Whitepaper

Rare Pathogens – Culture-Independent Eubacterial 16S rRNA Gene Diagnosis

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Summary

Rare pathogens are associated with critical conditions of patients in many cases demanding timely identification for adjustment of the antibiotic regime. Cultural diagnosis of rare pathogens, however, can be laborious and time-consuming, which lowers the prognosis of successful treatment. A prominent reason for this is the enormous diversity of rare pathogens that may be identifiable only with delay or even not identifiable by usual phenotyping methods. A review of clinical studies conducted in five countries from Europe, Asia and the USA reveals that 90.7 to 98.2% of systemic infections were caused by strains belonging to only 7 bacterial classes and one yeast genus. With less than 1% incidence in the majority of the countries, the taxonomic diversity of rare pathogens markedly increased adding another 13 bacterial classes to the list of aetiologies of sepsis. Even more, a survey of 54 case studies uncovered further unusual species that belonged to 27 classes. In order to break through the obstacles of poor cultural diagnosis, the use of culture-independent, pan-bacterial 16S rDNA PCR and sequencing analysis has entered routine as a helpful method of precise strain identification within hours instead of days.

Introduction

Latest 2016 recommendations of the Surviving Sepsis Campaign guide to the immediate (within one hour) administration of empiric broad-spectrum antimicrobials after diagnosis of sepsis or septic shock. The problem with gold standard blood culture is that cultures are often negative with blood draws after initiation of antibiotic treatment. Also, as demonstrated in a recent study (1), cultures grow positive with bacteria and yeasts only after 12 to 61 hours which delays targeted antibiotic treatment and increases the risk of poor prognosis of the patients. According to the recommendations, empiric antimicrobials should include combinations of two antimicrobials of different antibiotic classes targeting the most probable, i.e., common pathogens.

Common pathogens

In clinical practice in the Western and Eastern hemisphere, *Escherichia coli*, *Staphylococcus aureus* and coagulase-negative staphylococci have been shown in clinical studies to make up 43.1 to 65% of cases of bacteraemia in sepsis (Table 1). In large studies from Germany (4380 patients), France (842), USA (1585), Saudi Arabia (1626) and Pakistan (521), further common pathogens or representatives of the genus were identified with incidences of sepsis cases between 1 and 10%, including streptococci, *Salmonella typhi/paratyphi*, *Pseudomonas* spp., *Acinetobacter* spp., *Klebsiella* spp., enterococci, *Candida* spp., *Enterobacter* spp., *Proteus* spp., *Serratia* spp., *Bacteroides* spp., *Clostridium* spp., and, in USA, *Mycobacterium* spp. (Table 1). Depending on the country, these 16 pathogens/groups cause sepsis in 32.9 to 47.6% of cases. Together, common pathogens account for 90.7 to 98.2% of systemic infections.

Rare pathogens

Other pathogenic bacteria were associated with infections at lower than 1% incidence. This arbitrarily defined group of rare pathogens accounted for 1.4 to 13.2% of bacteraemias in the studies of the 5 countries (Table 1). The pathogens represent 13 taxonomic classes, including Enterobacterales (*Citrobacter* spp., *Yersinia enterocolitica*), Pasteurellales (*Haemophilus influenzae*), Vibrionales (*Vibrio* spp.), Neisseriales (*Neisseria meningitidis*), Clostridiales (*Peptostreptococcus* spp., *Peptococcus* spp.), Rhizobiales (*Brucella* spp.), Burkholderiales (*Burkholderia* spp.), Propionibacteriales (*Propionibacterium* spp.), Bacillales (*Listeria monocytogenes, Bacillus* spp.), Fusobacteriales (*Fusobacterium* spp.), Campylobacterales (*Campylobacter fetus*), Corynebacteriales (*Corynebacterium* spp.) and Veillonellales (*Veillonella* spp.). For comparison, the 16 common patho-

Table 1: Pathogens identified in blood cultures of septic patients.

Strain	Culture incidence (%)							Incidence
	Germany	France	USA	Pakistan	Saudi Arabia	Range		
	(2)	(3)	(4)	(5)	(6)	lower	upper	in majority of countries
Coagulase-negative staphylococci	9.3	10.7	9.2	36.4	24.7	9.2	36.4	
E. coli	23.9	29.2	15.0	18.9	9.1	9.1	29.2	≥10%
Staphylococcus aureus	20.4	21.4	18.9	9.7	18.6	9.7	21.4	
Sum	**53.6**	**61.3**	**43.1**	**65.0**	**52.4**	**43.1**	**65.0**	
Streptococcus pneumoniae and other *Streptococcus* spp.	12.4	16.7	8.7	1.5	7.7	1.5	16.7	
Salmonella typhi/paratyphi	2.1	1.1		8.9	0.7	0.7	8.9	
Pseudomonas aeruginosa and other *Pseudomonas* spp.	4.9	3.8	6.3	4.4	8.7	4.4	8.7	
Acinetobacter baumanii and other *Acinetobacter* spp.	2.2	1.1	1.4	3.4	7.8	1.1	7.8	
Klebsiella pneumoniae and other *Klebsiella* spp.	5.4	4.3	6.9	4.0	6.7	4.0	6.9	
Enterococcus faecalis and other *Enterococcus* spp.	5.5	5.5	6.7	5.7	4.8	4.8	6.7	
Candida albicans and other *Candia* spp.			5.9	5.0	5.9	5.0	5.9	≥1% to <10%
Enterobacter cloacae and other *Enterobacter* spp.	3.6		2.6		1.2	1.2	3.6	
Proteus mirabilis and other *Proteus* spp.	2.3	2.7	1.7		0.7	0.7	2.7	
Serratia marcescens and other *Serratia* spp.	0.8		2.3		1.4	0.8	2.3	
Bacteroides spp.	1.2		1.7		0.2	0.2	1.7	
Clostridium perfringens and other *Clostridium* spp.	0.5		1.6			0.5	1.6	
Mycobacterium spp.			1.8				1.8	
Sum	**40.9**	**35.2**	**47.6**	**32.9**	**45.8**	**32.2**	**47.6**	
Cryptococcus neoformans			0.8				0.8	
Citrobacter spp.	0.7			0.6	0.3	0.3	0.7	
Lactobacillus spp.	0.1		0.6			0.1	0.6	
Haemophilus influenzae	0.5		0.3		0.4	0.3	0.5	
Stenotrophomonas maltophilia			0.5	0.2		0.2	0.5	
Vibrio spp.				0.4			0.4	
Neisseria meningitidis	0.3	0.2				0.2	0.3	
Peptostreptococcus spp.	0.3						0.3	
Yersinia enterocolitica	0.1			0.2		0.1	0.2	
Brucella spp.					0.2		0.2	<1%
Burkholderia spp.					0.2		0.2	
Propionibacterium spp.	0.2						0.2	
Listeria monocytogenes	0.2		0.1			0.1	0.2	
Bacillus spp.	0.1		0.1		0.2	0.1	0.2	
Fusobacterium spp.	0.1						0.1	
Campylobacter fetus	0.1						0.1	
Corynebacterium spp.	0.3		0.1			0.1	0.3	
Peptococcus spp.	0.1						0.1	
Veillonella spp.	0.02						0.02	
Aspergillus fumigatus	0.02						0.02	
Other Gram-negatives (cumulated, not further specified)	1.5	6.2	4.3			1.5	6.2	
Other Gram-positives (cumulated, not further specified)		1.9	0.6		0.5	0.5	1.9	
Unspecified bacteria (cumulated)		3.2					3.2	
Unspecified fungi (cumulated)	1.3	1.7				1.3	1.7	
Sum	**5.7**	**13.2**	**7.6**	**1.4**	**1.8**	**1.4**	**13.2**	
Total	**100.2**	**109.7**	**98.3**	**99.2**	**100.0**			

gens (Table 1) fall into only 7 classes (Bacillales, Enterobacterales, Lactobacillales, Pseudomonadales, Bacteroidales, Clostridiales and Corynebacteriales).

There is a long record of further bacteria involved in rare cases of sepsis. A Google Scholar search under the term “rare pathogens in sepsis” resulted in 54 case reports uncovering an enormous diversity of unusual aetiologies of bacteraemia. Most of the identified bacteria normally live in the environment, on plants and in animals, and can give rise to sepsis in patients with neutropenia, meningitis, endocarditis, diabetic foot ulcers or catheterised, in the course of transplantations and surgeries, in neonates, after cat and dog bites and in other incidences. Table 2 sorts the pathogens into 40 families and 27 classes to underline the enormous diversity of bacterial species involved in septicaemia. This list does not claim to be comprehensive. Nonetheless, these case reports emphasise the notion that unusual bacteria stem from taxonomically highly diverse groups.

This diversity of pathogenic bacteria can pose problems to the cultivation and the identification of strains in clinical routine, because standard media not always meet their growth conditions and phenotypic tests may not represent their characters for identification purposes. In fact, the diagnostic procedure of unexpected aetiologies of sepsis can be laborious and time-consuming and standard phenotypic identification practices may fail or lead to misidentification. For instance, in their report Malkan et al. (15) described a case of an anaemic patient supported with iron by a venous infusion port catheter. The patient developed sepsis by a methicillin-sensitive S. aureus which was treated with daptomycin. At day 3, the catheter was removed. Cultures of the device were positive with Gram-positive cocci and Gram-negative

Table 2: Cultured rare strains causing sepsis and detectability by 16S rRNA gene PCR and sequencing.

Class / family	Species	Diagnosis	Reference	Strains found by direct 16S rDNA on taxonomic level [a]	No. species found (no. genera) [b]	Primers binding [c]
Actinomycetales						
Actinomycetaceae	*Trueperella bernardiae*	sepsis, diabetic foot ulcer	(7)	Actinomycetaceae	16 (3)	yes
Aeromonadales						
Aeromonadaceae	*Aeromonas hydrophila*	paediatric sepsis	(8)	*A. hydrophila*	1	yes
Succinivibrionaceae	*Anaerobiospirillum succiniciproducens*	sepsis after cat bite	(9)	Aeromonadales		yes
Alteromonadales						
Shewanellaceae	*Shewanella algae*	neonatal sepsis	(10)	*Shewanella*	2	yes
Bacillales						
Paenibacillaceae	*Paenibacillus amylolyticus*	bacteraemia	(11)	*Paenibacillus*	2	yes
Bacillaceae	*Lysinibacillus fusiformis/sphaericus*	bacteraemia	(11)	Bacillaceae	79 (10)	yes
Bacteroidales						
Prevotellaceae	*Prevotella buccae*	sepsis	(12)	*Prevotella*	7	yes
Burkholderiales						
Burkholderiaceae	*Burkholderia cepacia*	neonatal sepsis	(13)	*B. cepacia*	10	yes
	Ralstonia picketti	neonatal sepsis	(14)	*R. picketti*	5	yes
Comamonadaceae	*Acidovorax avenae*	catheter-related	(15)	*A. avenae*	6	yes
	Comamonas terrigena	sepsis, endocarditis	(16)	*C. terrigena*	5	yes
	Delftia acidovorans	sepsis	(17)	*D. acidovorans*	3	yes
Bifidobacteriales						
Bifidobacteriaceae	*Gardnerella vaginalis*	sepsis, post partum	(18)	*G. vaginalis*	1	yes
Campylobacterales						
Campylobacteraceae	*Campylobacter jejuni*	maternal sepsis	(19)	*C. jejuni*	4	yes
Caulobacterales						
Caulobacteraceae	*Brevundimonas vesicularis*	neonatal sepsis	(20)	*B. vesicularis*	6	yes
Chlamydiales						
Chlamydiaceae	*Chlamydophila abortus*	septicaemia	(21)	none	0	no result
Coriobacteriales						
Atopobiaceae	*Atopobium* spp.	sepsis	(22)	*Atopobium*	2	yes
Corynebacteriales						
Nocardiaceae	*Nocardia* spp.	sepsis	(23)	*Nocardia*	11	yes
	Rhodococcus equi	neonatal sepsis	(24)	*R. equi*	4	yes
Tsukamurellaceae	*Tsukamurella paurometabolum*	catheter-related	(25)	*Tsukamurella*	6	yes
Enterobacterales						
Enterobacteriaceae	*Cronobacter* spp.	neonatal sepsis	(26)	*Cronobacter*	2	yes
	Leclercia adecarboxylata	sepsis	(27)	Enterobacteriaceae	71 (19)	yes
	Shigella sonnei	sepsis, neutropaenia	(28)	*S. sonnei*	4	yes
Hafniaceae	*Edwardsiella tarda*	neonatal sepsis	(29)	Hafniaceae	1	yes
	Hafnia alvei	sepsis	(30)	*H. alvei*	1	yes
Morganellaceae	*Morganella morganii*	neonatal sepsis	(31)	*M. morganii*	1	yes
	Providencia stuartii	sepsis	(32)	*P. stuartii*	3	yes
Erwiniaceae	*Pantoea dispersae*	neonatal sepsis	(33)	*P. dispersae*	6	yes
Yersiniaceae	*Rahnella aquatilis*	sepsis, contaminated intravenous fluid	(34)	*R. aquatilis*	1	yes
Unclassified	*Plesiomonas shigelloides*	sepsis, meningitis	(35)	*P. shigelloides*	1	yes
Erysipelotrichales						
Erysipelotrichaceae	*Erysipelothrix rhusiopathiae*	sepsis, endocarditis	(36)	Erysipelotrichaceae	1	no result
Flavobacteriales						
Flavobacteriaceae	*Capnocytophaga canimorsus*	sepsis, dog bite	(37)	*Capnocytophaga*	2	yes
	Chryseobacterium indologenes	neonatal sepsis, meningitis	(38)	*C. indologenes*	5	yes
	Elizabethkingia (Chryseobacterium) meningosepticum	sepsis, diabetic patient	(39)	*E. meningosepticum*	1	yes
	Empedobacter brevis	neonatal sepsis	(40)	Flavobacteriaceae	12 (6)	yes
Fusobacteriales						
Fusobacteriaceae	*Fusobacterium necrophorum*	sepsis	(41)	*F. necrophorum*	7	yes
Leptotrichiaceae	*Leptotrichia trevisanii*	sepsis,	(42)	*Leptotrichia*	1	yes
Lactobacillales						
Leuconostocaceae	*Leuconostoc* spp.	neonatal sepsis	(43)	*Leuconostoc*	2	no result
	Weissella confusa	bacteraemia	(44)	*W. confusa*	4	yes
Micrococcales						
Micrococcaceae	*Kocuria kristinae*	paediatric sepsis	(45)	*K. kristinae*	7	yes
	Rothia dentocariosa	paediatric sepsis	(46)	*R. dentocariosa*	5	yes
Promicromonosporaceae	*Cellulosimicrobium cellulans*	neonatal sepsis	(47)	*Cellulosimicrobium*	1	yes
Neisseriales						
Chromobacteriaceae	*Chromobacterium violaceum*	paediatric sepsis	(48)	none	0	yes
Neisseriaceae	*Eikenella corrodens*	postanginal sepsis	(49)	Neisseriaceae	19 (4)	yes
Pasteurellales						
Pasteurellaceae	*Pasteurella multocida*	neonatal sepsis, meningitis	(50)	*P. multocida*	5	yes

Table 2: Continued.

Class / family	Species	Diagnosis	Reference	Direct 16S rDNA result on taxonomic level [a]	No. species found (no. genera) [b]	Primers binding [c]
Pseudomonadales						
Moraxellaceae	*Moraxella catarrhalis*	sepsis, cellulitis	(51)	*M. catarrhalis*	5	yes
Rhizobiales						
Rhizobiaceae	*Agrobacterium tumefaciens (radiobacter)*	catheter-related sepsis	(52)	*A. tumefaciens*	2	yes
Brucellaceae	*Ochrobactrum anthropi*	biliary sepsis	(53)	*O. anthropi*	7	yes
Sphingomonadales						
Sphingomonadaceae	*Sphingomonas paucimobilis*	paediatric sepsis	(54)	*S. paucimobilis*	22	yes
Tissierellales						
Peptoniphilaceae	*Peptoniphilus lacrimalis*	sepsis, diabetic foot ulcer	(7)	*P. lacrimalis*	10	yes
Veillonellales						
Veillonellaceae	*Veillonella parvula*	sepsis	(55)	*V. parvula*	5	no result
Vibrionales						
Vibrionaceae	*Vibrio vulnificus*	septicaemia	(56)	*V. vulnificus*	2	yes
Unclassified						
Gammaproteobacteria	*Ignatzschineria indica*	sepsis	(57)	none	0	yes
	Wohlfahrtiimonas chitiniclastica	sepsis	(57)	none	0	yes

[a] Results from evaluations of Molzym's products involving other diseases and clinical fluid and tissue materials; products tested: SepsiTest™-UMD CE, UMD-SelectNA™ CE or Micro-Dx™ CE (all include DNA extraction and PCR assay); amplicons from positive tests were Sanger-sequenced and Blast-analysed (sepsitest-blast.net and https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch)

[b] See also the complete list of organisms detected directly in clinical and other materials (https://www.molzym.com/images/products/Flyer_App_Notes/molzym_list-of-microbes_03_2018.pdf)

[c] Binding to 16S rRNA gene of cultured strain; Primer designing tool, NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/); specificity stringency: target sequences with 6 or more mismatches to forward or reverse primers or with 2 or more mismatches at 3' end were excluded (no result); 16S rRNA gene sequences were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/gene/)

rods. Same day blood cultures grew only Gram-negative rods and treatment was changed to gentamicin. Subculturing was performed on various media resulting in very small colonies, and Vitek 2 (bioMérieux) compact Gram-negative identification resulted in Cupriavidus pauculus. The bioMérieux API 20E system could not assign the organism to a certain species. At the end, definitive identification of the strain as Acidovorax avenae was reached by sequencing analysis of a 485 bp PCR amplicon of the 16S rRNA gene.

Ribosomal RNA gene sequencing in clinical practice

Ribosomal RNA gene sequencing is used in routine laboratories as a reliable method of strain identification. Mahlen and Clarridge (58), for instance, elaborated a strategy of broad-range 16S rRNA gene sequencing analysis of Gram-negative rods and coccobacilli as aid for routine identification methods. For strain identification, the rRNA genes or parts thereof are amplified in a PCR reaction. The reaction includes PCR primers that bind to sequences highly conserved among the kingdom of bacteria, while the sequences in-between vary among species. Identification of an unknown strain is achieved by comparison of its determined sequence to a library of sequences of known strains. Online tools are available that perform the assignment by an elaborated algorithm. Examples are the NCBI Basic Local Alignment Search Tool (BLAST®) leBIBI and Molzym's SepsiTest™BLAST.

Culture-independent diagnosis by rRNA gene sequencing

rRNA gene sequencing approaches, including Sanger and NGS sequencing of amplified and cloned 16S rRNA gene sequences, respectively, have been used for the direct, i.e., culture-independent analysis of bacterial strains and multiple infections for quite a while. Many of the reported methods are used for research purposes to answer specific scientific questions rather than for use of surveillance and in daily routine diagnostic practice.

rRNA gene sequencing promises to add benefit to culture diagnosis in two clinical situations: diagnosis of i) fastidious and anaerobic bacteria with unusual growth requirements and ii) growth-inhibited, but not cleared pathogens as a result of antimicrobial treatment of patients. Reported applications of the use of rRNA gene sequencing comprise of diseases like pneumonia, meningitis, thrombosis, pleuritis, septic arthritis, septic coxitis, intra-abdominal infection, intra-amniotic infection, brain abscess, recurrent cholecystitis, spondylitis, osteomyelitis, septicaemia, sepsis, subcutaneous abscess, spinal abscess, empyema (69), endophthalmitis (70), cystic fibrosis (71), infective endocarditis (72), urinary tract infections (73), airway inflammation (74), chronic venous leg ulcer (75), prosthetic joint infections (76) and others. Unlike other as-

says for direct analysis of a limited number of selected common pathogens, the unbiased nature of broad-range 16S rRNA gene PCR and sequencing follows a universal approach of the identification of common, rare and unusual pathogens.

Standardisation

The majority of methods for direct rRNA gene sequencing analysis of pathogens in clinical specimens is in-house developed tests and thus are not comparable among laboratories. Such tests differ in their pre-analytical and analytical procedures. A variety of commercial kits used in the laboratories are usually for DNA extraction from clinical samples and amplification. The problem is that chemicals, reagents and consumables are generally not produced under control of the absence of microbial DNA (77), because they are designed for applications where DNA contamination is irrelevant. Under these conditions the avoidance of false positive results in the diagnosis of low bacterial load materials is difficult to reach if not impossible. Moreover, the sensitivity and specificity of tests can be lowered by the vast load of human DNA in many clinical specimens (78). Another consequence is that Sanger and NGS sequencing analysis can be disturbed by human sequences from unspecific amplification (78).

The only unbiased 16S rRNA gene PCR and sequencing tests, which are CE-approved for the in-vitro diagnostics of hundreds of pathogens are provided by Molzym. The products are complete solutions for the whole process, including enrichment (depletion of human DNA) and extraction of DNA from bacteria present in samples at low loads (<100 cfu/ml), detection by Real-Time PCR amplification and sequencing analysis of the amplified hypervariable V3/V4 region of the 16S rRNA gene. By employing highly active and contamination-free PCR reagents, amplification runs of 40 cycles are performed without signals in negative PCR controls. Solutions are available for manual extraction, SepsiTest™-UMD, semi-automated extraction, UMD-SelectNA™, and fully automated extraction, Micro-Dx™ in the SelectNA™*plus* robot. Other, contamination-free research-use-only products provide options for the extraction of bacterial DNA from fluid (MolYsis™ kits) and tissue samples (Ultra-Deep Microbiome kit) and amplification of custom targets as well as defined parts of the 16S rRNA gene for qualitative, quantitative Real-Time PCR (Mastermix 16S/18S kits) and library preparation purposes (NGSeq 16S V3/V4). Validations in clinical studies and routine use have proven that unbiased 16S rRNA gene analysis can reliably detect and identify a great variety of microorganisms, including rare pathogens directly in fluid and tissue biopsies (59-68).

All of the common and rare species found in the studies discussed above (Table 1) have been shown to be detectable in other evaluations. Among the 54 unusual species listed in Table 2, 31 have been identified by Molzym's direct 16S rRNA gene sequencing approaches in studies employing other specimens. In 19 of the cases, 16S rRNA gene sequencing evaluations have not yet identified the same strains, but other species of the same genus, family or class of the cultured strains. No relatives of *Chlamydophila abortus*, *Chromobacterium violaceum*, *Ignatzschineria indica* and *Wohlfahrtiimonas chitiniclastica* have been found at the family or class level so far (Table 2). However, except for *C. abortus*, *in silico* testing of primer binding to the 16S rRNA gene targets was positive, indicating that the strains are detectable by the assay. Thus, 16S rRNA gene sequencing as represented by Molzym's molecular diagnostic kits have the potential to identify essentially all pathogens directly found in samples. The kits are CE-marked for the *in-vitro* diagnosis of pathogens.

Conclusions

Ribosomal RNA gene amplification and sequencing analysis is a frequently used method for the unbiased identification of rare and unusual pathogenic bacteria in clinical routine. In the past years, the method has been adapted to the culture-independent diagnosis of infected clinical materials at low loads of pathogens. With this approach, however, a variety of problems have been faced that disturb the precise diagnosis. Factors negatively influencing sensitivity, specificity and sequencing identification include, among others contamination of extraction and amplification reagents and consumables with microbial DNA and a great excess of human DNA. Molzym addressed these problems by the supply of contamination-free kits and reagents that enable the processing of human DNA-depleted preparations from a variety of clinical samples by just one protocol and the amplification over 40 cycles without background in negative PCR controls. By this solution hundreds of rare and unusual pathogens can be identified within hours without the need of cultivation.

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