Main S & T results and Foregrounds

From the public section of the Final Report (figures cannot be entered online)

The logo for the TEMPO-test-QC project is 

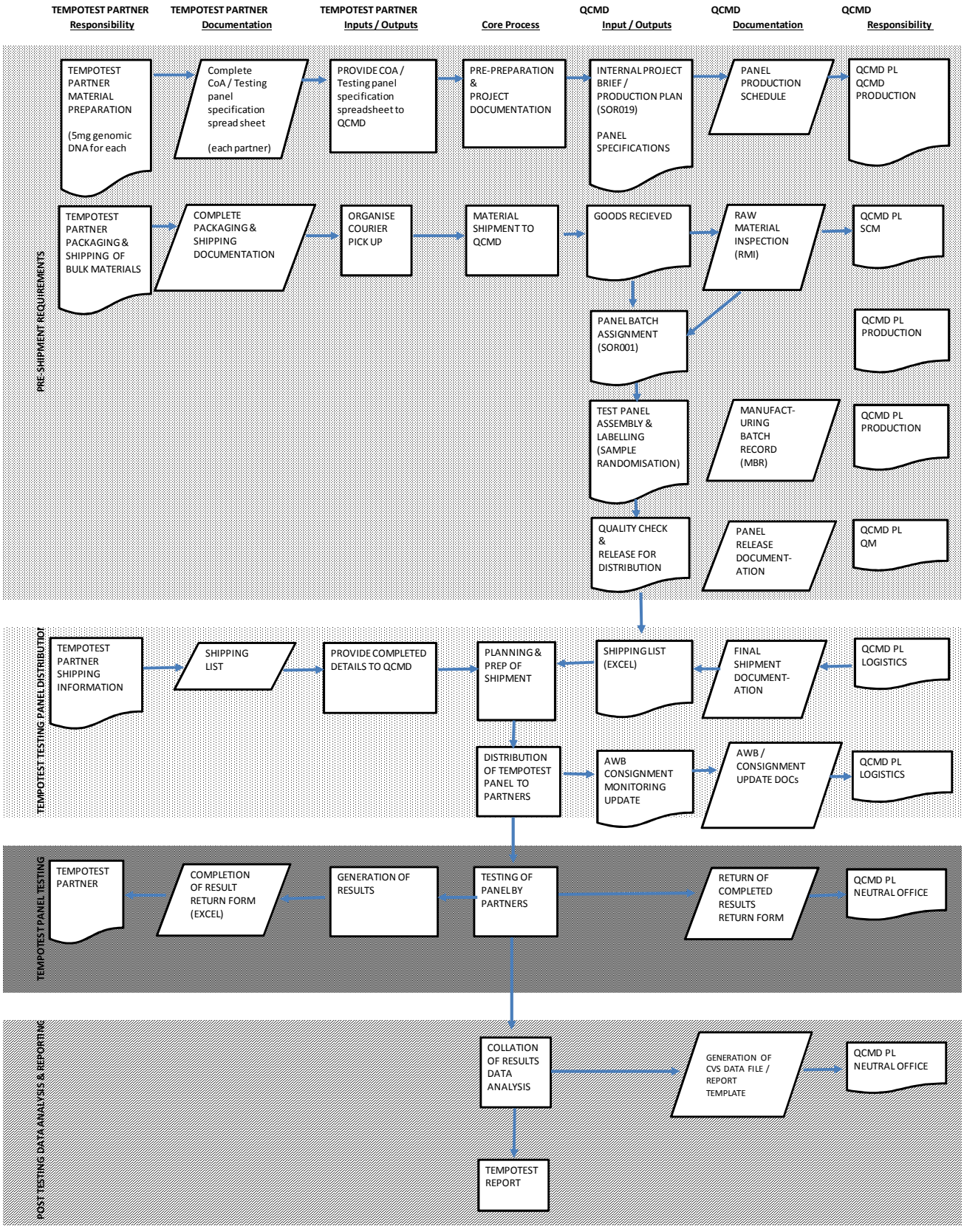
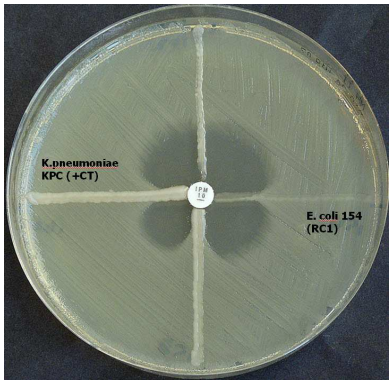


Figure 1: TEMPOtest-QC "Testing Study" Process

Technologies Evaluated and Validated - Publishable

1) Novel Microbial Culture Media

In a preliminary evaluaton study, 7 NDM-positive *Acinetobacter baumannii* isolates of worldwide origin were studied to evaluate the best technique for their identification. The detection of *A. baumannii* carbapenemase producers based on the measurement of carbapenemase activity by UV spectrophotometry, or by the modified Hodge test, failed (in contrast to results normally observed for carbapenemase producing Enterobacteriaceae). Considering that many microbiology laboratories do not possess the facilities for the molecular detection of these organisms, it was recommended that carbapenem-resistant *A. baumannii* isolates should be first screened using EDTA inhibition-based techniques followed by further PCR -based techniques in a reference laboratory.



In a separate evaluation study, the sensitivity of the modified Hodge test for detecting NDM-1 producers (low detection rate of 50%) was increased to 85.7% by adding ZnSO4 (100 µg/ml) to the culture medium, though the resulting test had a low specificity and was found to be time-consuming.

2) PCR and Sequencing

The carbapenemase NDM-1 has been identified recently in Enterobacteriaceae and *Acinetobacter baumannii* as a source of multidrug resistance, including resistance to carbapenems. Partner UPS analyzed the immediate genetic environment of the blaNDM-1 carbapenemase gene among a series of NDM-1-producing enterobacterial isolates, identifying a novel gene (bleMBL, for ble gene associated with the metallo-β-lactamase NDM-1). The bleMBL gene encodes a novel bleomycin resistance protein (BRP), named BRPMBL, that shares weak similarities with known BRPs (less than 60% amino acid identity). The expression of BRPMBL conferred resistance to bleomycin and to bleomycin-like molecules in Enterobacteriaceae and A. baumannii. The blaNDM-1 and bleMBL genes were coexpressed under the control of the same promoter, located upstream of the blaNDM-1 gene and at the extremity of the insertion sequence ISAba125. Most of the NDM producers possessed the bleMBL gene. Although BRPMBL did not modify the growth or death rates of *Escherichia coli* under experimental conditions, it suppressed the mutation rate of hypermutable *E. coli* and therefore may stabilize the plasmid-borne blaNDM-1 gene. This study suggests that the emerging carbapenemase NDM-1 is selected by bleomycin-like molecules, and that BRPMBL producers (and consequently NDM producers) are better suited to various environments. The addition of bleMBL gene/protein detection to Point-of-Care test kits may provide advantages in helping detect antimicrobial resistance and a potential marker for bacterial virulence.

Partner UPS was also involved in characterizing a novel group IIC intron targeting the core site of the aadA1 gene cassette attC site (aadA1-qacEΔ1 gene cassette junction) from enterobacterial isolates. Intron mobility (retrohoming) was analyzed using a two-plasmid assay performed in *Escherichia coli*. Intron mobility assays confirmed the mobilization-integration of the group II intron into the core site of the aadA2, blaVIM-2, blaCARB-2,

aac(6′)-Ib, dfrXVb, arr2, cmlA4, and aadB gene cassettes but not into the attI site. This mobility was dependent on maturase activity. This element was linked to the blaVEB-1 extended-spectrum β-lactamase gene in a high number of enterobacterial isolates. These findings could be useful in the development of Point-of-Care kit testing technology that may be used to follow and predict the "flow" and "construction" of mobile integrons that are associated with the spread of antimicrobial resistance.

3) Spectrophotmetry

Carbapenem-hydrolyzing ß-lactamases are the most powerful ß-lactamases being able to hydrolyse almost all ß-lactams. They are mostly of the KPC, VIM, IMP, NDM, and OXA-48 type. A spectrophotometry technique based on analysis of the imipenem hydrolysis was developed by partner UPS that differentiated carbapenemase- from noncarbapenemase producers. This inexpensive technique can be easily adapted for the screening of bacterial carbapenemase producers may be implemented in any reference laboratory worldwide.

4) Microarray (CheckPoints)

A new commercial low-density microarray Check-Points ESBL/AmpC/KPC/NDM-1 assay (Check-MDR CT101; Check-Points B.V., Wageningen, The Netherlands), which identifies common extended-spectrum β-lactamase plasmid-mediated cephalosporinase genes, as well as carbapenemase (blaKPC and blaNDM) genes, was evaluated. Partners UPS and UCL helped test 207 clinical and reference/collection isolates of the Enterobacteriaceae that possessed different beta-lactamase (*bla*) genes. Overall, the sensitivity and specificity of the microarray were 100% for the detection of the plasmid-mediated blaAmpC, blaKPC, and blaNDM genes using *bla* gene sequencing as the reference method.

Partners UPS and UCL were also involved in evaluation and validation of the Check-MDR CT102 microarray, aimed at identifying bacteria producing extended-spectrum β-lactamases (ESBL) (SHV, TEM, and CTX-M) and carbapenemases (KPC, OXA-48, VIM, IMP, and NDM-1). This commercial microarray was evaluated using a total of 144 Gram-negative bacterial strains expressing various β-lactamases. The sensitivity and specificity were found to be 100% for most of the tested genes, suggesting that this assay allows accurate identification of common ESBL and carbapenemase producers from bacterial cultures.

Check-MDR CT 101

5) MIC test strips (Liofilchem)

MIC Test Strips are commercial products from Liofilchem (Italy) for the quantitative assay of the Minimum Inhibitory Concentration (MIC) of antimicrobial agents. The product consists of porous strips impregnated with a predefined concentration gradient of antibiotic, across 15 two-fold dilutions of a conventional MIC method. UNISI have helped the company to optimize their products by testing, among others, a number of well-characterized gram negative microbial isolates, including several multiresistant strains with emerging resistance mechanisms, from the TEMPOtest-QC bacterial archive. The results from this testing,

presented at the 21st European Congress of Clinical Microbiology and Infectious Diseases held in Milan in May 2011 (Conte et al., Poster P572), demonstrated the overall good performance of the product, that was at least good as the comparators (determination of MIC according to standard microdilution method or by using a commercial, plastic based similar product) in the determination of antibiotic MICs.

6) <u>Automated bacterial count and sensitivity analyzer</u> (Alifax)

HB&L (Alifax) is an automated system able to perform bacterial count and susceptibility testing directly on liquid clinical samples (such as urine) using light scattering technology providing quantitative bacterial count results in CFU/ml.

UNISI evaluated the performance of this system both for antimicrobial susceptibility testing and bacterial count on respiratory samples (bronchoalveolar lavage).

A step of validation of the antibiotic compounds and of colony count has been needed before working on clinical samples. Some of the isolates selected from the UNISI microbial archive of the TEMPOTEST-QC project were used in this validation step.

The HBeL system has demonstrated good performance in colony count and in the characterization of resistance pattern using reference strains. Using clinical samples the performance was high with ability to detect and perform colony count within 3 hours and susceptibility testing within 6 hours.

7) <u>MicroScan WalkAway-96</u> (UCL)

The detection of extended-spectrum β-lactamases (ESBL) is a challenging task for diagnostic laboratories. Parner UCL evaluated the performance of the MicroScan WalkAway-96 (MScan) instrument (Siemens, Figure 13) for identification and detection of ESBL-producing EB (ESBLE).

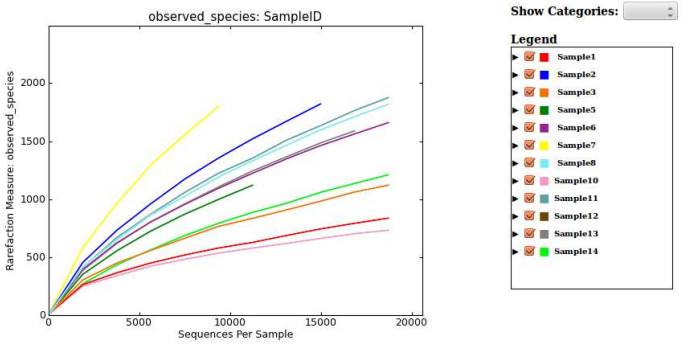
Partner UCL showed that species identification of *Enterobacteriaceae* by MScan is excellent (98% correct). Concerning ESBL detection, the system seems more adapted to non-AmpC expressing strains (95.8 % sensitivity) than for AmpC producing species (66% sensitivity). Differentiation between AmpC and ESBL producing strains remains challenging especially among AmpC producing strains.

8) Automated Microbiology System (UNISI/ Becton Dickinson)

The Phoenix™ Automated Microbiology System (BD Diagnostics, Sparks, MD, USA) is designed for the rapid bacterial identification at the species level and for the determination of Antimicrobial Susceptibility Testing (AST) of clinically significant human bacterial pathogens. Performance of this system has been previously evaluated using the breakpoints from Clinical Laboratory Standard Institute (CLSI) but not with those from the European Committee of Antimicrobial Susceptibility Testing (EUCAST). UNISI, together with the Laboratory of Microbiology of the Ramón y Cajal Hospital (Madrid, Spain), have evaluated the performance of the Phoenix system for the determination of bacterial AST using the EUCAST breakpoints. The library of tested isolates with well-defined phenotypes and well-characterized resistance mechanisms included, among others, bacteria from the TEMPOTest-QC archive. The tested system have shown accuracy assessment in accordance with the ISO standards when using EUCAST breakpoints. Results from this study were published in Giani et al., Clin Microbiol Infect 2012.

9) Next Generation Sequencing (Erasmus MC/UCL/BioMérieux)

Next Generation Sequencing (NGS) is a novel technology that is very sensitive for the detection of bacterial pathogens in clinical samples, with current research involving the identification of novel microorganisms associated with disease (Rigsbee et al doi:10.1038/ajg.2012.287). The technique may also be evaluated for routine use in diagnostic microbiology laboratories (Köser et al doi:10.1371/journal.ppat.1002824) and for the detection of novel antimicrobial resistance mechanisms and virulence factors (Pehrsson et al doi: 10.3389/fmicb.2013.00145; Mellmann et al doi:10.1371/journal.pone.0022751.g001), which once identified could be used to generate novel diagnostic kits and the treatment therapies in cases of multidrug resistant bacteria. However, a critical factor in the detection of bacterial pathogens using a non-selective technique such as NGS, is specimen quality, as incorrect specimen storage can lead to the overgrowth of potential bacterial pathogens by non-pathogenic bacterial species. The most microbiology laboratories there is a delay between accessing a sample, delivery to the laboratory and finally processing of the sample. In this respect, we investigated the effect of storage conditions (immediate freezing at -80 oC, storage at room temperature and storage at room temperature with a "stabilising" buffer) and methodology (bead beating versus no bead beating) using feces as an example of a clinical material that could be examined using NGS. The results showed a wide variation in the apparent ratio of different bacterial families dependent on sample storage conditions and that the use of a bead beater has an impact on the detection of Gram positive bacteria. These results should be taken into consideration, if NGS is to be used for microbiological diagnosis and/or the detection of novel antimicrobial resistance and virulence genes.



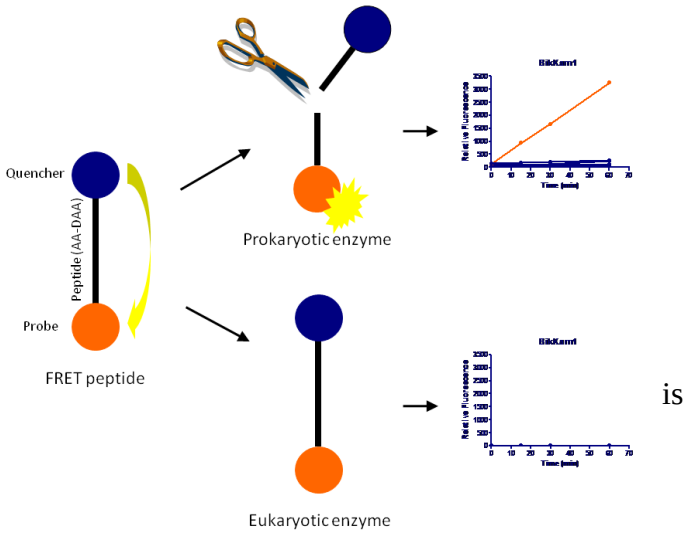
SampleID	Seqs/Sample	PD_whole_tree Ave.	PD_whole_tree Err.	chao1 Ave.	chao1 Err.	equitability Ave.	equitability Err.	observed_species Ave.	observed_species Err.	shannon Ave.	shannon Err.	simpson Ave.	simpson Err.
Sample1	10.0	2.768	nan	28.000	nan	0.982	nan	8.900	nan	3.094	nan	0.876	nan
Sample1	1880.0	19.903	nan	502.096	nan	0.800	nan	263.600	nan	6.433	nan	0.977	nan
Sample1	3750.0	24.713	nan	765.725	nan	0.765	nan	364.800	nan	6.512	nan	0.977	nan
Sample1	5620.0	28.463	nan	980.611	nan	0.743	nan	449.400	nan	6.551	nan	0.977	nan
Sample1	7490.0	31.507	nan	1196.403	nan	0.728	nan	518.500	nan	6.568	nan	0.977	nan
Sample1	9360.0	33.998	nan	1371.393	nan	0.717	nan	578.900	nan	6.582	nan	0.977	nan
Sample1	11230.0	36.157	nan	1523.469	nan	0.710	nan	627.000	nan	6.600	nan	0.977	nan
Sample1	13100.0	38.263	nan	1682.240	nan	0.701	nan	686.700	nan	6.607	nan	0.977	nan
Sample1	14970.0	40.417	nan	1878.933	nan	0.694	nan	743.400	nan	6.619	nan	0.977	nan
Sample1	16840.0	42.450	nan	2013.517	nan	0.688	nan	791.400	nan	6.626	nan	0.977	nan
Sample1	18710.0	44.343	nan	1963.159	nan	0.683	nan	836.300	nan	6.632	nan	0.977	nan
Sample1	10.0	2.209	nan	23.900	nan	0.966	nan	8.200	nan	2.924	nan	0.854	nan
Sample1	1880.0	26.800	nan	1423.387	nan	0.694	nan	454.000	nan	6.125	nan	0.925	nan
Sample1	3750.0	36.941	nan	2131.836	nan	0.663	nan	730.900	nan	6.304	nan	0.925	nan

Example of Results Obtained using Next Generation Sequencing on Feces.

The identification of the resistance mechanisms to beta-lactams in *Pseudomonas aeruginos*a by phenotypic tests is a challenging task because of the frequent overlap and interplay between enzymatic (acquired beta-lactamases, resistance mutations in chromosomal genes) and non enzymatic resistance mechanisms (overexpression of active efflux pumps, porin deficiencies etc). Partner UCL collected 10 multidrug resistant *P. aeruginosa* clinical strains for which conventional phenotypic and molecular testing could not identify the underlying resistance mechanism. UCL explored the potential of next generation sequencing to identify the unknown mechanisms of resistance. BioMérieux was especially interested to identify the role of some peptides (known or suspected) to be linked to resistance genes and to explore the involvement of several porins especially in resistance to carbapenems. Sequencing was hence performed by DNAVision (Gosselies, Belgium) and data were analysed in collaboration with the laboratory of Genomics at the Hospital Universitaire de Genève (HUG, Prof. Jacques Schrenzel) linked to BioMérieux. The whole genome 'including plasmid" of the 10 strains was obtained and reassembled. In a first step, comparison with other known genomes/genes focused on the search of new, (as yet unidentified beta-lactamase) coding genes. Based on homology comparison in PFAM, 241 beta-lactam hydrolysing candidates were selected for further investigation. Among these, 2 were cloned and expressed in *E. coli* but did not confer resistance to the recipient strains. Other candidates are currently being screened for beta-lactamase activity. All the sequences were also investigated in relation to specific peptides, porins in order to identify new targets for resistance diagnostic tests. The strains are also being analysed by Electron Spray, Mass spectrometry (EIS-MS-MS).

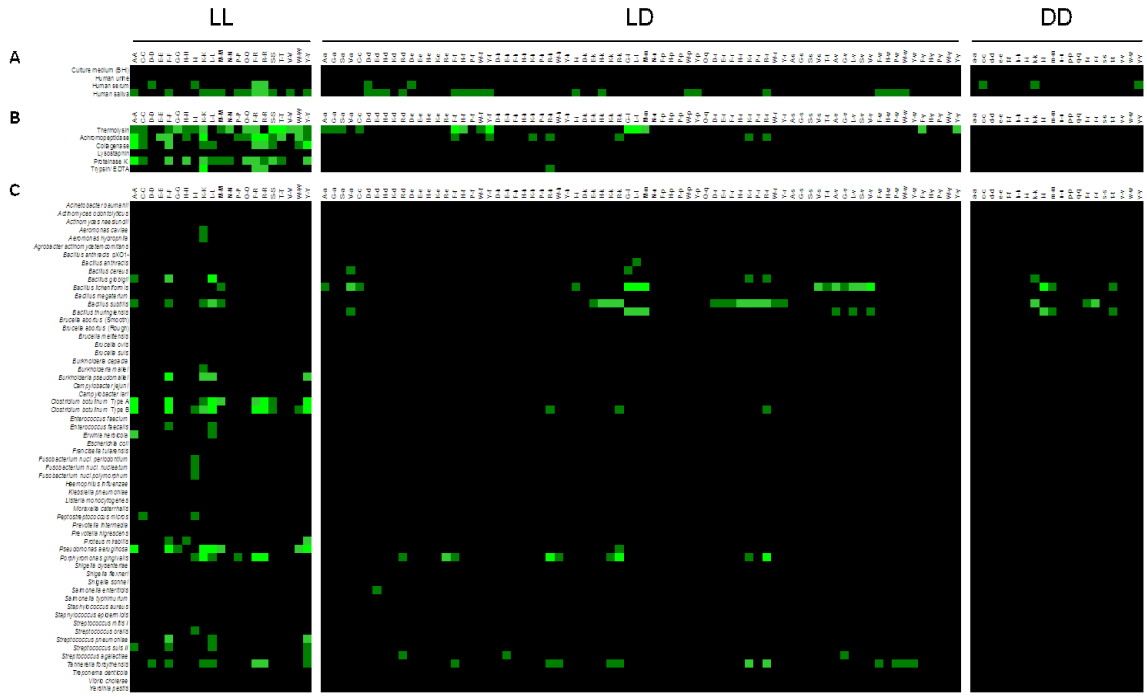
10) <u>Peptide-based Fret probes</u> (Academisch Centrum Tandheelkunde Amsterdam)

Within the project the use of peptide-based Fret probes was evaluated and validated as a rapid and simple assay for the diagnosis of bacterial infections. The simplicity of the technique would make the test well suited for development into a point-of-care device. The assay is based on the detection of bacterial proteases using FRET-labeled and quenched di-peptide probes. If the peptide sequence recognized by a bacterial protease, the peptide probe is cleaved and the distance between the quencher and the probe increases, resulting in spatial separation of the Fret reporter and quencher molecules and an increase in fluorescence signal. Incorporation of D-amino acids in the peptide sequence could enhance the bacterial specificity by decreasing the ability of proteases of eukaryotic origin to cleave the substrate (Figure 3). This makes the assay suitable for diagnosis *in situ*; without the need of pre-isolation of the protease of interest.



Principal of the FRET-peptide Substrates. Bacterial proteolytic activity is detected using di-peptide FRET probes. Bacterial specificity could be measured via the incorporation of bacteria-specific D-amino acids, which are not recognized by eukaryotic proteases.

In order to search for novel, bacteria-specific, substrates a FRET-peptide substrate library was screened using culture supernatants from a broad spectrum of bacterial spp. including bacteria preset in the TEMPOtest-QC bacterial archive. This revealed several species-specific substrates. For example 5 substrates were found to specifically recognize he periodontal pathogen *Porphyromonas gingivalis* (Table 1, BikKam9-13).



Heat Diagram of High-Throughput Screening of Bacterial Isolates for FRET-probe Cleavage Activity

Table 1. *P. gingivalis* specific substrates.

Fret Probe	Sequence
BikKam9	FITC-Arg-DAsp-KDbc
BikKam10	FITC-Arg-DGlu-KDbc
BikKam11	FITC-Arg-DHis-KDbc
BikKam12	FITC-Arg-DLys-KDbc
BikKam13	FITC-Arg-DArg-KDbc

The Point-Of-Care applicability of these substrates in the diagnosis of *P. gingivalis* related periodontitis was tested using patient material, so called paper points. The sensitivity and specificity of the substrates was compared to gold standard conventional anaerobic culture. Results were also compared to the activity of a commercially available test used in the diagnosis of periodontitis, L-BApNA. In evaluation testing, all five BikKam substrates had a higher sensitivity and specificity compared to L-BApNA. At the current moment in time research is ongoing regarding the the development of BikKam13 substrate into a rapid and Point-Of-Care “dental chair” test.

11) <u>High-throughput MultiLocus Sequence Typing – HiMLST</u> (Streeklab Haarlem)

A novel high-throughput bacterial genotype characterization technique has recently been described (Boers SA, van der Reijden WA, Jansen R (2012) High-Throughput

Multilocus Sequence Typing: Bringing Molecular Typing to the Next Level. PLoS ONE 7(7): e39630. doi:10.1371/journal.pone.0039630). This technique will be very useful in helping facilitate genotype characterization during screening, outbreak and detection/identification studies of bacterial pathogens. Further, advances in miniaturization and the reduced costs of next generation sequencing technologies and apparatus means that the type of apparatus required for HiMLST-related Point-of-Care diagnostics (including hospital-based Point-of-Care diagnostics), is already available within many academic hospitals. Here we showed the applicability of the HiMLST technique to identify and characterize genotypes of bacterial pathogens circulating within a hospital environment, thereby providing infection control experts with a means to identify, monitor and ultimately prevent the spread of antibiotic resistant bacteria within hospitals. This technique will replace standard (time-consuming and relatively expensive) MLST sequencing technologies in the near future.
Extended spectrum beta-lactamase (ESBL) expressing Enterobacteriaceae represent a major threat to global health and are particularly associated with nosocomial infections. Phenotypic, genetic and genotypic screening was performed to determine the dynamics of ESBL-positive *E. coli* isolated from a Dutch university medical centre between 2008 and 2010. First isolation *E. coli* isolates were collected during 2008 and 2010 from the Erasmus University Medical Centre and an arbitrary selection of 194 phenotypically ESBL-positive isolates was genotyped using a recently described high-throughput multi-locus sequence typing technique (HiMLST). PCR screening was used to determine the distribution of ESBL genes CTX-M (groups 1, 2 and 9). Our results (in preparation) indicate that HiMLST can be utilized as a high-throughput diagnostic test for screening, identifying and following bacterial pathogen genotypes over time within the nosocomial environment. This work is being followed by Roche (Diagnostics Division), Almere, the Netherlands.

12) <u>Optical Mapping</u> (Piext BV)

Optical mapping is a technique for constructing ordered, genome-wide, high-resolution restriction maps from single, stained molecules of DNA, called "optical maps". By mapping the location of restriction enzyme sites along the unknown DNA of an organism, the spectrum of resulting DNA fragments collectively serve as a unique "fingerprint" or "barcode" for that sequence (www.opgen.com). This technique is not only useful for genotypically characterizing bacterial isolates, but may also be used to detect the presence of large insertion sequences and bacteriophages present within the genome of a particular bacterial clone. More specifically, for diagnostic purposes, the presence of large insertion sequences and bacteriophages could indicate increased virulence associated with particular genetic clones of bacteria, a fact that may be useful for decisions relating to patient treatment and personalized medicine. Moreover, if such virulence-related regions are discovered, either: 1) optical mapping per se may be utilized to detect these regions in future microbial pathogen outbreaks, or 2) simple PCRs may be developed focusing on the virulence regions identified using optical mapping. This work is ongoing. Using this technique will allow rapid and specific Point-Of-Care diagnostic technologies e.g. PCRs, to be developed to detect otherwise hidden virulence traits.

13) <u>RNA sequencing</u> (Erasmus MC)

The detection of virulence and antimicrobial resistance in bacteria has been traditionally performed using phenotypic, culture-based methods, or more recently using

DNA detection-based methods. However, the expression of virulence factors and antimicrobial resistance genes, as well as accessory genes that play a role in the development of antimicrobial resistance, for example the expression of previously uncharacterized porin genes, may also be important contributing factors. In this respect, an RNA sequencing approach was used to try to identify genes that were overregulated in a bacterial isolate with reduced sensitivity to ceftazidime. Unfortunately, experiments indicated that though RNA sequencing is frequently used in eukaryotic biology e.g. in human oncology experiments, the technique itself has to be rigorously optimised for use in prokaryotic RNA sequencing methodologies. In particular, problems were encountered with respect to the removal of ribosomal 16S rRNA, possible environmental contamination, and loss of RNA during specimen processing. Further experiments will be required, outside of the TEMPOtest-QC project in order to further optimise the results obtained using RNA sequencing. However, this technique still offers the possibility of detecting novel virulence and antimicrobial resistance associated accessory genes, that may be important in future diagnostic test kits.