MINISTRY OF AGRICULTURE, FISHERIES AND FOOD

CSG 15

Research and Development

Final Project Report

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Research Policy and International Division, Final Reports Unit
MAFF, Area 6/01
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Project title	Studies on false positive Brucella test reactions and methods to improve diagnosis in cattle and pigs
MAFF project code	**SE0302**
Contractor organisation and location	Veterinary Laboratories Agency Woodham Lane, New Haw ADDLESTONE, Surrey.
Total MAFF project costs	£ 637,187
Project start date	01/04/97
Project end date	31/03/00

Executive summary (maximum 2 sides A4)

SE0302 has pursued several avenues of research to understand more about the problem of false positive serological reactions (FPSR's) in the UK and to develop tests that aim to improve the specificity of *Brucella* diagnosis. The project has looked at the problem in the field as well as studies on both serological and cellular aspects of diagnosis. In addition molecular approaches (e.g. PCR, surrogate antigens) to improving diagnosis have been explored. The motivation for this project was primarily the increase in FPSR's in the *Brucella* Eradication Scheme during the years proceeding the start of the project. Improvements in *Brucella* diagnosis underpin MAFF's statutory obligation to control and eradicate brucellosis from farm animals in Great Britain for reasons of animal and public health under the Animal Health Act of 1981, the Brucellosis (E&W) Order (as amended), the Brucellosis (Scotland) Order 1979 (as amended), the Zoonoses Order 1989 and various EU Directives (e.g. 64/432/EC - trade in cattle and pigs). Through the development of better diagnostic tests for brucellosis, the consumer will benefit in terms of improved welfare since *Brucella* specific diagnostic tests will ensure the rapid identification of diseased animals if they are imported. i.e. a faster response time will lead to a reduction in spread, thereby reducing the risk of entering the food-chain. In addition, MAFF will benefit by the reduced cost of clean-up procedures necessary following an outbreak. MAFF will also benefit by a reduction in the cost of re-sampling and re-testing false positive BES reactors. The Farming community will benefit from a reduction in the husbandry costs associated with the imposition of unnecessary restrictions in the movement of livestock. In addition, the international reputation of British livestock will be reinforced by tests to rule out *Brucella* infection in herds failing the current statutory export tests.

Main findings of the project:

- *Y.enterocolitica* O:9 was isolated once (Carlisle region) in a field study on FPSR herds.
- *Y.enterocolitica* in more prevalent in cattle from FPSR regions (Carlisle, Stafford, Truro) than the control region (Chelmsford), but there appears to be no association between any particular serotype and the FPSR problem.
- A protocol for use when investigating FPSR herds has been developed and evaluated.
- It was demonstrated that it is feasible to get blood samples for use in the bovine γ-IFN test to VLA-Weybridge within the specified time.
- Proteins extracted from *Brucella* are better than recombinant forms of *Brucella* proteins for use in the bovine γ-IFN test.
- Using a multiplex PCR no difference in the cytokine profile of cows experimentally infected with either *Brucella* or *Yersinia enterocolitica* O:9 could be demonstrated.
- A test to detect Poγ-IFN has been developed for use in the specific diagnosis of porcine brucellosis.
- A sensitive PCR for *Brucella* has been developed, detecting 20fg of *Brucella* DNA.
- ELISA tests to detect antibodies to *Yersinia* (MyfA, YopD) do not provide additional information useful when investigating FPSR herds.

Scientific report (maximum 20 sides A4)

Objective 01:

Determine cause of FPSR's in cattle by isolation of bacterial isolates from affected herds.

A field study was conducted which aimed to determine the cause of the false positive serological reactions (FPSR's) seen in cattle by the isolation and study of bacterial isolates from affected herds. This objective aimed to establish whether the FPSR's seen in the iELISA and CFT/SAT of the *Brucella* eradication Scheme (BES) were due to infection with the cross-reacting bacteria *Yersinia enterocolitica* serotype O:9. Three regions that were affected by FPSR problems (Carlisle, Stafford, Truro) and a control region (Chelmsford, where there had been no FPSR's) were identified and a protocol for investigating problem herds was drawn up in association with AHVG, AHDO and VID staff. The protocol involved taking additional blood samples and faecal swabs from those cattle in the herd that had FPSR problems and a representative proportion of the other cattle at the farm within 30 days of the original failure. Serum was tested using the statutory serological tests as well as a competition ELISA. The faecal swabs were enriched for *Yersinia enterocolitica* by cold enrichment at 4°C for two weeks after which the sample was plated onto a range of *Yersinia* media and the isolates typed. In addition, a questionnaire was completed at each farm visit for use in the statistical analyses to determine risk factors. The results of the serology, bacterial culture and the questionnaire were analysed by the statistics section of the Epidemiology Department at VLA-Weybridge. In some instances blood samples were collected for analysis in the bovine γ-IFN test. A workshop was held at VLA-Weybridge and an Animal Health Circular (98/45) was drafted and circulated to increase awareness of the field study.

Unfortunately, the demands on staff associated with the BSE problem, other regional work priorities and the poor MAFF profile with farmers have made it difficult to get sustained support for the study. Despite continued monitoring of potential FPSR herds and requests for them to be resampled there was limited support. The exception was the Chemlsford control region which gave full support, achieving the target sample figure of approximately 1000 animals in the region. The number required was dictated by the statistical requirements identified prior to commencing the study. In all Carlisle has resampled 5 herds (145 animals in total) of the 53 requested, Stafford has resampled 2 herds (42 animals in total) of the 21 requested, Truro has resampled 2 herds (142 animals total) of the 3 requested and the control region Chelmsford has sampled 14 herds (961 animals in total). Whilst *Yersinia enterocolitica* was recovered fairly frequently from some herds in all the regions studied, only one isolate was of the cross-reactive O:9 serotype. This isolate was recovered from a herd in the Carlisle region. The majority of the isolates were of the O:6,30 serotype (15 isolates, distributed amongst all the study areas), with O:5 (6 isolates) the next most prevalent. Whilst there is some evidence from the literature of a weak cross-reaction between O:6 and *Brucella*, a cross-reaction could not be demonstrated between the O:6,30 isolates recovered during this field study and *Brucella* (by reciprocal sero-agglutination studies using monospecific rabbit sera).

In most cases, when serum samples were positive by iELISA, the cELISA was negative. The cELISA therefore offers a greater degree of specificity. The most notable exception was a herd in the Carlisle region which had a high number of FPSR's by the BES primary screen iELISA (8 out of 23 samples submitted) of which 6 were also cELISA positive. This region had the most FPSR herds and was the only region from which we isolated the O:9 serotype of *Yersinia enterocolitica*. Unfortunately, the lack of support from farmers and field staff has meant that this possible association could not be explored further.

The poor uptake has also meant that there has been a limited opportunity to assess the bovine γ-IFN test for brucellosis in the field. Preliminary observations show that some field samples from the control region (where there is no brucellosis and no FPSR problem) are positive in the γ-IFN test. In addition, some samples gave high background γ-IFN levels in the diluent control sample used to calculate the stimulation index (optical density of sample with *Brucella* antigen added/optical density of sample with diluent added). This means that the stimulation index is artificially low and could disguise a true *Brucella* positive animal.

This problem of non-specific γ-IFN production has been noted previously in our lab and by collaborators both in Ireland and on the continent.

The numbers of herds with an FPSR problem have reduced over the life of this field study. This is probably due to changes in the age at which the animals are now tested and the change in the bovine population in response to BSE control initiatives. This has made it increasingly more difficult to find herds that satisfy our criteria for investigation.

However, the approach taken in the FPSR investigative protocol has confirmed that samples for γ-IFN testing can be collected and delivered to VLA-Weybridge within the specified time frame and the tests can be performed satisfactorily in this time. This study gave the opportunity to evaluate the investigative protocol for use when dealing with FPSR herds. The techniques used and the information flow were very effective. The protocol developed as part of this field study has been used subsequently to investigate problem herds in other regions of the country.

A full statistical analysis correlating the FPSR problem with cultural isolation of *Yersinia enterocolitica* O:9 was not possible because of the lower than anticipated numbers of samples taken. However, the results of the questionnaire completed at each investigation were analysed in association with the serological data. Given the relatively small numbers (329 cattle sampled in the FPSR regions and 961 in the control region) this analysis must be viewed cautiously. Using Fisher's exact test the questionnaire results were analysed comparing the replies for the test and control herds. There were several statistically significant differences, although the most significant of these was the observation that sheep were kept less frequently ($p<0.001$) and were in contact with the cattle less frequently ($p=0.005$) in the control region. This observation could suggest that sheep are a reservoir for a causative agent or could have a basis in regional differences in husbandry practices. A statistical analysis was done to determine whether there was any association between the other *Yersinia enterocolitica* serotypes isolated from FPSR farms and the FPSR problem. Although there was a significantly higher prevalence of *Yersinia enterocolitica* of all serotypes in the FPSR herds there was no association with a particular serotype.

Objective 02:

Develop improved sero-tests based on surrogate antigens from display "random shape" library.

The pFliTrx library (Invitrogen) displays peptides on the surface of *E.coli* using the major bacterial flagella protein (FliC) and Thioredoxin (TrxA). A diverse library of dodecapeptides has been inserted into the active loop site of Thioredoxin which has itself been inserted into the dispensable region of the flagella gene (fliC). Expression of the peptide fusion protein (FLITRX) is driven by a bacteriophage lambda promotor and is inducible through the addition of tryptophan to the growth medium. When induced, the fusion protein is exported and assembled into flagella on the bacterial cell surface, allowing display of the constrained peptide.

The library contains a vast number of random peptides, one of which may function as a surrogate antigen for the diagnosis of *Brucella*. Identification of potentially useful clones was conducted by a panning procedure, using the *Brucella* specific monoclonal antibody BM40 (specific for the non-cross-reactive M-epitope of the *Brucella* LPS O-chain) to isolate clones bearing the appropriate flagella 'shapes'. The rationale behind this approach being that 'shapes' recognised by BM40 would mimic this *Brucella* specific antigen, providing a starting point for the development of a more specific serological test.

The anti-M monoclonal was immobilized and used to bind the clones that expressed the shape that best fitted the antigen binding site of the monoclonal antibody. The clones that bind to the monoclonal were grown up and the panning procedure repeated for a total of seven times. A consensus motif was established from 4 sequenced clones and was RRGSILFCHFDH. Clones were analysed for their reactivity with a range of serological reagents to evaluate the potential as surrogate antigens to mimic the M-epitope of the *Brucella* LPS O-chain. Whilst it was possible to demonstrate reactivity of the flagella of the selected clones with BM-40 it was not possible to demonstrate recognition of the flagella by other M-specific reagents by either western blotting or ELISA using semi-purified flagella.

Objective 04:

Study CMI of cattle to *Brucella* to identify antigens and cytokine's for use in diagnostic tests.

The bovine γ-IFN test was evaluated using a range of antigens. Recombinant *Brucella* p39 and recombinant *Brucella* bacterioferritin (the latter is the main component of our usual cytoplasmic protein extract of strain B115 used in the γ-IFN test) were evaluated for specificity and sensitivity as antigens in the γ-IFN test on *Brucella* negative and *Brucella* infected cattle respectively. Whilst the bacterioferritin was better than the p39 antigen neither was as sensitive as the B115 antigen. In addition, there was more non-specific γ-IFN production when using the recombinant antigens with some test samples. This may have been due to the presence of lipopolysaccharide (LPS, likely to contaminate recombinant antigen preparations) from the *E.coli* strains used to produce the recombinant antigen. Recombinant forms of the *Brucella* 10 kDa, 16.5 kDa lipoprotein, 19 kDa and 25 kDa proteins were also evaluated as test antigens in the γ-IFN test on cattle experimentally infected with *Brucella*. None of these recombinant antigens functioned in the γ-IFN test. This suggests that there is a need for mixed protein extracts for good sensitivity in the γ-IFN test. However, this brings problems of reduced specificity and non-specific activity. In addition, it was confirmed that *Brucella* LPS (likely to contaminate any antigens extracted directly from *Brucella*) does not cause non-specific stimulation in the γ-IFN test. Therefore antigens purified directly from *Brucella* offer better specificity than recombinant antigens produced in *E.coli* for use in the γ-IFN test.

A multiplex PCR has been developed comprising primers to amplify from cDNA for γ-IFN, TNF-a, Il-1b, Il-2, Il-4, Il-6, Il-10 and Il-12 as well as β-actin as a housekeeping gene. The objective was to provide a cytokine profile when applied to cDNA produced by RT-PCR from mRNA isolated from blood lymphocytes recovered from cattle infected with either *Brucella* or *Yersinia enterocolitica* O:9 after stimulation with *Brucella* antigen. It was reasoned that because *Brucella* is principally a systemic disease and *Yersinia enterocolitica* an intestinal disease there may be differences in the characteristics of the immune response raised to each pathogen. The cytokine profile is one means of characterising the immune response. The methodology is relatively complicated and there were technical problems with the standardization and optimization of mRNA extraction, RT-PCR and multiplex PCR. Individual PCR reactions were evaluated for all primer pairs after which the pairs were combined sequentially and re-optimised to provide a successful multiplex PCR. When applied to peripheral blood mononuclear cells isolated from cattle infected with either *Yersinia enterocolitica* O:9 or *Brucella abortus* the profiles could not provide any reliable marker for either infection.

Objective 05:

Develop *in vitro* test for CMI to *Brucella* in pigs, based on detection of PoγIFN.

One objective of this project was to develop an *in vitro* test for cell-mediated immunity to *Brucella* in pigs, based on the detection of porcine gamma interferon (Poγ-IFN). The gene encoding Poγ-IFN was successfully cloned using the reverse-transcriptase PCR. The gene has been used to construct a DNA vaccine in the pcDNA3.1 vector, which was subsequently used to immunise rabbits and mice to induce the production of serological reagents specific for Poγ-IFN. Rabbit serum to Poγ-IFN was successfully used as the basis for an 'antigen capture' ELISA assay to quantify Poγ-IFN. In this assay, an unlabelled polyclonal antibody to Poγ-IFN is coated to microtitre plates and subsequently washed free of unbound material and unreacted sites blocked. The test sample is then added and incubated to allow any Poγ-IFN present to be bound by the capture antibody. After washing unbound material from the wells the presence of bound γ-IFN is established by the addition of a biotinylated gamma-globulin fraction purified from polyclonal rabbit serum to Poγ-IFN. The addition of a streptavidin peroxidase conjugate enables the amount of bound biotinylated antibody to be determined colourimetrically. The sensitivity of the test was evaluated using tissue culture fluid from porcine peripheral blood mononuclear cells (PBMC's) cultured in the presence of the mitogen concanavilin A (ConA), known to induce the expression of γ-IFN. The sensitivity was compared to a recently available Poγ-IFN test kit (this test kit is very expensive and erratic in it's supply, there is still therefore a good case for the development of MAFF's own Poγ-IFN test kit to reduce the cost

and ensure availability if the test proves useful in differential diagnosis). The Poγ-IFN test was able to detect this cytokine down to 50pg/ml.

Objective 06:

Evaluation of diagnostic tools developed under EU FAIR project (FT0393).

As part of an EU collaboration an inter-lab trial was conducted comparing the results of a range of PCR tests for the detection of *Brucella* available in a number of the collaborating labs. Initial studies compared the sensitivity of the PCR's in each lab on a range of dilution's of genomic DNA, prepared, aliquoted and frozen at VLA before distribution to each lab under code such that the trial was blind. The most sensitive PCR's used primers to target insertion sequence (IS711) of *Brucella*. Two protocols were able to detect 20 fg of *Brucella* genomic DNA. These were the IS711 PCR protocols from VLA and AFFSA. The PCR targets also included 16S RNA, 16S RNA spacer and *DnaK*. The PCR protocols and all necessary reagents for the VLA IS711 PCR and AFFSA IS711 PCR were then distributed amongst the collaborating labs and a different encoded range of *Brucella* genomic DNA sent to each lab. This study investigated the robustness of each PCR and showed that both PCR tests could be applied reliably in a number of different labs, although the sensitivity was lower in some labs. This was believed to be due to the type of PCR block used and suggests that any diagnostic PCR for use in a number of labs requires extensive standardization.

An indirect ELISA based on the detection of antibodies to a recombinant form of the *Brucella* cytoplasmic 26 kDa protein (rBP26) has been developed for use with bovine serum samples. The BP26 iELISA has been evaluated with sera from cattle experimentally infected with *Brucella abortus* (10^9 CFU intra-occularly) and cattle experimentally infected with *Yersinia enterocolitica* O:9 (10^{12} CFU orally). A good serological response was seen in those cattle experimentally infected with *Brucella*. In these cattle, antibodies to rBP26 were seen as early as 10 DPI and were still positive in excess of 1 year PI in one cow. In two of the four *Brucella* infected cows the serological response to BP26 rose rapidly following infection and then declined from between 6 weeks PI and 10 weeks PI. One of the *Brucella* infected cattle failed to seroconvert to BP26 despite being positive by all other tests, including standard serological tests, competitive and indirect LPS ELISA's and the γ-IFN test. Following infection, none of the *Yersinia enterocolitica* O:9 infected cattle gave OD's of the same magnitude as the *Brucella* infected cattle. However, at 36 weeks PI, one of the *Yersinia enterocolitica* O:9 cows showed a rise in antibody levels to rBP26 which rose steadily and remained elevated at 53 weeks PI. This could not have been due to *Brucella* infection since the two groups of cattle are housed on different farms several miles apart and we do not have *Brucella* infection in the UK. In addition, none of the *Yersinia enterocolitica* O:9 cows showed signs of *Brucella* infection. There is some indication that the OD's seen in this *Yersinia enterocolitica* cow to *Brucella* rBP26 are due to a low level of contaminating *E.coli* protein or carbohydrate antigens.

Tests to establish whether FPSR animals are infected with *Yersinia enterocolitica* O:9 potentially offer some insight into the problem. Tests have been developed that use two different *Yersinia* antigens as their basis, the MyfA ELISA and the YopD ELISA. Indirect ELISA assays using purified MyfA (mucoid Yersinia fibrillae) or recombinant YopD (an outer membrane protein) were used to test serum samples from cattle experimentally infected with *Brucella* or *Yersinia enterocolitica* O:9 as well as field samples from around the UK and on the continent. In the MyfA iELISA a monoclonal specific for MyfA was used to capture and immobilize the protein onto the surface of microtitre plates, thereafter the test is processed as an indirect ELISA. The YopD iELISA uses recombinant YopD in a traditional indirect ELISA format. These tests have been evaluated on panels of sera from FPSR, negative and *Brucella* infected cattle from both the UK and the continent.

In cattle experimentally infected with *Yersinia enterocolitica* O:9, antibodies to MyfA were detected as early as 2 weeks PI and remained high for at least one year post infection. All four *Yersinia* infected cattle were positive for anti-MyfA antibodies whilst none of the *Brucella* infected cattle demonstrated antibodies to this protein. However, in the field there did not appear to be a correlation between seropositivity in the MyfA ELISA and cross-reactivity in the standard *Brucella* serodiagnostic tests. There was some correlation in localized regions (SW England), however this was not universal. Interestingly, whilst there appeared to

be a weak correlation between the number of positive animals by the YopD ELISA and those affected by FPSR problems on sera from the continent, this association was not true of British herds. The level of YopD positivity was generally higher in the UK in both FPSR and negative herds. There was also little correlation between the results of the YopD ELISA and the MyfA ELISA. Neither of these *Yersinia* specific tests gave reliable additional information about the cause of the FPSR problem in UK herds. The MyfA ELISA successfully detected cattle experimentally infected with *Yersinia enterocolitica* O:9. None of the *Brucella* infected cattle were positive by MyfA ELISA at any point during the experimental infection. However, the MyfA ELISA did not appear to be sensitive enough to detect *Yersinia enterocolitica* infection in the field, since the positive rate for the FPSR group and the *Brucella* and negative groups was largely similar. Therefore, the MyfA ELISA was not a reliable predictor of exposure to *Yersinia enterocolitica* O:9. An alternative explanation could be that the majority of FPSR seen in Britain are not caused by *Yersinia enterocolitica* O:9, but are caused by another, as yet unidentified agent. Similarly, whilst the YopD iELISA clearly identified cattle infected experimentally with *Yersinia enterocolitica* O:9 it failed to correlate with FPSR samples from the field in the UK.

This project has been successful in conducting a field trial to investigate the association between *Yersinia enterocolitica* O:9 and the FPSR problem in cattle in the UK. Through this study a protocol for investigating problem herds has been developed and evaluated and is now used when requested to investigate cattle with an FPSR problem. The questionnaire which was completed as part of this field study has suggested some risk factors, although these should be treated cautiously due to the lower than anticipated population sample size.

A number of *Brucella* antigens have been evaluated as antigens in the bovine γ-IFN test to try and address the problems associated with the test. None of these antigens work better than the *Brucella* cytoplasmic antigen used currently. Another approach was to investigate the other cytokines produced by cattle infected with either *Brucella* or *Yersinia enterocolitica* O:9 to determine whether any additional cellular parameters could provide the basis for a differential test. Using a multiplex PCR approach there did not appear to be any additional cytokine signals that should be pursued to form the basis of a cytokine ELISA test for differential diagnosis. In addition a test for Poγ-IFN has been developed which is able to detect this cytokine down to a concentration of 50 pg/ml, easily within a biologically significant range.

One other objective was to develop a serological test based on a surrogate antigen that mimics the *Brucella* specific M-epitope on the otherwise cross-reactive LPS O-chain. The project successfully selected a clone reacting with the M-epitope specific monoclonal antibody BM-40. However, this surrogate antigen failed to be recognised by other M-specific reagents.

This project has also been successful in developing a sensitive PCR, able to detect 20fg of *Brucella* genomic DNA. At present this works well as a typing tool to rapidly identify an isolate as *Brucella* within hours of receipt of a pure culture. The project also looked at the benefit of using two ELISA tests, based on MyfA and YopD *Yersinia* proteins, to determine the presence of antibodies to *Yersinia* in sera from cattle with FPSR problems. Unfortunately these tests did not provide any valuable information that could be of use in measures to investigate FPSR herds.

These scientific achievements have been in pursuit of improved, more specific *Brucella* diagnosis in cattle and pigs (Section A.2.1 ROAME A). The field study, and studies with *Yersinia* specific tests, have indicated that the FPSR problem may be associated with *Yersinia enterocolitica* O:9 in some cases, although there appear to be other factors which contribute to the problem in the UK. These have not been defined. Refinements to the antigens used in the bovine γ-IFN test for brucellosis were not able to resolve the problem of non-specific γ-IFN test results encountered in the field. The development of an γ-IFN test for use in pigs has made good progress, but still needs refinement and validation in infected animals. The collaboration with EU partners has provided an opportunity to assess a range of tests under field conditions both in the UK and on the continent. This has been valuable in providing both a wider perspective of the

FPSR problem and the opportunity to consider the results, their validity and the use of the data in the context of an EU wide strategy to addressing the problem.

If the PCR can be applied successfully to tissue samples, blood samples as well as environmental and food samples it will be an excellent means to deliver a rapid answer whilst awaiting cultural confirmation and enable control measures to be applied more rapidly and effectively. This is one area where research should focus, possibly through the use of capture immobilized target specific oligonucleotides to concentrate the *Brucella* DNA from these samples, where the organism may be present at low levels and where the test sample may contain factors which present problems for the PCR reaction. When providing a test which forms the basis for control measures and epidemiological investigations it is important that a negative result can relied upon to mean a negative result. So far as is possible one area where research should be directed is in the development of an internal control for the PCR. This control confirms that the individual PCR test has worked, thereby ruling out the possibility that the result is negative because the PCR did not work. However, it should be acknowledged that this cannot be a categorical negative, despite the fact that the PCR reaction has been shown to work, i.e. since, for example, *Brucella* DNA could be present but below the threshold of PCR detection.

The test for Poγ-IFN has potential for generic use at the VLA for studies on the porcine immune response to other pathogens as well as having application where a test to determine the presence of a cellular response to a pathogen is of diagnostic importance. Therefore this is also an area where research should take the test to the stage where it has been validated under conditions of experimental infection, in this case as a differential test to distinguish between pigs infected with *B.suis* and those with *Yersinia enterocolitica* O:9. The Poγ-IFN test has the potential for commercial exploitation when further refined and validated.

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