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FAPIC Final report

FAPIC			
Fast Assay for Pathogen Identification and Characterisation			
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Contributors	
Beneficiary	Name(s)
UCBL	Christophe Marquette
AIT Austrian Institute of Technology	Ivan Barisic – Michaela Hendling – Rick Conzemius
University of Warwick	Elisabeth Wellington – Rui Abreu-Pereira – Séverine Rangama – Chiara Borsetto
University of Zagreb – School of Medicine	Branka Bedenic - Saša Likić
Radboud University Medical Center / Hasselt University	Valentino D'Onofrio - Inge Gyssens
AXO Science	Benjamin Corgier – Laetitia Villiers
Molzym	Michael Lorenz – Christine Baussmerth – Lara-Antonia Beer
Bee Robotics	Steve Jones – Llifon Osian Jones – Euros Jones
LIP	Patricia Odet

Reviewers		
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1. Conclusions on the project

Sepsis, a life-threatening organ-dysfunction caused by a dysregulated host response to infection, is life threatening with high mortality. Fast identification of patients most at risk for severe outcomes is crucial to start appropriate therapy. In addition, the precise diagnosis is of outstanding importance because the optimal antibiotic can only be prescribed if the pathogenic agent and its susceptibility have been identified.

Bacterial pathogens frequently encountered in human infections are genetically diverse but can share phenotypic traits in common hence the need for DNA-based identification. In addition, bacteria can acquire many traits via horizontal gene transfer, clinically important examples include antibiotic resistance and virulence factors that can have a significant impact on the infection and the therapy of a patient. Thus, to enable an optimal treatment, the underlying genetic mechanisms have to be identified using molecular detection technologies. However, the high multiplexing of DNA-based detection reactions is a challenging task because all relevant interactions of oligonucleotides cannot be computed in a reasonable time scale. Thus, the FAPIC partner AIT with the help of expertise at Warwick and AXO developed *in silico* and *in vitro* techniques that allowed us to improve the sensitivity and specificity of DNA-based assays. A key feature in design of such arrays is the understanding of bacterial diversity and the need for targeting both virulence and antibiotic resistance genes (ARGs) in addition to genes that have taxonomic value. Thus, we were able to provide markers for bacterial pathogens and determine if resistance genotypes were present. Warwick, Hasselt and Zagreb all contributed expertise on the key ARGs to be targeted based on experience from the study of clinically important resistance phenotypes and the genes responsible for resistance to antibiotic therapy in many life-threatening bacterial infections. In addition, the dissemination of such bacterial pathogens into the environment via human waste disposal is a further public health threat so an environmental array was developed at Warwick in collaboration with UCBL and AXO, with DNA extraction technique successfully designed in collaboration with Molzym.

In the road to fast and performant diagnostic detection using molecular biology, DNA extraction is one of the important steps. To solve the problem of lowering as much as possible the time-to-result of sepsis diagnosis Molzym's has developed, together with BEE Robotics, an automated method of enrichment and extraction of the DNA from bacteria present in whole blood samples. In particular, the approach was integrated into an overall concept of a near bedside molecular device for rapid pathogen analysis. This device, PathoDoc, consisted of a bench top robotic system that directed 1 ml blood samples through extraction, PCR amplification and hybridisation-based detection of pathogen DNA in a completely automated solution. Although molecular features of the systems were suboptimal in terms of diagnostic sensitivity against blood culture, their progressed state provides Molzym with a valuable technical basis and collaborative network of short- to mid-term development and marketing of molecular tools for rapid diagnostics in routine applications. The PathoDoc and array were subjected to detailed preclinical evaluation using spiked blood samples at Warwick which provided further data for optimisation.

From the clinical point of view and perspective, the FAPIC project allowed us to perform two clinical studies at Jessa hospital. The second and largest study included approximately 2000 patients with suspected sepsis presenting at the Emergency Department. Blood samples were collected from all patients together with clinical and laboratory parameters. Risk factors and innate immune response of these patients were analysed to allow for fast identification of high-risk patients. The study has also allowed for a clinical evaluation of the FAPIC diagnostic system, to rapidly identify causative pathogens. Both Hasselt University, and Radboudumc benefitted from these studies. Furthermore, the clinical study has resulted in an extensive biobank collection of blood samples from these patients which allows for future research on this life-threatening condition.

2. and their exploitation and dissemination

The development and evaluation of an ultraplex assay targeting the genetic mechanisms of the clinically relevant antibiotic resistance genes and virulence factors has been one of the main results. AIT is now investigating how the assay can be used to analyse the bacterial transcriptome which is important to understand the clinically observed phenotype. The *in silico* tools concerning the automated assays design are being published and will be further developed to meet other use cases such as the design of oligonucleotides for human diagnostics. Further work at Warwick was done to develop additional assays for environmental monitoring of pathogen dissemination and survival which will provide a basis for studying public health threats posed by inappropriate disposal of human waste.

The chemical and physical conditions of pre-analytical processes and their integration into robotic functions were adapted and consolidated in collaboration with the partner, Bee Robotics. In particular, processing of blood samples encompassed the optimisation of human cell lysis, human DNA degradation, filtration of the lysate, retention and on-membrane lysis of bacteria and purification of their DNA. This DNA

served for further analytical processes, including project-developed hybridisation-based array identification of pathogens. The prototypes were characterised in regard to limits of detection of selected pathogens (10^2 to 10^4 cfu/ml) and failure rates of processing of samples. An innovative protocol of monitoring of the correct function of the DNA extraction process with interruption and signalling of malfunction was developed in collaboration with Bee Robotics, introduced to PathoRobot and successfully tested with blood samples from healthy and sick individuals.

Pre-clinical and clinical validations of PathoRobot and Molzym's commercial SelectNA™*plus* (1 ml sample extraction) were done in comparison to culture. For this, EDTA-stabilised blood samples were taken from patients under suspect of sepsis at partners Hasselt and Zagreb. In a series of practical approaches, samples were shipped to Molzym for extraction or extracted on clinical site. Analysis was done by specific PCR amplification and DNA array analysis and, as a reference, universal 16S rRNA gene Real-Time PCR with sequencing analysis of amplicons from positive runs. The main result was that the sensitivity of molecular analysis of the blood of septic patients was at an inacceptable low level. The hypothesis was that the load of bacterial pathogens in the blood of septic patients was below the limit of detection (LOD) of the molecular systems. However, with other body fluids and tissues from another population of 29 endocarditis patients, SelectNA™*plus* coincided with all culture-proven positive results and at 91% at the species or genus level, including Gram-positive and Gram-negative bacteria as well as fungi (18S rRNA gene analysis). Here, the microbial load appeared to be above the LOD of the molecular device. While potentially applicable to other specimens, conceptual modifications and technical adaptations of the systems are still needed to be done for their use in blood stream analysis.

Two automated instruments were developed based on specifications and assay protocols developed during the project by the consortium and these were namely the **PathoDoc** and **PathoRobot**. The PathoDoc was developed to offer full automation for single samples providing DNA extraction, PCR cycling, microarray processing and imaging of the developed arrays at the end of the assay thus integrating four processes into one instrument. The instrument is operated from a separate PC with software developed by Bee as the operator interface where the operator is prompted on how to set up and run the assay. Once the sample and reagents are loaded onto the instrument all the assay steps are automated with the developed arrays being imaged at the end and sent to the PC for further analysis.

Although the instrument performs within specification some further testing is necessary to optimise the assay parameters for automation particularly reducing the overall time of the assay from start to finish to make the instrument a more viable product for the market. The PathoDoc can be further exploited as an open system for microarray assays where single samples need to be tested in a timely manner where the software can be adjusted to accommodate different assay protocols.

The PathoRobot was developed as a stand-alone DNA extractor particularly optimised for larger sample volumes (5ml). The concept of the DNA extraction method is the same as for the PathoDoc but can process up to 8 samples per run. The extraction is based on using filtered columns and vacuum where each channel has its own miniature vacuum pump and a blockage detection system which can shut off individual channels if a blockage is detected to avoid carry-over. The washing and incubation steps are fully automated where disposable tips are used to transfer reagents during the assay process and remove the extracted DNA from the columns to PCR tubes at the end of the assay. The assay protocol went through several iterations to optimise the process and further optimisation may be necessary around the mixing steps due to the higher reagent/sample volumes which may result in the peristaltic pump being replaced with piston pumps to avoid drift in the lines. This modification would further enhance the performance of the instrument and make it a more viable product for the market.

The clinical validation of a new multiple pathogen detection system (PathoRobot - AXOBot) was performed using 320 fresh blood samples collected from patients with suspected sepsis/bacteraemia in Hasselt and 405 fresh blood samples in Zagreb. The inclusion of a large number of patients and samples and the accompanying clinical data, which facilitated correlation of discrepant results to identify possible missed cases with blood cultures is a major strength. Furthermore, a comparison with routine blood cultures and with a commercially available MDx (SelectNA™plus) strengthened the validation. However, additional optimization of the system is necessary. After optimization, a clinical trial should be performed in a controlled setting to test the safety and efficacy of this diagnostic test. This study should also focus on the impact on antibiotic therapy management and patient outcomes. In addition, a second aim of this study was the discovery of novel biomarker profiles for the characterization of bacterial or viral etiology in sepsis/bacteremia patients. 406 patients, where the need for a fast characterization of the etiology of the infection exists, were selected from the large cohort in Hasselt for proteomics analysis by another partner, Radboudumc. This has also resulted in a new collaboration with the Interuniversity Institute of Biostatistics and Statistical Bioinformatics of Hasselt University, (Prof. Dr. Dirk Valkenborg) and the Flemish Institute of Technological Research (VITO, dr. Gohkan Ertaylan) for statistical analyses (final results are pending).

In total, 15 peer-reviewed publications in well-known international journals were published or are currently in press.

3. Socio-economic impact of the project

In FAPIC, we have developed **tools that allow us to better understand the genetic mechanisms that bacterial pathogens use to evade an antibiotic therapy**. The combined expertise on antimicrobial resistance (AMR) at Hasselt/Radboud, Zagreb and Warwick provided a unique training opportunity to train four PhD students and enrich three postdoctoral fellows with extensive expertise in diagnostics and the challenges of rapid pathogen ID for patients. This was usefully compared with the requirements for environmental pathogen monitoring which will meet future public needs for monitoring risks of pathogen dissemination and exposure. At the AIT, the project has made it possible to finalise three PhD theses and two Master theses. All of the team members involved in FAPIC, have continued to work in the field of molecular diagnostics and infectious diseases at the institute, a university or at companies.

The progressed state of the extraction systems gives reason to continue optimisation efforts after the end of the project. Further adaptions and validations of the prototypes of PathoDoc and PathoRobot employing specimens other than blood offer the opportunity of using them as tools for routine application at hospital and privately run laboratories that are among Molzym's customers in a short- to mid-term time frame. Thereby, diseases such as infectious endocarditis, bacterial meningitis, joint infections and others can be diagnosed within a day when culture needs much longer times. Moreover, the results indicate that molecular analysis uncovers infections where culture is negative. **By this way, patients can be optimally treated by a targeted chemotherapy in the absence of positive culture results.**

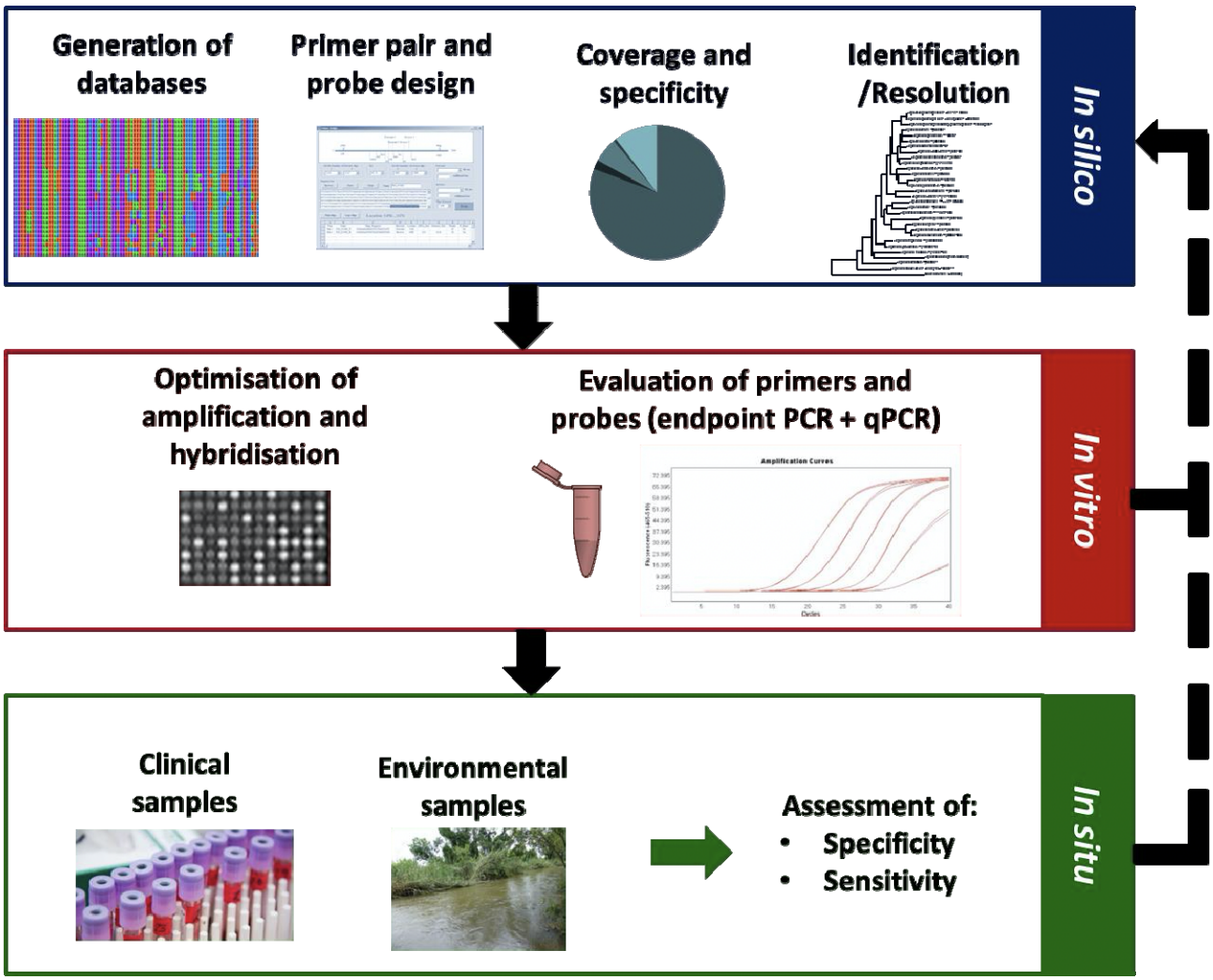
During the project the number of employees more than doubled at Molzym and the turnover increased by 39%. The project contributed to this positive development by implementing an innovative sample process monitoring protocol and other robotic optimisations in the current CE diagnostic tool, SelectNA™*plus*. This in turn drove sales and consolidated customers' satisfaction with Molzym's products.

Both the PathoDoc and PathoRobot once fully optimised could offer useful automated techniques for single sample testing and DNA extraction from larger sample volumes. With a big emphasis now on automation for laboratories where minimal sample handling by the operator is desirable automated instruments are becoming more important. Both instruments are prototypes and together with collaboration with other consortium parties further **R&D efforts would make both products viable to bring to market** which would have a positive impact on jobs and increased turnover.

At the emergency department, **ensuring a rapid diagnostic result in patients with life-threatening infections can help in a fast change to appropriate antibiotic therapy**, thereby reducing selective pressure for AMR. Assessment of the host response and risk factors early in the disease can help in triage and isolation measurements, and in better patient management. Rapid pathogen identification directly from blood can provide rapid microbiological results and thus in earlier start of more appropriate antibiotics, reducing the continuation of broad-spectrum antibiotics and thus reducing the selective pressure for AMR. An audit of empiric antibiotic therapy has also been performed. This analysis showed that adherence to antibiotic treatment guidelines in the Emergency department resulted in more patients receiving effective antibiotic therapy and was associated with better patient outcomes. The analysis also showed that changes in empiric antibiotic therapy were different (more de-escalation) when a microbiologist or ID physician was consulted. These results can provide Feedback and will allow improvement of guidelines and the existing antimicrobial stewardship program at Jessa hospital, leading to better patient management and outcomes. At UHasselt and Radboudumc, the project results have resulted in sufficient scientific publications for a joint PhD thesis between both universities. More importantly, a large biobank collection exists at Hasselt University. In the future, this collection provides opportunities for further research and development and can help in answering new research questions to further improve patient management in sepsis or to help tackle the threat of AMR.

4. Public project diagrams, photographs and videos illustrating the work done

The overall approach is summarised as follows:



The three stages in FAPIC for in silico, in vitro pre-clinical evaluation and the actual samples taken from patients and contaminated environments.

Patho-Robot loaded with consumables for enrichment and extraction of microbial DNA from 5 ml samples.

PathoDoc – Bench top unit providing automation for microarray assays from a single sample to imaging of the developed arrays at the end of the assay protocol.

PathoDoc Modules

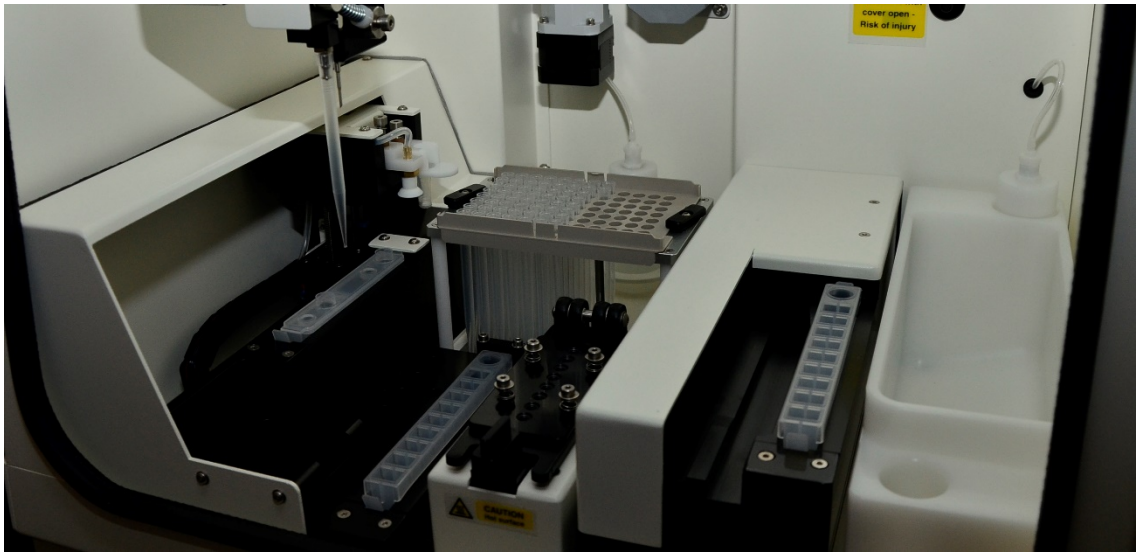
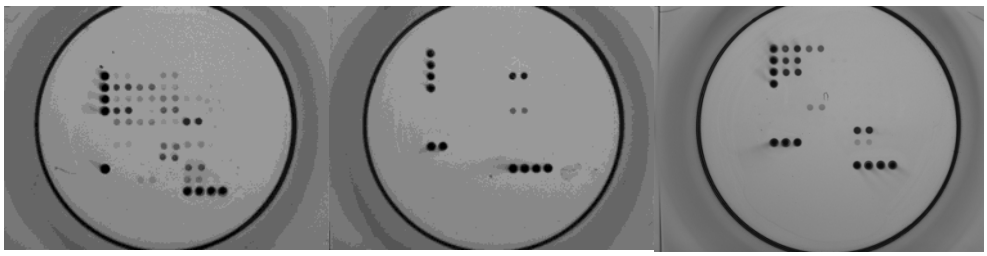


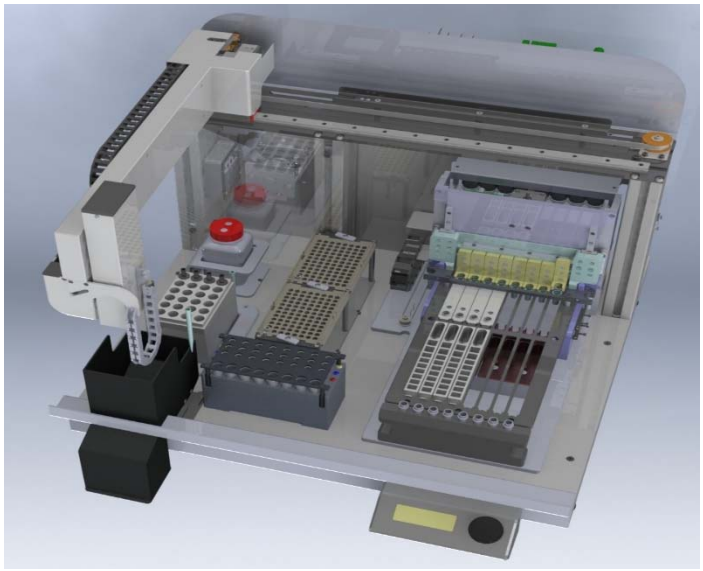
Image showing DNA extraction modules on the left with PCR Cycler module center and microarray processing module on the right. The microarray strip with the developed microarrays at the bottom of the well would move automatically into an imaging chamber at the back of the unit for imaging and typical images of developed arrays are indicated below: -



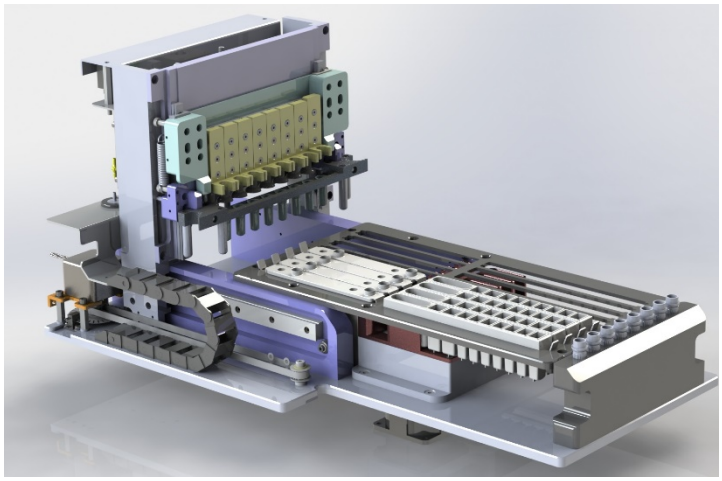
The interpretation software would grid the array and look for the position and intensity of the individual spots for analysis.

PathoRobot - Bench top unit providing automation for DNA extraction for larger samples volumes (5ml).

PathoRobot deck layout below showing the various modules with positions for disposable tips, samples, reagents and vacuum system.



PathoRobot Extraction Module showing the disposable cartridges and reagent holders. The mechanism would move the filtered cartridges in the Z axis to engage the columns with the disposable vacuum cartridges for adding reagents, washing and vacuum steps. The final DNA eluate would be transferred from the column into a PCR tube at the front of the instrument using disposable tips to avoid carry-over.



Both the PathoDoc and PathoRobots have integrated UV lamps for disinfection at the end of the assay.