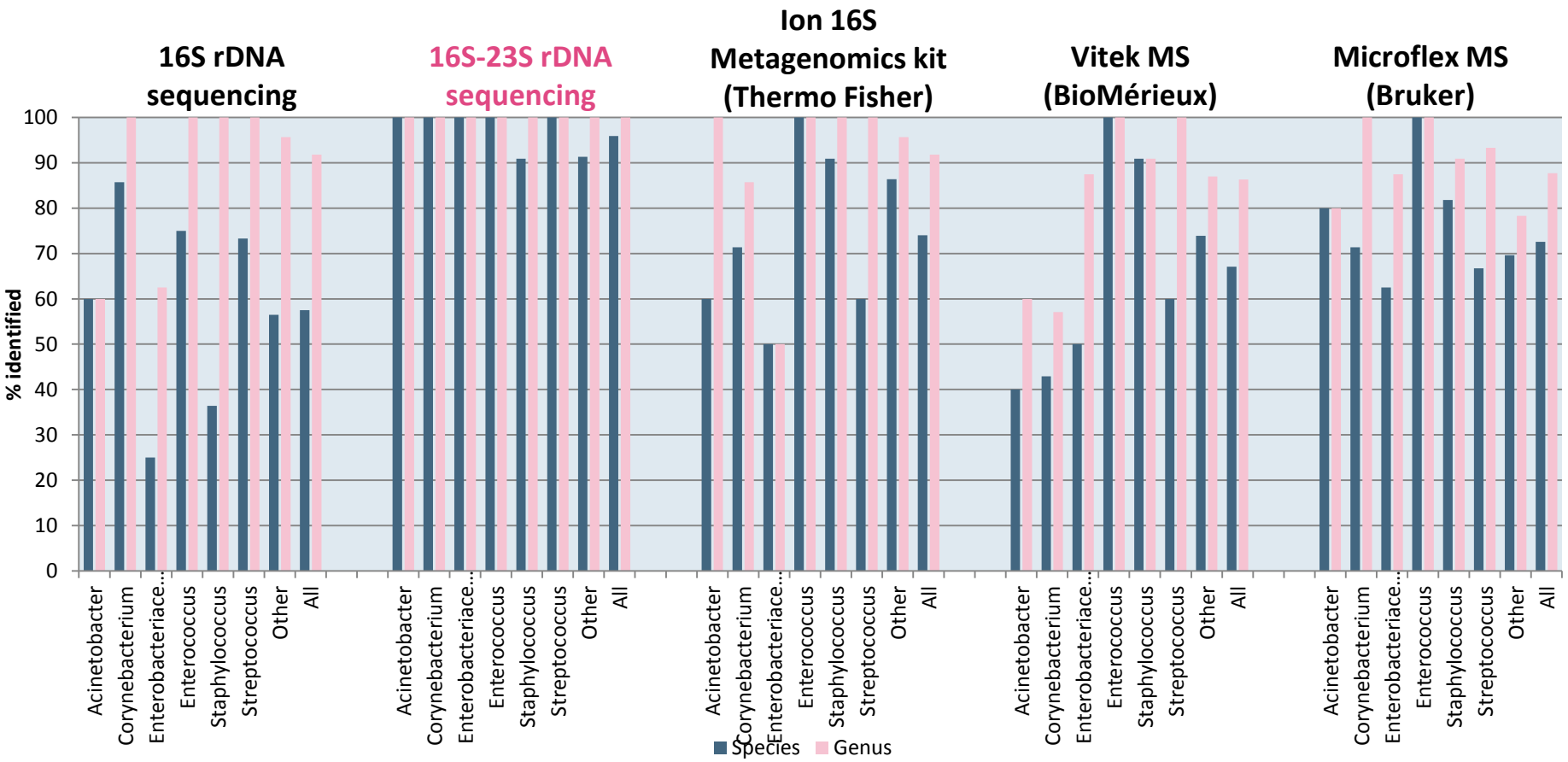
2. To compare 16S-23S rDNA NGS with 4 commonly used identification methods.

3. To evaluate the feasibility of 16S-23S rDNA NGS to detect and identify bacterial species in clinical specimens.

Species identification using 16S-23S rDNA NGS

73 difficult to identify clinical isolates were subjected to five methods.

16S-23S rDNA NGS: higher discriminatory power



The discriminatory power of species level identification using 16S-23S NGS is significantly higher than 16S rDNA Sanger Sequencing, Ion 16S Metagenomics kit, Vitek MS and Microflex MS ($p=0.0001$, $p=0.0003$, $p=0.0001$ and $p=0.0002$, respectively).

16S-23S rDNA NGS: clinical materials

Proof of principle

Materials:

- urine samples from patients suspected for urinary tract infection (n=60)
- positive blood culture samples (n=23)
- biopsies and punctates from orthopedic patients (n=21)

Methods:

- conventional culture
- 16S-23S rDNA NGS

Species ID from clinical samples using 16S-23S rDNA NGS

TABLE 1. Bacterial identification results from 60 urine samples based on culture and NGS of 16S-23S rRNA region.

Sample	Reported cause of UTI by conventional culture identification	Growth CFU/ml	Identification of additional colonies by culture methods	Species content by NGS of 16S-23S rRNA region (% of total reads)
UR1	*Pseudomonas aeruginosa*	10^5		*Lactobacillus gasseri* (97.2%), *Pseudomonas aeruginosa* (2.4%), *Corynebacterium amycolatum* (0.4%)
UR2	*Proteus mirabilis*	10^4		*Proteus vulgaris* (70.3%), *Proteus mirabilis* (29.7%)
UR3	*Escherichia coli*	10^5		*Escherichia coli* (99.1%), *Lactobacillus delbrueckii* (0.9%)
UR4	*Escherichia coli*	10^5		*Escherichia coli* (99.1%), *Peptoniphilus lacrimalis* (0.3%), *Bacteroides sp.* (0.6%)
UR5	*Escherichia coli*	10^5		*Escherichia coli* (93.2%), *Undibacterium oligocarboniphilum* (6.3%), *Pseudomonas saccharophila* (0.3%), *Phenylobacterium sp* (0.2%)
UR6	No clinical significance	10^4	*Bifidobacterium sp*	*Actinobaculum schaalii* (100%)
UR7	*Escherichia coli*	10^5		*Escherichia coli* (80.7%), *Lactobacillus crispatus* (19.3%)
UR8	*Escherichia coli*	10^5		*Escherichia coli* (98.7%), *Enterococcus faecalis* (1.0%), *Aerococcus sanguinicola* (0.3%)
UR9	*Escherichia coli* *Staphylococcus aureus*	10^5 10^5		*Escherichia coli* (53.7%), *Klebsiella oxytoca* (43.6%), *Staphylococcus aureus* (2.4%), *Enterococcus faecalis* (0.3%)
UR10	No clinical significance	10^2		*Ezakiella peruensis* (28,6%), *Fenollaria massiliensis* (4,1%), *Helcococcus sp.* (2,6%), *Peptoniphilus duerdenii* (2,6%), *Mobiluncus curtisii* (2,4%), *Varibaculum cambriense* (2,2%), *Peptoniphilus harei* (1,3%), *Actinobaculum urinale* (0,8%), *Peptoniphilus lacrimalis* (0,8%), *Propionimicrobium sp.* (0,7%), *Facklamia sp.* (0,7%), *Finegoldia magna* (0,6%), *Anaerococcus obesiensis* (0,6%), *Anaerococcus prevotii* (0,4%), *Anaerococcus degenerii* (0,4%), *Actinobaculum sp.* (0,4%), *Aerococcus urinae* (0,3%), *Fastidiosipila sanguinis* (0,2%), *Fastidiosipila sanguinis* (0,2%), *Bacteroides coagulans* (0,2%), Unidentified species (50,2%)
UR11	No clinical significance	10^2	*Staphylococcus epidermidis*	No PCR product
UR12	No clinical significance	10^3	*Proteus mirabilis*	*Proteus mirabilis* (75.5%), *Proteus vulgaris* (17.5%), *Undibacterium oligocarboniphilum* (4.8%), *Aerococcus urinae* (1.2%), *Corynebacterium striatum* (0.3%), *Pseudomonas saccharophila* (0.3%), *Enterococcus faecalis* (0.2%), *Ralstonia pickettii* (0.1%)
UR13	No clinical significance	10^2		No PCR product
UR14	No clinical significance	10^2		*Undibacterium oligocarboniphilum* (36.4%), *Fenollaria massiliensis* (15.6%), *Mobiluncus curtisii* (10.6%), *Peptoniphilus lacrimalis* (5.9%), Unidentified species (5.3%), *Peptostreptococcus anaerobius* (4.4%), *Peptoniphilus koenoeneniae* (4.1%), *Pseudomonas saccharophila* (3.1%), *Atopobium deltae* (3.7%), *Candidatus Peptoniphilus massiliensis* (2.6%), *Anaerococcus sp.* (1.8%), *Jonquetella anthropi* (1.1%), *Peptoniphilus harei* (1.1%), *Streptococcus anginosus* (0.8%), *Ralstonia pickettii* (0.7%), *Dialister propionicifaciens* (0.6%), *Methylobacterium oryzae* (0.6%), *Asinibacterium lactis* (0.5%), *Methylobacterium jeotgali* (0.4%)

Sabat *et al.*, Sci Reports 2017

TABLE 2. Bacterial identification results from 23 positive blood culture bottles based on culture and NGS of 16S-23S rRNA region.

Sample	Patient	Bottle	Culture (Maldi-TOF MS)	NGS of 16S-23S rRNA region (% of total reads)
BC01	Patient A	anaerobic	*Escherichia coli*	*Escherichia coli* (100%)
BC02	Patient B	aerobic	*Streptococcus dysgalactiae*	*Streptococcus dysgalactiae* (100%)
BC03	Patient C	anaerobic	*Klebsiella oxytoca*	*Klebsiella oxytoca* (100%)
BC05	Patient D	aerobic	*Staphylococcus heamolyticus*	*Staphylococcus haemolyticus* (100%)
BC06	Patient E	anaerobic	*Staphylococcus hominis*	*Staphylococcus hominis* (100%)
BC07	Patient F	aerobic	*Staphylococcus capitis*	*Staphylococcus capitis* (100%)
BC08	Patient G	anaerobic	*Streptococcus pneumoniae*	*Streptococcus pneumoniae* (100%)
BC09	Patient H	aerobic	*Staphylococcus epidermidis*	*Staphylococcus epidermidis* (100%)
BC10	Patient H	anaerobic	*Staphylococcus hominis*	*Staphylococcus hominis* (100%)
BC11	Patient I	anaerobic	*Bacteroides sp.*	*Bacteroides fragilis* (100%)
BC12	Patient J	aerobic	*Staphylococcus hominis*	*Staphylococcus hominis* (100%)
BC13	Patient K	aerobic	*Staphylococcus aureus*	*Staphylococcus aureus* (100%)
BC14	Patient L	aerobic	*Klebsiella oxytoca*	*Klebsiella oxytoca* (100%)
BC15	Patient M	anaerobic	*Streptococcus pneumoniae*	*Streptococcus pneumoniae* (100%)
BC16	Patient N	aerobic	*Escherichia coli*	*Escherichia coli* (100%)
BC17	Patient O	anaerobic	*Staphylococcus aureus*	*Staphylococcus aureus* (100%)
BC18	Patient P	anaerobic	*Streptococcus pneumoniae*	*Streptococcus pneumoniae* (100%)
BC19	Patient Q	aerobic	*Escherichia coli, Streptococcus infantis*	*Escherichia coli* (69.3%), *Streptococcus lutetiensis* (30.7%)
BC20	Patient Q	anaerobic	*Escherichia coli*	*Escherichia coli* (100%)
BC21	Patient R	aerobic	*Escherichia coli*	*Escherichia coli* (100%)
BC22	Patient R	anaerobic	*Bacteroides vulgatus*	*Bacteroides dorei* (100%)
BC23	Patient S	aerobic	*Staphylococcus hominis*	*Staphylococcus hominis* (100%)
BC24	Patient S	aerobic	*Staphylococcus epidermidis*	*Staphylococcus epidermidis* (100%)

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TABLE 3. Bacterial identification results from 21 clinical orthopedic samples based on culture and NGS of 16S-23S rRNA region.

Sample	Patient	Material	Culture	NGS of 16-23S rRNA region (% of total reads)
KM1	Patient A	biopsy (tissue)	Negative	*Propionibacterium acnes* (9.1%)[A], *Haemophilus parainfluenzae* (2.3%), eukaryotic DNA (88.6%)
KM2	Patient A	punctate (fluid)	Negative	eukaryotic DNA (100%)
KM3	Patient A	punctate (fluid)	Negative	*Sediminibacterium salmoneum* (0.3%), eukaryotic DNA (99.7%)
KM4	Patient A	punctate (fluid)	Negative	*Gemella sanguinis* (1.3%), *Haemophilus parainfluenzae* (1.0%), eukaryotic DNA (97.7%)
KM5	Patient A	punctate (fluid)	Negative	*Herminiimonas sp.* (10.5%), *Propionibacterium acnes* (9.7%)[A], *Moraxella catarrhalis* (7.5%), eukaryotic DNA (72.3%)
KM6	Patient B	pus	Negative	*Streptococcus intermedius* (100%)
KM7	Patient C	biopsy (tissue)	Negative	eukaryotic DNA (100%)
KM8	Patient C	biopsy (tissue)	Negative	No identification
KM9	Patient D	joint puncture (fluid)	Negative	*Enhydrobacter aerosaccus* (49.8%)[B], *Acinetobacter septicus* (18.1%)[B], *Moraxella osloensis* (14.0%), *Staphylococcus sp.* (5.8%), *Rheinheimera soli* (3.1%), *Staphylococcus epidermidis* (2.6%), *Psychrobacter sp.* (2.4%)[B], *Propionibacterium acnes* (1.3%)[A], *Alkanindiges sp.* (0.6%), *Acinetobacter sp.* (0.4%)[B], *Chryseobacterium sp.* (0.3%)[B]
KM10	Patient D	joint puncture (fluid)	Negative	No identification.
KM11	Patient D	biopsy (tissue)	Negative	*Propionibacterium acnes* (9.8%)[A], *Bacillus nealsonii* (6.7%)[B], *Pseudomonas fluorescens* (0.6%)[A], eukaryotic DNA (82.9%)
KM12	Patient D	biopsy (tissue)	Negative	eukaryotic DNA (100%)
KM13	Patient D	biopsy (tissue)	Negative	*Undibacterium oligocarboniphilum* (3.5%)[B], *Propionibacterium acnes* (0.7%)[A], eukaryotic DNA (95.9%)
KM14	Patient D	biopsy (tissue)	Negative	*Propionibacterium acnes* (1.4%)[A], eukaryotic DNA (98.6%)
KM15	Patient D	biopsy (tissue)	Negative	*Veillonella parvula* (0.9%), eukaryotic DNA (99.1%)
KM16	Patient D	biopsy (tissue)	Negative	eukaryotic DNA (100%)
KM17	Patient E	blood	n.d.	*Bacillus cereus* (0.5%)[B], eukaryotic DNA (99.5%)
KM18	Obduction material A	formaline captured, biopt (tissue)	n.d.	*Propionibacterium acnes* (64.4%)[A], *Staphylococcus epidermidis* (25.4%), *Paracoccus sanguinis* (10.1%)[B]
KM19	Obduction material B	formaline captured, lung biopt (tissue)	n.d.	*Staphylococcus epidermidis* (36.0%), *Propionibacterium acnes* (34.6%)[A], *Pseudomonas fluorescens* (29.4%)[A]
KM20	Patient F	joint puncture (fluid)	Negative	eukaryotic DNA (100%)
KM21	Patient F	biopsy (tissue)	Negative	*Acinetobacter sp.* (18.6%)[B], *Paucibacter sp.* (12.8%), *Herminiimonas arsenicoxydans* (5.2%), eukaryotic DNA (63.4%)

[A]Species present in negative control(s) and regarded as contamination introduced during sample preparation.
[B]Genus absent in negative controls but previously reported as contamination of DNA extraction kits, PCR and other laboratory reagents[10].

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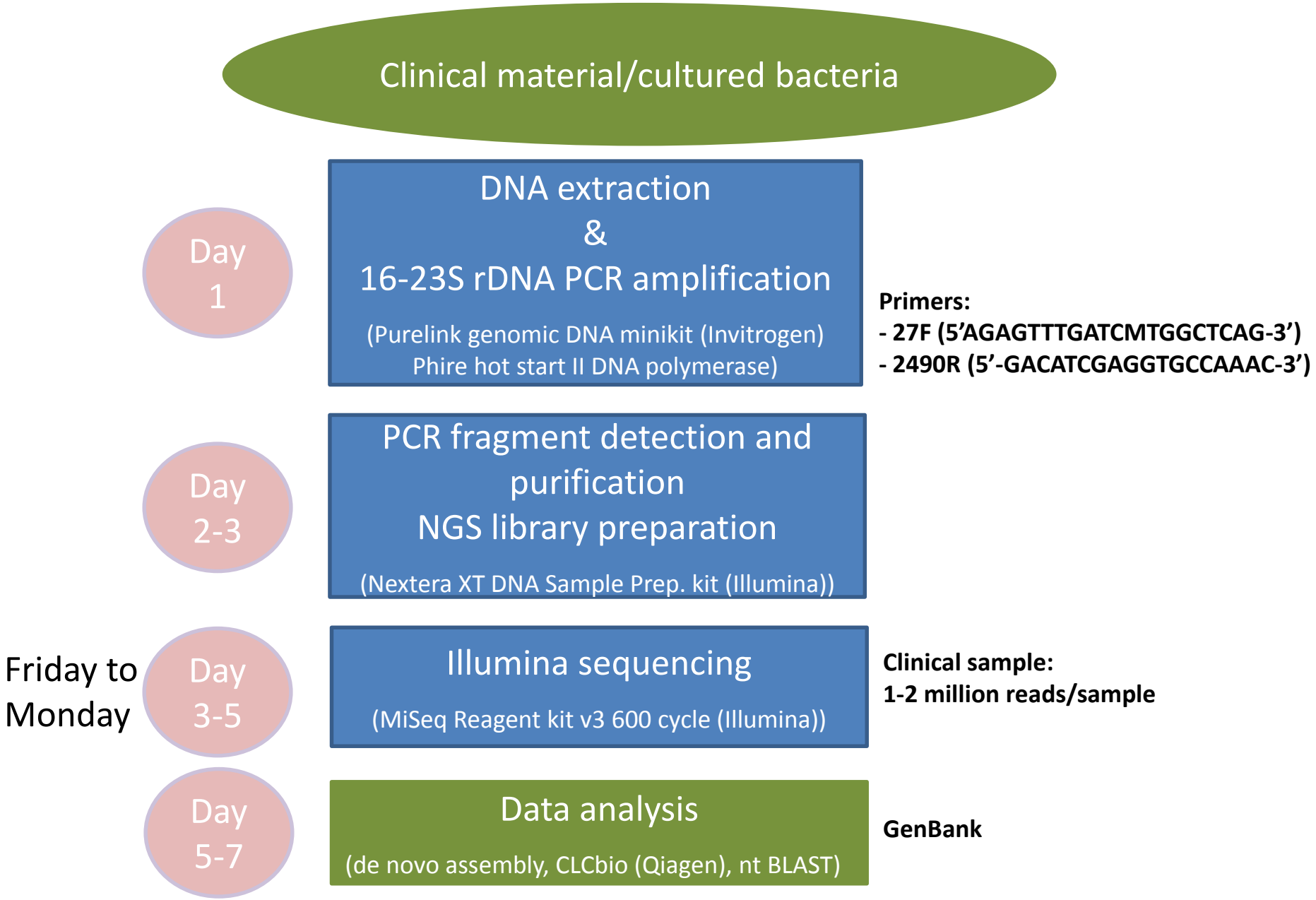
NGS of the 16S-23S rRNA region enables culture-independent detection and identification of multiple bacteria in complex polymicrobial samples.

WORKFLOW 16S-23S rDNA NGS (n= 21 samples, 3 controls)

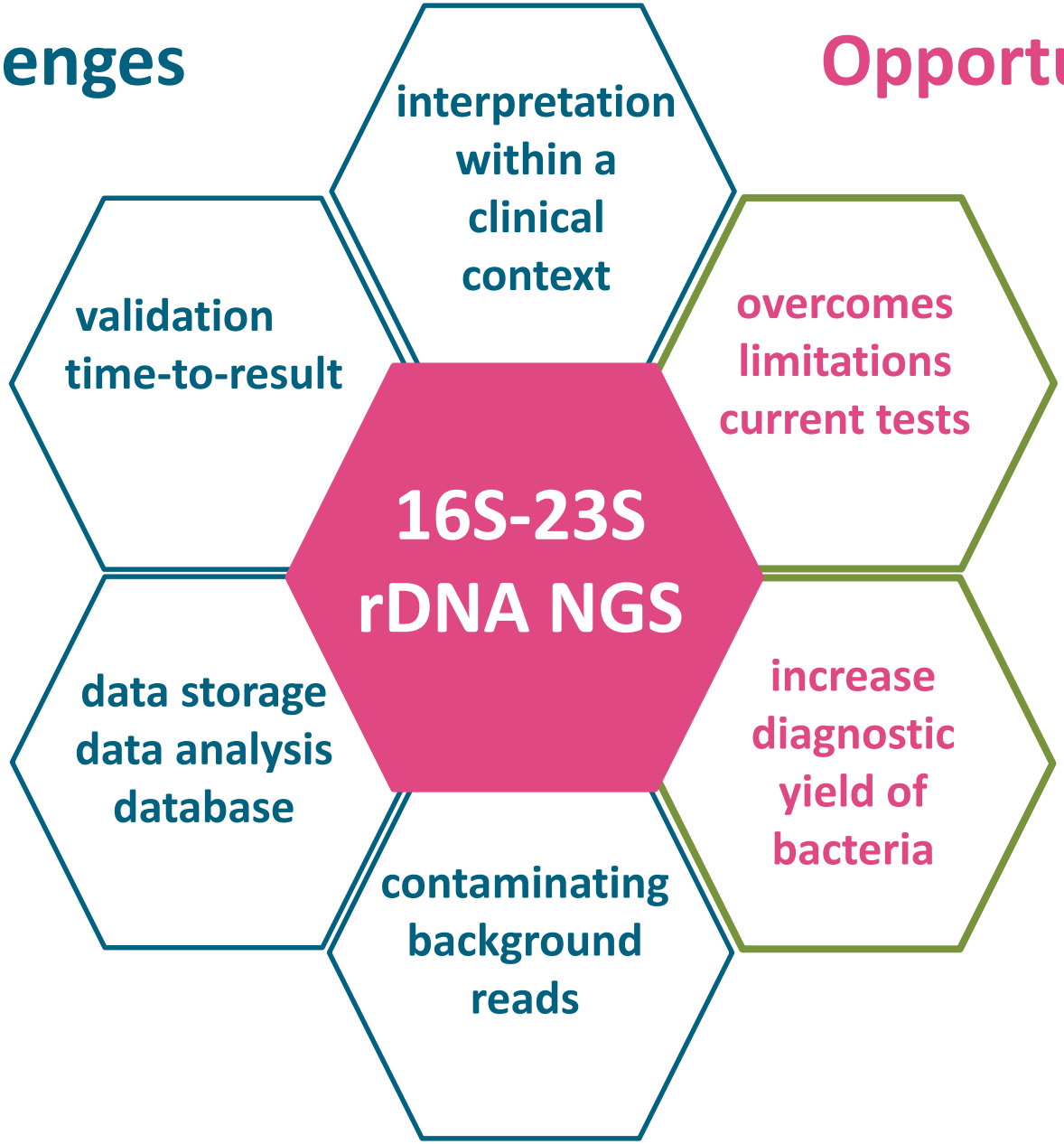


Patient	Age (year)	Clinical symptoms	Clinical sample	Culture	16S-23S rDNA NGS Species (fraction %*)
A	68	- fever: temp 39^5 - ill - necrotizing fasciitis	pus	negative	*Streptococcus pyogenes* (99.7 %)
B	63	- speaking is more difficult - fever - Paresis of left arm and leg MRI: Brain abscess	pus	*Streptococcus anginosus* *Aggregatibacter aphrophilus*	*Streptococcus intermedius* (89.4 %) *Parvimonas micra* (9.0 %) *Fusobacterium hwasookii* (1.6 %)
C	30	- confused - nauseous and vomiting - cardiac arrest on admission CT-scan: Brain abscess	pus	*Porphyromonas asaccharolytica* *Porphyromonas somerae* *Parvimonas micra*	*Fusobacterium necrophorum* (96.0 %) *Porphyromonas asaccharolytica* (3.0 %) *Porphyromonas somerae* (0.5 %) *Parvimonas micra* (0.1 %)

*%=percentage of identified reads

Challenges

Opportunities



Challenges 16S-23S rDNA NGS for diagnostic microbiology

- validation of the method
  - efficiency of extraction methods-depletion of human DNA
  - use of an internal control and a mock sample
  - NGS: read length, sequence depth
  - reproducibility

- time to results

- data storage, analysis and databases
  - development of a software-pipeline for data-analysis (track and trace!) to obtain data in a meaningful timeframe
  - development of a curated 16S-23S rDNA sequences database

Challenges 16S-23S rDNA NGS for diagnostic microbiology

- contaminating background reads (specimen collection, laboratory reagents)

- colonization versus infection

  how to discriminate between the two?

- interpretation within a clinical context

  multidisciplinary teams

  prospective studies

Conclusions

- The 16S-23S rDNA NGS method proved to be superior to commonly used identification methods.
- 16S-23S rDNA NGS has the potential to increase the diagnostic yield of bacteria involved in complex infections.
- 16S-23S rDNA NGS needs further validation.
- Studies focusing on clinical relevance are necessary to determine the applicability of this NGS-based approach in routine diagnostics.
- Multidisciplinary teams are needed to share their knowledge, in order to translate the results of 16S-23S rDNA NGS in a report that meets the needs of treating physicians.

Projectgroup 16S-23S rDNA NGS

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