Research Stimulus Fund

Final Report

Development of Novel Diagnostic strategies for the anti-mortem immunodiagnosis of bovine tuberculosis and Johne's disease: MYCOBACTDIAGNONOSIS

DAFM Project Reference No: **11/RD/EMIDA/1**

Start date: 01/06/2012

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Please place *one* "x" below in the appropriate area on the research continuum where you feel this project fits

Basic/Fundamental ⟶			Applied ⟶			Pre Commercial
1	2	3 X	4	5	6	7

Please specify priority area(s) of research this project relates to from the National Prioritisation Research Exercise* (NRPE) report;

Priority Area (s)	Sustainable Food Production and Processing

Key words: *(max 4)*
Tuberculosis; Paratuberculosis; Diagnostics

1. Rationale for Undertaking the Research

This section should outline the rationale for carrying out the research and identify the need / problem to be addressed

Mycobacterial infections of livestock such as bovine tuberculosis (bTB) or Johne's disease (JD) exact a high cost on European agriculture. bTB and JD are chronic inflammatory diseases caused by *Mycobacterium bovis* (*M. bovis*) and *M. avium paratuberculosis* (MAP), respectively. Detection and slaughter of *M. bovis* infected animals is required under EU law but JD control relies on voluntary cooperation. Both diseases can affect multiple domestic animal and wildlife species. The mainstay of bTB control is the skin test often combined with blood based interferon-γ (IFNG) release assays (IGRA); and serology. Detection of JD relies on serology (ELISAs). The diagnostics based on cellular immunity (CMI) measure responses to bovine, avian and johnin tuberculin (aka PPD), or similar crude cell or antigen extracts which have severe specificity and sensitivity limitations. These preparations share common antigens between different species of mycobacteria and their efficacy in the various tests can vary. With respect to bTB diagnosis, sensitivity and specificity of the comparative tuberculin skin test or the IGRA is severely compromised in animals that are dually infected with *M. bovis* and MAP as MAP infection results in high avian PPD responses masking bovine tuberculin responses. Vaccination of animals with current commercially available JD vaccines similarly produces immune responses that confound the diagnostic tests. Further, due to cross reactivity, PPD-based reagents in *M. bovis* skin testing elicits immune responses that may confound subsequent immunological detection of both diseases when complex antigen reagents such as whole bacterial extracts are being applied. Clearly there is an urgent need for specific diagnostic reagents for these important diseases and a requirement to validate diagnostic tests multi-nationally against a background of common mycobacterial infections.

The overall project aim was to improve the diagnosis of BTB and JD by generating more specific tools not compromised for sensitivity or specificity by co-infection and to increase the knowledge base of these two important livestock disease. The underlying philosophy of our consortium was based on a multi-pronged translational research approach combined with a fundamental and basic research arm. To deliver this goal, a consortium was formed of 11 partners from 7 countries (Czech Republic, France, Germany, Italy, Netherlands, United Kingdom, Republic of Ireland) through this 'MycobactDiagnosis' ERA-NET, with funding from individual national governments. This report will focus on the research work of UCD funded by DAFM.

2. Research Approach

Specify the research methodologies employed, emphasising novel techniques and also outline any modifications from the original approved project proposal

Our objective was to predict a set of *M. bovis* epitopes that reflect the allele-specific immune response to this pathogen. To achieve the goal of novel antigen identification our first step was to ensure that the annotation of the *M. bovis* 2122/97 genome, that would form the basis for this work, was accurate and up-to-date (the last update was 2002). To update the *M. bovis* AF2122/97 genome we pursued an approach that integrated a combination of *de novo* DNA, RNA and protein sequencing datasets. Then, since experimental binding data specific to BoLA-DRB3 (MHC-II) alleles is not available, we used pan-computational methods to approximate predictions based on already known human HLA-DR alleles. 'Pan' approaches are designed to allow methods trained on known alleles (those with available binding data sets) to be extrapolated to unknown alleles. Despite very limited validation, it has also been shown that MHC class II pan-specific predictions can be applied to cattle using human alleles. Our approach provided a significant enrichment for identification of T-cell epitopes, and underlines the potential of computational methods to accelerate antigen identification.

3. Research Achievements/Results

Outline main results achieved

Our re-annotation of the *M. bovis* 2122/97 genome resulted in identification of 16 high confidence nucleotide polymorphisms within the original *M. bovis* AF2122/97 genome sequence and disclosed the presence of the (supposedly deleted) large sequence polymorphism RD900. Next, a re-annotation project using proteogenomics in conjunction with transcriptomic data analysis resulted in the identification of a total of 44 novel CDS for the *M. bovis* AF2122/97 genome along with modifications to 14 existing gene annotations (manuscript in preparation).

Our next step was therefore to use the updated *M. bovis* 2122/97 genome annotation to predict a set of M. bovis epitopes that reflect the allele-specific immune response to this pathogen using a whole-genome screening approach. We used pan-computational methods (TEPITOPEpan and NetMHCIIpan) to approximate predictions based on already known human HLA-DR alleles. We used a selection of eight bovine alleles, which were based on published allele frequencies of Polish and US Holstein-Friesian cattle and BoLA alignments from the IPD database (https://www.ebi.ac.uk/ipd/mhc/bola/index.html). For each protein the set of all binders above appropriate cut-offs in at least *n* alleles were found and passed to a clustering algorithm that detected areas of high epitope density. This resulted in a ranked list of potential epitope sequences that could then be further filtered (based for example on evidence of protein expression from our proteogenomics datasets) so as to reduce the number of peptides that needed to be synthesised and screened in infected cattle.

For validation, a total of 376 peptides were synthesised: 182 of the top ranked predicted binders were selected along with 12 positive control peptides representing known epitopes recognised by bovine $CD4^+$ T cells from infected animals. A further random selection of 94 peptides of low predictive scores constituted a 'non-predicted' control set. All peptides were tested in 11 field reactor cattle naturally infected with M. bovis and peptide-specific IFN-γ responses determined by ELISA. Our computational approach provided a significant enrichment for identification of T-cell epitopes, and underlines the potential of computational methods to accelerate antigen identification. The work was recently published in Microbial Genomics (doi: 10.1099/mgen.0.000071).

To facilitate the dissemination of this computational approach, we developed EpitopeMAP, a web-based application for integrated execution, visualisation and analysis of MHC binding predictions in a flexible and user-friendly way (http://enzyme.ucd.ie/epitopeMAP).

4. Impact of the Research

A summary of the tangible impact of the research project should be provided under the outcomes' and 'outputs' heading below. In addition, please provide a short narrative synopsis of the benefits / improvements the research has made to the area under investigation particularly as regards end users, e.g. industry, consumers, regulatory authorities, policymakers, the scientific community, etc

Our work delivered two major scientific impacts:

- Firstly, we updated the genome annotation of *M. bovis* 2122/97 and made it available to the scientific community via the EMBL DNA database. This is the type strain for *M. bovis* genetic analysis and updating the functional information on the genes encoded in the genome will be of major benefit to researchers across the world.
- Secondly, our computational pipeline successfully enriched for peptides containing promiscuous epitopes, far in excess of what would be expected by chance. Our work increases considerably the hitherto known set of potential *M. bovis* antigens, and proves the utility of computational approaches to T-cell antigen-identification for infectious diseases. As such our computational pipeline could have major positive impact on the design of new vaccines and/or diagnostics.

4(a) Summary of Research Outcomes

(i) Collaborative links developed during this research

- This work involved direct collaborations with the groups of Prof Martin Vordermeier (APHA, UK); Dr Franck Biet (INRA, France); Dr Karen Stevenson (Moredun Research Institute, UK); Dr Jim McNair (AFBI, UK); Prof Stewart Cole (EPFL, Switzerland), Prof Ruedi Aebersold (ETH Zurich, Switzerland).

(ii) Outcomes where new products, technologies and processes were developed and/or adopted

- Our technological advance was to use two contrasting computational strategies, based primarily on MHC-II binding predictions, to select potential mycobacterial peptidic epitopes recognised by bovine T cells from the very large sequence space of the *M. bovis* proteome.

Our methods were successful in capturing epitope rich sequences using this computational approach, and our strategy based on finding regions of high promiscuity binder clusters seems the most promising. These epitopes are excellent medium-term candidates for use in future *M. bovis* diagnostics or potential sub-unit vaccines, while the computational methods presented here have general immediate impact for application in epitope selection across multiple infectious diseases.

(iii) Outcomes with economic potential

- As stated above, the novel promiscuous epitopes of *M. bovis* identified through our computational approach have medium-term potential for economic in that they could be used in next generation vaccines and/or diagnostics against bTB. An Invention Disclosure was submitted to UCD Research in 2015 to initiate the process of protecting the peptide sequences and computational approach.

(iv) Outcomes with national/ policy/social/environmental potential

- Improved control options for bTB would have significant long term impacts on the control of a major endemic disease.

4 (b) Summary of Research Outputs

(i) Peer-reviewed publications, International Journal/Book chapters.

- Dinan AM, Tong P, Lohan AJ, Conlon KM, Miranda-CasoLuengo AA, Malone KM, Gordon SV, Loftus BJ "Relaxed selection drives a noisy noncoding transcriptome in members of the Mycobacterium tuberculosis complex" MBio. 2014 Aug 5;5(4):e01169-14. doi: 10.1128/mBio.01169-14
- Farrell D, Gordon SV 'Epitopemap: a web application for integrated whole proteome epitope prediction'. BMC Bioinformatics. 2015 Jul 14;16:221. doi:10.1186/s12859-015-0659-0.
- Farrell D, Shaughnessy RG, Britton L, MacHugh DE, Markey B, Gordon SV 'The Identification of Circulating MiRNA in Bovine Serum and Their Potential as Novel Biomarkers of Early Mycobacterium avium subsp paratuberculosis Infection' PLoS One. 2015 Jul 28;10(7):e0134310. doi: 10.1371/journal.pone.0134310.
- Shaughnessy RG, Farrell D, Riepema K, Bakker D, Gordon SV "Analysis of Biobanked Serum from a Mycobacterium avium subsp paratuberculosis Bovine Infection Model Confirms the Remarkable Stability of Circulating miRNA Profiles and Defines a Bovine Serum miRNA Repertoire" PLoS One. 2015 Dec 16;10(12):e0145089. doi: 10.1371/journal.pone.0145089
- Farrell D, Jones G, Pirson C, Malone K, Rue-Albrecht K, Chubb AJ, Vordermeier M, Gordon SV "Integrated computational prediction and experimental validation identifies promiscuous T cell epitopes in the proteome of *Mycobacterium bovis*." Microbial Genomics, in press doi: 10.1099/mgen.0.000071

(ii) Popular non-scientific publications and abstracts including those presented at conferences

- Kerri M. Malone 'A Proteogenomic approach to identify novel T-cell antigens from Mycobacterium bovis for the development of new diagnostics and vaccines for Bovine Tuberculosis' Oral Presentation, AVTRW Irish Region 47th meeting, Agri-Food & Biosciences Institute (AFBI) Hillsborough. 4th October 2013

- Kerri M. Malone 'A Proteogenomic approach to identify novel T-cell antigens from Mycobacterium bovis for the development of new diagnostics and vaccines for Bovine Tuberculosis' Poster Presentation, Animal Health Ireland Conference, Rochestown Park, Cork 23rd October 2013
- Kerri M. Malone 'Proteogenomics: A dynamic tool for strengthening our knowledge of non-model organisms' Oral Presentation, Society for General Microbiology Irish Branch Meeting, 21st March 2014
- Stephen Gordon 'Exploring host tropism across the Mycobacterium tuberculosis complex' Oral Presentation, Irish Society for Immunology 12th Sept 2014
- Stephen Gordon 'Exploring host tropism across the Mycobacterium tuberculosis complex' Oral Presentation, Society for General Microbiology Irish Branch Meeting, 21st March 2014
- Stephen Gordon "Genomic Insights into the Biology of *Mycobacterium bovis*" Invited plenary lecture, VI International Mbovis meeting, Cardiff, UK 16th-19th June 2014
- Stephen Gordon "Comparative analyses of the human- and animal-adpated tubercle bacilli" Invited Plenary Lecture, International Union of Microbiological Societies (IUMS) 2014, Montreal, Canada 27th July-1st August 2014
- Stephen Gordon "Tuberculosis and One Health: Insights from *Mycobacterium bovis*" Invited plenary openign lecture, 4th MycoClub meeting, Marseille, France, 6th-7th May 2015
- Kerri M. Malone "Proteogenomics: A dynamic tool for strengthening our knowledge of non-model organisms" Poster Presentation, Genomes 2014 Conference, Paris France 24th-27th June 2014
- Damien Farrell "Computational selection of novel antigenic targets in the *Mycobacterium bovis* proteome" Poster Presentation, Genomes 2014 Conference, Paris France 24th-27th June 2014
- Kerri Malone "An integrated 'omics approach to define functional variation between the human and bovine tubercle bacilli" Acid Fast Club Meeting, Univeristy of Birmingham, 3rd July 2015
- Stephen Gordon "The *Mycobacterium tuberculosis* complex as a One Health paradigm" 3rd Sapporo Summer Seminar for One Health Hokkaido Univeristy, Japan, 16-17/09/2015

(iii) National Report

None

(iv) Workshops/seminars at which results were presented

- Stephen Gordon Departmental Seminar, National Institute for Medical Research, London, UK. Invited Seminar, "Tuberculosis and One Health: Insights from Mycobacterium bovis" 8th Jan 2015
- Stephen Gordon "Tuberculosis and One Health: adventures with Mycobacterium bovis" Departmental seminar National Animal Disease Center, Ames, Iowa, USA, 22nd June 2015

(v) Intellectual Property applications/licences/patents

- As stated above, an Invention Disclosure was submitted to UCD Research in 2015 with a view to protecting intellectual property contained in the peptide sequences and computational approach used in their discovery.

(vi) Other

N/A

5. Scientists trained by Project

Total Number of PhD theses: 1

Please include authors, institutions and titles of theses and submission dates. If not submitted please give the anticipated submission date

- Kerri M. Malone, PhD thesis, UCD: "An intergrative 'Omics approach to define functional variation between the human and bovine tubercle bacilli", submitted January 2016, graduated June 2016.

Total Number of Masters theses: 0

Please include authors, institutions and titles of theses and submission dates. If not submitted please give the anticipated submission date

6. Permanent Researchers

Institution Name	Number of Permanent staff contributing to project	Total Time contribution (person years)
UCD	3	0.76
Total	**3**	**0.76**

7. Researchers Funded by DAFM

Type of Researcher	Number	Total Time contribution (person years)
Post Doctorates/Contract Researchers	1	2.0
PhD students	1	3.1
Masters students	0	
Temporary researchers	0	
Other	0	
Total	**2**	**5.1**

8. Involvement in Agri Food Graduate Development Programme

Name of Postgraduate / contract researcher	Names and Dates of modules attended
N/A	

9. Project Expenditure

Total expenditure of the project:	€281,508
Total Award by DAFM:	€281,905
Other sources of funding including benefit in kind and/or cash contribution(specify):	€0

Breakdown of Total Expenditure

Category	UCD	Name Institution 2	Name Institution 3	Name Institution 4	Total
Contract staff					
Temporary staff					
Post doctorates	89,840.46				89,840.46
Post graduates	65,799.88				65,799.88
Consumables	46,133.00				46,133.00
Travel and subsistence	7,052.94				7,052.94
Sub total	208,826.28				208,826.28
Durable equipment	1,229.58				1,229.58
Other	8,804.62				8,804.62
Overheads	62,647.88				62,647.88
Total	**281,508.36**				**281,508.36**

10. Leveraging

Summarise any additional resources'/funding leveraged by this award from other sources e.g. Additional Staff, National/EU funding secured, EI Commercialisation Fund, etc.

- Dr Damien Farrell submitted an application for an Irish Research Council Government of Ireland Postdoctoral Fellowship 2015 on 27th Nov 2014, and this was awarded on 7th May 2015. This work will expand his bioinformatics approaches to improved disease diagnostics, focusing on MAP infection in cattle.

11. Future Strategies

Outline development plans for the results of the research.

- The potential for our computational approach to identify novel T-cell epitopes from other infectious agents will be continued, with an initial focus on *Mycobacterium avium* subsp *paratuberculosis*, the agent of Johne's Disease/Paratuberculosis in cattle. This work is being pursued in Dr Damien Farrell's IRC Postdoctoral Fellowship, and further funding has been sought through a pending application to the DAFM 2015 Research Stimulus Fund.