Final Report 2016

AHDB
BEEF & LAMB

Project Title Pilot study to assess the performance of PCR to detect Jaagsiekte Sheep Retrovirus (JSRV) infected sheep as part of a "thin ewe" diagnostic package.

Summary sheet (up to two pages)

Project number	6120022011		
Start date	01 Nov 2015	**End date**	30th Oct 2016

Project aim and objectives

The aim of the project was to assess whether the JSRV PCR test, when used on a subset of animals that are exhibiting poor body condition, one of the clinical signs that may represent early OPA, is an effective flock test for OPA. The study also aimed to determine whether samples of peripheral blood or of nasal secretions performed differently and which performed best.

The objectives were refinement of the PCR test previously used in a research setting for transfer to a commercial laboratory, and analysis of the results obtained testing known OPA-positive and negative flocks in order to establish the usefulness of the test as a flock test for OPA. The final objective was to disseminate the results of the study to veterinary and farming audiences.

Key messages emerging from the project

The nasal swab test proved better than the blood test and was more suitable for testing in a commercial laboratory setting. Although, like the blood test, it does not identify every OPA-positive individual, it performed well enough as a flock test applied to thin ewes to have potential as the basis of a flock assurance scheme.

Summary of results

Optimal extraction and PCR protocols were established in the commercial laboratory and samples were obtained for testing from 3 OPA-negative and 6 OPA-positive flocks. Postmortem examination(PME), thoracic ultrasonography or wheelbarrow test data made available from a concurrent study added value by enabling us to assess the efficacy of testing at an individual level as well as for a flock test.

Initial testing on samples of known OPA-positive and negative individual sheep showed that the nasal swab test was better than the blood test and therefore the remaining work focused on the nasal swab testing.

In 5 of the 6 known OPA-affected flocks tested a positive result was obtained from at least one nasal swab sample of the 20 samples tested. There were no positive results in animals from OPA-negative flocks or in OPA-negative individuals from affected flocks. That is, there were no false positives with the nasal swab test.

Statistical analysis estimated the sensitivity of the nasal swab test on individuals as approx. 40% and the specificity as 98-100%. The sensitivity as a flock test is dependent on the number of true positives within the sample group; If only 1 of the 20 ewes has OPA then the probability of the flock test being positive is 0.65 whereas if 5 of the 20 have OPA the probability of the flock test being positive is 0.9. Pooling the samples as groups of 5 and doing single rather than replicate PCR reactions can increase the lab throughput and reduce reagent usage, and therefore cost to the end user, but this balances against a small reduction in the sensitivity of the flock test. A preliminary test of transport conditions did not indicate a significant difference when samples were stored at 4°C or ambient temperature for 24 or 48 hours.

Lead partner	Moredun Research Institute
Scientific partners	BioSS
Industry partners	Biobest Laboratories Ltd
Government sponsor	Agriculture and Horticulture Development Board, Hybu Cig Cymru, Northern Ireland Agricultural Research and Development Council and the Animal and Plant Health Agency

Has your project featured in any of the following in the last year?	
Events	***Press articles***
Moredun Animal Health Road Show, Lockerbie, 17 Nov 2015 Quality Meat Scotland, Scottish Sheep Industry Group, Perth, 15 Jan 2016 Scottish sheep health group 25 Jan 2016 Sheep Veterinary Society 'Talking Sheep' meeting, Penrith, 8 Jun 2016 Moredun Animal Health Road Show, Skipton, 8 Nov 2016	NSA Scotland magazine Dec 2016.
Conference presentations, papers or posters	***Scientific papers***
Abstract submitted and accepted for the International Sheep Vet Congress, Harrogate, May 2017	In preparation
Other	
APHA Merrythought newsletter Biobest HiHealth Flockcare newsletter Moredun website update: http://www.moredun.org.uk/research/research-@-moredun/respiratory-diseases/opa	

Full Report

Q1: Financial reporting –

	Yes	No	N/a
Was the project expenditure in line with the agreed budget?	yes		
Was the agreed split of the project budget between activities appropriate?	yes		
If you answered no to any of the questions above please provide further details:			

Q2: Milestones – were the agreed milestones completed on time?

Project milestones	Proposed completion date	Actual completion date
1: Sample collection on farm	31/12/2015	31/05/2016
2: Optimisation of extraction from sample types and PCR protocols including test transfer from Moredun to the Biobest laboratory	31/01/2016	31/01/2016
3: Laboratory testing of samples	30/03/2016	31/07/2016
Additional: Investigation of sample storage and sample pooling	31/09/2016	31/08/2016
4: Postmortem examination and histopathology & interim progress report	31/04/2016	31/04/2016
5: Analysis of results	31/09/2016	31/09/2016
6: Write up of final report and scientific paper, knowledge exchange	30/10/2016	Final report 1/11/16 Scientific paper Feb 2017 KE on-going (some completed, some still to do).
If any of the milestones above are incomplete/delayed, please provide further details:		

Milestone 6:

An outline of the scientific paper has been prepared and each of the authors has been tasked with writing different sections of the paper. An additional experiment will be run in Jan 2017 to enable an improved paper and the first draft of the paper will be completed soon after. We expect to submit the paper in Feb 2017.

Q3: Results – what did the work find?

1.Optimisation of extraction from sample types and PCR protocols including test transfer to the commercial laboratory

For optimisation of nucleic acid extraction and JSRV detection, samples were obtained from sheep additional to the final sample sets.

Blood: Two extraction kits were compared to determine which obtained the greatest DNA yield. PCR for JSRV was duplexed with a housekeeping gene (GAPDH) as internal control GAP-DH. However we had difficulty getting positive PCR results for JSRV on known OPA-affected animals. Using a nested PCR technique improved results but this method is not suitable for a commercial laboratory due to cost and greater potential for cross contamination.

Nasal Swabs: Two RNA extraction kits were compared to determine which obtained the better results. A multiplex RT-qPCR was developed for JSRV together with an exogenous RNA control to enable the presence of inhibitors to be monitored. Various steps were taken to try to maximise sensitivity. The final assay gave a total of 6 positive samples from 8 expected positive samples (OPA confirmed at PME) and therefore was deemed suitable for testing of the farm samples.

2. Collection of samples on farm

Blood samples (EDTA) and nasal swab samples were collected from 20 sheep per flock selected specifically because they were thin(ner) (one of the early signs of OPA). A total of 9 flocks were tested. Three were presumed OPA-free flocks on the basis of history and laboratory investigations over the previous three years including postmortem examinations in the case of two of the flocks. Six flocks were known OPA-affected in which a number of OPA cases had been diagnosed during the previous year from clinical signs of OPA with histological confirmation following postmortem examination of the lungs. The body condition score (scale 0-5) was recorded and mouths checked to exclude ewes with incisor loss. The aim was to select for sampling those ewe with body condition score 1.5 or less in hill flocks and 2 or less in lowland flocks.

As originally proposed, in order to facilitate the sample collection for this project most of the sheep used were part of a different project which was funded by the Scottish Government. This was useful as it enabled us to use one or more method to confirm the OPA status of the individual sheep sampled as summarised in table 1 below. This provided much more information than simply knowing whether or not the flock should test positive. Note that farms A and B were selected groups of animals pre-screened by ultrasound and known to contain an unusually high proportion of OPA-positive sheep; farms E, G and K were the thinner sheep and were not pre-selected by screening. Farm M had, for at least the past 5 years been culling any sheep suspected to have OPA and had very few thin sheep, so after selecting the thin ones the others were picked on the basis of a mix of positive and negative ultrasound scan results.

Table 1: range of testing undertaken in the OPA-affected flocks ND = not done

Farm	Nasal swab & blood sample	Transthoracic ultrasound	Postmortem & lung histology	Wheelbarrow test
A	√	√	√	√
B	√	√	√	√
E	√	√	Ultrasound positives only	Ultrasound positives only
G	√	√	All ultrasound positives plus 1 negative	All ultrasound positives plus 1 negative
K	√	ND	√	√
M	√	√	ND	ND

3. Testing of samples

Due to the problems with the blood sample test it was decided at the mid-term review not to pursue this further and instead to concentrate on the nasal swab assay including to add an additional objective (3a, below).

The optimised RNA extraction and RT-qPCR was applied to the nasal swab samples collected from the 3 presumed negative flocks and the 6 known OPA-affected flocks. The results are summarised in table 2.

Table 2:

Farm identity	PCR positive	PCR negative	Flock status based on PCR findings	True flock status
Farm A	4	16	POSITIVE	POSITIVE
Farm B	5	15	POSITIVE	POSITIVE
Farm E	1	19	POSITIVE	POSITIVE
Farm G	1	19	POSITIVE	POSITIVE
Farm K	1	20	POSITIVE	POSITIVE
Farm M	0	20	NEGATIVE	POSITIVE
Farm A(Negative)	0	20	NEGATIVE	NEGATIVE
Farm B(Negative)	0	20	NEGATIVE	NEGATIVE
Farm C(Negative)	0	20	NEGATIVE	NEGATIVE

At least one sample was positive by RT-qPCR from the 20 tested in each of the known OPA-affected flocks, with the exception of flock M. There were no positive results by RT-qPCR in animals from flocks assumed to be OPA-negative or in individual animals in affected flocks where PME findings were negative for OPA. That is, there were no false positives.

3a. Sample transport conditions assessment

Samples from 9 animals were tested after storage at room temperature or 4°C for 24 hours or 72 hours to simulate time in transit from farm to the testing laboratory. The results are shown in Table 5. The results suggest that room temperature for 72h was the least acceptable storage regime as there were 3 internal control failures in this set of samples. However, Fishers exact test gave a p-value of 0.12, indicating that there is no evidence of differences between the proportion of positive samples under any of these storage conditions. As the group sizes were very small, additional testing would be advisable before recommending transport conditions for nasal swab samples.

	4°C 24h	4°C 72h	RT 24h	RT 72h
ICF	0	0	0	3
negative	6	7	7	6
positive	3	2	2	0

Table 3: Results of assay on nasal swab samples stored at different temperatures and for different times. (RT: room temperature. ICF:internal control failure)

4. Postmortem examination of animals with unexpected PCR results

Only one sheep from the OPA negative flocks died during the course of the study. This sheep was subject to PME and no gross or histological evidence of OPA was found.

As shown in table 1 many more of the sheep from the OPA-affected flocks were subject to postmortem examination. This allowed us to assess the efficacy of testing at an individual level as well as for a flock test.

5. Analysis of results

Statistical analysis of sensitivity and specificity of the nasal swab assay on individual animals.

Sensitivity and specificity was calculated by Gibbs Sampling compared with PME as the gold standard (Table 3). This showed that the sensitivity was fairly low but the specificity was very good.

Table 4: Sensitivity and specificity estimates for three diagnostic tests for OPA

	Sensitivity		Specificity	
Parameter	Median Estimate	95% Credible Interval	Median Estimate	95% Credible Interval
Nasal swab	**0.38**	**0.18-0.66**	**0.98**	**0.95-1.00**
Wheelbarrow test	0.53	0.42-0.64	0.99	0.97-1.00
Ultrasound	0.93	0.86-0.98	0.75	0.66-0.82

These estimates for the nasal swab test are in broad agreement with a sensitivity of 44% and a specificity of 100% calculated in a more basic way by estimating the sensitivity using the calculation TP/TP+FN and specificity as TN/TN+FP where TP is the number of true positives, TN is the number of true negatives, FN false negatives and FP false positives.

The relationship between internal control failure (ICF) and sample PCR status

There were internal control failures in the case of 19 of the 352 samples tested. These were re-extracted and in all cases the repeat assay was without internal control failure. Analysis of the data indicated that ICF occurred independently of the final PCR result. The presence of internal control failures adds to reagent costs and staff time and will need to be addressed in any future validation work.

Analysis of within- versus between-sample variability in Ct values

Diagnostic labs often use single qPCR reactions to reduce costs if appropriate, whereas triplicate PCR reactions are recommended by the reagent manufacturers and were used during test development and validation. The Ct values for positive animals showed little difference between each of the three replicates. The standard deviation between observations within sample is 0.22. This means that we can be 95% confident that 2 independent observations on a single sample will differ by less than 0.6 (0.62). Thus it would not compromise test performance if the test were run singly.

The Ct values for the lung fluid positive control used in all the testing showed little variation, Ct range 22.63–23.96 over all the testing undertaken. The standard deviation of the differences between positive samples was 2.9, about 10 times larger than the within-sample variation.

Estimating the effects of sample pooling and flock testing

Pooling of samples would also reduce the cost of the test. Using the data on test sensitivity and specificity together with the Ct values the effect of pooling 5 samples compared to testing 5 individual samples was modelled (Table 4), and showed that there was very little loss of sensitivity.

Table 5: Probability of a positive pooled test, or at least one positive individual test out of 5 tests, when 1, 2, 3, 4, or 5 samples out of the 5 are truly positive.

Number true positives	Probability of positive pooled test	Probability of positive individual test.
1	0.33	0.37
2	0.56	0.60
3	0.69	0.75
4	0.80	0.84
5	0.87	0.90

To extend this, the flock level sensitivity was calculated. Assuming the 20 thin ewes contain at least 1 positive animal, the mean sensitivity using 4 pools of 5 samples was 0.53 (95% S.I. 0.50 - 0.57) compared to mean 0.65 (95% SI 0.40 - 0.46) using 20 individual tests to define farm OPA status. This shows some loss of sensitivity from pooling which must be considered against a 5-fold reduction in the cost of consumables for RNA extraction and RT-qPCR and an increase in potential test throughput.

Q4: Discussion – what do the results mean for levy payers?

The results mean we are a step closer to having a commercial flock screening test for OPA. The test is now in a format that facilitates its use in a commercial laboratory setting and this study also revealed several ways to enable increased throughput and reduced reagent usage thereby making the test cheaper to run. The use of nasal swab samples also allows sample collection in a simple, quick, non-invasive way. This will be helpful in the next steps of validation where greater number of samples will be needed. In addition, preliminary results suggest that samples transported to the lab do not need to be frozen or on ice and therefore could be done inexpensively.

Q5: New knowledge – what key bit of new knowledge that has come out of this project?

The key bit of new knowledge obtained was the evidence that the RT-qPCR test on nasal swabs collected from 20 thin ewes may have a useful application as a screening test to determine flocks which have a low risk status for OPA. This will allow flocks that are seeking replacements to source low-risk sheep. A limitation of the test is its poor sensitivity for individual animals. Nevertheless we found it to be superior to the PCR on blood. It is highly specific meaning that the risk of false positive results is close to zero. Although a more sensitive test would be better it is possible to accommodate the limitations of a lower sensitivity diagnostic test when used to screen animal populations by incorporating into the rules and regulations appropriate criteria for number and frequency of qualifying tests and defining which animals and how many are tested as is the case in other assurance schemes for cattle and sheep. This is perhaps preferable to accepting a test with poorer specificity as the implications for a negative flock that falsely tests positive include loss of confidence of buyers and ejection from the scheme.

In summary, this nasal swab test is non-invasive, non-lethal and highly specific but its application within an assurance scheme will require careful communication of the test sensitivity so stakeholders understand the limitations.

Q6: Gaps in knowledge – what gaps in knowledge did this project identify?

The true prevalence of OPA in the UK sheep industry remains an important gap in knowledge. Whilst clearly it is perceived as a problem by many farmers, the extent of the problem will determine whether there will be sufficient demand for the test to make it commercially viable.

In addition we need more information on the prevalence of OPA either within whole flocks or in the thin ewe subset as this has a direct impact on the sensitivity of the flock test and the number of sheep that should be sampled per flock. We did not get that from this project as the subsets were especially biased to make sure that there were some positives present.

The number of farms that would be interested in paying for this test either as a flock health screen or for future accreditation is not known. This would depend upon price and perceived value of the test. To bring the test to market there will need to be scale-up of validation and further streamlining of processes to make the test more affordable and hence commercially viable. Final validation will require testing of flocks of unknown OPA status followed by purchase of sheep testing positive and negative in order to undertake postmortem examinations to corroborate results. In addition the number of years of OPA-negative test results required in order to indicate that a flock is OPA-free or low-risk OPA will need to be established.

Q7: Cost:benefit – what is value of this project?

The main benefit of this project has been that it has brought a commercial biotech company onto the scene which should make the possibility of a flock test for OPA more realistic. This first pilot study was facilitated by obtaining samples and additional information from a concurrent project funded by Scottish Government. This made the project affordable for the commercial company and the sponsors (grant awarding bodies) and opens the way to a larger project which should validate the test and take it forward to market.

Q8: Additional deliverables – what activity is planned with the results from this project?

Activity	What is planned?	When likely to happen?
Events	The results will also be presented at AHDB sheep breeders round table (Nov 2017), HCC research day (02/03/17) and for NFUS (25/01/17).	
Press articles	A press release will be published asap. In addition the project will be presented to journalists from the farming and veterinary press at Moredun Press Day on the 12th Dec 2017. We are expecting journalists from the Press and Journal, The Scotsman, The Glasgow Herald, The Courier, Veterinary Record, Farmers Weekly, Scottish Farmer, and Farmers Guardian.	Our Press day usually leads to articles about the work either in December or January issues of the papers or magazines.
Conference presentations, papers or posters	Presentation at the International Sheep Veterinary Congress	24th May 2017.
Scientific papers	A scientific paper describing the results of this project will be prepared for submission to Vet Record, The Veterinary Journal or Small Ruminant Research.	Feb 2017
Other	HiHealthFlockcare newsletter distributed to members and their vets; also on Biobest website	January 2017
Other	Vet Times article	February 2017