Final Publishable JRP Summary for JRP HLT08 INFECT-MET Metrology for monitoring infectious diseases, antimicrobial resistance, and harmful micro-organisms

Overview

Infectious diseases account for over 20 % of human deaths globally and 25 % of all morbidity. Respiratory tract infections (RTI) such as pneumonia, influenza and tuberculosis account for almost 50 % of all pathogen associated deaths. Accurate and rapid diagnosis alongside methods for monitoring transmission and spread in the community and resistance to therapeutic agents (e.g. antibiotics) are vital for public health protection. Consequently, the infectious disease testing market is one of the most rapidly growing segments of the in vitro diagnostics industry which is predicted to be worth $75.1 Billion by 2020 with advances in molecular diagnostic technologies being the main driving force behind the expected growth. However, measurement support for molecular approaches is lacking, with issues concerning quality, comparability and traceability of measurements widely highlighted.

Pathogens (e.g. bacteria and viruses) may be present in clinical samples at very low levels making accurate detection and measurement challenging. In many instances, tests are being used in non-commercial 'home-brew' formats of variable and undefined quality and even commercially available tests cannot always be directly compared between laboratories due to a lack of traceable reference standards and reference methodologies. This lack of consistency can lead to over- or under-diagnosis of infectious diseases, resulting in inappropriate treatment with concomitant financial/ quality of life costs associated with increased morbidity, mortality, disease spread and spread of antimicrobial resistance.

This project successfully developed highly accurate methods (towards SI traceability) and materials to support the accurate quantification of pathogens in clinical samples and assign traceable values to materials used in External Quality Assessment schemes. Furthermore, it developed a framework for the scientific community on how to establish suitable reference systems and contributed to developing international standards development activities in this area.

Need for the project

Improved comparability and traceability across Europe of approaches used for the surveillance and monitoring of infectious diseases and detection of antimicrobial resistance mutations is needed. Similarly, emerging approaches for the rapid detection of infectious agents need improved metrics for quantifying performance in order to facilitate faster regulatory approval. Full confidence in molecular measurements can only be achieved if the appropriate metrology framework, standards and higher order methods which have reduced uncertainties compared to standard approaches are developed. Currently, higher order methods with the potential for traceability to the SI or equivalent and reference materials, which are required for compliance with *In Vitro* Diagnostics regulation and ISO clinical testing standards and essential for assessing performance and improving comparability, are lacking for all but a small handful of pathogens. Without the development of such methods and materials for high-priority infectious agents such as influenza and tuberculosis healthcare providers and the biotechnology/diagnostics industry will be unable to reliably demonstrate the reproducibility of their assays (tests) in a traceable and comparable manner. This is critical for the implementation of assays deployed in a wide range of healthcare settings. Higher order methods and approaches are needed for assigning traceable values to reference standards in order to improve the quality and comparability of current and emerging molecular assays.

Scientific and technical objectives

The key aims of INFECT-MET were to:

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- **Develop quantitative, validated and highly accurate methodologies for the measurement of infectious agents, such as viruses and bacteria.** Develop and evaluate higher order methods based on enumeration (for example, digital polymerase chain reaction (PCR) and single molecule counting in flow) for accurate measurement of infectious agents with known uncertainties and investigation of sample extraction from different matrices (e.g. blood, plasma and sputum).
- **Quantitatively and comparatively evaluate new and emerging molecular approaches for the surveillance and monitoring of infectious disease load and detection of antimicrobial resistance mutations.** Investigate the measurement challenges associated with emerging methodologies such as next generation nucleic acid sequencing (NGS) for surveillance, epidemiology and antibiotic resistance screening.
- **Quantitatively and comparatively evaluate new and emerging diagnostic technologies for the rapid detection of infectious agents.** Investigate the measurement challenges associated with emerging methodologies for rapid, near-patient testing, including DNA/microfluidic surface interactions and isothermal nucleic acid amplification evaluations.
- **Develop methodologies for accurately quantifying the performance of commercially available diagnostic assays, 'in-house' clinical assays and novel emerging approaches.** Develop a reference measurement framework using higher order measurement approaches in collaboration with end-user communities to improve calibration and the quality assurance of current clinical PCR approaches.

Results

Highly accurate methodologies for the quantitative measurement of infectious agents, such as viruses and bacteria developed & validated.

Three model systems of infectious agents, tuberculosis as a bacterial model, cytomegalovirus as a viral DNA model and influenza as a viral RNA model, were selected for the development and evaluation of higher order measurement methods in consultation with key stakeholders. The performance of the commonly used nucleic acid (DNA and RNA) extraction methods for each of the chosen model systems were compared and differences in yield and precision between the approaches were evaluated. Higher order methods (digital PCR – which has the ability to accurately quantify nucleic acid targets by single molecule enumeration) for the identification and quantification of the chosen model systems were developed and a comprehensive assessment of the relative performance of these methods was completed. Well-characterised test materials of differing levels of complexity (nucleic acid molecule, nucleic acid extract and whole microbe preparation) were prepared to facilitate these evaluations. A series of inter-laboratory studies were completed between the project partners to validate the assays and determine the reproducibility of the higher order methods developed and to assign values to the corresponding panels of test materials. Results showed no systematic bias between the different laboratories or digital PCR platforms used and comparable measurement uncertainties were obtained in each study.

The project successfully achieved this scientific and technical objective. For the first time in Europe, digital PCR methods to enable absolute quantification of infectious agents with improved traceability to SI through the concept of enumeration were developed and validated for the three model systems with full uncertainty budgets. A suite of novel test materials was produced for each model system and traceable values assigned using the developed methods. In addition to supporting Reference Material development and calibration service work of the EU National Measurement Institutes (NMIs), such methods and materials could also be used by clinical research laboratories to evaluate their routine in-house assays and facilitate comparisons to other assay formats. They could also be used by in vitro diagnostic (IVD) developers to validate their diagnostic assays and facilitate faster regulatory approval. The methodologies could also be used for traceable value assignment of reference materials, calibrants and External Quality Assessment (EQA) materials by reference material developers and EQA providers.

New and emerging molecular approaches for the surveillance and monitoring of infectious disease load and detection of antimicrobial resistance mutations quantitatively and comparatively evaluated.

Model systems to develop and evaluate methodologies to monitor antimicrobial resistance mutations in infectious diseases were selected. A novel digital PCR method to detect rare single RNA mutations using

Influenza Oseltamivir (Tamiflu) resistance as a model system was developed. The utility of the method was demonstrated through application to 'blinded' clinical samples and the successful identification of samples containing the resistance mutation. Sensitivity was improved compared to the PCR method used clinically. A novel nucleic acid sequencing approach to detect known DNA mutations using multi-drug-resistant tuberculosis (MDR-TB) as a model system was also developed. The sequencing approach developed was able to detect the drug resistant genotypes that were present within a large population of non-resistant genotypes. Extensively drug-resistant tuberculosis (XDR-TB) was used as a model to determine the ability of whole genome sequencing to genotype and identify unique antibiotic resistance determining mutations in clinical samples and any measurement errors generated by the process. Results showed that the precision of the sequencing instrument was very high, with an error rate of 0.05 % when measuring 2000 DNA sequences. Work to develop a next generation sequencing metagenomic approach (i.e. using clinical samples) for the surveillance and monitoring of the pathogens involved in chronic obstructive pulmonary disease (COPD) was completed. Assays to target the common causative pathogens of COPD were optimised and working ranges established. A whole microbe control material containing a mixture of 6 COPD-causing bacterial species (*Haemophilius influenza, Moraxella catarrhalis, Streptococcus pneumoniae, Escherichia coli, Klebsiella pneumonia & Pseudomonas aeruginosa*) at clinically relevant levels was prepared and used to evaluate the performance of the sequencing process (including DNA extraction/purification). Results showed that it was the sequencing strategy rather than the extraction strategy that had the main impact on the data obtained and caused the most bias.

The project successfully achieved this scientific and technical objective. An evaluation of novel molecular approaches (digital PCR and NGS) was completed for all of the clinical model systems. A novel digital PCR method was developed to detect drug resistance in influenza which showed an increased sensitivity over the current clinical method. New knowledge of the potential of NGS to detect low levels of drug resistant mutations and identification of pulmonary disease causing pathogens in clinical samples was also developed. These findings have helped to define the metrology support needed for emerging technologies such as NGS which can be used to direct future standardisation and metrology development work.

New and emerging diagnostic technologies for the rapid detection of infectious agents quantitatively and comparatively evaluated.

Emerging isothermal approaches such as loop-mediated isothermal amplification (LAMP) which are faster (often less than 10-20 minutes, compared to ~1 hour for standard PCR approaches) and cheaper (due to simpler instrumentation) than PCR-based approaches were evaluated (and published) for rapid near-patient testing. These approaches were compared to standard quantitative PCR (qPCR) based near-patient instrumentation (i.e. used for the simultaneous detection and quantification of a targeted DNA molecule). The potential of LAMP as a quantitative method for examining Cytomegalovirus (CMV) viral load was evaluated and results showed that transferring the LAMP assay to a digital format (i.e. so that it can accurately quantify nucleic acid targets by single molecule enumeration) increased the limit of quantification but it was found to be less sensitive than qPCR. However, as LAMP is faster and cheaper, for rapid applications where accurate quantification and ultra-low level detection is not needed, this approach could offer advantages over qPCR. In parallel, X-ray photoelectron spectroscopy (XPS) and time of flight secondary mass spectrometry (ToF-SIMS) methodologies were developed and used to characterise DNA binding to digital PCR microfluidic chips and to quantify the adhesion loss of DNA fragments and selected PCR reagents on different surface materials and coatings used in their manufacture. These measurements will aid in the uncertainty calculations when assessing chip-based approaches for absolute quantification.

The project successfully achieved this scientific and technical objective. New methodologies which offer a distinct advantage in terms of time and cost over standard qPCR approaches were tested and new knowledge gained into the adhesion loss of DNA in materials used in the manufacture of microfluidic devices which can lead to inaccurate measurement results.

Methodologies for accurately quantifying the performance of commercially available diagnostic assays, 'in-house' clinical assays and novel emerging approaches developed.

Wider inter-laboratory comparison studies for Tuberculosis (TB) and CMV, involving end-user laboratories participating in External Quality Assessment schemes (EQAS), were completed, demonstrating the suitability

of the digital PCR methods developed during the project for the analysis of clinical samples. For TB, combined data from multiple laboratories as well as the expected levels based on dPCR evaluation of the TB test materials were shared with all participating laboratories. This allowed, for example, the end-user laboratories to achieve a better understanding of variation in the bacterial load values reported by the GeneXpert® system, which is used by the majority of end-user clinical laboratories. For CMV, results showed that calibration with well-characterised test materials could improve the determination of the limit of detection (LOD) and increase the efficiency of diagnostic end-user methods.

The project successfully achieved this scientific and technical objective. The value of the test materials and methods developed in this project and their potential as prototype reference systems for increased standardisation of molecular diagnostics, particularly through adoption in EQAS, was demonstrated. Recommendations for the scientific community on how a suitable reference system could be established have been formulated into a best practice guidance document.

Actual and potential impact

Dissemination

The results and the quality and traceability guidance for infectious disease molecular diagnostics developed in the INFECT-MET project are currently being disseminated into the official international Standards system through the following routes:

1. ISO TC212 laboratory medicine WG2 - ISO 17511 revision (JRC & LGC committee participation & drafting)-developing examples of traceability schemes for pathogen detecting in vitro diagnostic devices.
2. ISO TC212 laboratory medicine WG4 - Technical Specification© ISO 2014 – ISO/TS 17822-1:2014(E) 63 Part 1: General requirements, terms and definitions In vitro diagnostic test systems — Qualitative nucleic acid-based in vitro examination procedures for detection and identification of microbial pathogen – publications in reference list.
3. ISO TC212 laboratory medicine WG4 - Consideration of submitting a PWI (Proposed Work Item) on quantitative PCR for infectious disease diagnostics, (inputting results from INFECT-MET). This proposed work item is being drafted by LGC/UK for consideration and approval at the November 2015 meeting of TC212 WG4.
4. ISO TC276 biotechnology WG3 analytical methods (LGC participation & lead on NWIP drafting committee) – input into ISO/PWI 20395: “Quality considerations for targeted nucleic acid quantification methods”. This approved PWI (Proposed Work Item) is currently being developed into a NWIP (New Work Item Proposal) for voting at the October meeting of ISO TC276.

Considerable dissemination to the relevant user communities has taken place during the course of the project. In total, 1 book and 7 peer-reviewed papers describing INFECT-MET scientific achievements have been published in peer-reviewed journals. Wider dissemination of outputs from the project was also achieved through an article published in the November 2014 issue of Laboratory News (UK). 39 Presentations have been given at relevant international professional and clinical symposiums and the consortium has also been active in organising and participating in a number of workshop/training events with the user community.

Early impact

At the end of the project there are many examples of the outputs being taken up by the relevant communities:

- As part of the project’s objective *to develop methodologies for accurately quantifying the performance of commercially available diagnostic assays*: ‘in-house’ clinical assays and novel emerging approaches our successful participation in External Quality Assessment schemes (EQAS) for Cytomegalovirus (CMV) highlighted the utility of the digital PCR method developed in the project. All samples analysed were designated as correct and in good agreement with the target values. Results from this scheme will support efforts to standardise quantitative detection of CMV in terms of replacing ‘consensus’ values with traceable reference values. This will ultimately lead to more reliable tests and

therefore more accurate/effective diagnosis. Discussions have begun with the organisers of EQAS to use the digital PCR method developed for CMV to value assign materials in future schemes.

- As part of the objective *to develop quantitative, validated and highly accurate methodologies for the measurement of infectious agents*: clinical laboratories collaborating on the project (e.g. Bolnisnica Golnik Klinicni oddelek za pljucne bolezni in alergijo (UCG)) have benefited from the step-wise approach to assessing the performance of commercial extraction kits in order to select the best kit to use in a clinical setting. These and other clinical laboratories will be able to benefit from the guidance provided in the best practice guidance document.
- As part of the project's objective *to develop highly accurate methodologies for the quantitative measurement of infectious agents*: the digital PCR method developed was used to value assign commercially available TB material from a microbiology-biotechnology company which develops, manufactures and markets ready-to-use PCR-based, and other, kits for the diagnosis of infectious diseases. The method used was able to provide the company with additional quantitative data on the amount of TB in their products, resulting in improved confidence in test performance.
- As part of the project's objective *to evaluate new and emerging molecular approaches for the surveillance and monitoring of infectious disease load and detection of antimicrobial resistance mutations*: the materials developed and donated by the project in prototype format for evaluation by end-users are already starting to have an impact on the way end-user laboratories perform their measurements and quality control. For example, the whole microbe control material has been used by a clinical laboratory to establish a next generation sequencing capability. Having a material that contains quantified amounts of bacteria has ensured that their laboratory and bioinformatic workflow was consistent and re-producible. The material is also being used by UCL to evaluate new sequencing technology being developed by a UK company.
- As an example of findings from the project being taken up by external laboratories, selection of the optimal TB extraction kit (part of the objective *to develop quantitative, validated and highly accurate methodologies for the measurement of infectious agents)* has helped UCL standardise their sample processing protocol in a clinical evaluation of Whole Genome Sequencing of TB directly from sputum samples.

Future and wider impact

Through the development of higher order methods, a metrology framework and standards, the output of INFECT-MET will help healthcare providers and the biotechnology/diagnostic industry to demonstrate the reliability of their assays in a traceable way. The outputs will have particular potential for impact in supporting both the activity of the proposed new network of Reference Laboratories for Class D (infectious pathogens) IVDs, and the requirements for demonstrating metrological traceability, mandated by the new EU IVD regulation, which has now received EU parliament approval for a partial general approach, and is now expected to enter into force by early 2016.

The IFCC (International Federation of Clinical Chemists and Laboratory Medicine) sub-committee on Molecular Diagnostics is currently focusing international effort on supporting increased measurement quality in infectious disease diagnostics. The Chair of the Committee has stated an interest in disseminating the project recommendations for reference systems for pathogen testing, through the IFCC member network.

The social impact of the project comes from supporting the provision of improved healthcare to citizens across Europe. The development of reference methodologies and materials with reduced uncertainties and improved traceability to the SI are now available and their uptake by commercial and clinical laboratories via routes such as External Quality Assessment schemes will lead to more robust and comparable diagnosis and monitoring of infectious diseases and antimicrobial resistance.

List of publications

Nixon, G., Garson, J., Grant, P., Nastouli, E., Foy, C., Huggett, J. (2014) A comparative study of sensitivity, linearity and resistance to inhibition of digital and non- digital PCR and LAMP assays for quantification of human cytomegalovirus. *Anal Chem*, 6; 86(9):4387-94.

Honeybourne, I. (2014) The Molecular Bacterial Load Assay replaces solid culture for measuring early bactericidal response to anti-tuberculosis treatment. *J. Clin. Microbiol,* 52(8):3064.

Huggett, J., O'Grady, J. (2014) Molecular Diagnostics: Current Research and Applications – Book. EAN: 9781908230416 9781908230645.

Nixon, G., Svenstrup, H., Donald, C., Carder, C., Stephenson, J., Morris-Jones, S., Huggett, J., Foy, C. (2014) A novel approach for evaluating the performance of real time quantitative loop-mediated isothermal amplification-based methods. Biomolecular Detection and Quantification 2: 4–10.

Devonshire, A., Honeyborne, I., Gutteridge, A., Whale, A., Nixon, G., Wilson, P., Jones, G., McHugh, T., Foy, C., Huggett, J. (2015) Highly reproducible absolute quantification of Mycobacterium tuberculosis complex by digital PCR. *Anal Chem,* 87(7):3706-13.

Neukammer, J., Hussels, M., Kummrow, A., Devonshire, A., Foy, C., Huggett, J., Parkes, H., Zel, J., Milavec, M., Schimmel, H., Unger, W., Akgoz, M., Mchugh, T., Tomic, V., Grunert, H.P., Zeichhardt, H. (2015) Survey results on nucleic acid tests of infectious diseases: Present status and need for rapid and patient near diagnostics. *GMS Zeitschrift zur Förderung der Qualitätssicherung in medizinischen Laboratorien*, Vol. 6, ISSN 1869-4241.

Pavšič J., Devonshire A., Parkes H., Schimmel H., Foy C., Karczmarczyk M., Gutierrez Aguirre I., Honeybourne I., Huggett J., McHugh T., Milavec M., Zeichhardt H., Žel J. (2015) Standardising clinical measurements of bacteria and viruses using nucleic acid tests. J. Clin. Microbiol, Volume 53, Number 7

Luznik, D., Kosnik, M., Tomic, V. (2015) Comparison of Seeplex PneumoBacter Detection ACE assay with in-house multiplex PCR for identification of Streptococcus pneumoniae. *New Microbiol.* 38(1):51-8.

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