



An Roinn Talmhaíochta,
Bia agus Mara
Department of Agriculture,
Food and the Marine

13 S 472 - Early Diagnosis of Postpartum Uterine Disease for Enhancement of Reproduction and Improved Cow Health

Final Report

This project was funded under the Department of Agriculture,
Food and the Marine Competitive Funding Programme.

SUMMARY

The consumer wants a product (milk) that is coming from healthy cows that are kept in a sustainable farming environment. With milk production in Ireland intensifying uterine disease will increase. Postpartum uterine infections are a leading cause of compromised fertility, which is the single biggest threat to the Irish cattle sector. While considerable progress has been made in understanding uterine infection at multiple levels, no definitive early prognosis and intervention strategies exist to aid farmers in getting cows back in calf.

Our research objective was to integrate key multidisciplinary specialties to comprehensively address infertility in cattle by providing validated tools for early prognosis and treatment of uterine disease.

Having identified biomarkers that can determine on day 7 post calving if a cow will develop a uterine infection (endometritis) on day 21 will help farmers and vets to be able to intervene early and thus preventing cows from getting sick and not being able to produce the amount of milk they could. This will ensure that the consumer will continue to be able to avail of a product coming from a healthy cow and will prevent welfare implications for the cow and economic implications for the farmer.

KEYWORDS

Dairy cows, endometritis, biomarker

ACRONYM

ENRICH

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Dr. Andrew Cromie	Irish Cattle Breeding Federation

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23/10/2020

Section 1 - Research Approach & Results

Start Date

01 January 2014

End Date

30 September 2019

Research Programme

Research Stimulus Fund

TRL Scale

TRL 3: Experimental Proof of Concept

NRPE Priority area

Sustainable Food Production and Processing

Total DAFM Award

€1,224,817.30

Total Project Expenditure

€1,065,230.94

Rationale for undertaking the Research

Infertility is the biggest issue for the Irish dairy industry and postpartum uterine infection is a major contributor to this problem. After calving the uterine microbiome changes and this can lead to a form of uterine disease in majority of cows. Clinical uterine disease within 3 weeks of calving is defined as metritis, and the incidence is between 36%-50%. In 15%-20% of cows, infection persists beyond 3 weeks postpartum as endometritis and a further 30% of cows suffer from sub-clinical endometritis. Uterine infections decrease cow fertility: calving to conception is 30 days longer and conception rate to first service 20% lower with 3% cows remaining infertile even after clinical resolution of disease. The consequences of infertility, together with production losses and treatment costs associated with uterine disease, costs farmers an estimated €292/cow/year. With a national herd of approximately 1.3 million dairy cows and disease incidence ranging from a conservative 20% to the more recently reported 75% we can calculate that uterine infection costs the Irish dairy herd between €76 €284 million annually. Diagnosis of uterine infection typically occurs after clinical observation of disease between 2-5 weeks after calving. However, by this point substantial damage to the cow's health, productivity, and fertility has already occurred. A reliable early test to accurately diagnose uterine disease in the first few days after calving would be of considerable benefit toward tackling reduced fertility; this would enable early therapeutic intervention, and development of management strategies to reduce the substantial economic and welfare impacts.

Methodology

Research Methodologies employed:

- A. Detailed recording on 5 farms across 2 years resulting in the collection that were collected both at multiple time points. This sampling schedule demanded intensive sampling windows and specialist expertise from veterinary practitioners. Disease diagnosis was performed at 21 days after calving both in the field (by vaginal mucus score) as well as in the laboratory (cytology).
- B. This extensive sample database was complemented by the collection of reproductive and production records from ICBF on these farms resulting in a final dataset of 441 records for analysis.
- C. An association analysis was performed using reproduction and production data with disease diagnosis in 440 cows
- D. Multiple laboratory analyses were performed on these samples which included:
 - a. Measurements of metabolic and health status
 - i. Haematology analysis was performed to assess blood cell populations
 - ii. Disease diagnosis was performed for both clinical and sub-clinical disease
 - iii. Metabolites were measured
 - b. Measurement of nucleic acid (in subsets of samples)
 - i. microRNA expression levels were assessed in serum
 - ii. mRNA gene expression levels were assessed in epithelial cells collected from the uterus
 - c. Measurement of protein expression levels
 - i. Multiple inflammatory markers were measured in serum
 - ii. Multiple inflammatory markers were measured in vaginal mucus
 - d. Assessment of glycosylation levels of specific proteins in serum and milk
 - i. Milk is not the best sample matrix to use for quantification of glycoprotein biomarkers
 - ii. Glycoproteins AGP and IgG are analysable in serum and can be used as biomarkers
 - e. Assessment of the microbiome using
 - i. 16S amplicon Illumina-sequencing (in a subset of samples)

ii. Detection of endometritis associated microbial biomarkers in vaginal mucus using Taqman probes that were developed during this project.

Project Results

- A. We demonstrated that the occurrence of clinical disease in Irish dairy cattle results in a significantly longer calving to conception period (PMID: 31733846).
- B. We showed in the same publication that higher milk yield significantly increases the chances of clinical uterine disease in the subsequent lactation. This concept of cumulative stressors is very relevant and is not often considered.
- C. We have characterised the occurrence of both clinical and sub-clinical disease, as well as the overlap in occurrence between both conditions in the Irish dairy system.
- D. We characterised both the local (uterine) and systemic (in blood) response to disease.
- E. This characterisation has yielded valuable insights into the biology of infection but has also identified a number of useful inflammatory markers for disease diagnosis (PMID: 31233901).
- F. The identification of differentially glycosylated proteins in serum is very novel and may have potential utility as biomarkers for uterine disease, with animals going on to develop disease having different IgG glycosylation than healthy animals.
- G. We characterised the vaginal and uterine microbiota in cows during the postpartum period. We found that the loss of compartmentalisation of the reproductive tract during parturition results in the mixing of vaginal and uterine microbiota. Further differentiation of vaginal and uterine microbiota is apparent at 7 DPP in healthy cows, but it is delayed in cows that develop postpartum endometritis at 21 DPP. In addition, we showed that at 7 DPP, there are at least three different microbiome types associated with the development of postpartum endometritis (PMID: 30629579).
- H. We designed and characterised three Taqman probes-based assays for detection of early biomarkers associated with the different endometritic microbiome types. From these, we developed an assay that detects an endometritis-specific biomarker, called EFUP1452, present in two of the three microbiome types. Amplicon sequencing of the 16S rRNA of a subset of samples validated the above findings and at the same time allowed to correct for microbiome type. This resulted in an assay with sensitivity of 94%, specificity of 90% and accuracy of 91%.
- I. The development of an optimised technique for the measurement of inflammatory proteins in vaginal mucus (PMID: 30268128, PMID:30117040 and PMID: 28780482).
- J. We have developed an optimised method for the reliable isolation of cells from post-mortem tissue for immunological studies (PMID:32086740). This is very valuable achievement as it reduced the requirements for animal sampling in accordance with the NC3Rs.
- K. We have performed detailed mechanistic analysis of the control of inflammation within the bovine uterus (PMID: 30804935). This work is published in the high impact journal *Frontiers in Immunology* and identified inhibitors which reduce inflammation in cattle for the first time. These could become useful therapeutic drugs of the future.
- L. Discussions with NOVA-UCD are ongoing to explore patenting of prognostic microbiological and vaginal mucus markers for the development of endometritis 7 DPP.
- M. We have various papers in preparation and under review based on RND-seq and vitamin D in relation to the characteristics of endometritic cows.

Section 2 - Research Outputs

Summary of Benefits / Improvements of Project Findings

Before this project commenced we did not know the impact of uterine disease under a primarily pasture based system of dairying in Ireland. This project has delivered a wealth of useful science-based information on which to base future work and direct disease prevention and control measures. Our study showed that vaginal microbiota postpartum harbours early biomarkers associated with the development of postpartum endometritis. In addition, we unveiled different microbiome types associated with endometritis, showing that this disorder is more complex than previously thought. Furthermore, we developed an early diagnostics test that detects about 90% of cows of two of the three microbiota types with high specificity. As multiple independent sources of evidence (incl PMID: 26304020 on Irish cattle with high and low genetic merit for fertility) now suggest that uterine health and specifically, the regulation of inflammation is key to fertility. Our study has not only identified the precise molecular mechanisms underpinning this inflammation (in terms of genes and pathways) but also identified means to measure that inflammation in a cow-side routine manner. Furthermore, we have shown in vitro that specific immunological compounds can block that inflammation. Therefore, this project has proven an impact of disease on reproduction traits and shown that disease occurrence is exacerbated by high milk yield. This is of relevant across the spectrum from policy makers including DAFM in relation to methods to prevent the occurrence of disease. It is relevant to farmers in terms of identifying precise targets for diagnosis as well as enabling the identification of at risk cows through our vaginal mucus assay, which will enable them to employ risk amelioration management strategies (including tailored diets for example). The results will now enable early disease diagnosis and interventions that will reduce disease development which is associated with production losses and compromised animal welfare.

Summary of Staff Outputs

Research Output	Male	Female	Total Number
PhD Students	2	1	3
Post Doctorates	1	0	1
MSc Students	1	1	2

Summary of Academic Outputs

Research Outputs	Total Number	Details
PhD Theses	3	<p>Dr. Amy Brewer Dr. Paul Kelly Dr. Nicholas Ryan</p>
Publications in Peer Reviewed Scientific Journals	7	<p>Miranda-CasoLuengo R, Lu J, Williams EJ, Miranda-CasoLuengo AA, Carrington SD, Evans ACO, Meijer WG. Delayed differentiation of vaginal and uterine microbiomes in dairy cows developing postpartum endometritis. PLoS One. 2019 Jan 10;14(1)</p> <p>Kelly P, Meade KG, O'Farrelly C. Non-canonical Inflammasome-Mediated IL-1β Production by Primary Endometrial Epithelial and Stromal Fibroblast Cells Is NLRP3 and Caspase-4 Dependent. Front Immunol. 2019 Feb 5;10:102</p> <p>Miller BA, Brewer A, Nanni P, Lim JJ, Callanan JJ, Grossmann J, Kunz L, de Almeida AM, Meade KG, Chapwanya A. Characterization of circulating plasma proteins in dairy cows with cytological endometritis. J Proteomics. 2019 Aug 15;205:103421.</p> <p>Ryan NJ, Meade KG, Williams EJ, O'Farrelly C, Grant J, Evans ACO, Beltman ME. Purulent vaginal discharge diagnosed in pasture-based Holstein-Friesian cows at 21 days postpartum is influenced by previous lactation milk yield and results in diminished fertility. J Dairy Sci. 2020 Jan;103(1):666-675.</p> <p>Adnane M, Meade KG, O'Farrelly C. Cervico-vaginal mucus (CVM) - an accessible source of immunologically informative biomolecules. Vet Res Commun. 2018 Dec;42(4)</p> <p>Adnane M, Kelly P, Chapwanya A, Meade KG, O'Farrelly C. Improved detection of biomarkers in cervico-vaginal mucus (CVM) from postpartum cattle. BMC Vet Res. 2018 Sep 29;14(1)</p> <p>Qualitative and quantitative differences in endometrial inflammatory gene expression precedes the development of bovine uterine disease, Brewer et al., resubmitted and under review July 2020.</p> <p>Circulating vitamin D levels in post-partum cows Ryan et al., 2020 in preparation</p>
Masters Theses	2	<p>Mr. Joe Lim Ms. Ysabel Wright</p>
Peer Reviewed Conference Papers	11	<p>Nicholas Ryan Effects of uterine infection on milk production and reproduction outcomes in dairy cows in Ireland. British Society for Animal Science 2019, Edinburgh, UK.</p> <p>Amy Brewer Defining the inflammatory gene signature that precedes the development of uterine disease in postpartum cattle. British Society for Animal Science Conference, Dublin</p> <p>Amy Brewer Defining the inflammatory gene signature that precedes the development of uterine disease in postpartum cattle. Teagasc Walsh Fellowship seminar</p> <p>Amy Brewer Defining the inflammatory gene signature that precedes the development of uterine disease in postpartum cattle. Immunology Research Froum, TCD. Oral presentation.</p> <p>Paul Kelly Inflammasome dependent production of IL-1β by bovine endometrial stromal cells and polarized epithelial cells. Irish Society of Immunology (ISI) meeting, Ireland. Poster</p> <p>Paul Kelly Inflammasome dependent production of IL-1β by bovine endometrial stromal cells and polarized epithelial cells. British Society for Animal Science Conference, Dublin</p> <p>Nicholas Ryan AGP concentrations at 7 days post partum as a predictor for uterine disease in dairy cows ESDAR conference Cordoba, Spain</p> <p>Nicholas Ryan Elevated risk of clinical endometritis and reduced fertility in high yielding Irish dairy cows AVTRW, Backweston, Kildare</p>

cows IPRC, Dublin

Miranda-CasoLuengo R Pre-symptomatic changes in the microbiome of cows developing postpartum endometritis. Flash poster: Microbiology Society Focused Meeting Microbes and Mucosal Surfaces, University College Dublin

Miranda-CasoLuengo R, Lu J, Williams EJ, Miranda-CasoLuengo AA, Carrington SD, Evans ACO, Meijer WG. Delayed differentiation of vaginal and uterine microbiomes in dairy cows developing postpartum endometritis. "Poster presentation: MedVet Pathogens. Prato, Italy"

Intellectual Property

Discussions with NOVA-UCD are ongoing to explore patenting of prognostic markers for the development of endometritis 7 DPP.

Summary of other Project Outputs

Project Outputs	Details	Total No.
New Technology	The combination of microbiological and circulating biomarkers to increase the sensitivity of our biomarker panel is a novel way to identify cows that are at high risk of becoming diseased.	1

Potential Impact related to Policy, Practice and Other Impacts

Impact	Details
Environmental Sustainability	Identifying cows that will develop a disease later on will allow for early treatment of these animals, therefore not only leading to a decrease in antibiotic use on the farms but also maintaining the cow in the herd and her being able to achieve her full lactation potential, thus not needing extra replacement heifer and thus more cow numbers.
Industry	Early identification of cows that will become diseased at a later stage will prevent an economic loss for the farmer, as there will be less of a decrease in milk production and less veterinary costs when the cow is identified early.

Dissemination Activities

Activity	Details
Workshops at which results were presented	The main results achieved to that date were presented at a stakeholder workshop in August 2018.

Seminars at which results were presented

All 3 students presented the results of their studies both at national and international research seminars:

Nicholas Ryan Effects of uterine infection on milk production and reproduction outcomes in dairy cows in Ireland. British Society for Animal Science 2019, Edinburgh, UK.

Amy Brewer Defining the inflammatory gene signature that precedes the development of uterine disease in postpartum cattle. British Society for Animal Science Conference, Dublin

Amy Brewer Defining the inflammatory gene signature that precedes the development of uterine disease in postpartum cattle. Teagasc Walsh Fellowship seminar

Amy Brewer Defining the inflammatory gene signature that precedes the development of uterine disease in postpartum cattle. Immunology Research Froum, TCD. Oral presentation.

Paul Kelly Inflammasome dependent production of IL-1 β by bovine endometrial stromal cells and polarized epithelial cells. Irish Society of Immunology (ISI) meeting, Ireland. Poster

Paul Kelly Inflammasome dependent production of IL-1 β by bovine endometrial stromal cells and polarized epithelial cells. British Society for Animal Science Conference, Dublin

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Miranda-CasoLuengo R, Lu J, Williams EJ, Miranda-CasoLuengo AA, Carrington SD, Evans ACO, Meijer WG. Delayed differentiation of vaginal and uterine microbiomes in dairy cows developing postpartum endometritis. "Poster presentation: MedVet Pathogens. Prato, Italy"

Other

Publications:

Miranda-CasoLuengo R, Lu J, Williams EJ, Miranda-CasoLuengo AA, Carrington SD, Evans ACO, Meijer WG. Delayed differentiation of vaginal and uterine microbiomes in dairy cows developing postpartum endometritis. PLoS One. 2019 Jan 10;14(1)

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Adnane M, Kelly P, Chapwanya A, Meade KG, O'Farrelly C. Improved detection of biomarkers in cervicovaginal mucus (CVM) from postpartum cattle. BMC Vet Res. 2018 Sep 29;14(1)

Qualitative and quantitative differences in endometrial inflammatory gene expression precedes the development of bovine uterine disease, Brewer et al., resubmitted and under review July 2020.

Circulating vitamin D levels in post-partum cows Ryan et al., 2020 in preparation

Knowledge Transfer Activities

Identify knowledge outputs generated during this project.

Vaginal microbiome from cows with or without clinical endometritis identified.
Effect of milk yield in previous lactation on subsequent chance of developing clinical endometritis identified.

Circulating markers on day 7 post partum identified in cows who subsequently developed endometritis
Local markers on day 7 post partum identified in cows who subsequently developed endometritis.

Identify any knowledge transfer activities executed within the project.

The results of all studies were presented at a stakeholder meeting in August 2018. This stakeholder meeting was attended by fellow scientists, vets, farmers and industry. On a smaller scale regular presentations on results of the project have been given by both PIs and students on the project to both national and international peers.

List any impacts resulting from the knowledge transferred during the project.

Markers identified have been validated during the last phase of the project and a combination of circulating markers does help identify cows that develop disease subsequently early. This helps with early intervention and therefore decreases losses via lack of milk production and increased veterinary costs.
Some of these results have already been published in international peer reviewed journals with more to come - leading to more knowledge transfer.

Section 3 - Leveraging, Future Strategies & Reference

Leveraging Metrics

Type of Funding Resource	Funding €	Summary
Additional Staff	€0.00	In collaboration with Ross University in St. Kitts, veterinary interns were recruited to the project which gave us additional valuable veterinary expertise for diagnosis and sampling on farms. This also led to two additional MSc's funded by Ross University.
Additional Staff	€0.00	Collaboration with another international vet (Mounir Adnane) enabled us to develop an optimised technique for the measurement of the inflammatory markers identified in vaginal mucus. This is a very novel and relevant development for application of our research findings on farm. It also led to three peer-reviewed publications.

Future Strategies

1. The results from this DAFM funded project have laid the foundation for the researchers to secure additional competitive funding and building critical mass in the area of uterine health research. The immediate aim is to complete the publication of outstanding manuscripts.
2. The project partners exploring patenting of microbiological and vaginal mucus based prognostic markers for the development of endometritis 7 DPP
3. The discovery of different microbiome types associated with the development of postpartum endometritis opens a new door in the field. Further research will need to address 1) whether they represent different states during the differentiation of the microbiota or different types of uterine disease that are not clinically apparent and 2) whether they have different impacts on fertility and production.
4. In the post-quota era of dairy herd expansion, the issue of optimising uterine health in the dairy cow will remain a critically important issue. We now have the tools (assays), markers (inflammatory proteins) and expertise (technological know-how) to monitor uterine health and for the early detection.
5. The interest of a commercial company in translation of these findings into a cow-side diagnostic assay remains to be assessed.

Project Publications

1. Adnane M, Meade KG, O'Farrelly C. Cervico-vaginal mucus (CVM) - an accessible source of immunologically informative biomolecules. *Vet Res Commun.* 2018 Dec;42(4)
2. Adnane M, Kelly P, Chapwanya A, Meade KG, O'Farrelly C. Improved detection of biomarkers in cervico-vaginal mucus (CVM) from postpartum cattle. *BMC Vet Res.* 2018 Sep 29;14(1)
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