FINAL PUBLISHABLE REPORT

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1 Overview

In 2014 a World Health Organisation (WHO) report stated that antimicrobial resistance (AMR) is so serious, that it threatens the achievements of modern medicine, and while new therapies to treat resistant pathogens are needed, the diagnostic tools required to guide their application are equally lacking. This clinically focussed project applied innovative metrological concepts for developing quantitative higher order methodologies and materials to support the development and application of diagnostic testing for the detection and management of AMR. The project developed the materials and methods to support the implementation of standardisation of a protein biomarker for bacterial infection, screening of resistance in bacteria using genetic methods and for screening of membrane targeting antibiotic action. A reference measurement procedure was developed to support higher order methods for quantitative monitoring in virology and guidelines were published to assist the implementation of new tools, such as next generation sequencing (NGS), in detection of unknown antibiotic resistance.

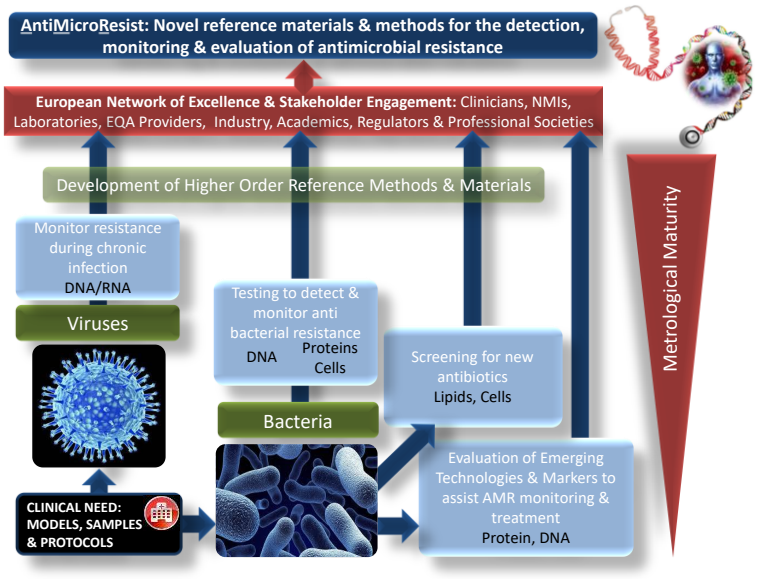
2 Need

Pathogens resistant to antimicrobial treatment threaten effective prevention of a range of infections. A recent review estimated AMR will account for a staggering 45 % of global deaths by 2050 (http://amr-review.org/). In recognition of this rapidly growing problem, several European activities to monitor detection and treatment of AMR have been initiated, including the European Centre for Disease Control (ECDC) interactive database for clinical detection of AMR antimicrobial resistance (EARS-Net).

Despite such initiatives, there is continued stakeholder need for methods to:

- More rapidly diagnose patients with infections that do need antimicrobials
- Detect infections that are already resistant
- Guide clinical practitioners with respect to correct and effective therapies, to reduce over prescription of antimicrobials
- Support testing of innovative antimicrobials

A recent report in association with The World Alliance Against Antibiotic Resistance (WAAAR) stated that "*Today, we do not have the diagnostic tools to effectively address AMR*" a fact that was further confounded by the lack of mechanisms to standardise the methods that do exist. At the start of the project the most advanced application of traceable measurement methods to manage infectious diseases was for viruses. While some internationally recognised reference materials do exist, AntiMicroResist developed reference measurement procedures that could improve the accuracy and reproducibility of reference material production. Standardisation for clinical testing associated with bacterial resistance was even less mature. It was acknowledged by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) that the development of reference measurement systems for infectious disease diagnostics and AMR was a key requirement to support both comparable clinical measurement and the industry compliance with the IVD regulation. The objectives of AntiMicroResist addressed these issues by progressing the development of reference methods and materials to underpin the development and application of diagnostic methods to identify and manage AMR, and to support the measurements required for the testing of new antimicrobials.



3 Objectives

This clinically focussed project applied innovative metrological concepts for the development of higher order quantitation methodologies, and it applied them to provide a step change in evaluating and treating microbial resistance.
The specific objectives of this project were:

1. *Identification of approaches for the management of bacterial antimicrobial resistance.*
   Metrological frameworks were developed for the management of bacterial antimicrobial resistance with a focus on protein biomarker monitoring, routine screening and cellular bioassays (Minimum Inhibitory Concentration (MIC) analysis). This included developing External Quality Assurance (EQA) materials and investigating candidate reference methods to improve reproducibility and better support of established measurements.
2. *Establishing higher order methods and candidate reference materials for quantitative monitoring of viral antimicrobial resistance.*
   A metrology framework based on digital PCR (dPCR) as an SI traceable reference measurement procedure was developed to support the use of higher order reference materials and methods for the quantitative monitoring of viral antimicrobial resistance. This approach brought the potential for SI traceability to the quantitative molecular approaches that are routinely used for the management of patients.
3. *Evaluation of reference materials and methods for the functional validation and screening of antibiotics.*
   Candidate reference materials and methods were evaluated for the functional validation and screening of last-resort and emerging antibiotics. Quantitative measurements of microbial cell walls and artificial membranes were assessed when challenged by antibiotics where intact membranes served as an indicator of resistance. These studies included the kinetics of antimicrobial action against resistant bacteria.
4. *Evaluation of future reference measurement needs.*
   The standardisation of innovative next generation approaches for the detection of emerging and more challenging antimicrobial resistance mechanisms was investigated. Preanalytical, analytical and informatics stages of the process were investigated to determine sources of error and recommendations derived from these findings.
5. *Uptake by reference testing laboratories, EQA scheme providers, health care professionals and diagnostic industry.*
   Uptake of the technology and reference measurement systems developed in the project by EU reference testing laboratories, EQA scheme providers, healthcare professionals (hospitals and health centres) and industry (diagnostic companies) opens up access to SI traceability to support the standardisation of comparable and traceable measurements in the field of antimicrobial resistance management.

4 Results

Objective 1: To determine the approaches needed for establishing a metrological framework for the management of bacterial antimicrobial resistance with the focus on protein biomarker monitoring, routine screening and cellular bioassays (Minimum Inhibitory Concentration (MIC) analysis). This will include developing external quality assessment (EQA) materials and investigating candidate reference methods to improve reproducibility and better support established measurements.

Work to achieve this objective was performed by LNE, LGC, NIB, PTB, TUBITAK, GOSH, UCG and UWH.

Protein biomarker monitoring

Today's clinicians need better tools to allow stratification between viral, bacterial or parasitic infections in order to guide the selection of therapy and to enable better antibiotic stewardship to reduce antimicrobial resistance. As an established protein biomarker for the early diagnosis of bacterial infections and antibiotic stewardship decision, procalcitonin (PCT) is a potentially ideal biomarker that may assist in achieving this objective. PCT concentration in human plasma or serum samples is currently measured in clinical and medical laboratories by a myriad of commercially available immunoassays. However, there is no SI-traceable higher order reference method and higher order reference material to support reproducible PCT measurement. Establishing a reference system for the measurement of PCT through the development of a primary reference method and the production of certified reference material will improve the accuracy and comparability of all routine assays and it will ensure the metrological traceability of the results to the SI units.

Within this project, the development of a candidate primary method for the quantification of PCT in human serum by Isotope Dilution Mass Spectrometry (IDMS) was initiated. The selection of primary calibrators, their characterisation and the optimisation of the analytical process were performed. In parallel to the development of the candidate primary method, LNE worked on the creation of an IFCC (International Federation of Clinical Chemistry and Laboratory Medicine) working group for the standardisation of PCT assays. This standardisation initiative, chaired by LNE, includes metrology institutes, academic laboratories, clinical laboratories and PCT assay or analyser providers and it started its activities in 2018. The aim of the working group is to support the development of metrological frameworks initiated within this project for the management of bacterial antimicrobial resistance. In addition to the development of a reference method, it appears to be essential to document and understand the variability of the results provided by the different commercially available PCT assays, to evaluate the need and the feasibility of standardising PCT assays, and to perform the standardisation if needed and feasible.

Depending on the calibration approach selected for the higher order reference method (protein-based or peptide-based calibration approaches), LNE identified two different providers of primary calibrators. Promise Advanced Proteomics was identified as a provider of recombinant labelled and unlabelled PCT proteins, and Pepscan Presto was identified as a provider of synthetic peptides. Within this project, efforts were focused on the peptide-based calibration approach as a first intention.

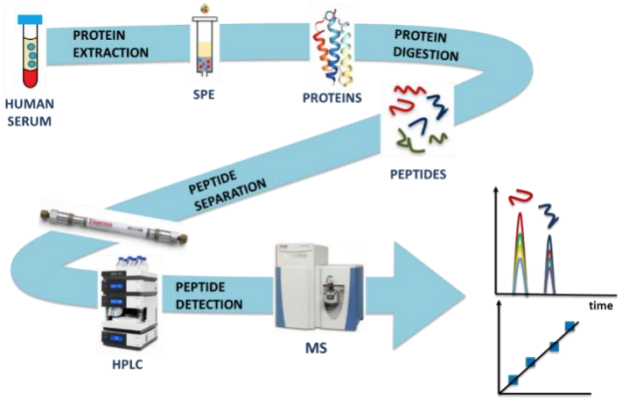


Figure 1.1: Process for the quantification of PCT in human serum.

The analytical process of the IDMS method developed for the quantification of PCT in a biological matrix is presented in Figure 1.1. This mass spectrometry bottom-up process includes extraction of the protein from human serum, enzymatic digestion with trypsin, spike of the internal standard after the digestion and analysis of the generated peptides by LC-MS/MS in PRM mode on a Qexactive Focus instrument (Thermo Fisher Scientific). The quantification of PCT is ensured through the quantification of selected peptides coming from the proteolysis of PCT. One tryptic

peptide appears particularly adapted for the quantification of PCT in serum by this IDMS method. Both the characterisation of the primary calibrator (Figure 1.2) and the validation of a quantification method for this peptide (Figure 1.3) were performed.

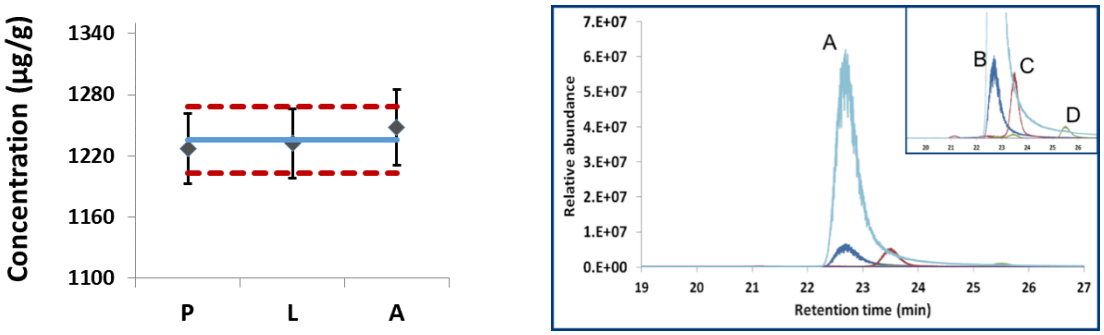


Figure 1.2: Amino acid analysis (left panel) and LC-MS analysis (right panel) of the selected peptide for the quantification of PCT in human serum. Left panel: proline, leucine and alanine amino acids were quantified. Blue spots represent, for each amino acid, the average concentration of the peptide (µg/g) from three independent experiments; Blue line represents the average concentration of the peptide (µg/g) from three independent experiments and the three quantified amino acids; Red dotted lines represent the uncertainties (k=2). Right panel: Some impurities were identified by LC-MS.

The peptide-based calibration method proved to be linear, with a regression coefficient above 0.997 for three independent calibration curves. The results show %CV lower than 3 % and a %Accuracy of 97.9 % between experiments for a medium-level QC sample. An LLOQ of 2.3 ng/mL was determined which is particularly interesting for the monitoring of patients on antibiotic therapy to help the clinicians to decide whether it is necessary to continue the treatment or not and thus reduce the unnecessary use of antibiotics. This developed method presents encouraging results for the quantification of PCT in human serum.

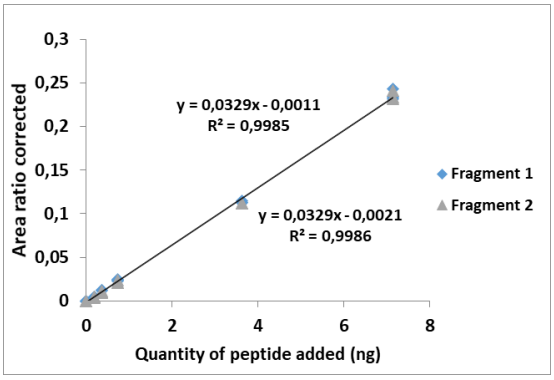


Figure 1.3: Calibration curves of the selected peptide for the quantification of PCT in human serum. These curves were obtained from the data points of one set of calibration solutions. Results of two different transitions in LC-MS/MS (PRM) are presented: Fragment 1 and Fragment 2.

As discussed, the peptide-based calibration approach may introduce imprecision in the quantification and large measurement uncertainties due to variability in the protein extraction or the digestion yield and the need to apply correct factors for these steps. The next steps will consist of the development of a protein-based calibration method that will compensate the issue coming from the peptide-based calibration method and this will improve the robustness, the uncertainty and the accuracy of the method. The quantification of samples with the two methods will allow the selection of the best calibration system for the development of a higher order reference method. In addition, further developments of the sample preparation (e.g. specific isolation of PCT from the serum) and the analytical system (e.g. reduction of the internal diameter of the analytical column) will improve the sensitivity of the method and thus reduce the LLOQ. This will allow covering cut-offs to be used in clinical practice to identify patients with sepsis and it will prevent the unnecessary prescription of antibiotics. Also, standardisation activities initiated within the IFCC WG-PCT will be pursued in close cooperation with clinicians, EQA providers and assay manufacturers.

Routine screening

In addition to the monitoring of infection using markers such as PCT, this objective also focused on screening for resistance genes using molecular tools to manage infection control in patients entering the

hospital. These approaches differ from the conventional approaches which require culture as they measure bacterial DNA directly from patient samples. This part of the project investigated the model of methicillin resistance in *Staphylococcus aureus* (MRSA) and multi-drug resistant tuberculosis (MDR-TB). For the purposes of the project the MRSA model system was used throughout and the MDR-TB model was still in early stages. These models were developed by LGC. The objective was to develop and assess the performance of candidate reference materials in this model system which would be needed to ultimately support routine and novel molecular screening in clinical practice. This work also investigated how it could support current and future external quality assessment (EQA) schemes in clinical microbiology. The impact of extraction was investigated as a major contributor of uncertainty when quantifying pathogens from clinical samples. Three methods were identified for the extraction of DNA from *S. aureus* and evaluated. Genomic DNA yield was determined by analysis of the extracts using species specific assays on a digital PCR (dPCR) platform and using fluorometric approaches (Figure 1.4).

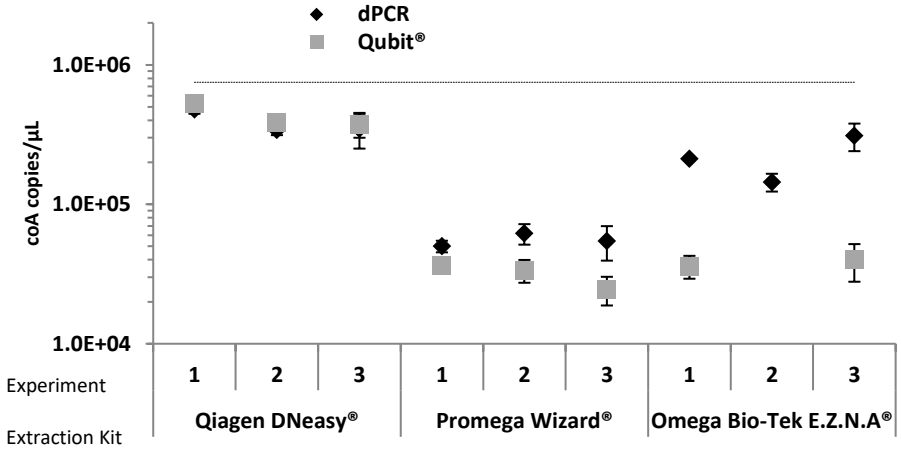


Figure 1.4: The data shown is from extraction of S. aureus materials. DNA yields expressed as coA copy number per µL were compared using dPCR, and converted from ng/µL to copy number per µL for the Qubit® fluorometric approach. Results are reported as mean values based on triplicate technical replicates for each analysis method, performed on three extraction replicates obtained across triplicate experiments. Error bars represent the 95 % confidence intervals.

Once the most suitable extraction procedure for upstream analysis was chosen based on these metrics, the development of the method of target quantification was initiated. In addition to the extraction step having an impact on the quantification of target there are considerations around gene choice and primer and probe chemistries which can also have an impact on the result. In order to address this concern different target genes were identified where possible (*coA* and *Sa442* for the identification of *S. aureus*) and different target sequences within the gene were identified (*mecA* for the indication of methicillin resistance and *femA* for identification of *Staphylococcus epidermidis*). These genes were used for assay design and they were then evaluated in terms of performance on the dPCR platform. Assays demonstrating high separation between negative and positive droplet populations, high correlation between observed versus expected target concentrations and good dynamic range were selected for going forward. An example of an assay (*coA*) which fulfilled these criteria is in Figure 1.5.

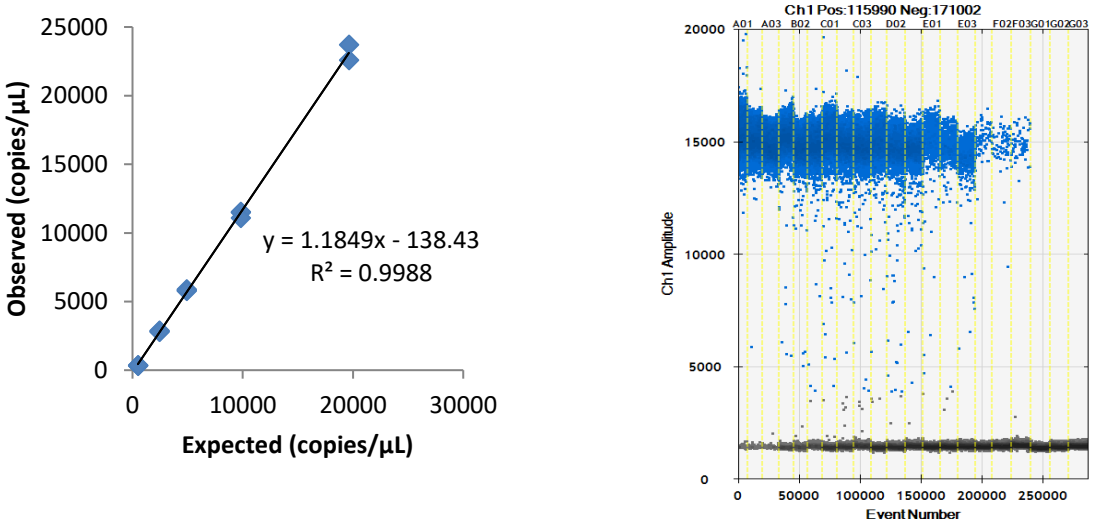


Figure 1.5: Correlation between the observed and expected concentration of the target coA and a dPCR plot showing the amplitude of fluorescence of the negative and positive droplets from the coA assay.

A further development of the method investigated performing the assays in duplex format, which was determined to perform comparably to the assays in uniplex. Prototype EQA materials were prepared and value assigned using the procedures developed, as already mentioned. Within laboratory, the measurement uncertainty for the whole process (assay and pre-analytical steps) was ≤ 34 % (Table 1). These materials were then analysed in an inter-laboratory study to determine if these methods were repeatable. It was observed that while methods performed well within laboratories this was not the case from the inter-laboratory study where there was greater variability. As an example the mean concentration of the *mecA* target in the MRSA high level material had a CV of 64 % and in the MRSE (methicillin resistant *Staphylococcus epidermidis)* in the MSSA (methicillin sensitive *S. aureus)* material had a CV of 97 % from the three laboratories. This finding indicates that an improvement in methods, especially in the reproducibility of the nucleic acid extraction step in the process, would be required in order to improve the harmonisation of methods. It could be argued that the purpose of producing a well characterised reference material which was performed in this project is needed to determine how well the methods perform across laboratories. It is only through this that EQA schemes can be improved and address reproducibility issues. This is especially important to meet the accreditation requirements which laboratories need to address. This part of the project defined the current landscape in terms of the reproducibility of AMR measurements and it set out the requirements needed in order to improve the accuracy of measurements made in clinical testing that are associated with the clinical management of AMR. The framework used in this part, could be used as a method for the investigation of other model systems and it could also be used to interrogate new materials and methods.

Material	Duplex	Target	Mean concentration of target (copies/µL) reported by Laboratory					
			Lab 1	Uncertainty (%)	Lab 2	Uncertainty	Lab 3	Uncertainty (%)
MRSA high	1	mecA	10.20	12	21.17	6.76	6.16	11
		femA_SE	<LOQ	NA	<LOQ	NA	n.d.	N/A
	2	mecA	10.08	13	21.37	7.90	5.90	13
		coA	11.61	8	21.64	8.01	7.20	11
MRSA low	1	mecA	1.42	19	2.48	0.77	1.02	6
		femA_SE	<LOQ	NA	<LOQ	NA	n.d.	N/A
	2	mecA	1.37	22	2.51	1.12	0.96	7
		coA	2.26	20	3.05	1.09	1.38	9

MRSE in MSSA	1	mecA	8.13	30	29.43	12.59	4.28	16
		femA_SE	7.23	25	24.72	11.35	5.04	14
	2	mecA	7.87	28	28.51	12.71	4.28	16
		coA	5.92	25	7.00	3.10	1.22	12
MRSA in MSSA	1	mecA	2.78	34	5.88	1.36	1.09	7
		femA_SE	<LOQ	NA	<LOQ	NA	0.52	6
	2	mecA	2.57	34	5.76	1.64	1.16	8
		coA	7.15	33	12.66	2.89	4.13	11

NA: not applicable, LOQ: limit of quantification

Table 1: Copies per µL reported from three laboratories obtained from two duplex reactions (mecA:femA_SE and mecA:coA) for four different materials. Each value, with its associated measurement uncertainty expressed as a percentage, represents the concentration obtained from the extraction of three different units of each material across three different days and triplicate reactions analysed using dPCR.

In addition to the need for improved reference materials to support EQA schemes, this project went beyond the state of the art to consider other needs which are currently unmet for novel molecular screening methods such as high throughput quantitative PCR (HT-qPCR). This method was investigated as it allows for the simultaneous screening of multiple genetic targets of resistance and for the identification of the likely causative pathogen. An RMG aligned to this work looked at developing an approach which could, in addition to screening the most common clinically important pathogens and resistance targets, investigate the detection of carbapenem resistance in Enterobacteriaceae (CRE). Reference materials were prepared in the form of plasmid controls and characterised using dPCR. Assays were designed for this work to target the most common gene families and were transferred onto a dPCR platform. The suitability of this method development and reference materials for CRE's were then checked by performing subsequent analysis of clinical samples. It is anticipated that this work, already presented at an international conference, will form a manuscript for submission to a peer reviewed journal. These applications of molecular tools represent a novel approach for the identification and quantification of resistance organisms in a clinical sample. The approach developed demonstrates a framework by which other reference materials and methods can be developed for other model systems and could hopefully improve the accuracy of these methods by using such an approach.

Cellular bioassays

Methods which determine the antibiotic susceptibility profile of a patient sample have traditionally been focused on the phenotypic determination of resistance. Traditionally these types of approaches determine the MIC, the lowest concentration of an antimicrobial agent which can inhibit the growth of a particular bacterial strain, but these methods are difficult to standardise and require growth of the bacteria which can be influenced by a large number of factors. While genotypic methods can be applied with a higher throughput and can offer a more rapid and simpler approach to determine the drug susceptibility profile, a given resistance genotype does not necessarily confer the phenotypic resistance. This project has looked at addressing novel state of the art approaches based on atomic force microscopy (AFM) nanomechanical cantilever measurements to determine the antimicrobial susceptibility profile of bacteria when exposed to an antimicrobial agent. The methods involved were thoroughly investigated to identify sources of variance, to improve the reproducibility of the technique and to determine the origin of the signal.

The sources of variance in the nanomechanical cantilever method were identified as follows:

- Bacteria immobilisation methodology (ex situ vs in situ)
- Bacteria immobilisation methodology (non-specific)
- Bacteria seeding density, including explicit counting of bacterial cell numbers
- Signal analysis (influence of windowing on the signal)
- Cantilever geometry and properties (stiffness, coating)
- Drift, as influenced by local and external heating
- Closed vs open fluid exchange within the AFM

The MICs for three reference strains of *Escherchia coli* (JM109, BL21, DH5α) using an established laboratory clinical protocol (liquid culture, optical density measured using a plate reader) was determined for both ampicillin and kanamycin. Ampicillin and kanamycin both showed MICs in the range expected (4-8 µg/mL and 2-4 µg/mL respectively). For the nanomechanical cantilever method measurements of the MIC for ampicillin, the *E. coli* strain used by EPFL (DH5α) was selected.

It was noted that the bacteria immobilisation step of the protocol led to the highest levels of uncertainty in both the seeding density (Figure 1.6) and the subsequent measurements. Improvements to the protocol were developed by EPFL (Ecole polytechnique fédérale de Lausanne), and kindly shared with UCL through a collaboration. Significant efforts were made to improve and standardise the immobilisation methodology and resultant seeding density for the nanomechancial cantilever experiments, but this remains an ongoing challenge. However, an alternate optical method was developed which shows considerable promise for rapid anti-microbial susceptibility profiling. This new approach overcomes the inherent limitations of bacterial immobilisation on cantilevers - it is also rapid, achieves single cell sensitivity and has been piloted with clinical samples. These findings are being submitted for publication shortly.

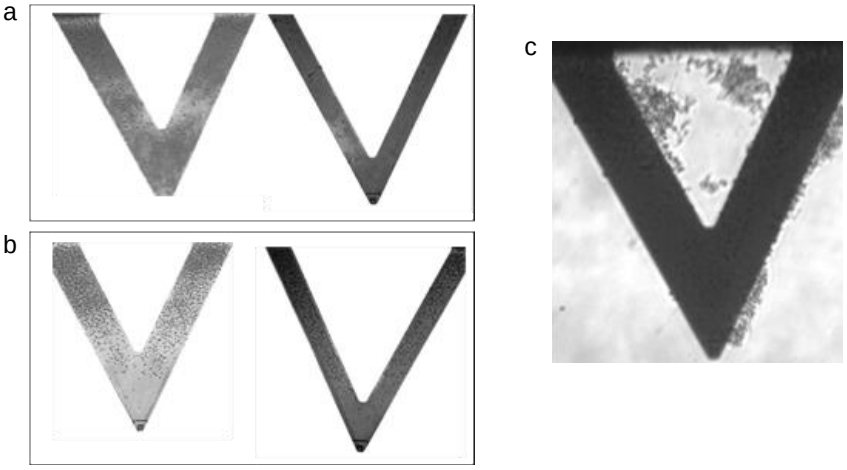


Figure 1.6: Variable bacterial coverage. A, Low bacterial coverage. B, "Optimal" bacterial coverage. C, "Clumpy" bacterial coverage.

*1. Longo, G. et al. Rapid detection of bacterial resistance to antibiotics using AFM cantilevers as nanomechanical sensors. Nat Nano **8,** 522–526 (2013).*
*2. Kasas, S. et al. Detecting nanoscale vibrations as signature of life. PNAS **112,** 378–381 (2015).*
*3. Stupar, P. et al. Nanomechanical sensor applied to blood culture pellets: a fast approach to determine the antibiotic susceptibility against agents of bloodstream infections. Clin. Microbiol. Infect. **23,** 400–405 (2017).*

The key outputs for the project in achieving objective 1 were the development of a SI traceable approach for quantification of PCT using a peptide-based calibrator, establishment of standardisation activities through the IFCC working group for PCT initiation, development of a reference system using dPCR for screening of MRSA and development of an alternate optical method which could improve cellular bioassays for determination of MIC's.

Objective 2: To establish a metrology framework to support the use of higher order accurate materials and methods for quantitative monitoring of viral antimicrobial resistance. This approach will focus on validation and on establishing the SI traceability of the quantitative molecular approaches that are routinely used for the management of patients with chronic disease.

This objective aims to address the lack of higher order reference methods that are available for viral load and resistance monitoring by describing sources of variance and bias, using dPCR which has been previously shown to offer high accuracy, and potential SI traceability, as it performs an absolute count of the number of molecules present within a defined volume. This objective was achieved involving PTB, LGC, NIB, TUBITAK and GOSH. For routine quantitative testing, which is most advanced in virology, EQA schemes are supported by internationally accepted reference materials which, however, are not traceable to the SI and do

not contain comprehensive, robust estimations of uncertainty making inter-comparison between technology platforms difficult to assess in terms of sources of technical error.

Secondary antimicrobial resistance may develop during the treatment of chronic viral infections. Human immunodeficiency virus (HIV) infections and chronic hepatitis C virus (HCV) infections require lifelong antiviral therapy with a substantial risk of developing antimicrobial resistance. Asymptomatic carriage of human cytomegalovirus (hCMV) is common but usually without health risks for immune competent adults. By contrast hCMV can become an issue for patients after organ transplants that require immunosuppression to avoid transplant rejection. Prolonged anti-CMV treatment (e.g. ganciclovir) is thus frequently necessary for such patients. Following literature research CMV was selected as example of a DNA virus and HIV was selected as an RNA virus with particular relevance for the development of antimicrobial resistance (Figure 2.1). The number of papers typically increases by 5 % per year (see https://www.ncbi.nlm.nih.gov/search/).

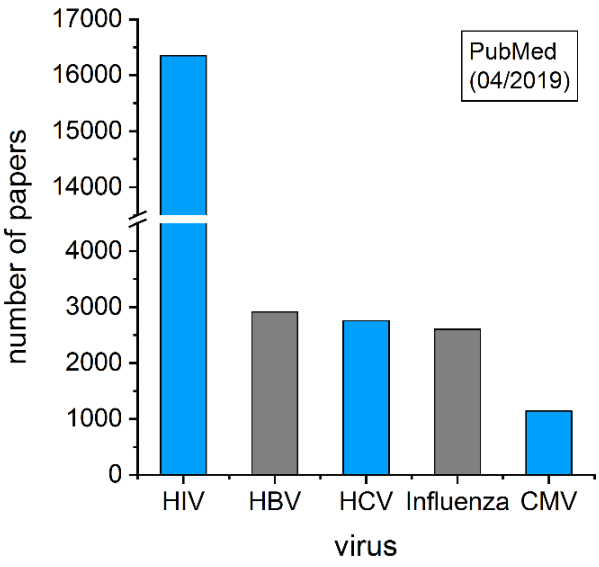


Figure 2.1: Number of journal paper abstracts found in PubMed searching antimicrobial resistance and certain viruses (cytomegalovirus, HIV, influenza, hepatitis B and C).

Quantification of virus concentration was performed with dPCR. Different commercial extraction methods were tested for each virus and the method with highest extraction efficiency was selected, since the variation between repeat extraction on consecutive days showed no clear preference. For extraction of DNA from CMV in plasma samples the "High Pure Viral Nucleic Acid Kit" from Roche was selected. For extraction of RNA from HIV in plasma samples the "QIAmp Viral RNA Mini Kit" from Qiagen showed best performance. Quantification of RNA by PCR requires reverse transcription. Two-step reverse transcription performing complementary DNA (cDNA) generation before droplet generation was tested with several kits but it showed inferior performance compared to one-step reverse transcription, in which this reaction is performed after droplet generation and partitioning of the viral RNA sequences (One-Step-RT-ddPCR advanced Kit for Probes, BioRad). While the extraction procedure used commercial kits, the primer and probes were selected from the literature.

Digital PCR directly counts target nucleic acids and can thus be potentially used for SI-traceable measurements of pathogen concentration. In the course of the project LGC, NIB and PTB participated in several ring trials for virus concentration measurement organised by the German INSTAND for routine virologic laboratories. The NMIs (National Measurement Institutes), PTB, LGC and NIB used dPCR in these ring trials. Other participants (from outside of this project) used qPCR. Results of the three ring trials are shown in Figure 2.2.

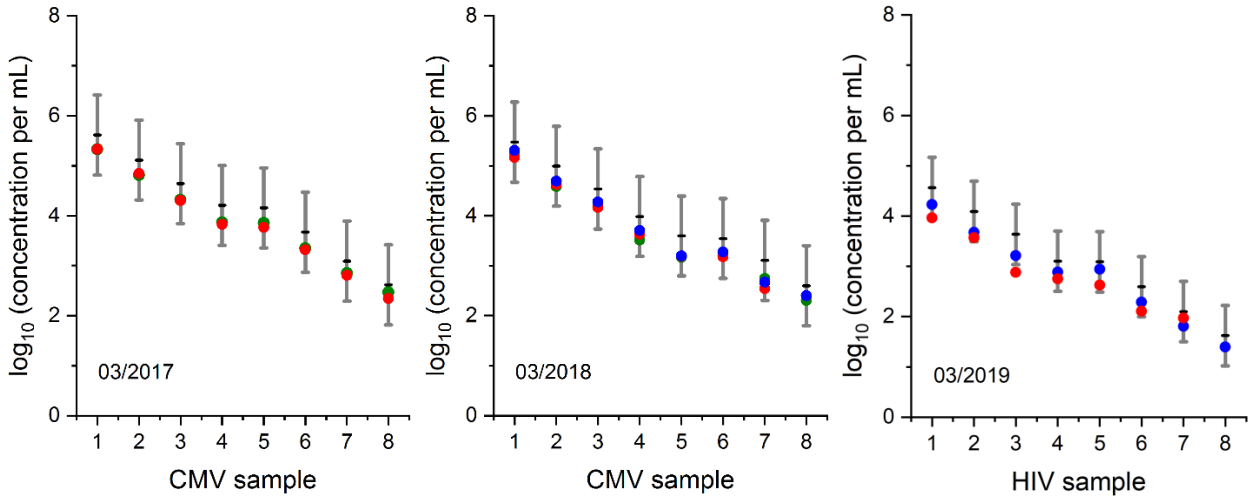


Figure 2.2: Selected ring trials contained eight samples each. Dots are the concentration values measured by dPCR. Grey bars give the acceptance range for the results reported by routine laboratories according to the Guidelines of the German Medical Association (±0.8 log10 for CMV, ±0.6 log10 for HIV). Samples are ordered with decreasing robust mean value of the results reported by participating labs.

The good agreement between the results obtained for hCMV by PTB, LGC and PTB underlines the high reproducibility of dPCR concentration measurements. The centre of the grey bars is calculated as a robust mean of the results obtained by the laboratories participating in the ring trial. The length of the acceptance interval is defined in the Guidelines of the German Medical Association for these ring trials. On a decadic logarithmic scale a variation of ±0.8 is allowed for CMV, which means a factor of 6.3 deviation from the robust mean of the results of all participants in the ring trial. For HIV the acceptance range is somewhat smaller. The deviation of ±0.6 on a decadic logarithmic scale corresponds to an allowed factor 4.0 deviation for the HIV concentration. As most participants of the ring trial used qPCR for virus quantification their results rely on their manufacturer provided calibrator, which may partly explain the systematic deviation of the traceable dPCR measurements to lower values than the robust mean dominated by qPCR methods. Another factor could be the limited extraction efficiency in the sample preparation step, and for HIV a potentially limited reverse transcription efficiency. Digital PCR has the distinct advantage that no calibrator is required for measuring the concentration of the target sequence qualifying it as a potential primary reference measurement method. Therefore, quantification of hCMV by dPCR was submitted to the Joint Committee for the Traceability in Laboratory Medicine (JCTLM) as a candidate reference method.

Comparison with other quantification approaches

The use of orthogonal methods for the traceable quantification of virus concentration is highly desirable for the validation of virus concentration measured by dPCR. The main challenge is that PCR techniques are much more capable of identifying specific targets than other methods, which makes it difficult to select a commutable sample material. The comparison of dPCR and flow cytometry produced favourable agreement in the CCQM (Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology) P154 study "Absolute quantification of plasmid DNA". AntiMicroResist performed a comparison using whole virus material measured by dPCR and a traceable flow cytometer developed in the previous EMRP JRP SIB54 BioSITrace.

PTB approached the depositor of ATCC VR-1578 (Prof. Sinzger, Univ. Ulm), who kindly made a sample from the UL32-EGFP-HCMV-TB40 strain available. This is a genetically modified hCMV that carries an EGFP on each of the capsid-associated tegument proteins pUL32 (pp150). This virus can be detected by its

fluorescence without further staining. The virus particles were grown and purified by ultra-centrifugation. The samples were inactivated using aldehyde treatment.

The atomic structure of the hCMV has recently been resolved by Yu et al. (Science 356, 2017, 1350). This study resolved 60 repeat asymmetric structure units each carrying 15 pp150 proteins for the hCMV capsid; the authors expect that 3 additional pp150 are likely to be present in each unit, but they were unresolved in their measurement due to symmetry issues.

Flow virometry was performed using a modified Partec-Sysmex Cube 8 flow cytometer, which is equipped with temperature and pressure sensors to allow the measurement of the gas buffer volume used to inject the sample suspension. The uncertainty of the sample volume measurement is less than 1 % and can be neglected here (see below). Particles are detected setting the trigger to FITC fluorescence. The fluorescence detection channel was calibrated using Rainbow beads. The trigger level was set to 270 MEFL. At this trigger level the negative control samples had a count rate of <50 Hz. The count rate for the sample measured at different values of dilution was in the range from 50 Hz to 2.3 kHz.

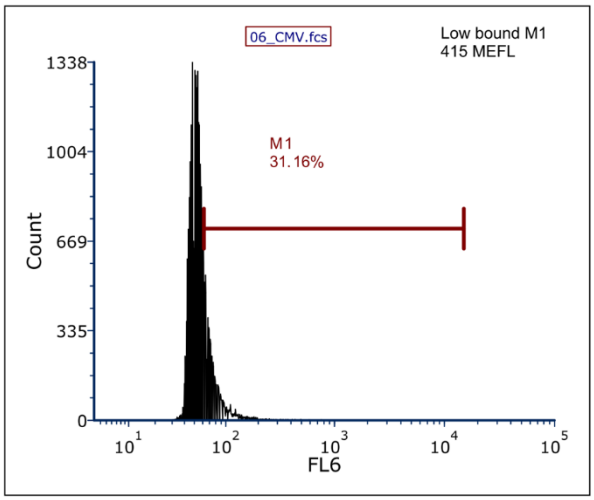


Figure 2.3: Sample histogram of CMV measurement by flow virometry (FL6 = FITC, dilution fraction 0.0025, sample volume (414.6±0.8) µL, average flow rate 3.3 µL/s, average count rate 218 Hz).

The sample consists of intact virions and a range of viral fragments. High viral fragmentation may result from the aldehyde inactivation that is used to obtain a material with long-term stability. For comparison with PCR, only intact virions should be counted. These are identified by setting the fluorescence gate at the equivalent fluorescence intensity of 60×15 (or 60×18) EGFP molecules (Figure 2.3). As the quantum yield for EGFP is 0.60 compared to 0.93 for FITC and the molar extinction coefficient for EGFP is 55000 $(M\ cm)^{-1}$ compared to 77000 $(M\ cm)^{-1}$ for FITC, a single EGFP has about 0.461 MEFL. So, the threshold to be chosen is 415 MEFL or 498 MEFL (for 60×18 pp150) respectively.

The observed concentration is plotted in Figure 2.4 as a function of the dilution fraction. Surprisingly, the observed concentration increases with dilution fraction. For monodisperse suspensions a decrease in the observed concentration is expected with increasing dilution fraction, due to coincidence loss.

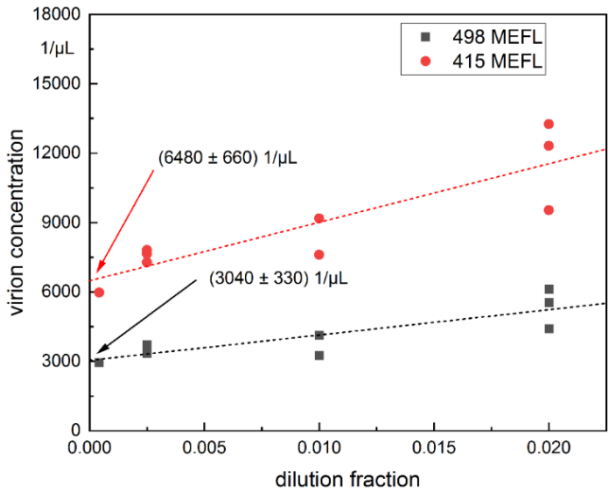


Figure 2.4: Concentration of virions determined with flow virometry and two different settings of the gate. The valid results are obtained at infinite dilution.

In the samples considered here, there is a high concentration of viral fragments. The coincident detection of these fragments leads to high signal events, that outnumber the coincidence loss of counted intact virions. This explains the apparent increase in the observed virion concentration with increasing dilution

fraction. The correct concentration is obtained by extrapolating to infinite dilution.

Setting the limit for intact virions to 498 MEFL leads to an extrapolated concentration of 6480 μL^{-1} ± 660 μL^{-1}. The quoted uncertainty is the standard uncertainty for the linear regression used for extrapolating to infinite dilution. Setting the limit for intact virions to 415 MEFL leads to an extrapolated concentration of 3040 μL^{-1} ± 330 μL^{-1}. The difference between the two concentrations indicates the uncertainty of the virion concentration resulting from difficulties to identify the target particles by flow virometry. This source of uncertainty dominates all other sources by far. The result obtained for the concentration by flow virometry from the above two values is 4800 μL^{-1} ± 2500 μL^{-1}.

Fixed plasma samples may contain free CMV DNA which, in addition, can be highly fragmented. Therefore, a large fraction of CMV DNA is unlikely to be associated with intact virions. To measure DNA from an intact CMV virion only, the CMV quantification assay was combined with DNase I treatment followed by dPCR quantification. Results from dPCR of DNase I treated CMV samples can reasonably be compared to results from flow virometry.

Results obtained by dPCR are shown in Figure 2.5. The error bars on the brown bars are the standard uncertainty for the concentration measured in individual wells. The horizontal dashed line is the weighted average of the dPCR measurements and the horizontal green band shows the standard uncertainty for the average value. The result for flow virometry and its uncertainty is plotted on the right for comparison. While the results between dPCR and flow virometry are not clearly separated in view of the error bars, there is a tendency that results found by dPCR are higher (decadic logarithm ratio for the averages is 0.24).

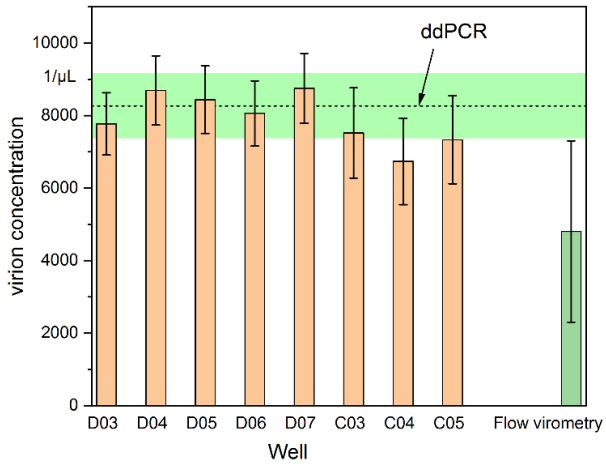


Figure 2.5: Concentration of virions determined with dPCR (brown bars, weighted average (8270±900) μL^{-1}). The result from flow virometry is show for comparison.

Flow virometry is an orthogonal method to (digital) PCR for quantification of virion concentration. For the artificial material considered in this measurement a reasonable agreement between the two methods is found. On the decadic logarithmic scale the observed difference is 0.24 which has to be compared to the acceptance range of external quality assurance for virology laboratories (±0.80 on a log-10 scale for CMV). However, this particular result should not be misinterpreted as a recommendation to use flow virometry for absolute quantification of virus concentration as a routine technique. The high specificity and robust detection of viruses by dPCR allows a much broader application of that technique.

Antimicrobial resistant viruses

The antiviral drug ganciclovir (GCV) was approved for the treatment of CMV in 1988 and has since remained the first-line treatment for hCMV infections in immunocompromised patients. GCV is targeting viral polymerase, thus preventing replication of CMV. In order to decrease the toxicity of GCV the drug is only activated in infected cells, where viral protein kinase, coded in unique long region 97 (UL97) phosphorylates GCV in to its active form ganciclovir monophosphate. Mutations in UL97 prevent the phosphorylation of the pro-drug and render the viral strains drug resistant.

NIB designed three dPCR assays targeting mutations on codons 460, 594 and 595, which present 70 % of GCV-resistant clinical isolates. All three codons are a part of the UL97. The developed assays were assessed for specificity, repeatability and robustness. Limits of detection (LOD) and quantification (LOQ),

and the working range were determined. The method for the quantification of hCMV, as developed within EMRP JRP HLT08 InfectMet (Pavšič et al., 2016), has been used as a reference method.

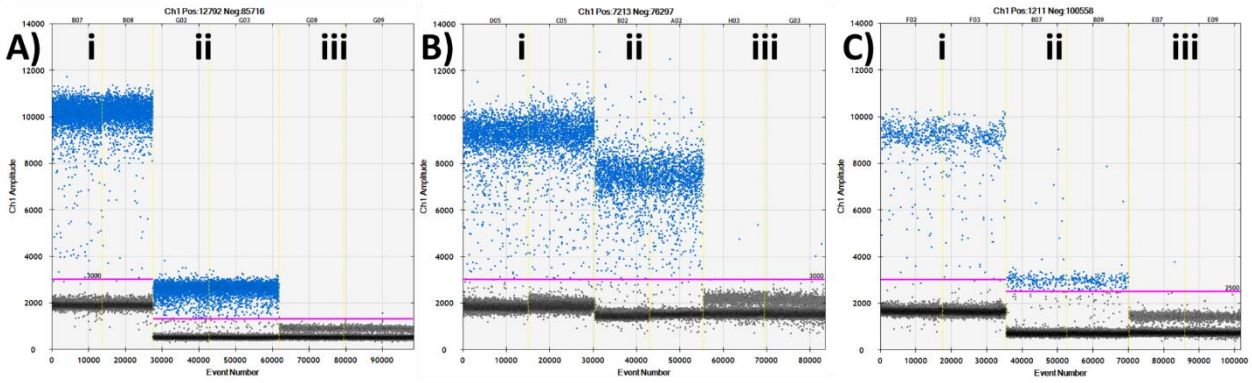


Figure 2.6: Comparison of the M460V, A594V and L595S assays (A ii, B ii and C ii respectively) with the reference method (A i, B i, C i). M460V and A594V show a bias <25 % within the dynamic range, and the L595S assay shows a consistent bias of ~26 % across the whole dynamic range. Cross-reactivity of the M460V, A594V, L595S assays (ii) to the wt template (iii), can be observed in the form of a false-positive cluster (iii – light grey).

The overall key outputs achieved by objective 2 were demonstration of dPCR as a potential higher order method for quantification using the model system of quantitative viral load testing . This approach was submitted as reference method for hCMV quantification by dPCR to the JCTLM database. Future research will allow the extension of the use of dPCR as a reference measurement method to other pathogenic viruses.

Objective 3: To evaluate candidate reference standards for the functional validation and screening of last-resort and emerging antibiotics. Quantitative measurements of microbial cell walls/membranes, intact, resistant and micro-organisms challenged by antibiotics, will be validated, as will the kinetics of antimicrobial action against resistant bacteria

Work to achieve this objective involved NPL, PTB, LGC, LNE, UCL and GOSH.

Current methods for drug susceptibility testing are limited to bioactivity tests and do not take into account the development of cell wall and membrane resistance. Reliable methods which can test both for antibacterial resistance as well as measuring phenotypic changes in bacterial membranes are lacking, therefore new materials and methods are required which can be used with metrological scrutiny. These methods are also needed also so that large numbers of new potential antimicrobial agents can be tested more rapidly.

Candidate reference materials for the screening of antibiotics have been developed. The materials are reconstituted phospholipid membranes mimicking resistant and susceptible bacterial membranes. The materials are offered in two formats, as unilamellar vesicles and supported lipid bilayers. The two formats allow for multi-modal measurements in solution, e.g. by isothermal titration calorimetry (ITC) and fluorescence; and on surface, e.g. by surface plasmon resonance (SPR). ITC and SPR have been assessed for suitability as reference methods in conjunction with reference membranes. Both methods measured the strengths of interactions between antibiotics and membranes (over 10 antimicrobial agents were tested). ITC was selected as a candidate reference method for further inter-comparability studies (project 3, VAMAS TWA40). The method and the materials have been applied in industry case trials to validate a protein antibiotic for MRSA treatments, currently in clinical trials (Figure 3.1), and for screening potential antimicrobial candidates (over 30 experimental compounds). The results obtained were perfectly consistent

with the results of the orthogonal measurements, biological and mechanistic, indicating the versatility of the proposed candidate reference materials and methods.

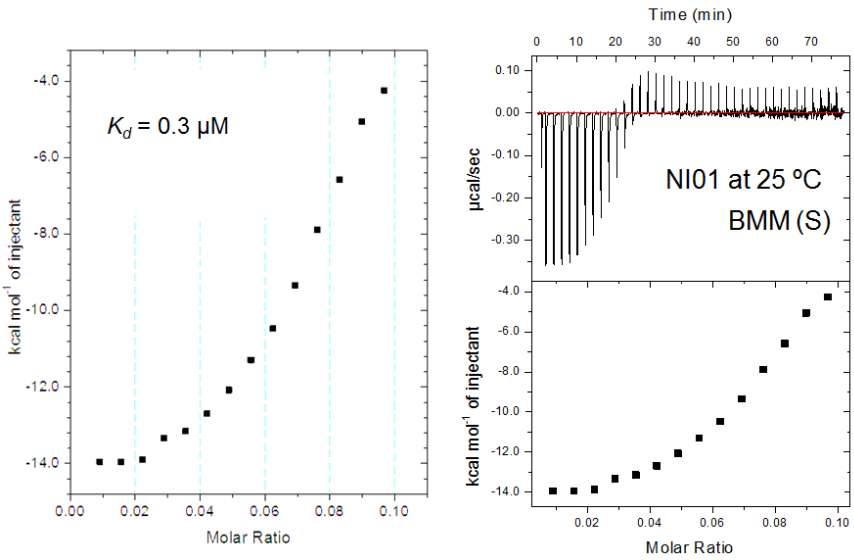


Figure 3.1: Strength of interactions between a protein antibiotic and reference membranes; bacterial mimetic membranes, susceptible – BMM (S), expressed in K_d derived from isothermal titration calorimetry traces. Heat absorbed (µcal/s) for each isotherm is plotted versus titration time (min) and shows endothermic binding (top right). Integrated heats (kcal/mol) are plotted versus antibiotic molar ratios (left and bottom panel).

Based on the results of this project, a series of follow-up inter-comparisons have been started to validate a protein antibiotic as a biophysical benchmark for macromolecular antibiotics and biologics in general. The first study focuses on the comparability in the thermal stability of the antibiotic (project 6, VAMAS TWA40).

The reference membranes in a unilamellar vesicle format have been assessed for the high throughput screening of membrane active antimicrobials using a microfluidic-based measurement platform (Figure 3.2).

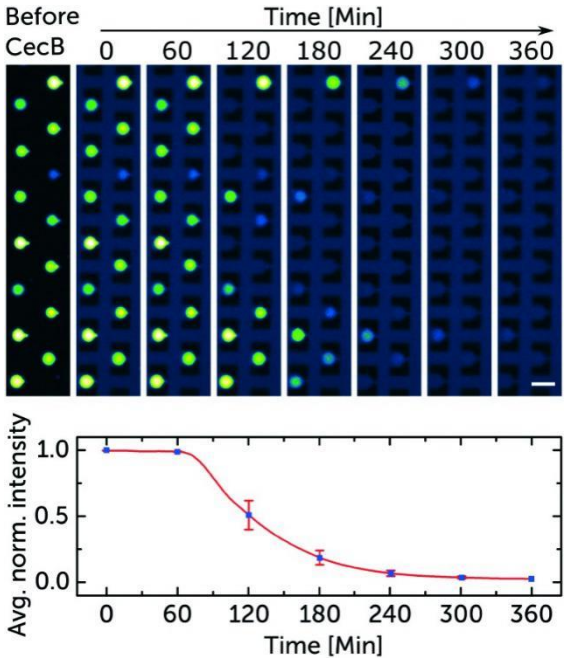


Figure 3.2: Fluorescence microscopy images of trapped vesicles treated with an antimicrobial. The trapped vesicles encapsulate a membrane impermeable dye. The dark blue background marks the arrival of the antimicrobial into the chamber. The averaged normalised fluorescence decrease in intensity from the 14 trapped vesicles is mapped (Al Nahas, K. Et al. Lab Chip, (2019) 19, 837).

This and similar microfluidics platforms have been demonstrated to industrial stakeholders and are being tested for the simultaneous screening of multiple antibiotics. Using the membrane materials, it was possible to score the efficacy of antibiotics with different modes of action, including antibiotics that have no ability to bind to membranes. Scores were expressed as dissociation constants (K_D, ITC) and the amount of antibiotics per unit area (SPR). As expected, membrane-active antibiotics showed strong binding. In a pilot study with industrial partners the materials were used to characterise a commercial protein antibiotic, currently in clinical trials as a treatment for nosocomial infections. Conversely, this study helped to successfully validate the candidate reference materials using an antibiotic whose primary mode of action was to porate bacterial membranes.

The candidate reference materials and methods form a part of NPL's measurement services that are already being exploited with end-users. The materials will be further developed to grade levels as per customer requirements, e.g. certified reference materials.

The overall key outputs achieved by objective 3 were the development of candidate reference materials for screening of antibiotics and development of candidate reference methods for screening of potential antimicrobial candidates which have been further investigated in industrial case studies and inter-laboratory comparability studies (VAMAS).

Objective 4: To investigate future reference measurement needs in order to support the standardisation of innovative next generation (sequencing, mass spectrometry) approaches for the detection of emerging and more challenging antimicrobial resistance mechanisms.

The main aim of this part of the project was to determine the reference needs for advanced next generation diagnostic tests that measure proteins and nucleic acids, from individual microbes and microbial communities (microbiomes) in order to enable the detection of unknown resistance mechanisms. The activities in this objective were achieved by GOSH, LGC, LNE, NPL, NIB, UCL, UWH and UCG.

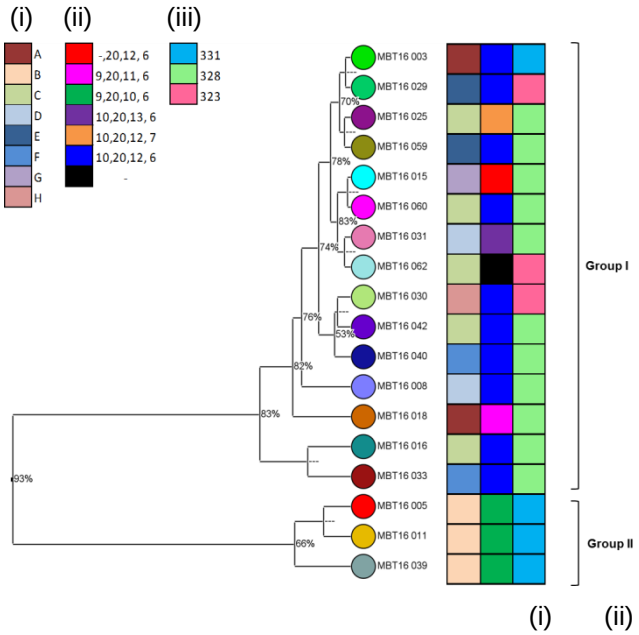
Mass spectrometry

Carbapenem resistant bacteria were used as a clinical model system to evaluate the accuracy, robustness and reproducibility of strain analysis using matrix-assisted laser desorption ionisation–time of flight mass spectrometry (MALDI-TOF MS). The Bruker MALDI Biotyper protocol was applied to the 19 *Acinetobacter baumannii* isolates associated with a drug resistant nosocomial outbreak. Although a single practical approach was followed, data were analysed using two methods; (i) the Bruker FlexAnalysis method, and subsequently (ii) using bioinformatics software (BioNumerics 7.6) for the analysis of exported peak data. To enable a rigorous evaluation of MALDI-TOF MS typing reproducibility, 27 spectra were included for each isolate across a total of three days. Aspects of the method that may impact upon typing reproducibility were assessed at a single centre, and a multi-centre analysis of typing output at two other laboratories was also performed. The results indicated that whilst MALDI-TOF MS is a technically robust method, upstream sample preparation and culture impact upon the reproducibility of the spectra that could in turn influence the bacterial strain typing result.

The two MALDI analysis methods were also used to identify peaks that may be suitable biomarkers for strain typing, and the outputs of each compared. In addition, whole-genome sequencing (WGS) and Fourier-transform infrared spectroscopy (FTIR) Biotyper analysis were applied to the samples. The typing result for these methods was compared with VNTR profiles for the isolates obtained from the reference laboratory (Figure 4.1). There was limited correlation between the MALDI Biotypes and the other methods, with the exception of the group B isolates (MBT16-005, MBT16-011 and MBT16-039) which consistently clustered together for all five methods. The bioinformatic MALDI analysis showed some similarities with the VNTR (variable number tandem repeats) and FTIR profiles, with discrepancies for those isolates that fell into

'Group I'. The genomic typing methods (WGS and VNTR) correlated reasonably well with some discrepancies; MBT16-015 and MBT16-062.

(a)



(b)

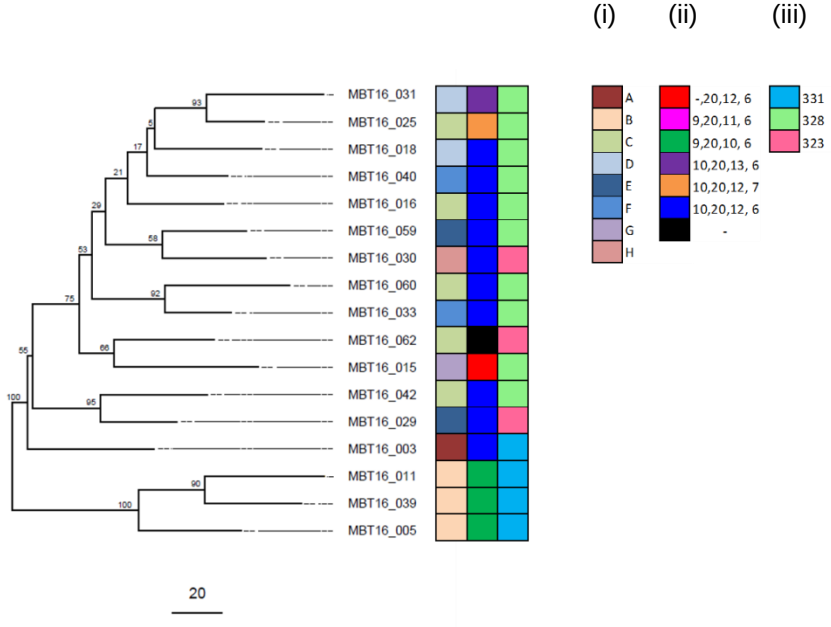


Figure 4.1: (a) UPGMA hierarchal clustering of MALDI-TOF MS spectra compared with the MALDI Biotype, VNTR profile and FTIR Biotyper cluster and (b) WGS SNV analysis compared with MALDI Biotype, VNTR profile and FTIR cluster. Coloured keys represent (i) MALDI Biotype (ii) VNTR profile and (iii) FTIR cluster.

The success of MALDI-TOF MS in strain typing *A. baumannii* is dependent on the reliability of peak calling and the data analysis method implemented. If MALDI and FTIR technologies are to be used for strain typing then they would require the support of other currently used MLST protocols, such as PFGE (Pulsed-field gel electrophoresis) and VNTR. A manuscript reporting this work has been drafted and will be submitted to the Journal of Clinical Microbiology in August 2019.

Next Generation Sequencing (NGS)

Whole-genome sequencing of isolated organisms

Carbapenem-resistant *Enterobacteriacae* (CRE) were used as model organisms for the detection of resistance genes using Next Generation Sequencing (NGS). Factors affecting the performance of a number of selected pipelines was evaluated at a single site. This data was used to design an interlaboratory study to identify the contributors to discordant AMR prediction results reported by different laboratories using similar NGS workflows and data analysis pipelines. The interlaboratory study used genuine clinical samples specifically chosen to be of varying quality and complexity. Each laboratory was blinded to information about the samples and was required to identify the species of each sample, to provide a list of the AMR associated genes identified and to predict whether the isolate would be resistant to four antibiotics: ciprofloxacin, gentamicin, amikacin and cefotaxime. The full characteristics of the samples included in the interlaboratory study are in Table 2.

Study ID	Same isolate	Isolate species	Sequencing method	Carbapenamase gene	Median depth of coverage	Comment
AMRIL_1	1	K. pneumonia	NEBNext Ultra II + NextSeq 150bp PE	*bla* OXA-48-like	190.2	Exact duplicate of AMRIL_6
AMRIL_2	2	E. cloacae complex	NEBNext Ultra II + NextSeq 150bp PE	*bla*OXA-48-like	1.4	Very low coverage duplicate of AMRIL_5
AMRIL_3	3	K. oxytoca	Nextera DNA + HiSeq 100bp PE	*bla*OXA-48-like	37.4	Same original isolate as AMRIL_9
AMRIL_4		K. pneumonia	NEBNext Ultra II + NextSeq 150bp PE	*bla*NDM	83.5	
AMRIL_5	2	E. cloacae complex	NEBNext Ultra II + NextSeq 150bp PE	*bla*OXA-48-like	142.9	High coverage duplicate of AMRIL_2
AMRIL_6	1	K. pneumoniae	NEBNext Ultra II + NextSeq 150bp PE	*bla*OXA-48-like	190.2	Exact duplicate of AMRIL_1
AMRIL_7		E. coli	Nextera DNA + HiSeq 100bp PE	*bla*IMP	20.6	
AMRIL_8		C. freundii	NEBNext Ultra II + NextSeq 150bp PE	*bla*VIM	32.5	
AMRIL_9	3	K. oxytoca	NEBNext Ultra II + NextSeq 150bp PE	*bla*OXA-48-like	156.4	Same original isolate as AMRIL_3
AMRIL_10		A. baumanii	NEBNext Ultra II + NextSeq 150bp PE	*bla*OXA-23-like + *bla*OXA-51-like	22.2	

Table 2. Sample characteristics.

Good consensus between the organism identified from the whole genome sequencing data and culture identification (82/88, 93.2 %) was observed and adequate pairwise consensus between laboratories (86.7 %, 298/344)

The number of AMR associated genes reported by each laboratory in each sample were compared by GOSH and they found a disparity between laboratories (Figure 4.2). Laboratory 1 reported two different methods for achieving this (1a and 1b). The choice of database, and to a lesser extent, the identity or coverage thresholds used to infer the presence of a gene effected the number of AMR associated genes reported. Laboratories 1a, 2, 3 and 5 all repeatedly reported the presence of the highest number of genes in each sample and all used CARD as their reference database. Laboratory 2 consistently reported the highest number of AMR genes in each sample. Both laboratory 2 and 8 used the lowest identity and coverage thresholds (75 % sequence identity and no coverage threshold) but laboratory 8 used Resfinder as its reference database, whereas laboratory 2 used CARD.

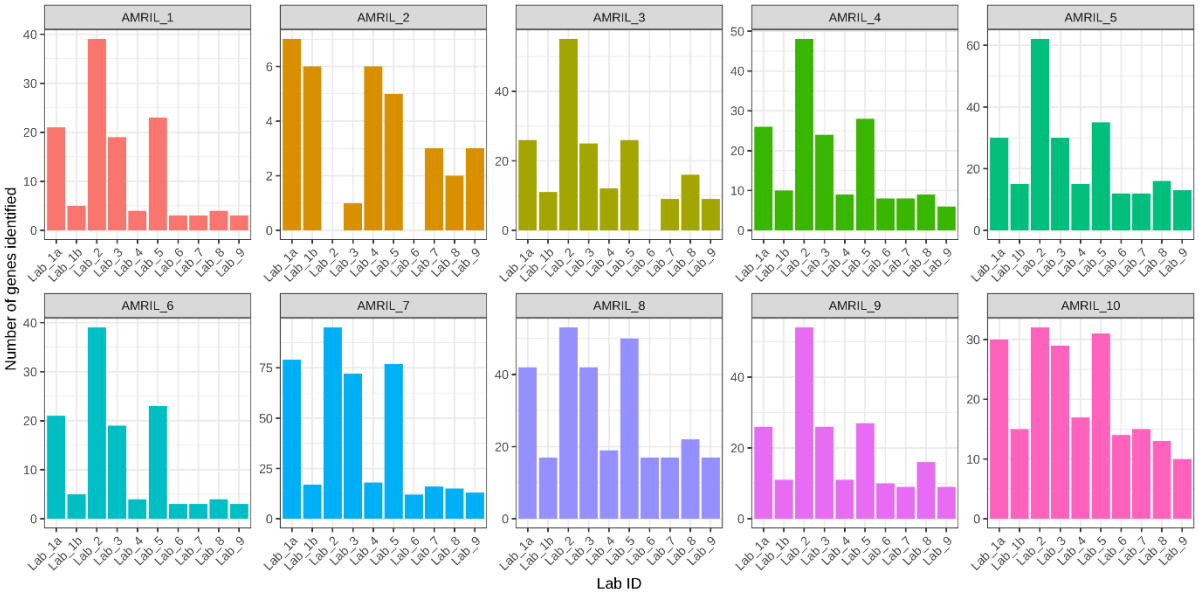


Figure 4.2: Number of antimicrobial resistance associated genes identified in each sample by each laboratory.

All samples included in this study were carbapenem resistant. The laboratories identification of carbapenemase genes from whole-genome sequencing matched the reference PCR result in 91 % of cases (91/100). Eight of the nine misidentifications occurred in the low coverage sample as expected. Differences between reported gene variants of *bla*$_{IMP}$ were seen in AMRIL_7. Five laboratories reported *bla*$_{IMP-1}$, whereas the other five reported *bla*$_{IMP-34}$. This discrepancy exactly matched the reference database used with those reported *bla*$_{IMP-1}$ having used CARD and those who reported *bla*$_{IMP-34}$ either having used Resfinder or ARG-ANNOT. Sequences for *bla*$_{IMP-1}$ and *bla*$_{IMP-34}$ differ by a single amino acid but reference sequences for both are included in all of the databases used by every laboratory in this study.

NGS of mock microbiome samples

As a follow on from the previous study which looked at the sequencing of cultured isolates, the sequencing of mixed community samples to represent a microbiome was investigated. The bioinformatic process was interrogated using two mock microbial community samples which had been previously prepared and characterised using molecular approaches (qPCR and dPCR) and mass-based approaches (the Nanodrop spectrophotometry and the Qubit fluorometer). These mock community samples were then sequenced using previously developed approaches and distributed to a number of laboratories which were then requested to report the presence and relative abundance of the micro-organisms with 97 % identity contained within the samples. Thirteen laboratories participated and submitted their results. In general, it was observed that the different bioinformatic approaches differed by less than threefold when predicting the relative abundance of organisms within both samples compared to dPCR. Differences greater than this were observed when reporting the abundance of *Pseudomonadaceae* and *Mycobacteriaceae* which differed up to 466 and 96-fold respectively when compared to dPCR (Figure 4.3).

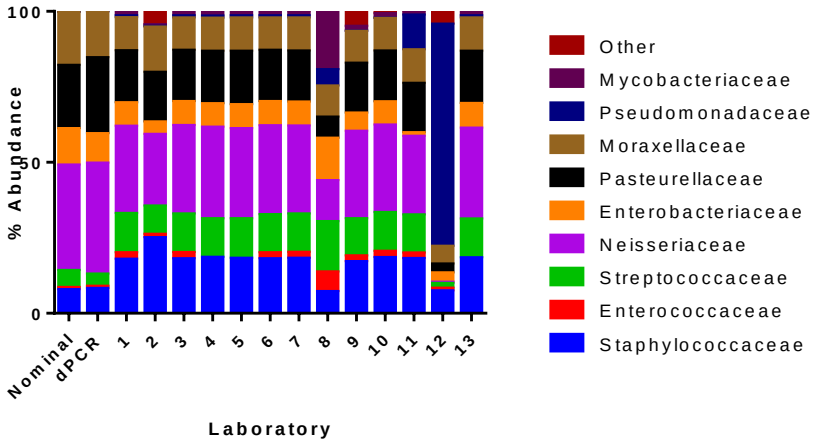


Figure 4.3: The % family abundance reported by each laboratory including the nominal and dPCR reported composition for one of the mock microbiome samples.

These results could be due to these families being present at very low abundance, 0.0015 % and 0.002 % respectively in the sample according to dPCR. However, the degree to which the prediction from sequencing differed compared to dPCR appeared to differ between the laboratories suggesting some types of bioinformatic approaches can deal better with this issue. When performing these types of analyses, it was noted that the bioinformatic tools could struggle to identify organisms at this abundance and therefore using these types of materials could give the community more confidence when making predictions about the composition and abundance of taxa in a sample. The findings of this study stress the importance for when users are performing sequencing experiments to use test data to determine any potential biases which could be introduced from their analysis step. The data from this study is planned to be made available through open access resources for other laboratories to use.

Performance of emerging sequencing technologies in the detection of antimicrobial resistance

This part of the project looked at evaluating the performance of the MinION portable sequencer (Oxford Nanopore) in the identification of antimicrobial resistance in *Mycobacterium tuberculosis*. The work evaluated the method in terms of accuracy using previously well characterised isolates using established phenotypic testing for resistance (DST: drug susceptibility testing). This work looked at the performance of the approach in terms of repeatability of the method from the clinical isolate to the sequencing data file. The results showed high concordance between the drug resistance mutations inferred by a widely used profiling tool for predicting drug resistance in *M. tuberculosis* (*TBProfiler*). The sensitivity of the tool in predicting drug resistance was reported to range from 95.9 % (rifampicin) to 23.8 % (Para-aminosalicylic acid) when compared to DST considered to be the gold standard for resistance determination. These findings were compared to other commonly used drug resistance prediction tools which were found to be inferior. The work within this project has enabled the development of this tool to improve its accuracy for resistance determination and its ability to process third generation next generation sequencing data. As the MinION technology could enable increased access for laboratories to perform whole genome sequencing, or even as a point of care device, there is an increased focus on tools to handle the data in terms of processing and drug resistance determination. Therefore, this work within the project facilitates moving this technology closer into a point of care setting.

The project has successfully achieved this scientific and technical objective. A MALDI typing method for tracking *A. baumanii* outbreaks in hospitals will be published in a peer-reviewed journal, and this paper will include recommendations for clinical laboratories and the wider scientific community on how to implement this test alongside current methodologies, and emerging methodologies such as NGS and FITR. The inter-laboratory study of WGS data from isolated organisms has demonstrated for the first time that the performance of NGS pipelines for the detection of AMR are affected by a number of technical factors. The consortium have identified that Bioinformatic analysis, and in particular the database of resistance genes, is the most important area to focus on in order to standardise the techniques. This has led to a good practice guide with recommendations for the steps in the sequencing and data analysis that are critical for the

standardisation of next generation sequencing approaches for the detection of emerging and more challenging antimicrobial resistance mechanisms. This work has also been written up as a paper and will be submitted for publication in a peer-reviewed journal in at the end of August 2019. There has already been stakeholder uptake of the recommendations, with GOSH implementing a WGS method for AMR detection in *Mycobacterium abscessus* and WGS for AMR detection in other organisms, including CRE, is planned later this year.

The overall key outputs achieved by objective 4 were the requirements for standardisation of emerging next generation tools (MALDI and NGS based) which were identified in the context of the model systems investigated (*A. baumanii*, CRE and mock microbiome samples).

Objective 5: To facilitate uptake of the technology and reference measurement systems developed in the project by healthcare professionals (hospitals and health centres) and industry (diagnostic companies) in order to support the standardisation of comparable and traceable measurements related to the management of antimicrobial resistance.

The primary aim of this project was to look at addressing the issues around developing a metrological framework for the identification of antimicrobial resistance pathogens. To achieve this, the project investigated the development of relevant procedures and standards which could contribute to this outcome. This objective was achieved by LGC, PTB, LNE, NPL, NIB, TUBITAK, UCL, GOSH, UCG, UWH and BSAC. In order to achieve impact within the microbiological community close connections were initiated and fostered through the project with stakeholders from industry, EQA providers, clinical laboratories and academia as well as close connections with standards committees and technical working groups. Throughout the course of the project relevant organisations that are representative of the diagnostic, clinical and healthcare stakeholder community and collaborations were consulted and engaged in the project.

Outputs from this project were represented at drafting committees for ISO guidelines. This project has inputted into a number of ISO draft documentary standards:

ISO/WD 20391-2: Biotechnology Cell counting - Part 2: Experimental design and statistical analysis to quantify counting method performance via TC 276, Biotechnology Working Group 3, Analytical Methods.

ISO TC 276/ WG 3 (Biotechnology) - Guidelines for evaluating the performance of quantification methods for nucleic acid target sequences: Part 1 qPCR and dPCR

ISO/TC 212 WG4 (In vitro diagnostics) - In vitro diagnostic test systems - Qualitative nucleic acid-based in vitro examination procedures for detection and identification of microbial pathogens (ISO/CD 17822-2)

ISO/TC 212 WG2 (reference systems) – Clinical Laboratory Testing and IVD's which aims to provide guidelines on standardisation in the field of laboratory medicine and in vitro diagnostic test systems.

As mentioned above, LNE have created an IFCC working group for the standardisation of PCT measurements. The aim of this group is to support the development of metrological frameworks which have been initiated within this project. The creation of this working group has shown the strong interest of clinicians, health authorities, IFCC, calibration laboratories and almost all IVD manufacturers of PCT assays or analysers (Thermo Fischer Scientific, Abbott, Roche Diagnostics GmbH, Siemens Healthcare Diagnostics, Beckman Coulter, bioMérieux, DiaSys Diagnostic Systems GmbH, Diazyme Laboratories, Ortho Clinical Diagnostics, DiaSorin S.p.A.) in the outcomes from objective 1 of this project. In addition to this IFCC activity, the consortium has a representation as the chair of the IFCC committee on Molecular Diagnostics which fosters exchange between the IFCC and molecular diagnostic laboratories and industry and provides a route for dissemination of the findings from this project with respect to the framework developed for achieving

objective 2. It is anticipated that good practice guides generated from the project could be disseminated through this committee.

The consortium has been heavily involved in CCQM working groups giving presentations and performing pilot studies with a number of presentations and workshops given at the bi-annual CCQM Working group meetings on protein analysis (PAWG) and nucleic acid (NAWG). In collaboration with the stakeholder GBD the consortium has repeatedly used dPCR to determine the concentration of hCMV for INSTAND EQA samples. The results are in good agreement with the robust mean when analysing the results from the studies that they coordinate. This finding was presented at the SoGAT (Standardisation of Genome Amplification Techniques) meeting in June 2018. NIB has submitted a reference method to the JCTLM database for review. This measurement method will be extended to quantification of the copy number of HIV RNA genomic sequences. Material based on synthetic RNA gene fragments was produced for the NAWG pilot study P199 that will proceed later in 2019.

The involvement of the project with EQA providers represents a key impact as it facilitates improvements for the wider clinical community by applying reference measurement procedures to support the reproducible clinical testing associated with AMR management. This is also evident with the participation of UWH who have developed and performed EQA studies with more challenging samples with greater characterisation which could improve the area of testing for AMR. This has then facilitated the performance of the key EQA studies to determine how the clinical community performs in an EQA of this type to set the current performance of the laboratories with these challenging and highly characterised materials. UWH have modified the standard MRSA EQA as a result of work within this project to improve material traceability.

Two molecular biology test kit manufacturers RTA Laboratuvarlari Biyolojik Urunler Ilaç ve Makine San.Tic. A.Ş. and Sentromer DNA Teknolojileri Ltd. Şti. have expressed an interest in the outputs of the project. The outputs of the project have been shared with these organisations and they have planned to directly integrate these into the development of new products. One manufacturer of LC-MS based diagnostic kits, Zivak Technologies, have expressed an interest in the outputs of the project and the outputs of the project will be shared with them to determine how they can integrate the findings into their product development. The project has shared its outputs with Ingenza and Amprologix who discover antibiotics and are developing manufacturing platforms for their production. The companies are interested in using the synthetic membranes to characterise and screen antibiotics as well as in a mechanistic understanding of their modes of action. In addition to this the microfluidics platforms have been demonstrated to industry stakeholders and the materials and methods are being used to screen experimental antibiotics: this is now offered as a measurement service by NPL.

The frameworks developed in this project and how measurement science can assist in improving AMR diagnostics research and development have been disseminated through workshops for early career researchers on diagnostics for Antimicrobial Resistance organised by the UK Learned Societies Partnership on Antimicrobial Resistance (LeSPAR) and the ESCMID courses on Principles of Molecular Microbiological Diagnostics in Maastricht, Netherlands. These types of activities enable the project to impact researchers in their early careers so that they can take these approaches to the chosen career pathway which could be industry/academia or clinical laboratories. The outputs from this project have also contributed to the formulation of analytical microbiology modules in the Medical Microbiology masters and undergraduate courses at the University of Surrey. Also through other activities within the consortium presentations have been given to a number of key international conferences as an example at EuroMedLab, ECCMID (European Congress of Clinical Microbiology and Infectious Diseases) and qPCR, dPCR and NGS Congress among others.

There have been a number of technology transfer examples from the consortium to the clinical community with best practice approaches being transferred for the application of dPCR in the Great Ormond Street Hospital and University College London Hospital as well as extraction methods used in this project being

utilised by Queen Mary University of London. An AntiMicroResist symposium in close collaboration with the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and BSAC took place within the BSAC Spring Conference in March 2019, Birmingham to showcase project findings and associated research and encourage uptake to members of industry, healthcare scientists and academia.

A further example of uptake from this project has been the implementation of a WGS method for AMR detection in *M. abscessus*, which has been mentioned following the recommendations generated within this project. It is also anticipated that this will be further extended in the implementation of a WGS method for the detection of CRE's later this year. It is anticipated that the good practice guide, a key deliverable from this project, will be further disseminated throughout the community so that the key observations can be visible and promote uptake in future studies. Further work from this part of the project have led to interaction with the key stakeholder Oxford Nanopore Technologies Ltd who have been part of the work investigating the use of their platform (MinION sequencer) to determine drug resistance mutations in *M. tuberculosis*. This part of the work contributed to the development of a bioinformatic tool hosted by one of the consortium collaborators, the London School of Hygiene and Tropical Medicine, which can facilitate the processing of the sequencing data for AMR detection which is ultimately contributing to a point of care format. It is anticipated that the data generated from this project (MinION, Cultured Isolates and 16S rRNA studies) will be freely available through the ENA (European Nucleotide Archive) hosted by EMBL-EBI and can be used by the community as an *in silico* tool for the verification of their bioinformatic pipeline.

Overall the consortium has managed to have a direct effect on the implementation and development of the approaches for AMR detection but ultimately highlighting and providing frameworks for determining traceability of materials and/or methods used. This has been highlighted through the development of a reference method for the quantification of hCMV, a reference system for the screening of MRSA as well as providing guidance for the implementation of reference systems for the quantification of PCT as examples. Through the close connections within the relevant international organisations that are active in the area of enhancing the comparability of laboratory medicine including IFCC, ESCMID and JCTLM as well as being part of the committees involved in the drafting of relevant ISO standards in the area of clinical laboratory testing and in vitro diagnostic test systems. The availability of materials from within the project including poster and oral communications, peer reviewed publications (currently 4 published open access papers and 9 drafted for submission), a good practice guide and recommendation reports will demonstrate to the industrial, clinical and scientific community how measurements in this field can be made with improved accuracy to improve accuracy in the measurements associated with identifying and managing antimicrobial resistance.

The key outputs from objective 5 were that the project achieved considerable activity and outputs in terms of facilitating uptake of the findings by end-users in healthcare and industry through production of a good practice guide, representation on relevant standards committees, establishment of new working groups, input into standardisation documents, workshops, meetings and publication of studies in peer reviewed journals.

5 Impact

More than thirty-six presentations/posters were made at conferences, including the 4th Joint EFLM – UEMS Congress, Poland, the 'qPCR dPCR & NGS 2017' Symposium, Germany and the European Congress of Clinical Microbiology. During the first half of the project four workshops were run including the LESPAR Early Career Researcher workshop on diagnostics for Antimicrobial Resistance in 2017 and the ESCMID (European Society of Clinical Microbiology and Infectious Diseases) 3rd Course on the Principles of Molecular Microbiological Diagnostics in 2018. Early project findings describing the first molecular reference measurement procedure for infectious diseases was also presented at the JCTLM Member's and Stakeholders' Workshop in December 2017. Three open access papers were published in international peer-reviewed journals, including Nature Communications and an additional seven papers drafted for peer review submission and planned open access publication. A project symposium was held at the British Society for Antimicrobial Chemotherapy (BSAC) Spring Conference in March 2019 to showcase project findings and associated research and to encourage uptake to members of industry, healthcare scientists and academia.

Impact on relevant standards
Impact on standardisation for AMR measurements was achieved through contribution to international activities including:

- A new work item proposal for ISO TC212 (Clinical laboratory testing and in vitro diagnostic test systems) WG4 (Microbiology and Molecular Diagnostics) on AMR measurement. Contribution to standard development in ISO TC276 (Biotechnology) WG3 (Analytical methods).
- A project proposal for a VAMAS inter-laboratory study for predicting an appropriate antibiotic treatment.
- A good practice guide on methods for the standardisation of next generation molecular methods for monitoring AMR was developed in association with the IFCC Committee for Molecular Diagnostics and this was submitted to the CCQM working group for nucleic acid measurements.
- A reference method for the quantification of microbial nucleic acids was submitted to JCTLM.

Impact on industrial and other user communities
AntiMicroResist generated early impact through the application of metrological concepts in a clinical context and the development of new measurement capabilities that includes novel, precise (and potentially SI traceable) reference methods for the measurement of surrogate biomarkers, resistant microbes and resistance levels. These concepts are applied by our clinical laboratory partners (Great Ormond Street Hospital (GOSH), The Royal Free Hospital (UCL), Helios Kliniken (UWH) and Klinika Golnik (UCG)). GOSH has already implemented a whole-genome sequencing method for AMR detection in the clinical laboratory that is based on the good practice guide delivered by the project.

Techniques for the development of reference materials to support a wide range of measurements associated with AMR management including methods to monitor nucleic acids, proteins and cell phenotypes were developed and incorporated into appropriate guidelines and disseminated (alongside the reference methods developed to characterise and quantify them) for use by the IVD industry and to support EQA provision. Early inclusion in relevant EQA schemes for both antiviral resistance and antimicrobial resistance testing was facilitated through our partnership with EQA scheme providers (both as partners, Helios Kliniken (UWH) and named collaborators Gesellschaft für Biotechnologische Diagnostik mbH (GBD)).

Close liaison with our clinical and industrial stakeholders and partners on AntiMicroResist is allowing the validated next generation diagnostic methods to be applied in preclinical and translational research in order to perform accurate measurements, which enable a better understanding of antimicrobial resistance. Furthermore, close discussion is progressing based on the materials and methods developed in the project to support near patient point of care AMR testing with a number of industrial collaborators including Oxford Nanopore, a leading sequencing technology provider, regarding the development of reference materials.

Impact on the metrology and scientific communities
AntiMicroResist has increased the focus of the metrology community on this medical priority by ensuring that AMR is part of the discussions amongst the CCQM and JCTLM and of the research portfolio of many European and global NMIs conducting bioanalysis. The impact on the wider scientific community was achieved by demonstrating how antimicrobial resistant measurement comparability can be improved. This

was ensured through peer reviewed papers in high impact journals, conference presentations, workshops and other dissemination activities, including best practice/required information guidelines for clinical research which are needed to assist in its translation to the patient.

Longer-term economic, social and environmental impacts
AntiMicroResist inherently responds to the needs of healthcare sectors, where its long term impact will be strongest. Although different sectors can benefit from the project results, the diagnosis and treatment of AMR remains the main impact domain where this coordinated metrological programme will have major long-term contributions.

Longer term social impact
In response to specific criteria, which WHO set in 2009-2011 and which retain their urgency today, in the longer term, the successful uptake of the project's outputs will improve the measurements that are required to understand the underlying reasons behind a high percentage of hospital acquired infections, monitor infections resistant to first line medicines and support the use of effective antimicrobials for the treatment and prevention of infections.

Longer term economic impact
The scale of the challenge is compounded by the fact that the most severe gaps in European biotechnology and pharmaceutical sectors remain at the early, high risk stages, making it difficult for small and medium enterprises to survive. The outputs of this project will aid in reducing the associated risks by improving the quality of the materials required to support accurate measurements. Given the growing market size in the diagnostics, and therapy of AMR, this will assist even small contributions in bringing significant benefits.

Longer term environmental impact
The emergence of virulent bacterial strains has adverse ecotoxicological consequences on environmental organisms, which is one of the reasons for the spread of antimicrobial resistance. While drugs and their breakdown products can be harmful or mutagenic to microorganisms, plants and animals, new medicines must be as specific as possible to provide the desired effects on human health without undesired consequences on other organisms. Consequently, many of the measurement findings will directly impact on the study and management of antimicrobial resistance in the environment by supporting both better application of antimicrobials, but also by demonstrating the accuracy of some of the technologies that are also used in environmental microbiology such as metagenomic analysis. More confident measurement and monitoring of AMR is also directly applicable to animal husbandry in both the agricultural and veterinary industry.

6 List of publications

Phelan, J, O'Sullivan, DM, Machado, D, Ramos, J, Campino, S, O'Grady, J, McNerney, R, Hibberd, M, Viveiros, M, Huggett, JF, Clark, TG (2019) Integrating informatics tools and portable sequencing technology for rapid detection of resistance to anti-tuberculosis drugs. Genome Medicine, 11:41 https://doi.org/10.1186/s13073-019-0650-x

Shaw LP, Doyle RM, Kavaliunaite E, Spencer H, Balloux F, Dixon G and Harris KA (2019). Children with cystic fibrosis are infected with multiple subpopulations of Mycobacterium abscessus with different antimicrobial resistance profiles. Clin Infect Dis, ciz069, https://doi.org/10.1093/cid/ciz069

De Santis, E., Alkassem, H., Lamarre, B., Faruqui, N., Bella, A., Noble, J. E., Micale, N., Ray, S., Burns, J., Yon, A. R., Hoogenboom, B. W. & Ryadnov, M. G. Antimicrobial peptide capsids of de novo design. Nature Commun, 8, 2263 (2017) https://doi.org/10.1038/s41467-017-02475-3

7 Contact details

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