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Evaluation and Application of PCR Tests for Mycoplasma hyopneumoniae

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Executive Summary

Conventional PCR assays designed to detect *Mycoplasma hyopneumoniae*, the causal agent of porcine mycoplasmosis, do so by amplifying specific target DNA that can be demonstrated on a gel-based detection system to yield a specific-sized product. Many PCRs were originally developed in research laboratories using cultures of *M. hyopneumoniae*, possibly lung samples from infected pigs and usually limited numbers of cultures of other organisms to prove specificity of the test. In Australia, different PCRs have been used for assessment of *M. hyopneumoniae* infection and disease-freedom status, but results have been conflicting. These PCRs are normally utilised in testing clinical samples such as suspect infected lungs or nasal swabs.

In this study, five published PCRs (Verdin, Kurth, Baumeister, Stark, Calsamiglia PCRs) that target different genes within *M. hyopneumoniae* were initially evaluated against a battery of mycoplasmal isolates of pig origin. For such evaluations, the most important organisms, besides *M. hyopneumoniae*, are mycoplasmas that commonly frequent the respiratory tract of normal pigs. Such organisms include *Mycoplasma flocculare*, which is considered non-pathogenic but very closely related to *M. hyopneumoniae*, and *Mycoplasma hyorhinis*, which is generally of low pathogenicity but can cause clinical conditions in young pigs. Both these organisms are found in the nasal cavity of normal pigs, but can also co-inhabit pneumonic lesions with *M. hyopneumoniae*.

It was found that all five assays as originally described in the literature showed some or many false positive results with one or several isolates of *M. flocculare* or *M. hyorhinis*. To overcome this specificity problem, the assays were then 'optimised' by increasing the annealing temperatures used during the PCR procedure. The aim of this process was to reduce the amount of cross-reactivity with organisms other than *M. hyopneumoniae*, but retain reactivity with both *M. hyopneumoniae* strains and infected lung samples. Some PCRs were found to show no improvement (Stark PCR), or some improvement (Kurth, Calsamiglia, Baumeister) in specificity without loss in sensitivity. Only one (Verdin) was found to have high specificity and retain sensitivity for detection of infected lung samples by this process.

Several further PCRs that specifically target *M. hyorhinis* or *M. flocculare* alone or in conjunction with *M. hyopneumoniae* in a duplex (2 in 1) or multiplex (3 in 1) format were also assessed against a range of mycoplasmal isolates and samples, and similarly optimised if required. In addition, a PCR targeting *Mycoplasma hyosynoviae* (a pig pathogen that can reside in the pharynx) was assessed and optimised at that time. Not only did these generally prove useful in assessing the purity and identity of a number of cultured isolates, but one multiplex PCR (Stakenborg) was shown to be particularly valuable and was chosen for evaluation with field samples in the next phase of the study.

Subsequently, the five original *M. hyopneumoniae* PCRs (i.e. under test conditions as originally published) plus the four 'optimised' *M. hyopneumoniae* PCRs were evaluated on field samples from seven pig herds that included two known infected herds (BB, HT), one suspect infected herd (WW), three herds of high health/low risk status (MU, HF, WE) and a further herd of doubtful status but negative lung histopathology at slaughter (DHG). The field samples comprised 11-12 lungs per herd from slaughter age pigs from all seven herds and 80 nasal swabs per herd from weaner and finisher pigs from six herds. The multiplex Stakenborg PCR was also applied to lung samples from all herds.

Using dilutions of *M. hyopneumoniae* DNA, the quantitive sensitivity of all 10 assays described above was determined, and revealed a range of sensitivities, with the nested PCRs (Stark, Calsamiglia,

Kurth, Verdin) being the most sensitive. PCR reactions were compared among the field samples across herd status, type of DNA extract procedure used, and age of pigs sampled by nasal swabs.

The overall results indicated that:

1. Instagene extraction procedures can be used routinely for efficient detection of *M. hyopneumoniae* from all sample types, and can replace a more expensive and labour-intensive procedure.
2. The optimised Verdin PCR and the Stakenborg multiplex PCR are the only assays that can be recommended for routine applications in Australian laboratories. The Stakenborg assay was 100% specific in all applications assessed and showed moderately high (75%) sensitivity in infected lungs. The Verdin assay was more sensitive and able to detect slightly more infected lung samples (90%), and showed high specificity in lungs from high health herds.
3. Although the more sensitive optimised Verdin PCR would be the preferred assay for nasal swab PCR, it was still not sensitive enough for detection of many carrier animals from nasal swabs in infected herds, and showed variable reactor rates in two of three high health herds. In one herd, over 50% of weaner pigs were PCR reactors but the majority of these were weak reactions. The presence of weak cross-reactivity to *M. hyorhinis* (as found in one of 11 strains studied) in the Verdin optimised PCR could explain these findings. Alternatively, the possible presence of low virulent strains of *M. hyopneumoniae* in some high health herds cannot be discounted.
4. Based on sequencing of Stark PCR products, the repeat sequence that forms part of the ISHp1 transposase gene (and the target of the Stark PCR assay) was found to be not specific for *M. hyopneumoniae*. This gene thus appears to have gained insertion into some strains of *M. flocculare* and *M. hyorhinis,* explaining the cross-reactivity encountered in the Stark PCR.
5. Genes targeted by the Kurth PCR (unique hypothetical gene segment) and the Calsamiglia PCR (16S rRNA gene segment) may similarly be present in other mycoplasmal species. Since REA profiles of amplified Kurth and Calsamiglia PCR products from nasal swabs from low and high health herds were similar, the organisms responsible for the reactivity in both herd types appear to be carrying the same gene target. However, one infected herd did show a different fingerpint in its amplified Kurth product from other herds of varying (low and high) health status, suggesting a different strain was present in that infected herd.
6. With the aid of the numerous PCRs examined in this study, purified strains of *M. hyopneumoniae* were able to be isolated and confirmed, and can now be used in future studies. Additionally, these assays were useful in confirming the identity of a range of *M. flocculare* and *M. hyorhinis* isolates that can be applied to new investigations.
7. In order to assess PCR inhibitors in clinical samples, a PCR targeting normal pig DNA (via a portion of the pig beta 2 microglobulin gene) was successfully developed. This was reactive with lung tissue samples and nasal swabs even in nested PCR reactions and under the higher annealing temperature conditions used elsewhere in the study. Further optimisation is required to run this as a duplex assay to demonstrate lack of PCR inhibitors in a given *M. hyopneumoniae* PCR, as the tested reagent concentrations currently overwhelm components directed against *M. hyopneumoniae* in two assays tested.

Summary

PCR Assay Development

This project evolved from the need of Australian producers and consulting pig veterinarians to have greater confidence in diagnostic assays used for the confirmation of *Mycoplasma hyopneumoniae*. Such tests are particularly important when applied to high health herds thought to be free of disease associated with this organism. In the project, nested and single step conventional PCRs were evaluated in several ways. Initially five assays were chosen, being four nested assays (Verdin, Kurth, Stark and Calsamiglia) and one single step assay (Baumeister), two of which had been used in Australian diagnostic laboratories in recent years (Stark PCR and a modified version of the Baumeister PCR). Initially, these five PCRs were examined for reactivity and specificity against cultures of a limited number of common porcine mycoplasmas, including four strains of *M. hyopneumoniae*, two of *Mycoplasma flocculare* and one of *Mycoplasma hyorhinis*.

Based on the initial testing, it was found that none of the five assays as originally published were specific, as all cross-reacted with one or more of the *M. flocculare* and *M. hyorhinis* isolates. Since cross-reactivity with the two *M. flocculare* isolates was most frequent, cultures of these two isolates were triple cloned at EMAI to ensure there was no contamination with *M. hyopneumoniae* that could explain the cross-reactivity. In addition, DNA from the two *M. flocculare* strains was forwarded to VLA Weybridge for denaturing gradient gel electrophoresis (DGGE) testing and 16S rRNA sequencing, which also confirmed their identity as *M. flocculare*.

PCR assays are published with specified primers, specified reactants and reactant concentrations, and specified temperatures and times for the three key steps of the PCR cycle: denaturation, annealing and extension. Since specificity of PCR is highly dependent on the annealing temperature chosen, the five assays were assessed by modifying the annealing temperatures of the first and second generation reactions. When cross-reactivity was able to be reduced and preferably eliminated, the 'optimised' parameters of the assay were selected for further testing. The tests were then run on a panel of 25-26 porcine mycoplasmal isolates, including the seven previously tested and two subclones of the *M. flocculare* strains but extended to 7 isolates of *M. flocculare*, 11 of *M. hyorhinis*, 7 of *M. hyopneumoniae* and one of *M. hyosynoviae*. These included several isolates of *M. hyorhinis* or *M. hyopneumoniae* sourced from Queensland but fully identified and cloned at EMAI, and recent NSW isolates of *M. flocculare* and *M. hyopneumoniae* cloned at EMAI. As a result, final optimisation was achieved (no cross-reactivity with non-*M. hyopneumoniae* isolates, and reactivity with all *M. hyopneumoniae* strains) with only one of the PCRs, being the Verdin assay. The Stark PCR remained cross-reactive with some *M. flocculare* strains at all increases of annealing temperature, while the Kurth, Calsamiglia and Baumeister PCRs were able to be partly optimised by increasing the annealing temperatures without significant loss in sensitivity, but did show evidence of cross-reactivity at those temperatures.

This provided a total of 9 *M. hyopneumoniae* PCR assays for application to field samples: original and optimised versions of the Verdin, Kurth, Baumeister and Calsamiglia PCRs, and the original version of the Stark PCR. To assess and compare the quantitative sensitivity of these assays, DNA from a reference strain of *M. hyopneumoniae* (strain 232) was diluted, and endpoints for each assay were determined. This was important to assess if the higher annealing temperatures in the optimised PCRs had any adverse effect on test sensitivity. The results indicated that the optimised assays had similar sensitivity to their original counterparts, while the nested assays were more sensitive (1-500 fg/uL) than the Baumeister one step assay (1 pg/uL). Among the nested assays, the most sensitive were the Stark (1 fg/uL), Kurth (10 fg/uL) and Calsamiglia (10 fg/uL) assays, while the Verdin assay

was the least sensitive (25-500 fg/uL) and also the only one with a slightly reduced sensitivity with an optimised protocol.

The battery of porcine mycoplasmal isolates was also assessed by additional PCRs described for identifying cultures of *M. hyorhinis, M. flocculare*, or *M. hyosynoviae*. These included duplex and multiplex assays designed for differentiating two or more porcine mycoplasmas, including *M. hyopneumoniae*. The tests differentiated *M. hyopneumoniae, M. hyorhinis* and *M. flocculare* in a single multiplex PCR (Stakenborg PCR), while two duplex PCRs differentiated *M. hyopneumoniae* from *M. hyorhinis* (Lin PCR) or from *M. flocculare* (Stemke PCR; Lin nested PCR) while single species PCRs detected *M. hyorhinis* (Caron/Assuncao PCR) and *M. hyosynoviae* (Ahrens/Assuncao PCR). These were found to be useful confirmatory tools and showed complete agreement with the identity of the 26 strains used in the specificity testing. The quantitative sensitivity of the multiplex PCR of Stakenborg was also assessed and found to be moderate, with an endpoint of 10 pg/uL.

Two factors that influence application of PCRs to field materials are the DNA extraction system used on the various sample types, and possible inhibition to the PCR reaction from sample components. An ideal DNA extraction method maximises target DNA and reduces inhibitory substances, while a system to confirm that negative results are not attributable to residual inhibitory substances is considered highly desirable. This study compared two commercially-available DNA extraction procedures (Qiagen DNeasy tissue kit, Biorad Instagene matrix), initially on stored nasal swab materials known to give PCR positive results with the Stark PCR. Initial findings indicated both commercial kits gave acceptable results, so all field testing was then based on extractions using both methods (which considerably increased the ultimate PCR workload).

In order to demonstrate that pig DNA could be amplified in lung and nasal swab samples and thus confirm a lack of PCR-inhibitory substances, the project also developed additional PCR assays for pig DNA. From tests developed to demonstrate two candidate DNA targets thought to be present in pig samples (beta actin and beta 2-microglobulin), only the assay for beta 2-microglobulin successfully detected pig DNA in lung and nasal swab samples. While the B2M PCR reliably amplified a 205 bp product in all pig lung and nasal swab samples tested, it also swamped the *M. hyopneumoniae* PCR when applied as a duplex assay with the optimised Kurth nested PCR. Thus the B2M assay showed distinct promise as a tool to investigate PCR inhibition, but as it would have required considerable optimisation to be used in a duplex format with the *M. hyopneumoniae* assays, it was not further developed.

Application of PCR Assays to Field Samples

For a practical comparison of the original and optimised assays, samples of nasal swabs and lung lesions were collected from six NSW herds, two of which were considered to have endemic mycoplasmal pneumonia (herds BB and HT) and four were high health herds (herds MU, WW, HF and WE) considered free of mycoplasmal pneumonia by their consulting veterinarian. In each of these herds, a cross-sectional sampling was undertaken on-farm on one day using nasal swabs from 40 weaners (8 weeks old) and nasal swab and blood samples from 40 finishers (20-24 weeks old). The nasal swabs were mixed with phosphate buffered saline and the resultant liquid was used in PCR analyses, while the sera from the finisher pigs were tested in two ELISAs for *M. hyopneumoniae* – the EMAI 43 kDa ELISA and the Dako competitive ELISA. In all six herds, additional pigs were monitored at slaughter, with up to 100 lungs examined for evidence of consolidation/pneumonia and scored on the abattoir chain using the Goodwin lung score system. From each herd, 11-12 lung lesions suspicious for mycoplasmal pneumonia were retained and each lesion was divided into a fresh tissue portion (for PCR) and a formalin fixed tissue portion (for histopathology). Slaughter pigs from an

additional herd (herd DHG, related to herd WW) were similarly examined and samples collected for PCR and histopathology.

(i) Lung Sample Testing

Routine testing of sera and fixed lung indicated endemic *M. hyopneumoniae* infection in two herds (BB and HT), with 21 of 24 lungs having histopathology consistent with mycoplasmal pneumonia. *Pasteurella*-like bronchopneumonia was evident in the remaining three pigs in these two herds. A further herd (WW) showed some reactivity in the Dako ELISA and suspicious histopathology in one animal. All other lung lesions in herd WW and the remaining herds (MU, HF, WE, and DHG) were consistent with non-specific collapse and/or inhalation of foreign matter, apart from one animal in herd WE which had lesions of bacterial bronchopneumonia suggestive of pasteurellosis.

For field testing of lung samples, 10 *M. hyopneumoniae* PCR assays (both original and optimised Verdin, Kurth, Baumeister and Calsamiglia, plus original Stark and the Stakenborg multiplex PCR) were applied to both Qiagen and Instagene extracts from all 7 herds.

Overall, more lung samples extracted with Instagene yielded positive PCR results than those extracted with the Qiagen DNeasy tissue kit. The two endemically infected herds showed most lungs tested positive in most tests, although the Qiagen extracts were particularly inferior in some tests in herd HT. While positive reactors were detected among the other herds, this was at much lower reactor rates. Lung samples from the two endemically-infected herds showed that the Baumeister assays failed to detect 40-70% of the lungs with histological lesions, while the other assays detected 70-100% of affected lungs. Two assays (Kurth and Stark) were less reactive in one of the two endemic herds (herd HT), which may have been due to the failure of some PCRs to detect some strains of *M. hyopneumoniae* , since recent studies have indicated that 25% of U.S. strains were not detected by primers in the Kurth or Verdin PCRs and 11% by those in the Stark PCR (Strait *et al.* 2008).

In lungs from three herds with high health status and no serological evidence of *M. hyopneumoniae* infection (herds MU, HF, WE), nil or few PCR reactors were detected with the single step PCRs of Stakenborg or Baumeister, or by the optimised versions of the Verdin and Calsamiglia PCRs, In these herds, the Stark PCR and the optimised Kurth PCR showed moderate to high reactivity, especially in Instagene extracts. This was also consistent with failure to achieve maximum specificity in preliminary development testing of the Stark and Kurth PCRs. Lungs from suspect herd WW and its relative herd DHG were variable among the tests. Herd DHG showed reactors in all tests at a prevalence rate of 9-60% depending on the extraction method and test applied. In contrast, herd WW lungs were negative in the Calsamiglia, Baumeister and Stakenborg assays and had a low to moderate prevalence of reactors in the other assays. Since herd WW was the first herd slaughtered at the abattoir on its kill day, cross-contamination of *M. hyopneumoniae* from another source herd via the water bath would have been minimal in lung samples from this herd.

Lung samples were also examined for the prevalence of strong PCR reactivity (as opposed to any PCR reactivity, weak or strong). While results for the endemic herds included high reactor rates of strong PCR signals, strong PCR reactivity still accounted for the majority of the PCR reactions detected among the other herds. Weak reactivity was only prominent among Qiagen extracts tested in the Verdin PCRs in two herds (WW and DHG). Among 21 lungs with histopathology indicative of mycoplasmal pneumonia, Instagene preparations were most frequently PCR positive in the following PCRs optimised for specificity (where possible) in the following order: optimised Verdin (90%),

optimised Calsamiglia (85%), original Stark (85%), original Stakenborg (75%), optimised Kurth (71%) and optimised Baumeister PCR (58%).

(ii) Nasal Swab Sample Testing

For nasal swab samples, 8 *M. hyopneumoniae* PCR assays (both original and optimised Verdin, Kurth and Calsamiglia assays, original Stark and optimised Baumeister assays) were applied to both Qiagen and Instagene extracts from all 6 herds sampled. The lesser number of tests for nasal swabs reflects the lower sensitivity of the Baumeister and Stakenborg PCR for this sample type, as indicated by the negligible reactor rate with samples tested with the optimised Baumeister assay, precluding the need to evaluate the original Baumeister or Stakenborg PCRs on nasal swabs.

The Baumeister optimised PCR was not reactive with any nasal swab samples from the four high health herds, but also yielded very few reactions (≤ 5%) in samples from the two endemic herds, and then only with Instagene preparations. Due to their expected increased specificity, the optimised versions of the Verdin, Kurth and Calsamiglia assays generally had fewer reactors in all herds for all tests compared to the original versions, with the exception of the Verdin assay which showed extremely high reactivity rates in one herd, although only among weaner age pigs in that herd (herd WE).

Nasal swabs had a noticeably higher reactor rate for Instagene extracted samples compared with Qiagen extracted samples. Surprisingly, the reactor rates were not noticeably different between the two endemically-infected herds and the four high health herds for any of the nested assays. In the Stark PCR, supporting earlier findings of a lack of specificity for *M. hyopneumoniae*. all herds showed relatively high reactor rates, but of interest was that the two endemically infected herds revealed lower reactor rates (18-25%) than the high health herds (35-51%). Among the other optimised nested PCRs with higher specificity, the Calsamiglia PCR detected moderate reactor prevalences in the two endemically-infected herds (25-28%), and variable reactor prevalences in the four high health herds (15-43%), with two herds (WE, WW) having low prevalences (15-16%) and two (MU, HF) high prevalences (28-43%). In contrast the less sensitive optimised Verdin PCR showed similar low to moderate reactor rates among all 6 herds (2.5-5% for infected herds, 0-28% for high health herds), while the optimised Kurth PCR showed a similar low reactivity in both infected herds (10%) and in high health herds (2.5-12%) and usually only with Instagene extracts.

The results for nasal swabs indicated that assays with lower sensitivity (Baumeister, Verdin nested) give little reactivity, and are therefore diagnostically limited for this application. The PCR with lower specificity but high sensitivity (Stark nested) was equally diagnostically limited as it gave relatively high reactor rates regardless of the health status of the herd. Of the remaining two assays with known high sensitivity (optimised Kurth and Calsamiglia), neither can be recommended as ideal. The optimised Kurth assay failed to differentiate between endemic and high health herds, and gave low numbers of reactors in endemic herds, while the optimised Calsamiglia assay was much more reactive, but showed equal prevalence of reactors in both the endemic and high health herds. In addition, the Qiagen extraction method performed poorly compared to reactivity rates among Instagene extracts, and there was no apparent advantage in the Qiagen technique in comparing results between infected and high health herds.

In comparing the two ages of pigs sampled, finisher pigs had the highest reactor rates from nasal swabs regardless of herd type, or PCR assay used. The only exception applied to one herd (WE) where only weaner pigs were reactive in the Verdin optimised assay. Between different tests within the same herd, the variable sensitivity between the optimised assays accounted for the variable levels

of reactivity found. Among the three more specific nested assays, the optimised Calsamiglia assay provided the greatest number of reactors in either endemic herds or high health herds. In contrast the optimised Kurth and Verdin assays performed poorly in the endemic herd HT (detecting mainly weaner pigs), and both generally yielded few strong PCR reactions in nasal swabs in the endemic herds.

Overall Performance of PCR Assays on Lungs and Nasal Swabs

The Stakenborg assay provided the highest specificity (100%) and showed good sensitivity (75%) for lung samples. It was superior to the optimised Baumeister PCR in both sensitivity and specificity. Neither of these assays are likely to be sensitive enough for nasal swabs or other samples where *M. hyopneumoniae* DNA concentrations may be low.

Overall, the optimised Verdin and Calsamiglia assays performed best on lung samples, with high proportions of strong PCR reactions and high reactivity rates in lesioned samples, and low reactivity in samples without typical lesions. However none of the PCRs proved reliable in identifying herds free of *M. hyopneumoniae* pneumonia from nasal swabs. The Calsamiglia assay detected the highest numbers of animals in nasal swabs in the two endemically infected herds, but also high numbers in high health herds. The Verdin assay detected fewer pigs from nasal swabs in the endemic herds, as well as in 3 of the 4 the high health herds, but had quite high reactor prevalences in weaners in one high health herd (WE). This may be because such herds have lowly virulent strains of *M. hyopneumoniae* that currently are not differentiated from those that cause lesions in endemic herds.

The reactivity of optimised PCRs in nasal swabs in the high health herds could have been attributed to genuine infection in one (WW), but the reactivity in the other three is either due to *M. hyopneumoniae* strains of low virulence, or cross-reactivity with strains of mycoplasmas from species other than *M. hyopneumoniae*.

Application of PCRs in Confirmation of Local Field Strains of M. hyopneumoniae

The development of the various PCRs in this study enabled the differentiation of a local strain of *M. hyopneumoniae* from isolates of *M. flocculare* that were co-cultured with it. Cultures of this strain (Hillcrest) were able to be differentiated after triple cloning into a single confirmed isolate of *M. hyopneumoniae*, while two of 11 mycoplasmal cultures received from interstate were able to be triple cloned and accurately confirmed as *M. hyopneumoniae*. These isolates will prove particularly valuable in future assessment of purified challenge systems for *M. hyopneumoniae*.

Recommendation

Instagene extraction can be used for lung and nasal swab samples for all *M. hyopneumoniae* PCRs tested, and provides superior results to Qiagen DNEasy. For lung samples, the multiplex Stakenborg and optimised Verdin nested PCR proved the most reliable, while for nasal swabs, only the optimised Verdin assay was considered of any merit.

These two PCRs target the ATP binding cassette (ABC) transporter gene (Verdin) and the 16S rRNA gene (Stakenborg) of *M. hyopneumoniae* respectively. While the optimised Verdin nested PCR may have greater quantitative sensitivity for *M. hyopneumoniae* DNA, and provided slightly better detection rates of affected lung samples from infected herds than the Stakenborg assay, it also revealed a low detection rate in nasal swabs from the two infected herds studied, so it may be unsuitable for routine application to such samples. Further, as recent studies of 36 U.S. strains of *M. hyopneumoniae* found the Stakenborg PCR primers superior to the Verdin PCR primers (which gave 25% false negatives) (Strait *et al.* 2008), the Stakenborg assay could prove to be the more reliable for

samples with moderate to high amounts of *M. hyopneumoniae* DNA, such as lung samples, despite its lower sensitivity and multiplex format. The Stakenborg PCR did also prove to be a very useful screening tool for pig mycoplasmal cultures.

Introduction

Mycoplasmal pneumonia of swine is caused by the exclusive pig pathogen *Mycoplasma hyopneumoniae (Mhp)*. This organism resides principally on the mucosal lining of the respiratory tract and is pivotal in paving the way for secondary pig pathogens (especially *Pasteurella multocida, Streptococcus suis, Haemophilus parasuis* or *Actinobacillus pleuropneumoniae*) to infect the lung and cause a more severe form of pneumonia, known as enzootic pneumonia (Thacker 2006). Since *M. hyopneumoniae* is difficult to isolate from affected lung tissue, serological and molecular-based assays have been developed to identify *M. hyopneumoniae* infections. While serological assays are relatively inexpensive and can be used in live animals, these are limited by the time delay required for seroconversion (typically 9 weeks after infection). Molecular assays which detect DNA, such as PCR, were originally designed for application to cultures, and some have been applied and evaluated on samples of lung from slaughtered pigs, or respiratory tract swabs or washings from live pigs.

The majority of PCR assays described are referred to as conventional or gel-based PCRs, which were the first type developed. Newer equipment and technology has also enabled the development of non-gel based PCRs, known as real-time PCRs, with potentially greater sensitivity and speed. However, such assays are currently limited by their availability, requiring expensive equipment only available in high throughput laboratories, and because their DNA target is usually much smaller than that of conventional PCR, specificity could be an issue. For these reasons, the present study focussed on conventional PCR assays.

In most PCR applications, test specificity and sensitivity has been measured relative to certain culture strains of *M. hyopneumoniae* and other organisms. On this basis, the quantitative sensitivity of PCR has been shown to be very high, particularly for nested PCRs. In a one step conventional PCR, a single PCR reaction seeks to amplify target DNA in a given sample. In a nested conventional PCR, two PCR reactions are required: the DNA from the sample reacts with a set of primers in a first generation reaction, whose amplified product then becomes the DNA substrate for reaction with a second set of primers. These target a sequence internal to that amplified by the first generation reaction. The quantitative sensitivity of a nested PCR can thus be of the order of 1000 fold greater than that of a single step PCR.

In recent years, several *M. hyopneumoniae* PCRs have been published, with most claiming high sensitivity and specificity. However, the specificity of these has been based on their failure to react with a limited number of cultures of organisms other than *M. hyopneumoniae*. Among the most closely related and potentially problematic of these are the porcine mycoplasmas *M. flocculare* and *M. hyorhinis* that are common inhabitants of the porcine respiratory tract. Cultures of such organisms are in limited quantity, so specificity testing of new PCRs are commonly directed against as few as one strain of each of these organisms. This is a serious limitation of such assays, and may seriously overestimate their specificity. Additionally, PCR assays for *M. hyopneumoniae* in field usage have generally been limited to their application in endemically-infected herds or experimentally-infected animals, such that any positive reaction was considered the result of amplified *M. hyopneumoniae* DNA and thus confirmatory for *M. hyopneumoniae* infection. In reality, potential cross-reactions with closely related mycoplasmas in field samples have been ignored.

The Australian pig industry utilizes a limited number of diagnostic facilities to investigate mycoplasmal status of herds, and does so particularly to investigate possible disease breakdowns, or confirm *M. hyopneumoniae* freedom, for example after herd depopulation/repopulation procedures. In recent years, the industry has been serviced through Government laboratories in Victoria

(Bendigo) and NSW (EMAI Menangle) which used different PCR and ELISA-based tests for this purpose. There has been concern that the PCR tests provide differing results, making veterinary certification of herd status difficult.

This project involved a combined laboratory and field-based study to provide information on the suitability of currently used and alternate PCR-based tests to characterize the infection status of Australian pig herds. The tests were also examined for their sensitivity and specificity by assessing their performance on both mycoplasmal cultures and on samples derived from pig herds of varying health status.

During the study, a range of fresh samples from pig farms of varying health status was obtained. Isolates of *M. hyopneumoniae* and other closely related mycoplasmas (*M. hyorhinis, M. flocculare*) were sourced from reputable culture collections with a low likelihood of containing other (contaminating) mycoplasmal species. In addition, we sought to isolate and clone *M. hyopneumoniae* from lung lesion material, which requires the application of PCR assays to exclude other contaminating mycoplasmas. A functional multiplex PCR was then established to differentiate mixed mycoplasmal cultures from pure ones, as this procedure is critical in attempts to produce purified isolates of *M. hyopneumoniae* for future evaluation of new vaccines or diagnostic assays. The overall process should progress our understanding of recent PCR positive results from high health farms – especially if they are likely to relate to isolates of differing virulence from those on endemically infected farms.

Hypothesis

Mycoplasma hyopneumoniae infection can be reliably detected in nasal swab, lung and culture materials based on published PCR protocols, but with varying sensitivity. Such protocols can exclude cross-reactivity with other closely-related mycoplasmas.

PCR technology can be utilised to test herd and animal status, and be applied to identify purified cultures suitable for efficacy testing of pig vaccines.

Apparent false positive PCR reactions in high health status herds can represent the presence of strains of *M. hyopneumoniae* of low virulence. The PCR technology can be further developed to characterise strains of *M. hyopneumoniae* present in commercial herds.

Project Objectives

Number	*Description*
1	Identify current and published PCR methods and review literature to determine limitations
2	Select and evaluate PCR methods on reliable culture sources and field isolations of *M. hyopneumoniae* from abattoir samples
3	Access materials from herds of high and low health status, and use these to compare four PCR methods on nasal swabs and lung lesions, and relate to herd seroprevalence
4	Validate positive PCR results in herds of high health status
5	Evaluate comparative sensitivity and specificity of the PCR assays selected; examine and identify procedures that maximise specificity and sensitivity
6	Using materials from culture collection and abattoir samples, develop single and multiplex PCR to determine mixed mycoplasmal infections in broth cultures and lung material
7	Using the above enabling technologies, obtain pure cloned isolates of *M. hyopneumoniae* for vaccine efficacy studies and further characterisation of virulence in Australian pig herds
8	Report and recommend test/s suitable for routine laboratory diagnostic testing

Methodology

General

This work was undertaken at EMAI in four phases:

1. An initial phase involved a literature review and obtaining culture materials and field samples for PCR evaluation
2. The second phase involved evaluation of selected PCR tests
3. The third phase involved fine tuning and further development of these tests
4. The final phase sought to select suitable materials to produce cloned *M. hyopneumoniae* isolate/s suitable for efficacy and virulence studies in the future

Objective 1 Identify Current and Published PCR Methods and Review Literature to Determine Limitations

To review the limitations to the routine application of current and new PCR tests for *M. hyopneumoniae* in diagnostic laboratories. This will include sourcing of Standard Operating Procedures for PCR tests for *M. hyopneumoniae* in current use in Australian diagnostic laboratories, and methods used in specialist overseas laboratories (e.g. USA).

Objective 2 Select and Evaluate PCR Methods on Reliable Culture Sources and Field Isolations of M. hyopneumoniae from Abattoir Samples

Obtain reference strains of *M. hyopneumoniae*, *M. hyorhinis*, *M. flocculare* and *M. hyosynoviae* from overseas specialist laboratories in the USA and Europe.

Collect samples of diseased lung from abattoir killed pigs from several originating herds and culture in modified Friis broth to isolate *M. hyopneumoniae* in mixed or single culture.

Select at least four PCR methods, including those in use at EMAI and Bendigo, to evaluate these cultures for sensitivity and specificity of the PCR assays.

Objective 3 Accessing Materials from Herds of High and Low Health Status, Compare Four PCR Methods on Nasal Swabs and Lung Lesions, and Relate to Herd Seroprevalence

Collect nasals swabs and lung lesions from herds of high and low health status relevant to *M. hyopneumoniae*, and store at -20°C prior to testing. Evaluate blood samples collected at slaughter for seroreactivity in two ELISA for *M. hyopneumoniae*, and compare the seroprevalence against prevalence of PCR positive results. Examine lung lesions histologically. The sample collections to approximate the following plan:

(i) Collect nasal swabs from 40 weaner and 40 grower pigs from each of 6 herds (3 high health, 3 low health/endemic). Tonsil swabs may also be collected from 20 weaner pigs per herd.
(ii) Collect 40 bloods per herd from slaughter pigs
(iii) Collect 10 lesioned lungs per herd at slaughter

Objective 4 Validate Positive PCR Results in Herds of High Health Status

Examine positive PCR results from herds of high health status by methods to confirm *M. hyopneumoniae*, such as sequencing and/or REA testing of PCR product, or PCR testing of the sample for alternate *M.hyopneumoniae* signatures

Objective 5 Evaluate Comparative Sensitivity and Specificity of the PCR Assays Selected; Examine Procedures that Maximise Specificity and Sensitivity

Compare the results of the (four) PCR assays for diagnostic and analytical sensitivity, and specificity. Where a lower than expected specificity or sensitivity is found, reaction conditions will be examined to determine optimal responses for these tests. Examine concentrations of Mg, annealing temperatures, extension enzymes and DNA extraction methods to optimise their performance.

Objective 6 Using Materials from Culture Collection and Abattoir Samples, Develop and Perform Uniplex and Multiplex PCR to Determine Mixed Mycoplasmal Infections

Develop PCR tests for *M. hyorhinis*, *M. flocculare* and *M. hyosynoviae*. This will include optimisation by examination of similar reactants as described for the *M. hyopneumoniae* PCR's in Objective 5. A multiplex PCR to detect *M. hyopneumoniae* concurrent with *M. hyorhinis* and *M. flocculare* will then be developed and applied to lung cultures from abattoir lung materials and reference culture alone and in mixtures. Studies to optimise this for reference cultures and lung tissues and culture mixtures will also be undertaken.

Objective 7 Using the above Enabling Technologies, Obtain Pure Cloned Isolates of M. hyopneumoniae for Vaccine Efficacy Studies and Further Characterisation of Virulence in Australian Pig Herds

M. hyopneumoniae cultures from lung materials will be sought for triple cloning and then tested with the battery of tests as described above. The aim is to provide a purified culture for efficacy testing and possible isolation of *M. hyopneumoniae* strains from various herds, including high health status herds. This study will cover a range of initial dilution and passage procedures required to produce suitable strains.

Part 1 - *M. hyopneumoniae* PCR Test Selection, Initial Evaluation And Optimisation

1.1 Materials and Methods

1.1.1 Literature Review

A 25 page literature review "*Application of PCR tests for Mycoplasma hyopneumoniae with reference to lung tissue and respiratory disease swabs*" was completed and is available at APL as an adjunct to this report.

1.1.2 M. hyopneumoniae PCR Assays

Five PCR assays for *M. hyopneumoniae* were selected based on the literature review and the requirement to compare assays previously employed by NSWDPI and Victorian DPI. These assays are listed in Table 1.1.

These included the nested PCR of Stark et al. (1998) (used by NSWDPI) and a modification of the single step PCR of Baumeister et al (1998) described by Carew (2004) which was used by DPI Victoria. The three other *M. hyopneumoniae* PCRs selected were the nested PCR assay developed by Dr Kobisch's group in France and described by Verdin et al (2000), the nested PCR developed at Iowa State University by Dr Eileen Thacker's group in collaboration with Prof Chris Minion, and previously reported by Kurth *et al* (2004), and the nested PCR developed initially at the University of Minnesota (Dr C. Pijoan's group) and described by Calsamiglia *et al* (1999). All the above five assays have been applied to lung tissue, and all but the Verdin and Baumeister assays have been proven to detect *M. hyopneumoniae* in nasal swabs (refer submitted literature review in separate report).

Table 1.1: M. hyopneumoniae PCR assays examined in this project

Assay name	Type	Gene target	Sensitivity reported	Reference	Location used in Australia	Developed at/ by
Verdin	Nested	ATP binding cassette (ABC) transporter gene	1 fg	(Verdin *et al.* 2000)		France – Kobisch et al
Kurth	Nested	Unique hypothetical genes mhp023 and mhp024	0.5-1 fg	(Kurth *et al.* 2002)		USA (Iowa State Uni) – Minion et al
Baumeister (modified)	Single step	Unknown gene	5-18.5 fg	(Baumeister *et al.* 1998) (Carew 2004)	DPI Victoria	Germany & Denmark (Uni of Hannover & DVL Copenhagen)
Stark	Nested	Repeated element MHYP/03-950 (REP sequence) and part of ISMhp1 transposase gene	1.2-2.5 fg	(Stark *et al.* 1998)	NSW DPI	Switzerland (Uni of Berne) – Frey et al
Calsamiglia	Nested	16S rRNA gene	1 fg	(Calsamiglia *et al.* 1999)		USA (Uni of Minnesota) – Pijoan et al

As summarised in Table 1.2 (which also appears in the literature review), the four nested assays have been rated to detect low levels of genomic DNA

Table 1.2: Claimed sensitivity of various PCR tests for M. hyopneumoniae genomic DNA

Test	Type	Sensitivity*	Sensitivity reference
PCRs selected for study			
Verdin *et al* 2000	nPCR	1 fg	(Verdin *et al.* 2000); (Kurth *et al.* 2002)
Kurth *et al* 2002	nPCR	0.5 – 1 fg	(Kurth *et al.* 2002)
Baumeister *et al* 1998	PCR	5-18.5 fg	(Carew 2004)
Stark *et al* 1998	nPCR	1.2-2.5 fg	(Stark *et al.* 1998); (Kurth *et al.* 2002)
Calsamiglia *et al* 1999	nPCR	1-96 fg	(Calsamiglia *et al.* 1999); (Kurth *et al.* 2002)
PCRs with lower sensitivity not selected for study			
Artiushin *et al* 1993	PCR	1-10 pg	(Artiushin *et al.* 1993); (Kurth *et al.* 2002)
Blanchard *et al* 1996	PCR	0.5-10 pg	(Blanchard *et al.* 1996); (Kurth *et al.* 2002)
Caron *et al* 2000	PCR	1-50 pg	(Caron *et al.* 2000); (Kurth *et al.* 2002)
Mattsson *et al* 1995#	PCR	6-100 fg	(Mattsson *et al.* 1995); (Kurth *et al.* 2002)

* 1 pg = 10^{-12} g; 1 fg = 10^{-15} g and 1 colony forming unit (cfu) is approximately 1.2 fg

Mattsson primers are equivalent to 1st generation reaction of Calsamiglia nested PCR

1.1.3 General PCR Methodology

PCR analyses were undertaken using the same work practices and facilities. All PCR reagent (cocktail) preparations were undertaken in a dedicated Class 2 Biological safety cabinet that was separate from a second dedicated Class 2 Biological safety cabinet used for 1st generation DNA template inoculations. Amplifications were undertaken in a physically separated building in 96 well thermocyclers (Corbett Research model 960C cooled thermal cycler or model 960G gradient thermal cycler). Transfer of 1st generation amplified product to 2nd generation cocktail reagent was undertaken in a third separate building, using a PCR/UV workstation (Cleanspot PCR/UV workstation, Laboratory Products, Michigan, USA).

Individual reagents except Taq polymerase were thawed at 37°C in a dry bath incubator (Major Science) and mixed by vortexing. Instagene extracts were centrifuged at 11,000 rpm for 3 minutes in a microfuge prior to inoculation. Cocktail preparations were mixed in sterile 5 mL plastic screw-top tubes and the PCR reactions were performed in 200 uL plastic tubes in 8-tube strips (Snap Strip #3240-09, Scientific Specialities Inc, USA). Dedicated Gilson pipettes that were calibrated regularly were used for all cocktail preparations. Reagent volumes for the PCRs examined in Part 1 of this study were either 50 uL (for the Verdin, Kurth, Stark and Calsamiglia PCRs) or 25 uL (for the Baumeister PCR). In Part 2, where a range of other PCRs were investigated, the reagent volumes varied according to the relevant published information from 25 uL (for the Assuncao and Lin PCRs) to 50 uL (for the Stemke and Stakenborg PCRs) and 100 uL (for the Caron PCR). Template addition was undertaken using new dedicated ergonomic Finpipettes with volume ranges of 0.5-10 uL for first generation templates and 0.5-2 uL or 0.5 -10 uL for second generation templates.

All samples and reagents were handled with disposable latex gloves that were changed regularly and between all steps of the PCR procedure. Reagents and template were pipetted with presterilised

aerosol resistant tips (ART 10 Reach, 20P, 200, 300 and 1000E tips, Molecular Bioproducts Inc, USA) with dedicated tips per work area. Cocktail volumes were dispensed into Snap Strip tubes using a digital multidose pipettor (Eppendorf Research Pro 300) with an ART 300 pipette tip. All reagents and samples were retained on ice during the various PCR procedural steps, and reagents stored at -20°C between steps. DNA extracts were stored at -20°C in 1.5 mL Eppendorf tubes, and all field samples were retained as single aliquots. Control positive DNA (strain J or 232) was retained at 5°C at 1 or 10 ng/uL. All assays were run with both positive and negative (MilliQ water) controls in the nested format (both generations) and the 2nd generation only stages, while DNA extraction controls were used for all field samples, with typically one extraction control per 20 samples extracted.

Amplified products in volumes of 15 uL were developed by electrophoresis at 94V x 1.5 h using 1% agarose containing 4.6 uL of ethidium bromide stock (10 mg/mL, Fluka 1% ethidium bromide solution, Sigma Aldrich, Switzerland) per 120 mL gel. The agarose gel was made up in a Tris/borate/EDTA buffer (Amresco TBE buffer 10X ready back, diluted to 0.5X), and the same 0.5X buffer was used for electrophoresis. Developed images were captured by fluorescence under UV light using a Bio-Rad Gel Doc 2000 analyser and Quantity One software.

1.1.4 Initial Screening Evaluation with Seven Mycoplasmal Strains

Seven mycoplasmal strains were used to investigate the reactivity and specificity of the five selected Mhp PCR assays, based on published primers, reagent mixtures and test conditions. Five of these strains were provided by Dr Eileen Thacker (Iowa State University, Ames Iowa) for use in the initial evaluation, and two were reference strains of *M. hyopneumoniae* from the EMAI collection. The seven isolates used in the initial screening evaluations of the PCR assays are described in Table 1.3.

Where specificity of *M.hyopneumoniae* PCRs was found to be less than 100% with these isolates, studies were undertaken to adjust the test conditions to improve specificity. In most cases, this was performed by increasing the annealing temperature (T_A) of the first and/or second generation PCR step.

To assist in optimisation of the Calsamiglia assay, DNA from *M. flocculare* strain MS42 and from five pigs confirmed to have typical lesions at slaughter were also used. The latter were DNA extracted from lungs of four pigs (152, 237, 161, 171) challenged with *M. hyopneumoniae* in earlier trials in 1995 (pigs 152, 237), 2004 (pig 161) or 2005 (pig 171) and from lung of one pig detected at routine slaughter in 2002 (pig 12).

Table 1.3: Mycoplasmal isolates used in initial PCR assay screening

Species	EMAI lab reference	Source	Strain identity at source lab
M. hyopneumoniae	J	Type strain, EMAI	
	232	Type strain, EMAI	
	CM07/04	Dr E. Thacker	95MP1509
	CM07/05	Dr E. Thacker	00MP1301
M. flocculare	CM07/06	Dr E. Thacker	94MF1501
	CM07/07	Dr E. Thacker	02MF1501
M. hyorhinis	CM07/08	Dr E. Thacker	SK76C

1.1.5 Sequencing and Alignment of Stark PCR Product

Stark PCR products that were considered false positives and could not be removed by increased annealing temperatures were further investigated by sequencing these products and aligning the product sequences with those of the *M. hyopneumoniae* target gene as described in Genbank. Sequencing was undertaken at Macquarie University and the sequences were compared with published sequences from NCBI blast searches using Bioedit software (Ibis Biosciences, Carlsbad USA).

1.2 Results

1.2.1 Effect of Varying Annealing Temperatures and Other Modifications on Test Specificity

The results of modifying the annealing temperature in each cycle of the first generation PCR (T_{A1}) and the second generation PCR (T_{A2}) for the seven mycoplasmal isolates (four being *M. hyopneumoniae* - strain J, strain 232, isolate 07/04, isolate 07/05; two being *M. flocculare* - isolates 07/06 and 07/07; one being *M. hyorhinis* - isolate 07/08) are shown temporally in Tables 1.7 – 1.11. In each table, the T_{A1} and T_{A2} as published in the original paper are shown next to the test name (shaded row), while the T_{A1} and T_{A2} found to give optimal specificity and sensitivity are shown in shaded double-edged cells near the base of each table.

Table 1.4 gives results for the Verdin and Kurth nested PCRs. In the Verdin PCR, the optimised T_A were found to be 62°C for both generations, compared to original published temperatures of 57°C and 58°C for T_{A1} and T_{A2}. In the Kurth PCR, the original published temperatures of 49 °C and 56 °C for T_{A1} and T_{A2} required an increase to 64 °C for optimal specificity.

Table 1.4: Effect of varying annealing temperatures...
..for first (T_{A1}) and second (T_{A2}) generation reactions in the M. hyopneumoniae nested PCR of Verdin et al and Kurth et al. Final row with highlighted, double edged cells indicates annealing temperatures giving optimal result.

Assay /Date	T_{A1}	T_{A2}	Strain or isolate						
			J	232	07/04	07/05	07/06	07/07	07/08
Verdin	57	58							
13/4/07	59	59	wk	+	+	+	+	+	-
13/4/07	59	63	-	-	+	+	wk	wk	-
13/4/07	59	66	-	-	+	+	-	-	-
3/7/07	60	58,60,62	NT	+			-	-	
3/7/07	62	58	NT	wk			-	-	
3/7/07	62	60	NT	-			-	-	
3/7/07	62	62	NT	wk			-	+	
3/7/07	64	58	NT	wk			-	+	
3/7/07	64	60, 62	NT	-			-	-	
5/7/07	60	58	-	+	+	+	-	+	-
11/7/07	60	60,62	-	+	+	+	wk	+	-
11/7/07	60, 62	60, 62, 64	NT	+			-	-	
11/7/07	64	60	NT	+			-	-	
11/7/07	64	62	NT	-			-	-	
11/7/07	64	64	NT	wk			-	-	
11/7/07	66	60	NT	+			-	-	
11/7/07	66	62, 64	NT	vwk to -			-	-	
17/7/07	60	60, 62	NT	+	+	+	-	+	-
17/7/07	62	60	NT	+	+	+	-	wk	-
17/7/07	62	62	NT	+	+	+	-	-	-
Kurth	49	56							
17/5/07	49	56	+	NT	+	+	-	+	-
23/5/07	49	56, 59, 62	+	NT			-	+	
23/5/07	52	56, 59, 62	+	NT			-	-	
8/6/07	52	56, 59	+	NT	+	+	+	+	-
8/6/07	54	56,59	+	NT	+	+	-	+	-
15/6/07	54	56, 59, 62	+				+	-	
15/6/07	56	56, 59, 62	+				+	+	
15/6/07	60	56, 59, 62	+				-	-	
20/6/07	60	56, 58, 60	+				-	+	
22/6/07	60	62, 64	+				-	+	
22/6/07	62	62, 64	+				-	-	
27/6/07	62	62	+	NT	+	+	-	+	-
29/6/07	62	62, 64	+	NT	+	+	-	wk to +	-
29/6/07	64	62	+	NT	+	+	+	-	-
29/6/07	64	64	+	NT	+	+	vwk	-	-

+ : clear positive specific band; wk: weak positive; vwk: very weak positive; - : negative; NT: not tested

Figure 1.1: Verdin PCR at optimal annealing temperature and showing PCR product... ...of 706 bp for M. hyopneumoniae. Lane marker indicates 500 bp as a thick line, with 100 bp intervals above and below this. Results are shown for DNA from M. hyopneumoniae isolates 07/04 and 07/05 and positive control strain 232 compared with results for M. flocculare isolates 07/06, 07/07 and M. hyorhinis isolate 07/08.

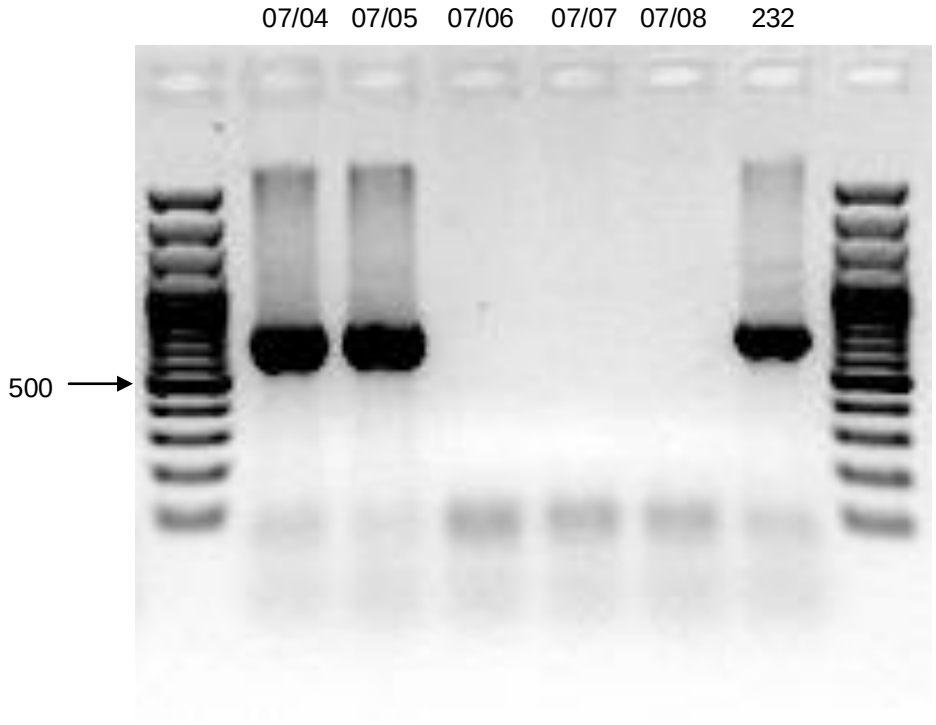
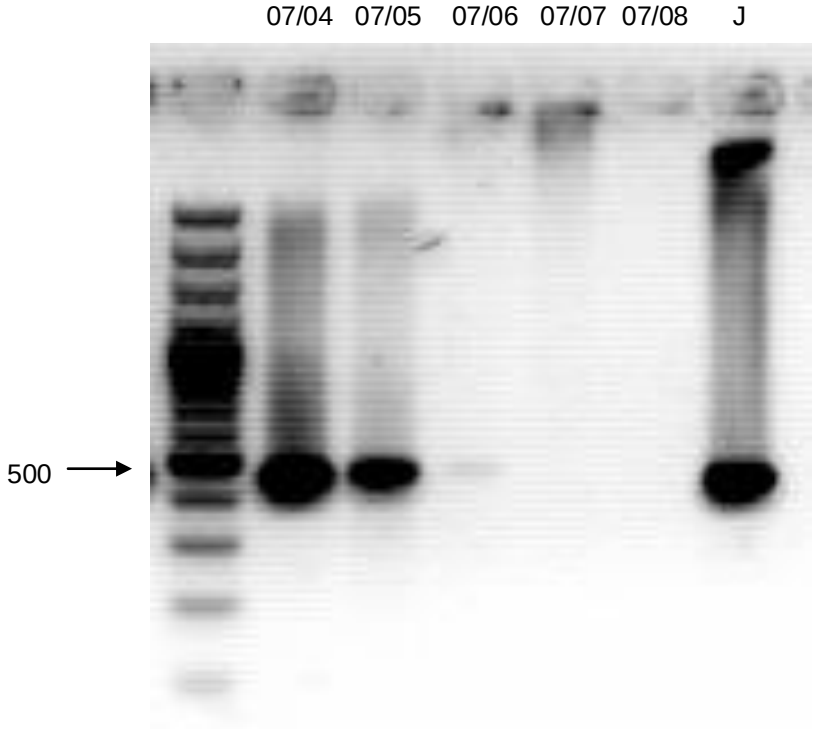


Figure 1.2: Kurth PCR at near optimal annealing temperatures (64/64°C)...
...showing PCR product of 456 bp for M. hyopneumoniae. Lane marker indicates 500 bp as a thick line, with 100 bp intervals above and below this. Results are shown for DNA from M. hyopneumoniae isolates 07/04 and 07/05 and positive control strain J compared with results for M. flocculare isolates 07/06, 07/07 and M. hyorhinis isolate 07/08.



In the Baumeister one step PCR, the original published annealing temperature of 55°C was found to give false positive signals and adjustment to 64°C was required to optimise this assay (Table 1.5). An earlier study (Carew 2004) suggested an annealing temperature of 62°C was necessary, but our results indicated a weak cross-reactivity with *M. flocculare* isolate 07/07 at that temperature.

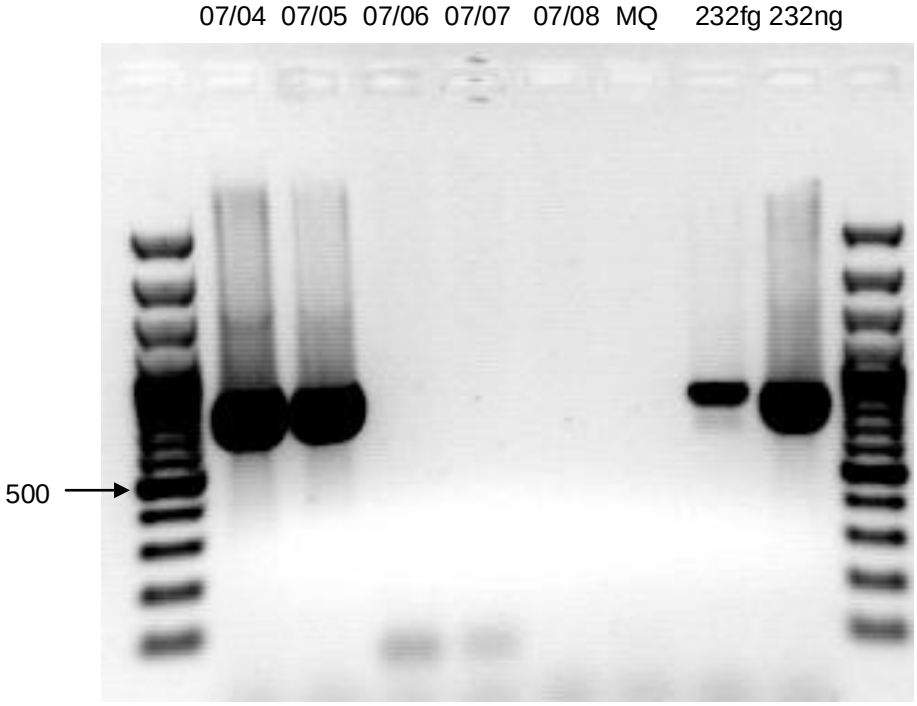
Table 1.5: Effect of varying annealing temperature (T_A) for M. hyopneumoniae one step Baumeister PCR. Row with highlighted, double edged cells indicate annealing temperature (T_A) giving optimal result.

Assay and date	**T_A**	**Strain or isolate**						
		J	**232**	**07/04**	**07/05**	**07/06**	**07/07**	**07/08**
Baumeister	55 (62 Carew)							
3/5/07	58	+	NT	+	+	+	+	-
3/5/07, 8/5/07, 14/5/07	62	+	NT	+	+	- to +	wk	-
8/5/07	63	+	NT			-	wk	
8/5/07, 14/5/07	64	+	NT			-	- to + (high load)	
8/5/07	65	+	NT			wk	+	
14/5/07	68	+ to wk	NT			-	-	
16/5/07	64	+	NT	+	+	-	-	-

+ : clear positive specific band; wk: weak positive; vwk: very weak positive; -: negative; NT: not tested

Figure 1.3: Baumeister PCR at optimal annealing temperature and showing PCR product....

....of 853 bp for M. hyopneumoniae. Lane marker indicates 500 bp as a thick line, with 100 bp intervals above and below this. Results are shown for DNA from M. hyopneumoniae isolates 07/04 and 07/05 compared with results for M. flocculare isolates 07/06, 07/07 and M. hyorhinis isolate 07/08. Other lanes are a negative MilliQ water control (MQ) and positive M. hyopneumoniae strain 232 controls for fg and ng amounts.



The nested Stark PCR produced cross-reactivity with the U.S. isolates of *M. flocculare* and *M. hyorhinis* (Table 1.6), and continued to cross-react particularly with *M. flocculare* even at high annealing temperatures up to 63-64°C, when reactivity with *M. hyopneumoniae* appeared compromised. Figure 1.4 indicates typical cross-reactivity with *M. flocculare* at annealing temperatures of 56°C (1st generation) and 62-66°C (2nd generation). Reduced sensitivity at high temperatures was seen with extracts of *M. hyopneumoniae* strain J where the nested fg control became weak with progressively higher annealing temperatures above 62°C in the second generation (Figure 1.4).

This assay could therefore not be optimised, so was only subsequently applied at the original temperature of 52°C for both generations of the assay.

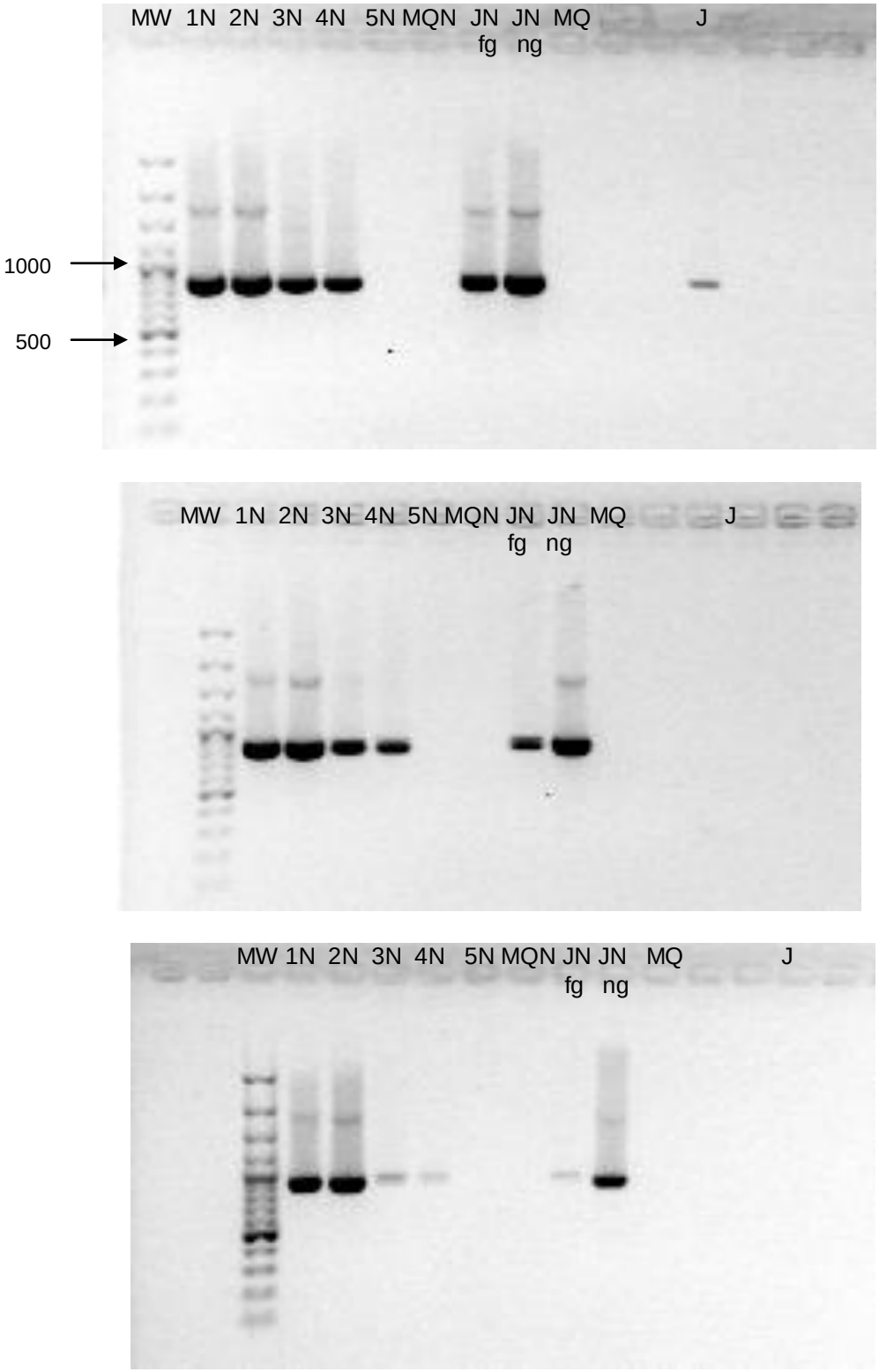
Table 1.6: Effect of varying annealing temperatures for first (T_{A1}) and second (T_{A2}) generation...
...reactions for Stark M. hyopneumoniae nested PCR. Rows with highlighted, double edged cells indicate best result, but not optimal. Where results of this assay or of subsequent assays with the same test parameters were not ideal, further testing may have been undertaken. Results are given as: positive: +, weak: wk, very weak: vwk or negative: -.

Assay /Date	T_{A1}	T_{A2}	Strain or isolate						
			J	232	07/04	07/05	07/06	07/07	07/08
Stark	52	52							
14/3/07	49	49, 52, 56	+	NT	+	+	+	+	+/-
20/3/07, 27/3/07	52	52, 56, 59	+	NT	+	+	+	+	+
21/3/07	56	62,64	+	NT	+	+	+	+	-
21/3/07	56	66, 68	wk to +	NT	+	+	wk	wk	-
22/3/07	60	62	wk to +	NT	+	+	+	+	+
22/3/07	62	62	- to +	NT	+	+	+	+	wk
22/3/07	64	62	- to +	NT	+	+	wk	+	-
20/7/07	56	60, 66	+	NT			-	+	-
20/7/07	56, 60, 63	63	+	NT			+	+	-
20/7/07	60	60, 66	+	NT			-	+	
20/7/07	63	60, 66	+	NT			-	+	
24/7/07	64	64, 66	+	NT			+	+	-
24/7/07	66	64, 66	+	NT			+	wk	
1/8/07	64	64	+	NT			+ to -*	+ to -*	
1/8/07	64	66, 68	+ wk	NT			wk to -*	vwk to -*	
1/8/07	66	64	wk	NT			-	-	
1/8/07	66	66, 68	-	NT			-	-	
14/8/07	60	66	+	NT	+	+	+*	+*	-
14/8/07	60	68	+	NT	+	+	+*	vwk*	-
14/8/07	63	66	+	NT	+	+	-*	wk*	-
14/8/07	63	68	wk	NT	wk	wk	-*	-*	-

+ : clear positive specific band; wk: weak positive; vwk: very weak positive; - : negative; NT: not tested

* indicates reactivity with triple cloned isolate; all others refer to reaction with original isolate.

Figure 1.4: Examples of cross-reactivity in Stark PCR with strains of M. flocculare... ...even at high annealing temperatures of 56/62°C (top), 56/64 °C (middle), and 56/66 °C (bottom) for the first and second generation reactions respectively. Lanes 1N & 2N are nested result for M. hyopneumoniae isolates 07/04, 07/05; lanes 3N and 4N are M. flocculare isolates 07/06 and 07/07; lane 5N is M. hyorhinis isolate 07/08. Lanes JN fg and JN ng are M. hyopneumoniae strain J nested controls. Lanes with MQ represent MilliQ water controls (nested and unnested). Lane J is an unnested strain J control. MW are molecular weight markers, and target product is 808 bp.



In the Calsamiglia PCR as published (Calsamiglia *et al.* 1999), excessive residual first generation product at the end of the second generation reaction was demonstrated. In the original test description, all reactions were undertaken in 25 uL reaction volumes. In an attempt to overcome the excess first generation product and improve accuracy in addition of the second generation template, the reaction volumes were adjusted to 50 uL but the first generation sample volume and the amount of primers and Taq was not increased relative to the higher volume of other components which were kept at their original published concentrations. The original and adjusted volumes of reagents were as follows:

Original	1st generation	5 uL sample + 20 uL cocktail
	2nd generation	0.5 uL template + 24.5 uL cocktail
Adjusted	1st generation	5 uL sample + 45 uL cocktail
	2nd generation	1 uL template + 49 uL cocktail

Initial testing also gave poor results with the *M. hyopneumoniae* strain J control. A comparison of the primer sequences against the published nucleotide sequence of the 16S rRNA gene of *M. hyopneumoniae* strains J, 232 and 7448 revealed that the forward primer for the first generation reaction (1F) was not an ideal match for strain J but did match the sequence of strains 232 and 7448. This primer (IF: GAG CCT TCA AGC TTC ACC AAG A) requires an AGG nucleotide sequence in *M. hyopneumoniae* at positions 3-6 from the end of the IF target sequence, but the published sequence revealed an AAG sequence at that site in strain J (Vasconcelos *et al.* 2005). To overcome this problem, primer IF was modified to a 21mer that aligned with a 7 nucleotide smaller segment of the *M. hyopneumoniae* 16S rRNA gene, thus targeting a 642 bp product for the first generation reaction. The modified IF primer (IF mod: CAA GCT TCA CCA AGA AAT GGG) was found to have 100% homology with known sequences of *M. hyopneumoniae* strains 7448, J, and 232 according to Genbank accessions AE017332.1 for strain 232 (Minion *et al.* 2004), AE017244.1 and AE017243.1 for strains 7448 and strain J respectively (Vasconcelos *et al.* 2005). It was also 100% homologous with the published 16S rRNA gene sequence of *M. hyopneumoniae* strain ATCC 27719 according to Genbank accession Y00149.1 (Taschke *et al.* 1987).

Based on testing the five mycoplasmal isolates listed in Table 1.3 and additional DNA from *M. flocculare* strain MS42 and from lung lesions of pigs experimentally or naturally infected with *M. hyopneumoniae*, an annealing temperature of 64°C and 62°C in the first and second generation assays respectively was considered optimal for specificity and sensitivity (Tables 1.7, 1.8). The modifications to remove excessive 1st generation product were not successful, and this PCR frequently showed both 1st generation (649 bp) and 2nd generation (352 bp) product in gels from *M. hyopneumoniae* isolates and lung samples.

Table 1.7: Effect of varying annealing temperatures for first (T_{A1}) and second (T_{A2}) generation reactions...
...in the Calsamiglia M. hyopneumoniae nested PCR. Row with highlighted double edged cells indicate best result.

Assay /Date	T_{A1}	T_{A2}	Strain or isolate						
			J	232	07/04	07/05	07/06	07/07	07/08
Calsamiglia	60	60							
4/4/07	60	60	- to +	- to +	+	+	-	-	-
2/11/07	60	60	+ #	NT	+ wk#	+ wk#	-	-	-
7/11/07	60	60	+#	NT	+#	+#	+	+	-
7/11/07	62	62	+#	NT	+#	+#	+	+	-
15/11/07 ϕ	60	62	wk#	NT	+ #	wk#	- #	- #	-
15/11/07 ϕ	62	62, 64, 66	wk#	NT	wk#	wk#	- #	- #	-
15/11/07 ϕ	60	64	+#	NT	+#	+#	- #	- #	-
23/11/07 ϕ	62	60	+#	NT	+#		-#	-#	
23/11/07 ϕ	62	62, 64	wk#	NT	wk,+#		-#	-#	
23/11/07 ϕ	64	60	+#	NT	+#		-#	-#	
23/11/07 ϕ	64	62, 64	wk#	NT	wk#		-#	-#	
23/11/07 ϕ	66	60, 62, 64	wk to vwk#	NT	wk#		-#	-#	
3/12/07 ϕ	62	60	vwk	NT			-#	-#	
3/12/07 ϕ	64	60	wk	NT			-#	-#	
5/12/07 ϕ	62	60	+	NT			-#	+#	
5/12/07 ϕ	64	60	+#	NT			-#	wk#	
10/12/07	60	60	+#	+#	+#	+#	-	+	+
29/2/08 ϕ	60, 62, 64	NT	+	+				+*	-
18/3/08 & 27/3/08	60	NT	+	NT	+	+	- to wk	-	-
27/3/08	60	60, 62, 64	+	NT	+	+	-	-	-
1/4/08 **	60	NT	+	NT	+	+	-	-	-
1/4/08 ϕ	60	NT	+	NT	+	+	+	+	-
1/4/08 **	60	60	+#	NT	+#	+#	-	+	-
1/4/08 ϕ	60	60	+#	NT	+#	+#	-#	-#	-
12/5/08 ϕ	64, 66	NT	+	NT			+	-	
12/5/08 ϕ	68	NT	+	NT			wk	-	
13/5/08 ϕ	68,66	64	wk#	NT			-	-	
13/5/08 ϕ	64	64	wk#	NT			-#	-	
13/5/08 ϕ	68, 66	60	+#	NT			-	-	
13/5/08 ϕ	64	60	+#	NT			-#	-	
13/5/08 ϕ	68	62	wk#	NT			-	-	
13/5/08 ϕ	66	62	+#	NT			-	-	
13/5/08 ϕ	64	62	+#	NT			-#	-	
26/5/08 ϕ	64	66	NT	NT			-#	-	
26/5/08 ϕ	66	66	wk#	NT			-#	-	
26/5/08 ϕ	64, 66	68	-#	NT			-	-	

+ : clear positive specific band; wk: weak positive; vwk: very weak positive; - : negative; NT: not tested

also contains 1st gen product

ϕ new IF primer assessed

** includes additional testing according to conditions specified in a related one step PCR (Mattsson *et al.* 1995) where same primer but different PCR conditions apply

Table 1.8: Effect of varying annealing temperatures for first (T_{A1}) and second (T_{A2}) generation reactions...
...in the Calsamiglia M. hyopneumoniae nested PCR for isolates of M. hyopneumoniae (strain J) and of M. flocculare (07/06, 07/07, strain MS42). Results are compared with those of lung extracts from five pigs (152, 237, 12, 161, 171) artificially or naturally infected with M. hyopneumoniae and confirmed to have typical lesions at slaughter. Row with highlighted double edged cells indicate best result.

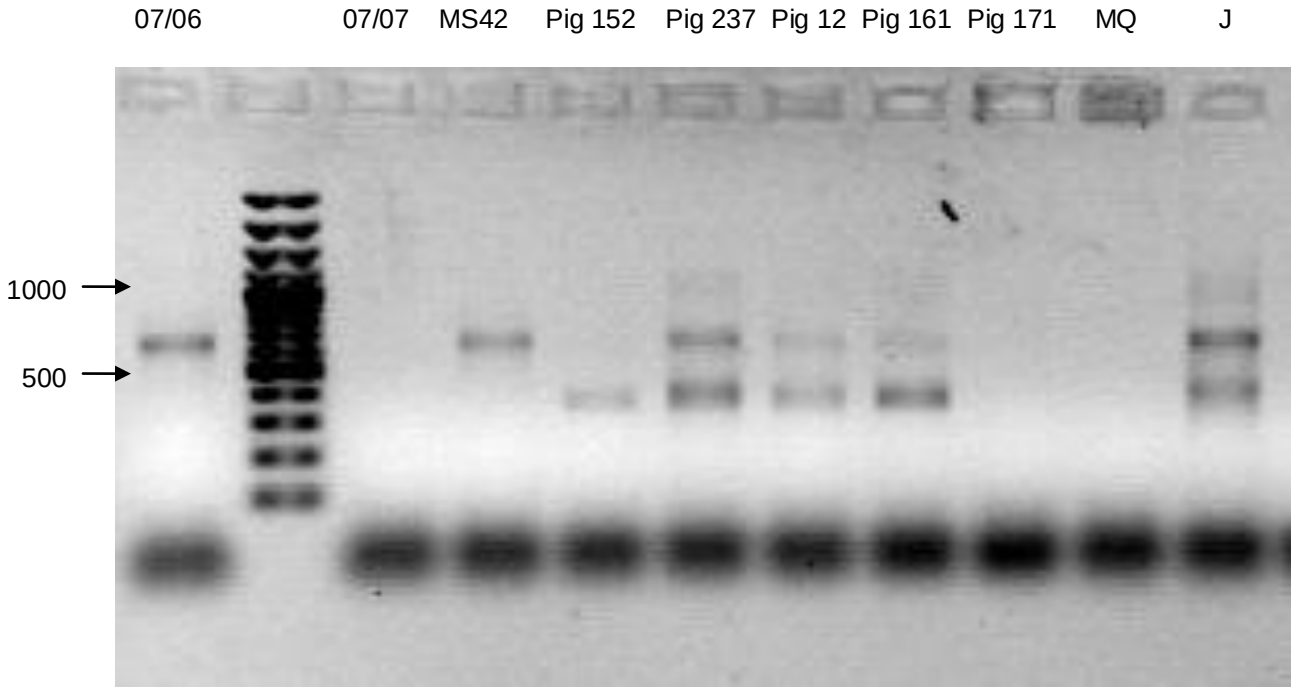
Assay /Date	T_{A1}	T_{A2}	Strain or sample								
			J	Pig 152	Pig 237	Pig 12	Pig 161	Pig 171	07/06	07/07	MS42
Calsamiglia	60	60									
12/5/08 ϕ	64, 66	NT	+	+	+	+	+	wk to -	+	-	wk to +
12/5/08 ϕ	68	NT	+	wk	+	+	+	-	wk	-	-
13/5/08 ϕ	68,66	64	wk#	-	wk#	wk#	wk#	-	-	-	-
13/5/08 ϕ	64	64	wk#	-	wk#	wk#	wk#	-	-#	-	-#
13/5/08 ϕ	68, 66	60	+#	-	+#	+#	+#	-	-	-	-
13/5/08 ϕ	64	60	+#	wk#	+#	+#	+#	-	-#	-	-#
13/5/08 ϕ	68	62	wk#	-	+#	+#	+#	-	-	-	-
13/5/08 ϕ	66	62	+#	wk#	+#	+#	+#	-	-	-	-
13/5/08 ϕ	64	62	+#	+	+#	+#	+#	-	-#	-	-#
26/5/08 ϕ	64	66	NT	wk#	+#	wk#	wk#	-	-#	-	-#
26/5/08 ϕ	66	66	wk#	-	-#	+#	+#	-	-#	-	-
26/5/08 ϕ	64, 66	68	-#	-	-#	- to -#	-#	-	-	-	-

+ : clear positive specific band; wk: weak positive; vwk: very weak positive; - : negative; NT: not tested

also contains 1st gen product

ϕ new IF primer assessed

Figure 1.5: Calsamiglia nested PCR at optimal annealing temperatures of 64°C and 62°C in the first and second generations...,
...showing residual 1st generation product of 649 bp and 2nd generation product for M. hyopneumoniae at 356 bp. Lane marker shows 500 and 1000 bp as thicker lines, with 100 bp intervals between. DNA from lung lesions of five infected pigs and from the J strain control are compared with results for M. flocculare isolates 07/06, 07/07 and MS42. MQ represents negative (MilliQ water) control.



1.2.2 Sequencing Alignment Studies

The Stark PCR was not able to be optimised and was run only at annealing temperatures of 52°C in both generations of the nested PCR. This yielded strong PCR product with all five U.S. isolates as shown in Figure 1.6, and the nested products from *M. flocculare* isolates 07/06 and 07/07 and *M. hyorhinis* isolate 07/08 were sequenced in both directions to investigate if the product was similar to that described as specific for *M. hyopneumoniae*. Sequence alignment with the *M. hyopneumoniae* repetitive sequence (ISMHp1 transposase) confirmed >99% homology with each of the products (Table 1.9), and indicated this repetitive element is not specific to *M. hyopneumoniae*.

Figure 1.6: Stark nested PCR products at an annealing temperature of 52°C for both generations of the assay...
...Lanes 1N & 2N are nested result for M. hyopneumoniae isolates 07/04, 07/05; lanes 3N and 4N are M. flocculare isolates 07/06 and 07/07; lane 5N is M. hyorhinis isolate 07/08. Lanes JN fg and JN ng are M. hyopneumoniae strain J nested controls. Lanes with MQ represent MilliQ water controls (nested and unnested). Lanes J fg and J ng are unnested strain J controls. MW are molecular weight markers, and target product is 808 bp. Products equivalent to lanes 3N, 4N and 5N were sequenced and aligned with the M. hyopneumoniae genome.

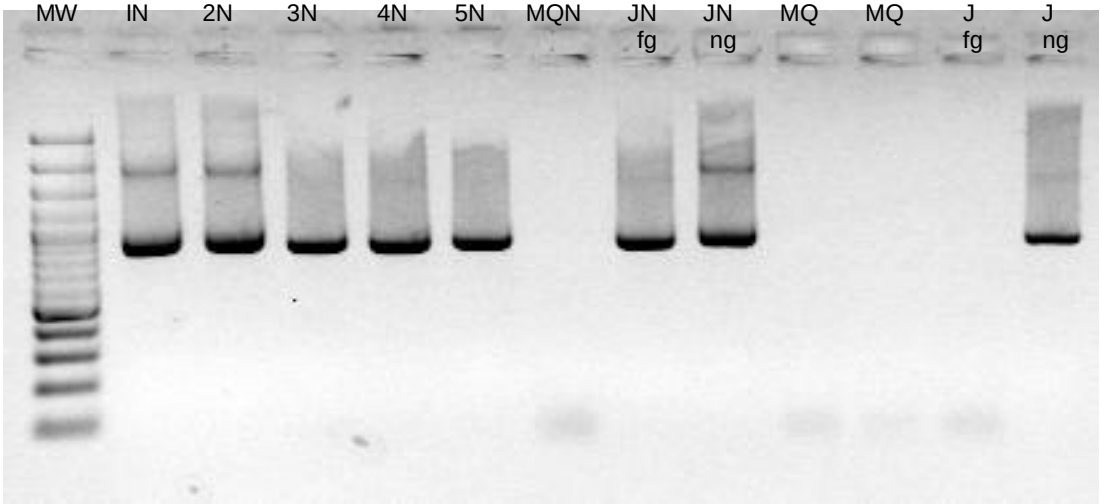


Table 1.9: Comparison of sequenced PCR product from M. flocculare and M. hyorhinis isolates with sequence...
...of Stark PCR target (ISMhp1 transposase) in M. hyopneumoniae strain 7448. The full size of the expected Stark PCR product for M. hyopneumoniae is 808 bp.

Isolate	Identity	Stark product-primer	Size of sequenced material (bp)	% homology with *M. hyopneumoniae* ISMHp1 transposase#
07/06	*M. flocculare*	3N-2L	724	99.7
		3N-2R	723	99.6
07/07	*M. flocculare*	4N-2L	719	99.7
		4N-2R	738	99.3
07/08	*M. hyorhinis*	5N-2L	693*	99.9
		5N-2R	732	99.9

* excluding 46 bp deletion after nucleotide 596
# based on Genbank accession AE017244 for *M. hyopneumoniae* strain 7448

Table 1.10: Initial selection of reaction times and temperatures for original and optimised versions of the five M. hyopneumoniae PCR...
...assays heavily based on reactivity with five mycoplasmal isolates. In the Stark PCR, the same conditions were applied to each PCR reaction, and no optimised annealing temperature was applicable.

Assay	Step	Original T (°C)	Optimised T (°C)	Time	Cycles	Product size for 1st generation/ 2nd generation PCR (*M. hyopneumoniae*)
Stark nested		94	NA	3 min	x 1	913/ 808 bp
	Denature	94	NA	30 s		
	Anneal	**52**	NA	30 s	x 35	
	Extend	72	NA	1 min		
		25	NA	1 min	x 1	
Baumeister		94	94	5 min	x 1	853 bp
	Denature	93	93	30 s		
	Anneal	**55**	**64**	30 s	x 50	
	Extend	72	72	1.5 min		
		72	72	10 min	x 1	
Calsamiglia nested	*1st generation*					649/ 352 bp
		95	95	2 min	x 1	
	Denature	95	95	30 s		
	Anneal	**60**	**64**	45 s	x 30	
	Extend	72	72	30 s		
		72	72	30 s	x 1	
	2nd generation					
		95	95	2 min	x 1	
	Denature	95	95	30 s		
	Anneal	**60**	**62**	45 s	x 30	
	Extend	72	72	30 s		
		72	72	30 s	x 1	
Kurth nested	*1st generation*					1040/ 456 bp
		94	94	4 min	x 1	
	Denature	94	94	1 min		
	Anneal	**49**	**64 (60*)**	1 min	x 30	
	Extend	72	72	1 min		
		72	72	7 min	x 1	
	2nd generation					
		92	92	2 min	x 1	
	Denature	92	92	1 min		
	Anneal	**56**	**64**	1 min	x 30	
	Extend	72	72	1 min		
		72	72	3 min	x 1	
Verdin nested	*1st generation*					1561/ 706 bp
	Denature	92	92	45 s		
	Anneal	**57**	**62**	45 s	x 20	
	Extend	72	72	1.5 min		

	2nd generation					
	Denature	92	92	45 s		
	Anneal	**58**	**62**	45 s	x 40	
	Extend	72	72	45 s		

* later modified to 60°C based on results with other samples

1.3 Discussion

Both of the *M. hyopneumoniae* isolates received from Dr Thacker's laboratory (CM 07/04 and 07/05) were recently evaluated for reactivity with the separate primers for, and under original test conditions of, the Verdin, Kurth, Stark and Calsamiglia PCRs (Strait *et al.* 2008), while the additional three mycoplasmal isolates from that laboratory (*M. flocculare* strains CM 07/06 and CM 07/07 and one *M. hyorhinis* strain CM 07/08) were evaluated for cross-reactivity but only with novel primers for a real-time assay in the same publication. Surprisingly, Strait *et al* (2008) reported that *M. hyopneumoniae* strain 00MP1301, equivalent to our CM 07/05 isolate, was not amplified by the inner primer set of the Kurth PCR yet we found this isolate was readily amplified by the nested Kurth assay under the same as well as under optimised conditions.

The Stark *et al* PCR has been applied to nasal swabs in overseas studies in recent years (Vicca 2002), and tested negative for cross-reactivity for a number of mycoplasmal species, including four *M. hyorhinis* and five *M. flocculare* strains (Stark *et al.* 1998), which is greater than all other published single conventional *M. hyopneumoniae* assays. In its original publication, one of the nested primers was unfortunately misprinted, missing a cytosine base in Primer 3 according to the published target repeat element MHYP1/03-950 (Frey *et al.* 1997), but this was rectified in the present study. Later submissions to Genbank have indicated that the above element makes up a 1023 bp portion of a larger transposase gene known as ISMhp1 which is described as a 'novel IS4 family element that duplicates target sites of variable length during insertion' (Genbank accession AF272977 Calcutt and Wise 2000). The latter is also documented in full genome sequencing for *M. hyopneumoniae* such as Genbank AE017244, which represents the strain 7448 complete DNA sequence.

Our study indicated that no adjustment to annealing temperature for the Stark nested PCR was able to remove the cross-reactivity found in some *M. flocculare* and *M. hyorhinis* strains, and sequencing information on products of the Stark PCR applied to such strains clearly showed why. These strains were found to possess elements with very high homology to the transposase gene ISMhp1 (of which MHYP/03-950 is part), so since this PCR target appears to be present in species other than *M. hyopneumoniae* it would be unsuitable for specific diagnostic application to *M. hyopneumoniae* in isolates or infected samples.

The Calsamiglia PCR was slightly modified because its primer set was not an exact match to published sequence information for strain J of *M. hyopneumoniae* (Vasconcelos *et al.* 2005). This was overcome by replacing the forward primer for the first generation PCR (IF) with one based on a slight shift in the target nucleotide sequence by 7 nucleotides, so the assay is not exactly equivalent to that originally published. This PCR was also slightly problematic in that it often produced two gel bands representing the nested target product of 356 bp as well as residual first generation product of 649 bp. It was also evident that some non-*M. hyopneumoniae* strains produced amplicon from the 1st generation product (including *M. flocculare* CM 07/06 and MS42 but not CM 07/07) indicating some *M. flocculare* strains are amplified in the first generation of this PCR. While the residual 1st generation product was a minor concern with isolates of mycoplasmal species, this is likely to be due to the high level of DNA obtained when assessing extracts from such samples. Despite this, some extracts from infected lungs were found to have sufficiently high DNA to produce the same phenomenon, as seen in Figure 1.5.

Part 2 - Application of Tests that Identify Non-M. hyopneumoniae Strains, Using Alternate and Multiplex PCRs

2.1 Materials and Methods

2.1.1 Assay Selection

From published assays, six PCRs that target porcine mycoplasmas to enable differentiation from *M. hyopneumoniae* in single, duplex or multiplex format assays were selected according to Table 2.1.

Table 2.1: PCR designed to distinguish M. hyopneumoniae from other common porcine mycoplasmas

Target species	Reference	Type	Target size (bp)	Target gene
hyopneumoniae vs hyorhinis	(Lin *et al.* 2006b)	Duplex	751 (*Mhr*) 430 (*Mhp*)	16S rRNA
hyopneumoniae vs *flocculare* vs *hyorhinis*	(Stakenborg *et al.* 2006)	Multiplex	1129 (*Mhr*) 1000 (*Mhp*) 754 (*Mf*)	16S rRNA
hyopneumomiae vs *flocculare*	(Stemke 1997; Stemke *et al.* 1994)	Duplex	400 (*Mf*) 238 (*Mhp*)	16S rRNA
hyopneumoniae vs flocculare	(Lin *et al.* 2006b)	Duplex nested	679 (common) then 396 (*Mhp*)	16S rRNA
hyorhinis	(Caron *et al.* 2000) as per (Assuncao *et al.* 2005)	Single	346	p37
hyosynoviae	(Ahrens *et al.* 1996) as per (Assuncao *et al.* 2005)	Single	398	16S rRNA

2.1.2 Evaluation Procedure

DNA extracted from isolates of *M. hyopneumoniae*, *M. flocculare*, and *M. hyorhinis* as used in initial testing of the *M. hyopneumoniae* PCRs described in Part 1 (and listed in Table 1.3) were applied. Some assays were tested with some additional strains whose DNA was stored at EMAI; these included DNA derived from a strain of *M. flocculare* (identified as F1), *M.hyorhinis* (type strain BTS-7) and *M. hyosynoviae* (identified as HYOSYN).

Initial studies indicated that the published Stemke duplex PCR information for the *M. hyopneumoniae* forward primer was incorrect in the 1997 publication, while the 1994 publication was not an exact match with the information on *M. hyopneumoniae* 16S rRNA in either Genbank (Accession Y00149) or an earlier publication describing the V2 region of this gene (van Kuppeveld *et al.* 1992). The primer was therefore modified to be equivalent to that described by Stemke *et al* (1994) but with the 5' end commencing with the sequence ATTCA rather than ATTTCA. The full MHP5' primer was therefore ATTCAAAGGAGCCTTCAAGCTTCA, despite claims that the lack of the additional thymidine may result in weak *M. hyorhinis* cross-reactivity (Stemke *et al.* 1994).

2.2 *Results*

Among the six PCRs designed to detect mycoplasmas other than *M. hyopneumoniae*, all except the nested duplex PCR of Lin *et al* (2006b) for *M . flocculare* vs *M. hyopneumoniae* were shown to function as claimed. The nested duplex PCR of Lin showed no amplified 679 bp product with the first generation PCR and the products of the second generation PCR were not the expected 396 bp size, instead yielding two products of 300 and 420 bp. The primers for this assay were possibly faulty. As the non-nested duplex Lin PCR was shown to be suitable for the differentiation of *M. hyorhinis* and *M. hyopneumoniae*, and others were found suitable for differentiation of *M. hyopneumoniae* from *M. flocculare*, the nested Lin PCR was not further examined.

Among the alternate Mycoplasma PCRs, the Stakenborg multiplex, Stemke duplex and Lin duplex assays were reliable at their published annealing temperature. Elevated annealing temperatures were required to optimise the Ahrens/Assuncao PCR for *M. hyosynoviae* and the Caron PCR for *M. hyorhinis*.

The results of the optimisation studies for all the above PCRs are shown in Tables 2.2 and 2.3. Additionally, these one step PCRs indicated the identity of isolates received from the U.S.A. and used in the optimisation studies for the nested and non-nested *M. hyopneumoniae* PCRs concurred with the original description of these isolates i.e. 07/04 and 07/05 are *M. hyopneumoniae* strains, 07/06 and 07/07 are both *M. flocculare* strains and 07/08 is an *M. hyorhinis* strain.

The only test that gave weak cross-reactivity with 07/04 and 07/05 was the Caron PCR at elevated annealing temperatures of 54 and 58◦C. Nevertheless, this PCR clearly detected *M. hyorhinis* at an annealing temperature of 54◦C, and was found to be reliable in later examination of isolates from Qld found to be *M. hyorhinis* (Table 2.2, Table 3.1). Further optimisation of the Caron PCR was not undertaken, as reliable alternatives were available with the Stakenborg and Lin duplex assays.

Typical results for the various PCRs after optimisation studies are shown in Figures 2.1-2.4.

Table 2.2: Results of multiplex/duplex/single PCRs in distinguishing M. hyopneumoniae from other porcine mycoplasmas... ....Numbers indicate PCR product sizes (bp). Rows with highlighted, double edged cell indicates annealing temperature (T_A) giving optimal result.

	T_A	J	232	07/04	07/05	07/06	07/07	Mfloc F1	07/08	Mhr BTS-7	Comments
Stakenborg	55										Designed to detect Mhp, Mflocc, Mhr
10/9/07, 13/9/07	55	-	NT	1000	1000	754	754		1129		Correct; faulty J; $MgCl_2$ increased from 1.5 to 2 mM
15/4/08	55	1000	1000	1000	1000	754	754	754	1129	1129	Correct
Lin duplex	60										Designed to detect Mhp, Mhr
25/9/07	56	-	-						-		
25/9/07	60	430	-						751 wk		Poor results at 2.5-4 mM $MgCl_2$
25/9/07	63	430	-						751		Better at 4 mM $MgCl_2$
26/9/07	56, 60, 63	430	NT						751		Variable results with Promega Taq & buffer; Good results with Roche Taq & buffer 2.5 mM $MgCl_2$ good
28/9/07	60	430	430	430	430	-	-		751		Correct, new 232 control good
Stemke	61										Designed to detect Mhp, Mflocc
4/10/07	61, 63	238	238					400			Primers described in literature incorrect. With redesigned primers gave correct size; better at 5- 6 mM $MgCl_2$ than 4 mM
8/10/07	61	238	NT	238	238	400	400		-		Correct - good at 4 mM $MgCl_2$
22/10/07	61	238	238	238	238	400	400		-		Correct. OK at 1.5 mM $MgCl_2$; good at 2 mM; Failed at 1 mM
Caron (Assuncao)	50	-									Designed to detect Mhr only
4/10/07	47, 50, 54	-							346		Correct

9/10/07, 10/10/07	50, 51, 54, 58	-	NT	346 wk	346 wk	346 vwk	346 vwk		346		
15/4/08	54	-									Reacted with 8 *M. hyorhinis* isolates from Qld (Table 3.1) in agreement with Lin duplex and Stakenborg PCRs
Lin nested	60/60										Designed to detect Mhp, Mflocc in stage 1 and Mhp in stage 2
19/10/07	Stage 1 60	-	-	-	-	-	-		-		Incorrect – should be 679 for Mhp, Mflocc
19/10/07	Stage 2 60	420, 300	420	420, 300	-	-	-		-		Incorrect – should be 396 for Mhp; primers faulty?

Table 2.3: Results from single-step PCR for detecting M. hyosynoviae....
...Row with highlighted, double edged cell indicates indicates annealing temperature (T_A) giving optimal result.

Assay and date	T_A	Strain/isolate ID (results indicate amplicon size in bp)								Comments
		J	232	07/04	07/05	07/06	07/07	07/08	Mhs	
Ahrens (Assuncao)	54									Designed to detect Mhs only
17/10/07	50, 54, 57								398	Correct but xs primer dimer
26/10/07	54	-	NT	-	-	-	-		398 thick	Minor non-specific bands at 800 bp for J, Mfloc, Mhr. Try reduced primer conc from 100 to 50 pmol and $MgCl_2$ 2 vs 4 mM
1/11/07	54	-							398	2 mM $MgCl_2$ and 50 pmol primer best. NS Mhp J line at 100 pmol/4 mM
1/11/07	58	-							398	All correct 50 pmol primer and 2 mM $MgCl_2$ very good
2/11/07	58	-	NT	-	-	-	-	-	398	Correct (50 pmol, 2 mM $MgCl_2$)

Figure 2.1: Optimal result for the Stakenborg multiplex PCR showing differing products with DNA...
...from strains of M. hyopneumoniae (07/04, 07/05, J, 232)(1000 bp), M. flocculare (07/06, 07/07, MS42)(754 bp) and M. hyorhinis (07/08, Mhr)(1120 bp)

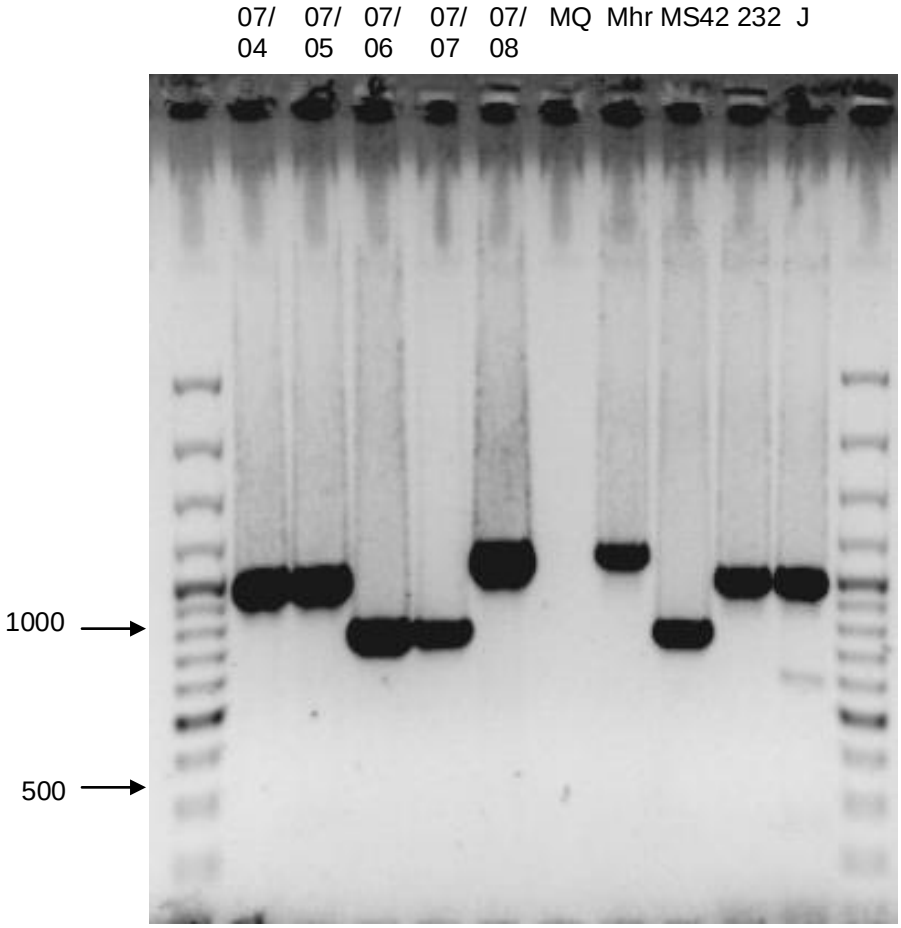


Figure 2.2: Optimal result for the Lin duplex PCR showing differing products with DNA from strains of M. hyopneumoniae (07/04, 07/05, J, 232)(430 bp) and M. hyorhinis (07/08) (751 bp) but not with M. flocculare isolates (07/06, 07/07)

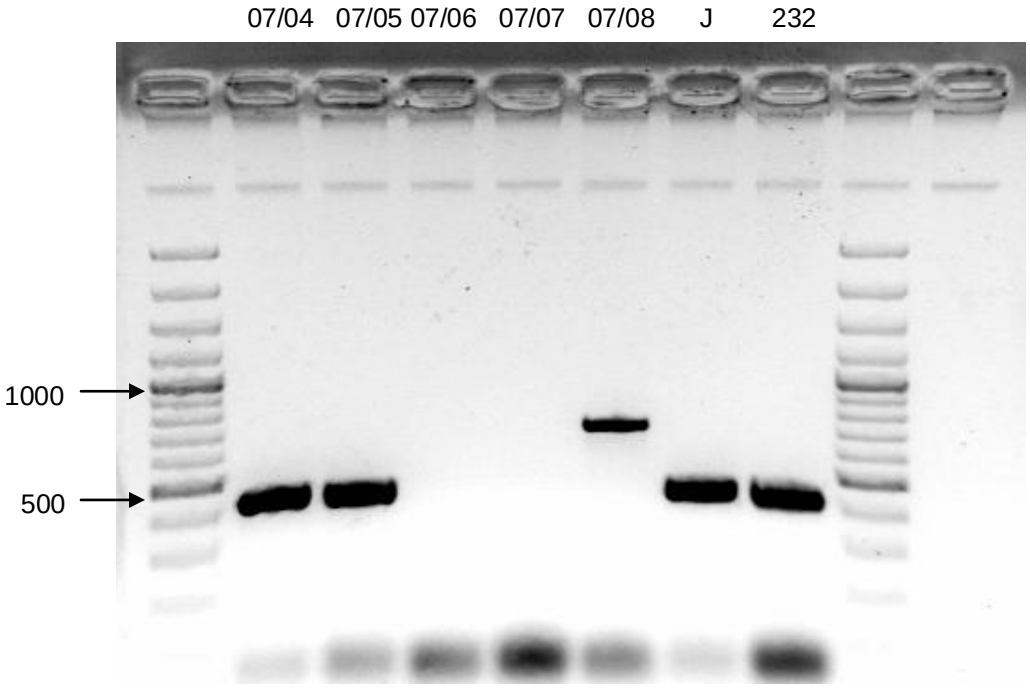


Figure 2.3: Optimal result for the Stemke duplex PCR showing differing products with DNA...
...from strains of M. hyopneumoniae (07/04, 07/05, J, 232) (target 238 bp) and M. flocculare (07/06, 07/07)(target 400 bp) but not with M. hyorhinis (07/08).

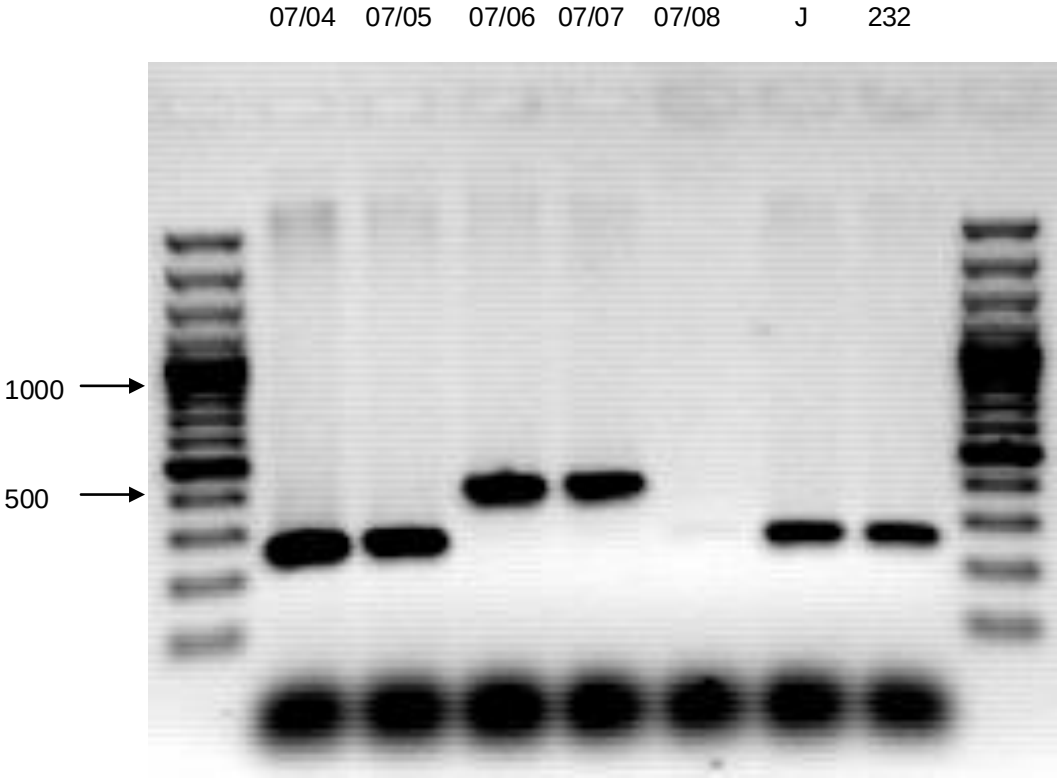
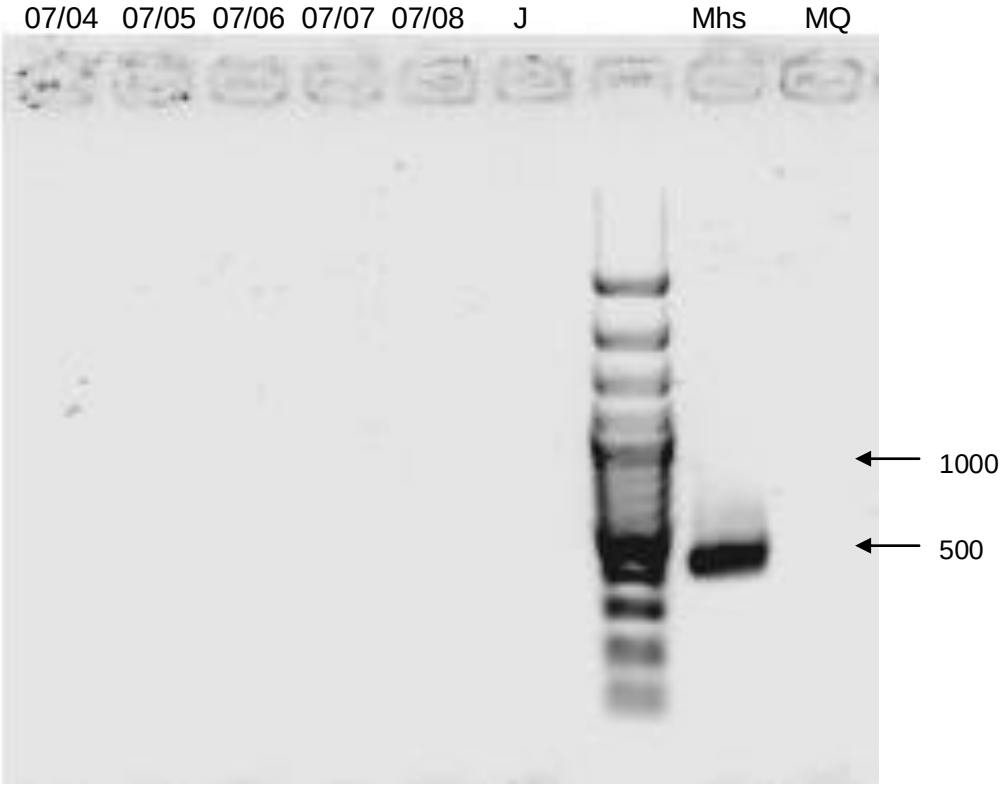
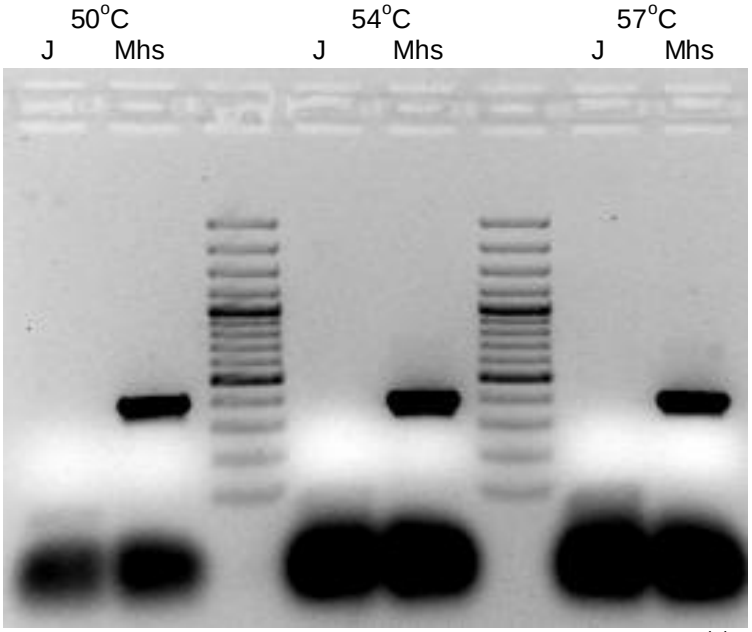


Figure 2.4: Top: Optimal result for the Assuncao/Ahrens PCR for M. hyosynoviae at an annealing temperature of...
...58°C showing no cross-reactivity with DNA from strains of M. hyopneumoniae (07/04, 07/05, J), M. flocculare (07/06, 07/07) or M. hyorhinis (07/08). M. hyosynoviae DNA is in the second last lane marked Mhs with expected product of 398 bp, while MQ is a negative MilliQ water control.
Bottom: Evaluation of same PCR at other temperatures, with evidence of non-specific banding at 54°C.





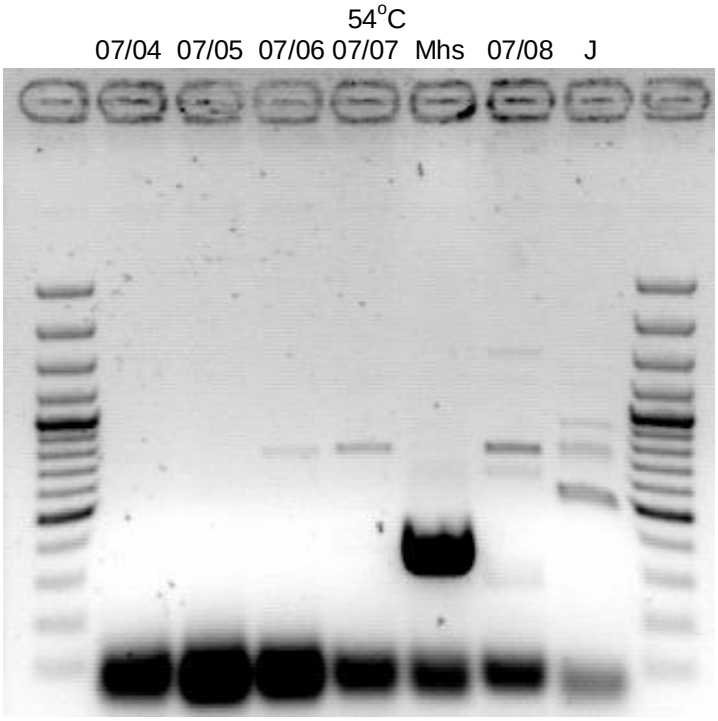


Table 2.4: Selection of reaction times and temperatures for original and optimised versions of PCR assays...
...designed to detect various mycoplasmal species (Mhp: M. hyopneumoniae; Mhr: M. hyorhinis; Mfloc: M. flocculare; Mhs: M. hyosynoviae). Optimisation was not proven for the Caron assay.

Assay	Step	Original	Optimal	Time	Cycles	Product sizes
Stakenborg multiplex	Denature	94	94	30s		Mhp 1000 bp
	Anneal	55	55	15s	x 30	Mhr 1129 bp
	Extend	68	68	68s		Mfloc 754 bp
Lin duplex		95	95	5 min	x 1	Mhp 430 bp
	Denature	94	94	1 min		Mhr 751 bp
	Anneal	60	60	30s	x 30	
	Extend	72	72	1 min		
		72	72	15 min	x 1	
Stemke duplex		96	96	5 min	x 1	Mhp 238 bp
	Denature	95	95	30s		Mfloc 400 bp
	Anneal	61	61	30s	x 25	
	Extend	72	72	90s		
Caron	Denature	94	94	60s		Mhr 346 bp
	Anneal	50	54	60s	x 35	
	Extend	72	72	90s		
		72	72	10 min	x 1	
Assuncao/ Ahrens		94	94	3 min	x 1	Mhs 398 bp
	Denature	94	94	1 min		
	Anneal	54	58	1 min	x 35	
	Extend	72	72	90s		
		72	72	10 min	x 1	

2.3 Discussion

Differentiation of *M. hyopneumoniae* from other mycoplasmas by molecular means is critical to the establishment of purified cultures for challenge studies or for specificity testing of new diagnostic tests. Among the several assays assessed for this purpose, and unlike most of the *M. hyopneumoniae*-specific assays, most performed well with their original published parameters. The most versatile appears to be the Stakenborg PCR, which despite its similar size product for *M. hyorhinis* (1129 bp) and *M. hyopneumoniae* (1000 bp) was found satisfactory if sufficient time was allotted for gel electrophoresis to distinguish the two band sizes in 1% agarose.

The Stemke and Lin duplex PCRs were reliable additional assays to confirm and differentiate *M. flocculare* and *M. hyorhinis* from *M. hyopneumoniae*. The only PCR that appeared to be unrealiable was a nested PCR described by Lin *et al* in the same paper where the duplex PCR was published. The former assay was not found to produce any product as described by those authors for detection and differentiation of *M. hyopneumoniae* from *M. flocculare*.

The size of the Stemke product for *M. hyopneumoniae* is claimed to be approximately 200 bp (Stemke *et al.* 1994) or 238 bp (Stemke 1997) but data in Genbank accession Y00149 which

describes the sequence of *M. hyopneumoniae* 16S rRNA indicates these primers would amplify a 254 bp sequence. Examination of the gel results in the present study (Figure 2.3) indicates that the latter size is likely to be more accurate.

Part 3 - Final M. hyopneumoniae PCR Test Selection Evaluation and Optimisation

3.1 Materials and Methods

3.1.1 Additional Mycoplasmal Strains for Full Specificity Evaluation

During the project, additional isolates of *M. hyopneumoniae, M. flocculare, M. hyorhinis* and *M. hyosynoviae* were obtained to provide a complete set of cultures from the sources described in Table 3.1. In the initial evaluation of PCR assays, seven of these isolates (HP1, HP2, HP6, HP7, MF1, MF2 and HR10) had been tested to examine the specificity of PCRs using published protocols.

Four additional cultures, provided from Dr P. Kuhnert at the University of Berne, Switzerland, were not viable as these were maintained in liquid culture form only and did not survive transportation. These strains were identified as *M. hyopneumoniae* strains Ue1221, Ue2235 and Ue860, and also *M. hyorhinis* strain NCTC 10130, which was equivalent to BTS-7 (Assuncao *et al.* 2005) and thus already available in the EMAI reference collection (Table 3.1). All isolates were triple cloned prior to testing.

Table 3.1: Mycoplasmal isolates used for final specificity testing

Species	Isolate no.	EMAI lab reference	Source	Strain identity at source lab
M. flocculare	MF1	CM07/06	Dr E. Thacker, (Iowa State University)	94MF1501
	MF2	CM07/06c	Cloned ex above	
	MF3	CM07/07	Dr E. Thacker	02MF1501
	MF4	CM07/07c	Cloned ex above	
	MF5	Hillcrest Mf1 (centred)	EMAI	
	MF6	Hillcrest Mf2 (uncentred)	EMAI	
	MF7	MS42	Type strain, EMAI	
M. hyorhinis	HR1	CM08/01	Cloned following receipt from QDPI (J Forbes-Faulkner)	
	HR2	CM08/02	" "	
	HR3	CM08/03	" "	
	HR4	CM08/05	" "	
	HR5	CM08/06	" "	
	HR6	CM08/07	" "	
	HR7	CM08/09	" "	
	HR8	CM08/10	" "	
	HR9	CM08/11	" "	
	HR10	CM07/08	Dr E. Thacker	SK76C
	HR11	BTS-7	Type strain, EMAI	
M. hyopneumoniae	HP1	J	Type strain, EMAI	
	HP2	232	Type strain, EMAI	
	HP3	Hillcrest Mhp	EMAI	
	HP4	CM08/04	Cloned following receipt from QDPI (J Forbes-Faulkner)	
	HP5	CM08/08	" "	
	HP6	CM07/04	Dr E. Thacker	95MP1509
	HP7	CM07/05	Dr E. Thacker	00MP1301
M. hyosynoviae	HS1	HYSYN	EMAI	

3.1.2 Final PCR Optimisation

Based on findings in Part 1 and 2, the PCRs of Verdin, Kurth, Stark, Baumeister, and Stakenborg were further evaluated at their 'initial' optimal annealing temperature (where applicable) for reactivity with mycoplasmal isolates described in Table 3.1. DNA from five infected lungs from pigs experimentally or naturally infected with *M. hyopneumoniae* (pigs 12, 152, 161, 171 and 237) were also assessed at this time in the Verdin, Kurth, Baumeister, and Stakenborg PCRs. The Calsamiglia PCR had been tested with all five lung samples described above in earlier testing, and was not further assessed at this time. However, the optimised Calsamiglia PCR was later tested against Instagene extracts of 11 isolates of *M. hyopneumoniae*, comprising all seven described as HP1-HP7 in Table 3.1 plus strains Beaufort, Sue, OMZ407 and C1735/2. The latter four were materials held at EMAI from former studies, of which C1735/2 is considered equivalent to the Qld isolate HP5.

In addition, the mycoplasmal isolates received from QDPI (HR1-HR9, HP4, HP5 in Table 3.1) were tested in the duplex PCR of Lin, while isolates MF5, MF6 and MF7 and HP1, HP3 and HP5 were tested in the duplex PCR of Stemke, and the nine Qld isolates HR1-HR9 were tested in the *M. hyorhinis* PCR of Caron.

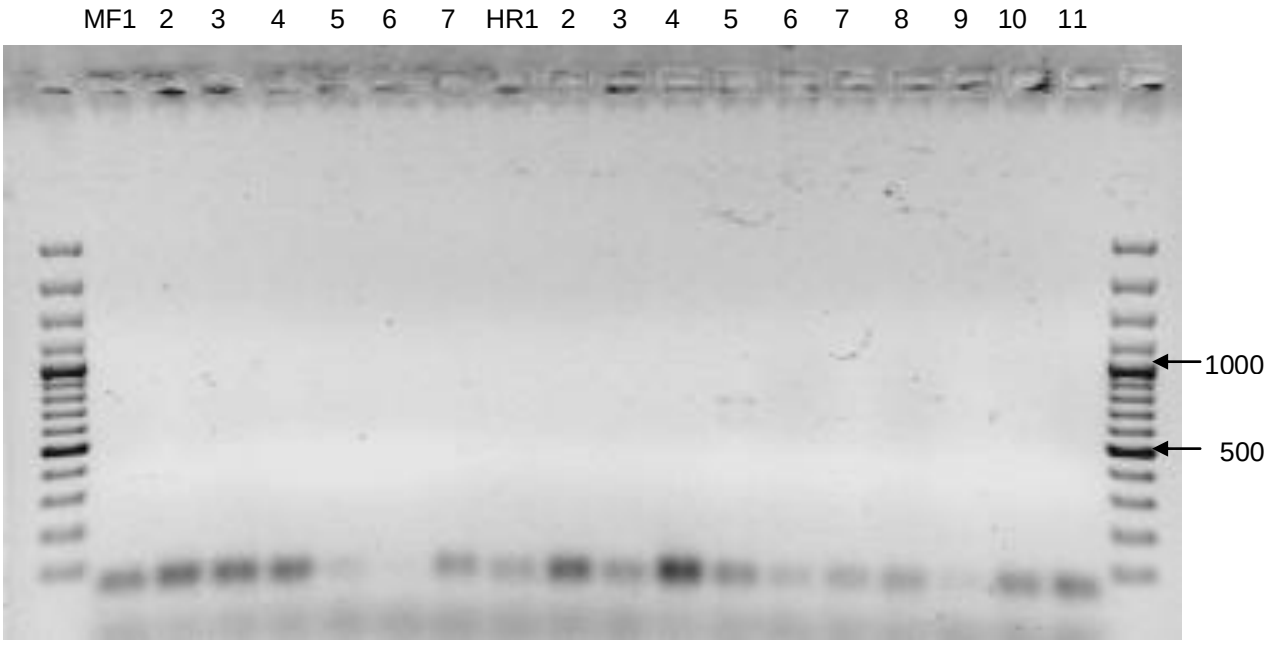
The Stakenborg and Lin duplex PCR were also evaluated with DNA extracted from 20 nasal swabs collected from grower pigs in an unrelated nutrition trial at EMAI in 2008, simultaneously with DNA from 8 of the Qld mycoplasmal isolates (HR1-4, HR 6-9), to assess whether this PCR could detect different mycoplasmal species in nasal swabs. The same swabs were then tested for evidence of *M. flocculare* and *M. hyorhinis* using the Stemke duplex PCR (for *M. flocculare* and *M. hyopneumoniae*).

3.2 Results

3.2.1 Verdin PCR

At the previously optimised annealing temperatures of 62°C in both generations (Tables 1.4 and 1.10), the Verdin PCR showed high specificity and sensitivity: none of 7 isolates of *M. flocculare* or of 11 isolates of *M. hyorhinis* gave a positive signal, apart from a single weak positive with *M. hyorhinis* isolate HR5. Concurrently, all 7 *M. hyopneumoniae* isolates gave a positive signal, as did all five infected lung samples (Figure 3.1). In repeat testing several months later, similar results were obtained, with HR5 again yielding a weak positive signal and all other non-*M. hyopneumoniae* strains remaining test negative (Figure 3.2).

Figure 3.1: Final specificity testing of Verdin nested PCR at optimised annealing temperatures of 62°C...
...in both the first and second generation reactions for 25 mycoplasmal isolates and five lung samples with typical lesions of M. hyopneumoniae pneumonia. Target product is 706 bp, relevant to MW markers of 1000 and 500 bp (darker bars) shown. Samples are from M. flocculare isolates MF1-MF7; M. hyorhinis isolates HR1 – HR11; M. hyopneumoniae isolates HP1-HP7, while L1-L5 are lung samples from pigs 12, 152, 161, 171 and 237 respectively



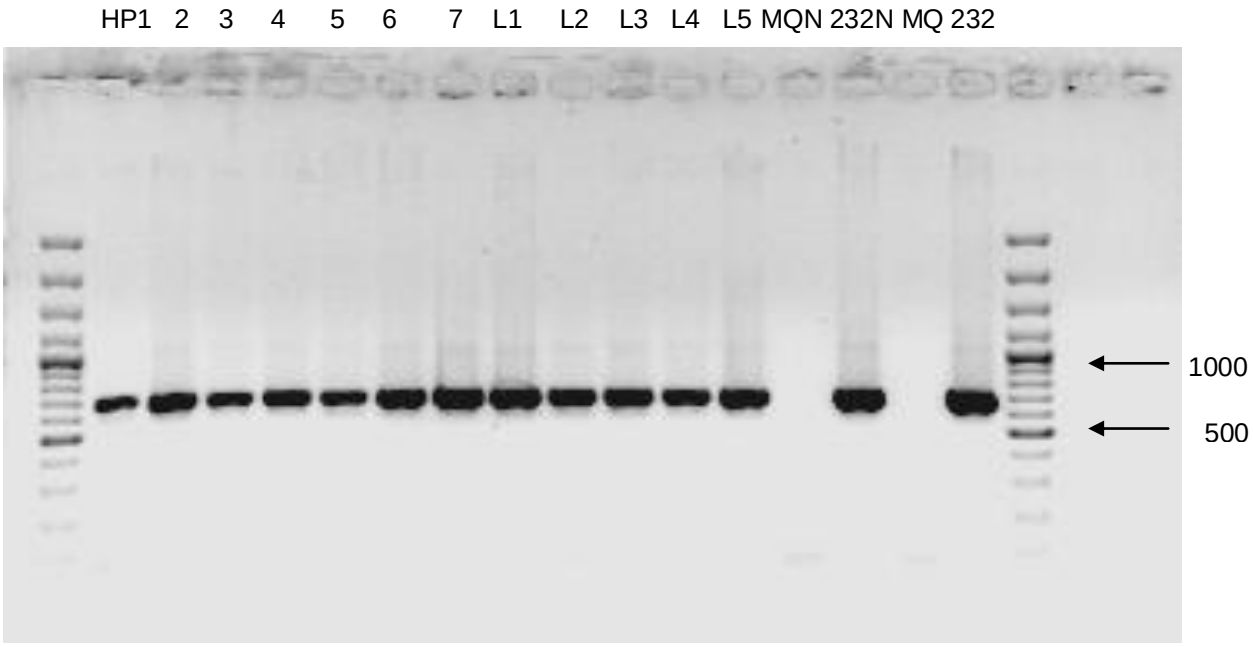
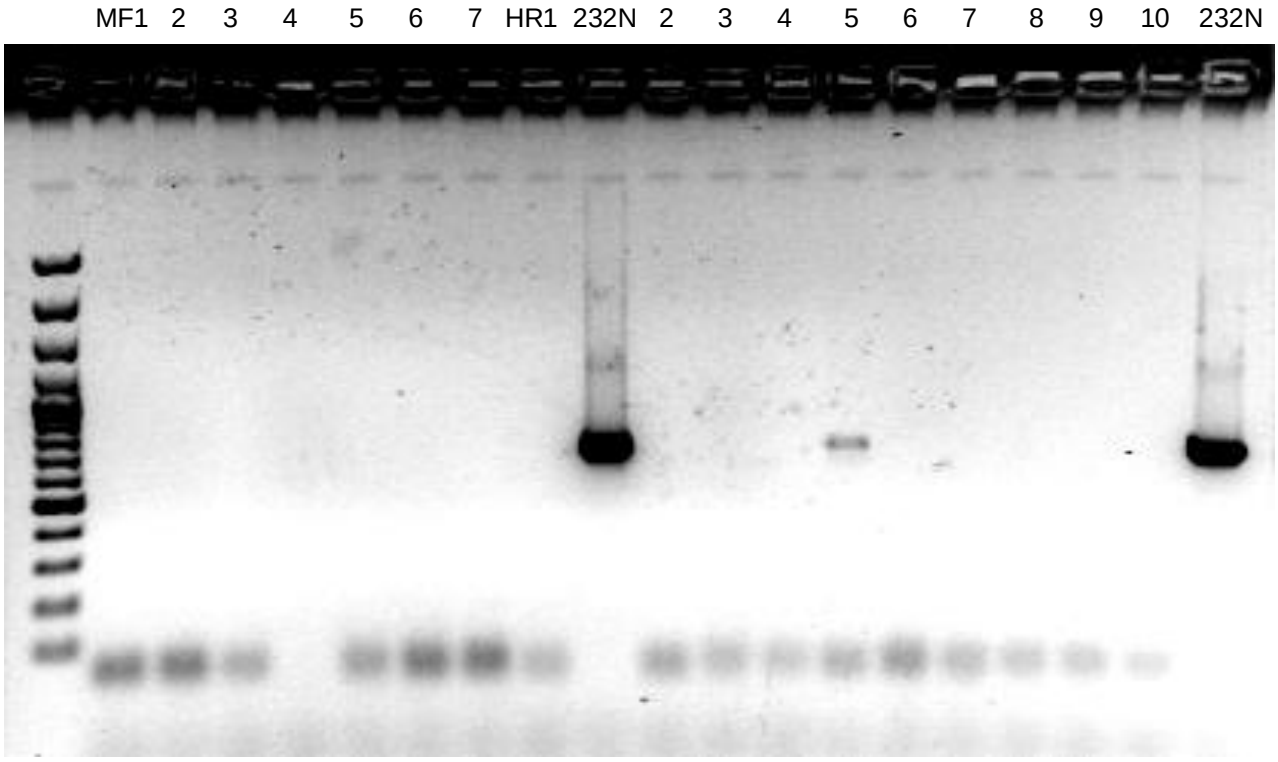
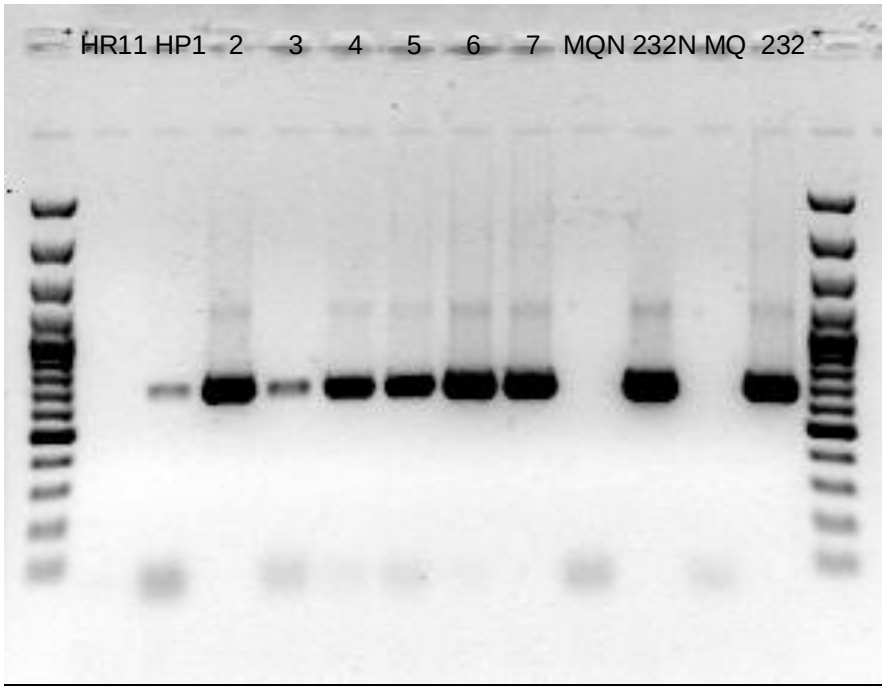


Figure 3.2: Repeat of final specificity testing of Verdin nested PCR at optimised annealing temperatures of 62°C in both the first and second generation... ...reactions for 25 mycoplasmal isolates. This assay was used to compare with results of Kurth, Calsamiglia and Stark assays undertaken at the same time. Target product is 706 bp, relevant to MW markers of 1000 and 500 bp (darker bars) shown. Lanes show M. flocculare isolates MF1-MF7; M. hyorhinis isolates HR1 – HR11 and M. hyopneumoniae isolates HP1-HP7, with positive (strain 232) and negative (MQ water) controls. N indicates nested control, tested in both generations of the PCR, while samples labelled 232 and MQ are tested in the second generation assay only.





3.2.2 Kurth PCR

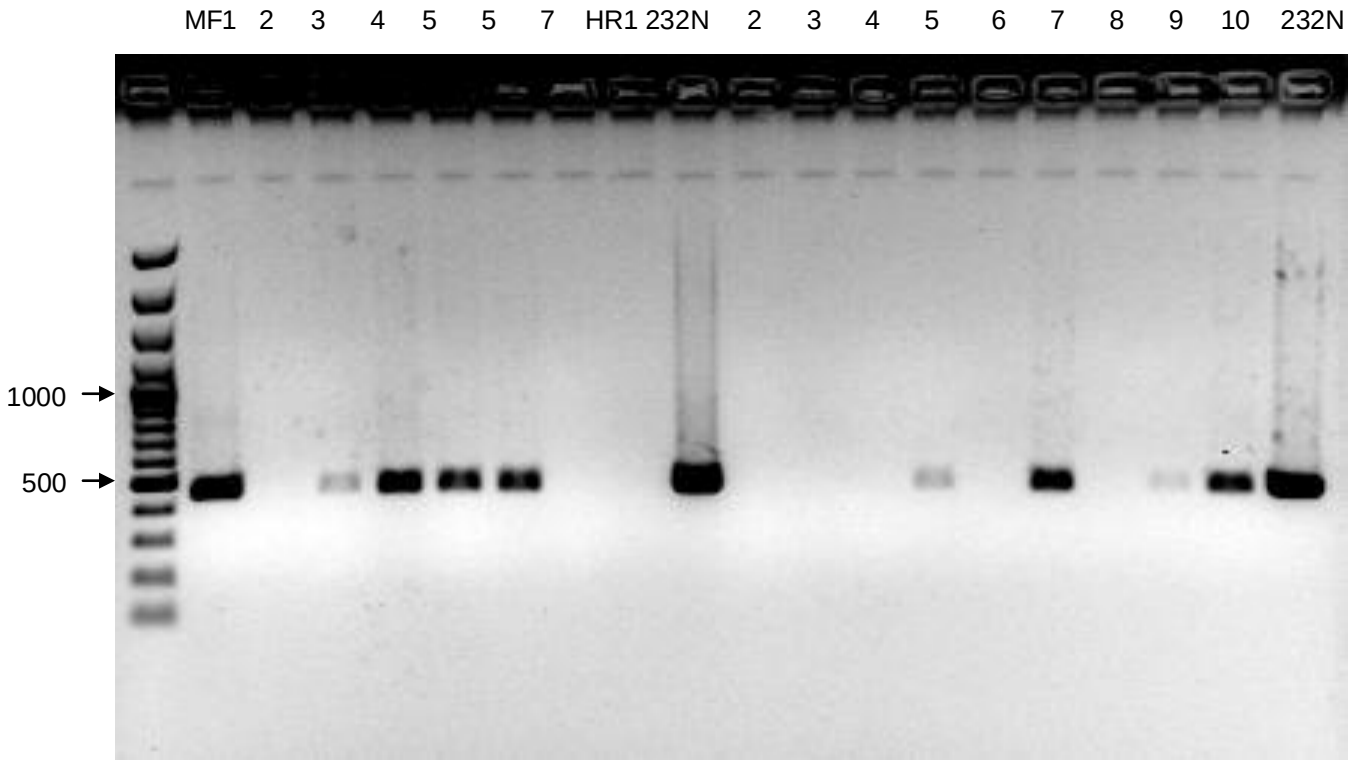
Although initial studies indicated annealing temperatures of 64°C for the first and second generation reaction were superior for specificity, a first generation annealing temperature of 60°C was required to maintain sensitivity for both *M. hyopneumoniae* isolates and infected lung samples (Table 3.2). However, despite testing across a wide range of 1st and 2nd generation annealing temperatures, none yielded maximal specificity with cross-reactivity still evident with at least three *M. flocculare* and two *M. hyorhinis* isolates (Table 3.2, Figure 3.3). The "optimised" Kurth PCR was therefore still likely to show false positive reactions on field samples containing *M. hyorhinis* or *M. flocculare*. Additionally, *M. hyopneumoniae* isolates (HP3, HP7) showed intermittent reactivity in the Kurth PCR (Table 3.2, Figure 3.3).

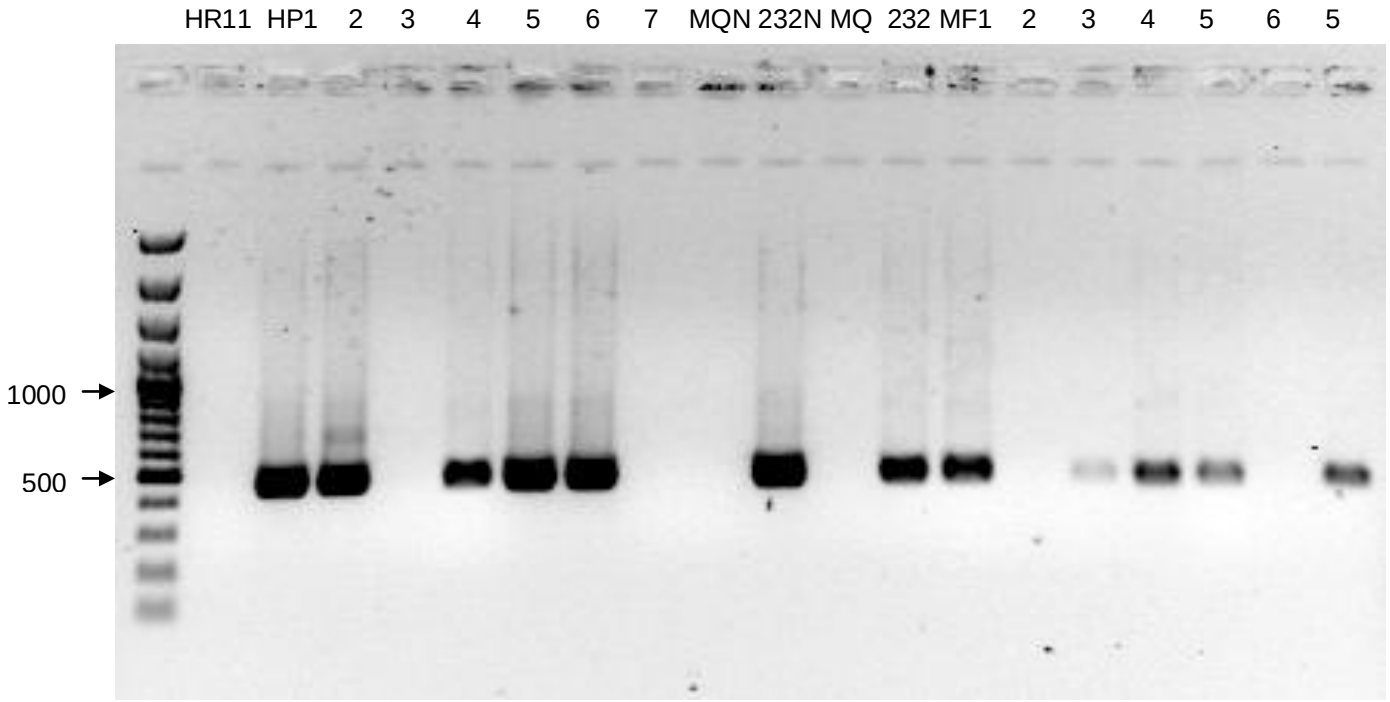
Table 3.2: Kurth PCR reactivity with various isolates of M. flocculare, M. hyorhinis and M. hyopneumoniae and with lung samples from pigs...
...ith suspected M. hyopneumoniae infection. Blank spaces denote certain isolates or lung extracts were not tested on that occasion. Highlighted cell indicates optimised annealing temperatures without significant loss in sensitivity.

Isolate or lung extract	Annealing temp for 1st/2nd generation PCR														
	62/60	62/62	64/60	64/62	64/64	62/64	63/63	64/62	60/62	60/66	58/62	58/64	58/66	60/64	60/64
	27/5/08		29 & 30/5/08			3 &13/6/08	3/6/08		6/6/08					6 &13/6/08	10/2/09
MF1	+	+	+	+	+	-/+	-	-	+	+	+	+	+	+	+
MF2	-	wk	-	-	-	-	-	-						-	-
MF3	wk	wk	-	-	-	-	-	-	+	wk	+	+	+	+/-	wk
MF4	-	-	-	-	-	-	-	-						-	+
MF5	-	-	-	-	-	-	-	-						+	+
MF6	wk	wk	-	-	-	-/wk	-	-	-	-	-	-	-	-	-
MF7	-	wk	-	-	-	-	-	-						+	-
HR1	+	+	-	-	-	-/+	-	-	+	-	+	+	-	+	-
HR2	-	-	-	-	-	-	-	-						+	-
HR3	wk	wk	-	-	-	-	-	-						-	-
HR4	-	-	-	-	-	-	-	-						+	-
HR5	+	+	-	-	-	-	-	-	+	wk	+	+	+	+	wk
HR6	-	-	-	-	-	-	-	-						+	-
HR7	-	-	-	-	-	-	-	-						-	+
HR8	wk	-	-	-	-	-	-	-						-	-
HR9	wk	wk	-	-	-	-	-	-						-	vwk
HR10	+	+	-	-	-	-/+	-	-	+	+	+	+	wk	+	+
HR11	wk	-	-	-	-	-	-	-						-	-
HP1	+	+	+	+	+	+	+	+						+	+
HP2	+	+	+	+	+	+	+	wk						+	+
HP3	+	+	-	-	-	-/+	-	-	wk	-	-	wk	-	wk/+	-
HP4	+	+	-	-	-	-/+	-	-	+	+	+	+	+	+	+
HP5	+	+	+	+	+	+	+	-						+	+

HP6	+	+	+	+	+	+	+	+						+	+
HP7	+	+	-	-	-	-/+	-	-	+	wk	-	-	-	+/-	-
Lung 12	-	+	-	-	-	-	-	-	+	wk	-	-	-	+	
Lung 152	-	-	-/wk	-/wk	-/wk	-	-	-	wk	wk	+	+	wk	+/-	
Lung 161	+	+	-	-	-	-/+	-	-						+	
Lung 171	+	+	-	-	-	vwk/+	wk	-						+	
Lung 237	**-**	**-**	**-/+**	**-/+**	**-/+**	**-**	**-**	**-**	+	+	+	+	wk	**+/-**	

Figure 3.3: Kurth nested PCR at optimised annealing temperatures of 60°C and 64°C... ...in the first and second generation reactions respectively for 7 M.flocculare isolates (MF1-MF7), 11 M. hyorhinis isolates (HR1-HR11) and 7 M. hyopneumoniae isolates (HP1-HP7) as described in Table 3.1. Molecular weight markers indicate 1000 bp and 500 bp as darker gel lines. The M. hyopneumoniae second generation target is 456 bp.

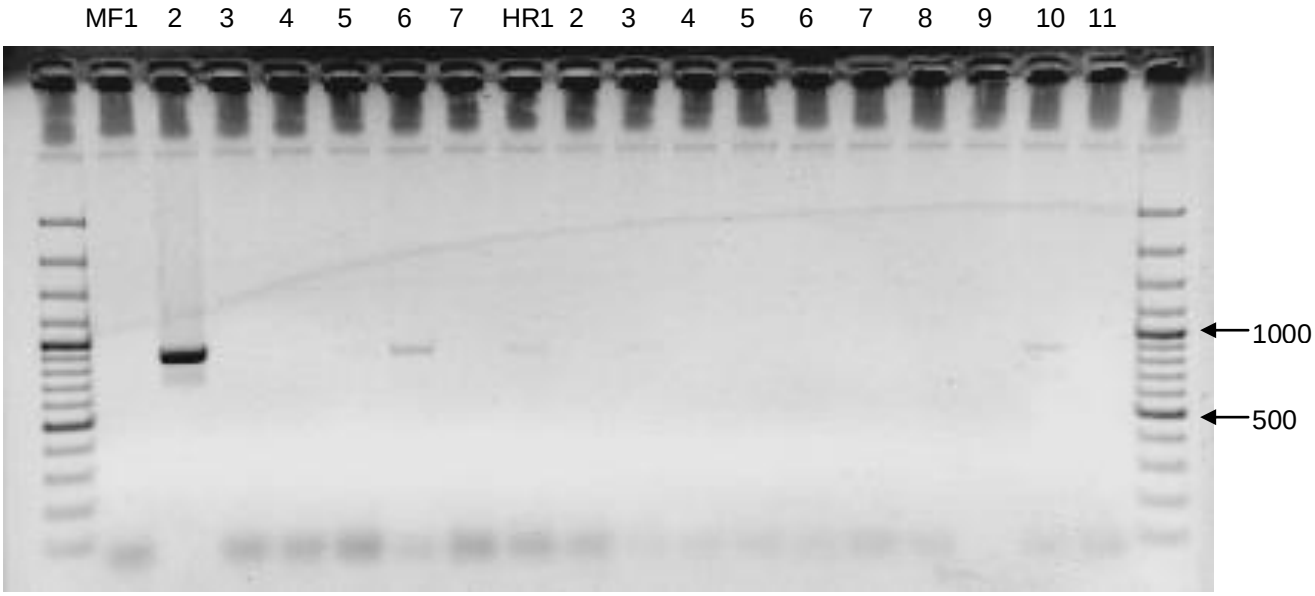


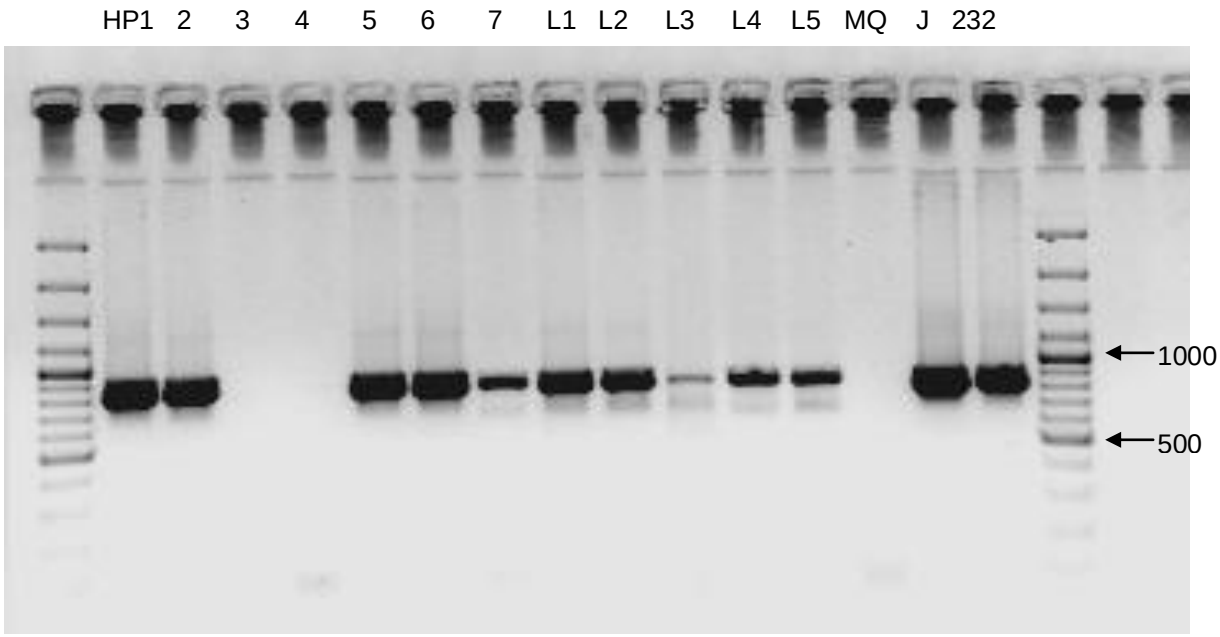


3.2.3 Baumeister PCR

The Baumeister PCR at an annealing temperature of 64°C was found to have good specificity, with two of the 7 *M. flocculare* isolates (MF2 and MF6) giving a positive or weak positive signal respectively and two of the 11 *M. hyorhinis* isolates (HR1, HR10) giving a weak positive signal (Figure 3.4). However, two *M. hyopneumoniae* strains (HP3, HP4) were not detected and a weak positive signal was detected in one of the infected lung samples (Pig 161)(Figure 3.4).

Figure 3.4: Final specificity testing of Baumeister PCR at an optimised annealing temperature of 64°C for 25 mycoplasmal isolates...
...and five lung samples with typical lesions of M. hyopneumoniae pneumonia. Target product is 853 bp, relevant to MW markers of 1000 and 500 bp (darker bars) shown. Samples are from M. flocculare isolates MF1-MF7; M. hyorhinis isolates HR1 – HR11; M. hyopneumoniae isolates HP1-HP7, while lung samples L1-L5) are from pigs 12, 152, 161, 171 and 237 respectively.

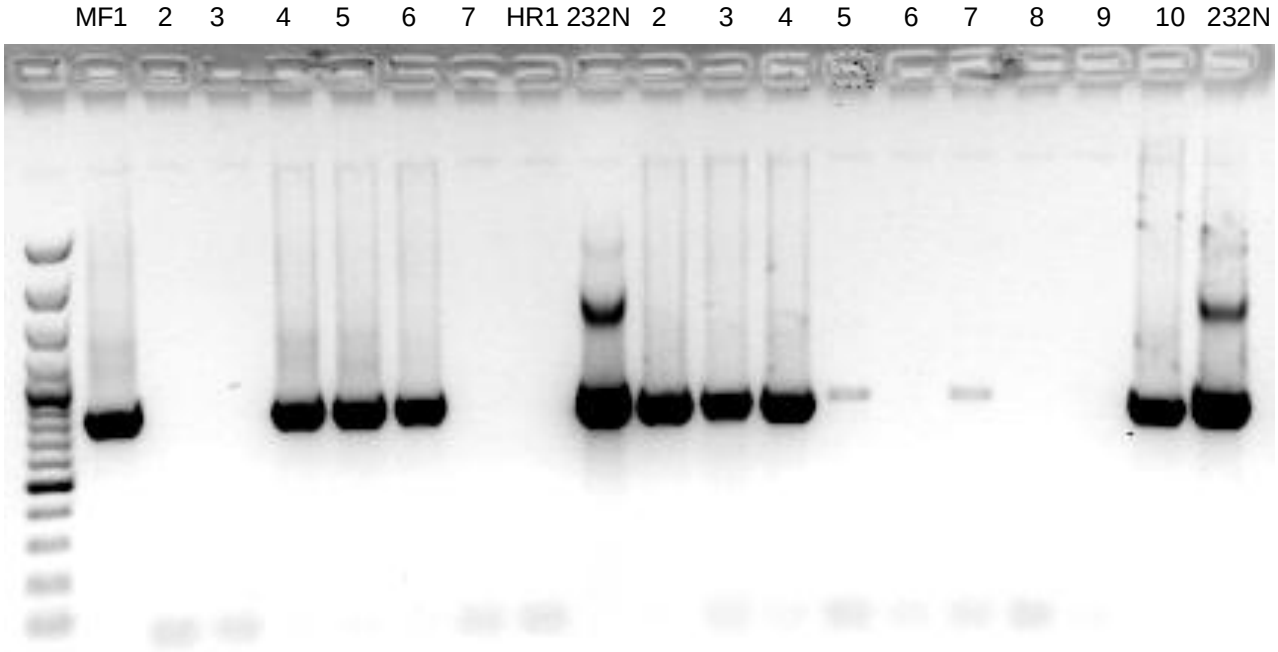


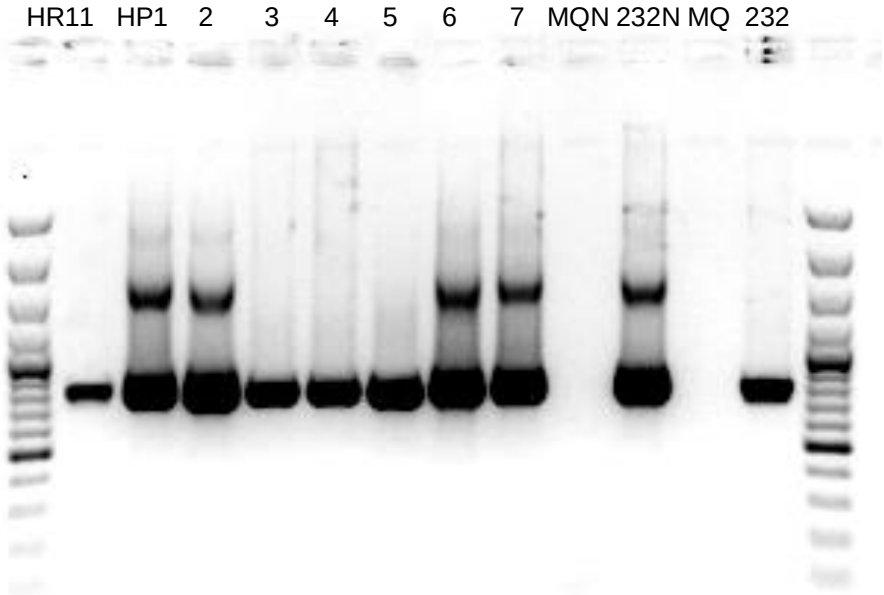


3.2.4 Stark PCR

The Stark PCR showed cross-reactivity with several strains of *M. flocculare* (4/7) and *M. hyorhinis* (5/11), while detecting all seven *M. hyopneumoniae* isolates examined (Figure 3.5).

Figure 3.5: Stark nested PCR at annealing temperatures of 52°C in the first and second generation reactions for 7 M.flocculare isolates...
...(MF1-MF7), 11 M. hyorhinis isolates (HR1-HR11) and 7 M. hyopneumoniae isolates (HP1-HP7) as described in Table 3.1. Molecular weight markers indicate 1000 bp and 500 bp as darker gel lines. The M. hyopneumoniae second generation target is 808 bp

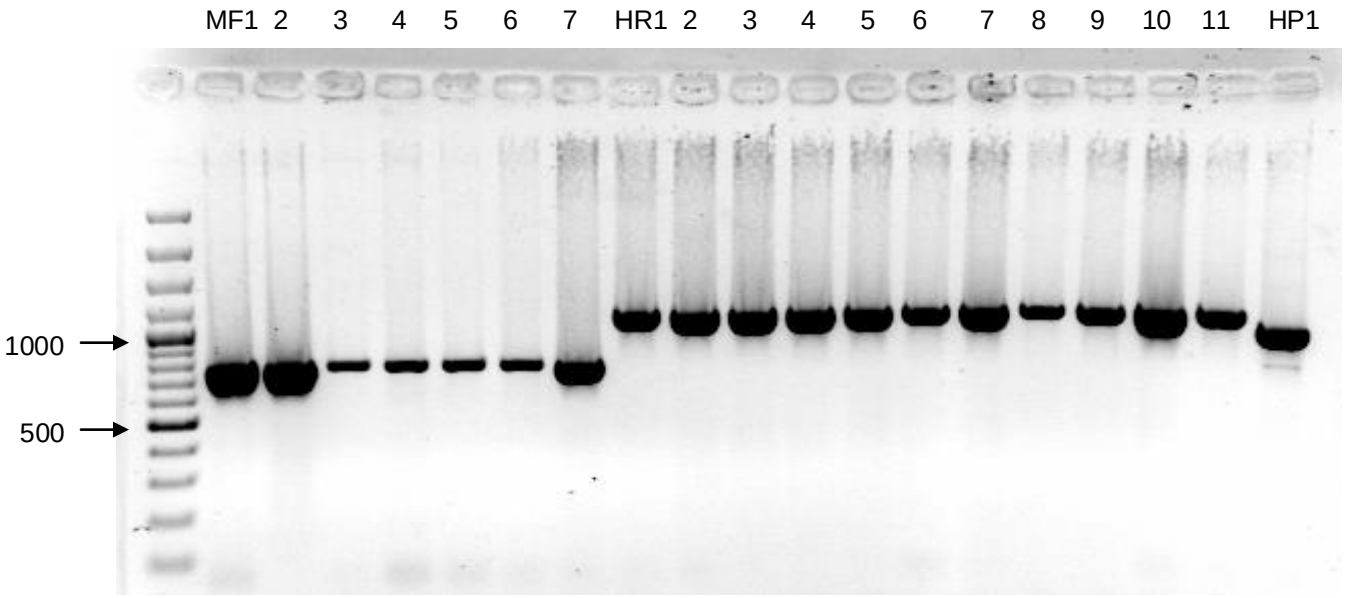


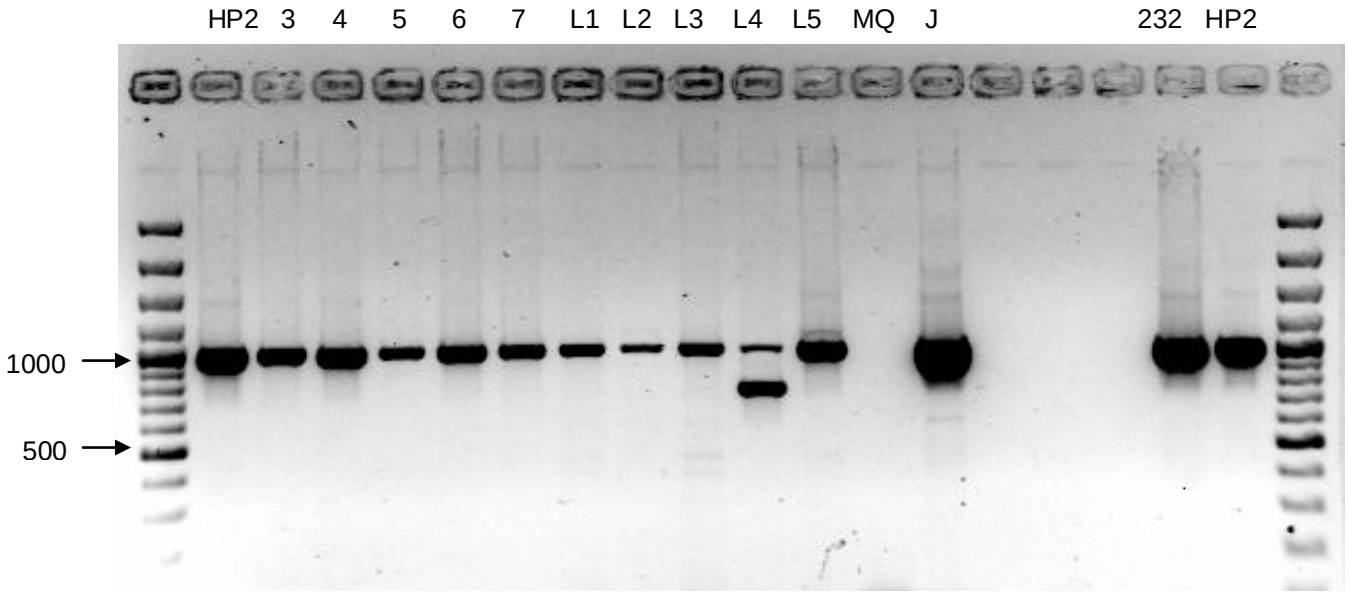


3.2.5 Stakenborg Multiplex PCR

The Stakenborg PCR performed very well on all isolates and samples at the published annealing temperature, as shown in Figure 3.6, giving 100% sensitivity and specificity. The lung sample from Pig 171 indicated DNA from both *M. flocculare* and *M. hyopneumoniae* was present, whereas all other samples showed evidence of only a single mycoplasmal species (Figure 3.6).

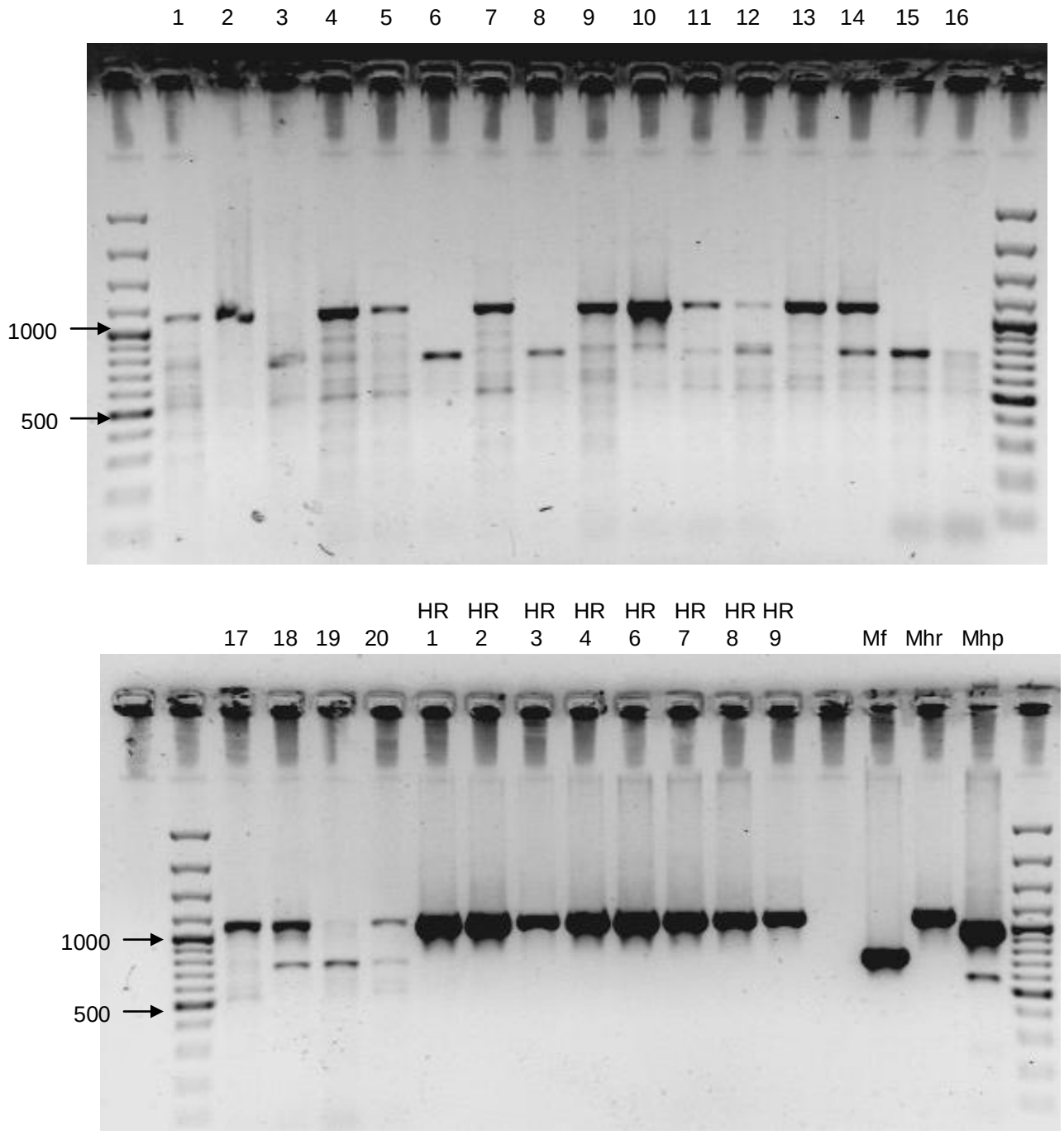
Figure 3.6: Final specificity testing of Stakenborg multiplex PCR at an annealing temperature of 55°C for 25 mycoplasmal isolates...
...and five lung samples with typical lesions of M. hyopneumoniae pneumonia. Target product is 754 bp for M. flocculare, 1000 bp for M. hyopneumoniae and 1129 bp for M. hyorhinis, relevant to molecular weight with 1000 and 500 bp shown as darker bars (arrows). Samples are from M. flocculare isolates MF1-MF7; M. hyorhinis isolates HR1 – HR11; M. hyopneumoniae isolates HP1-HP7 and lung samples L1-L5) are from pigs 12, 152, 161, 171 and 237 respectively.





When applied to DNA extracted from 20 nasal swabs with the Instagene matrix, the Stakenborg PCR was able to detect product consistent with *M. flocculare* in 14 swabs, and *M. hyorhinis* in 13 swabs, with both species being detected in 7 swabs (Table 3.3; Figure 3.7). No *M. hyopneumoniae* was detected in that sampling. The signal generated for *M. hyorhinis* from nasal swabs was variable in strength and marginally stronger than that for *M. flocculare*, but weaker than that for *M. hyorhinis* isolates (Figure 3.7).

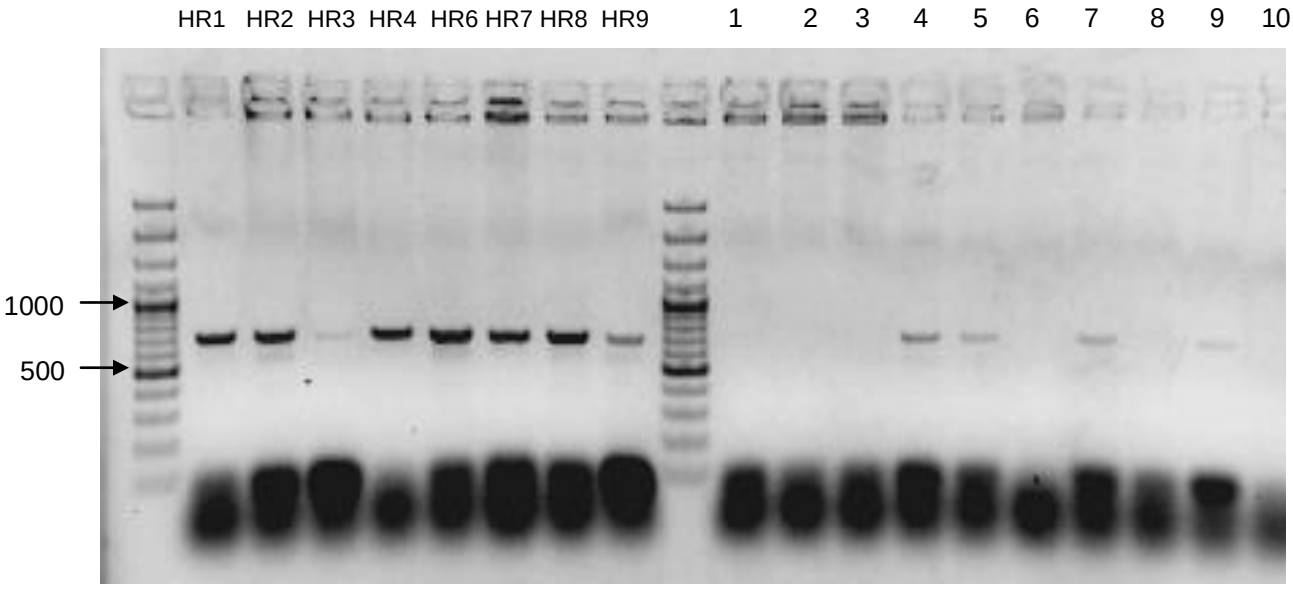
Figure 3.7: Stakenborg multiplex PCR at the published annealing temperature of 55°C applied to 20 nasal swabs from healthy grower pigs...
...and 8 isolates of M. hyorhinis (HR1-4, HR 6-9) derived from Qld. Target product is 754 bp for M. flocculare, 1000 bp for M. hyopneumoniae and 1129 bp for M. hyorhinis. Molecular weight markers indicate 1000 bp and 500 bp as darker gel lines.

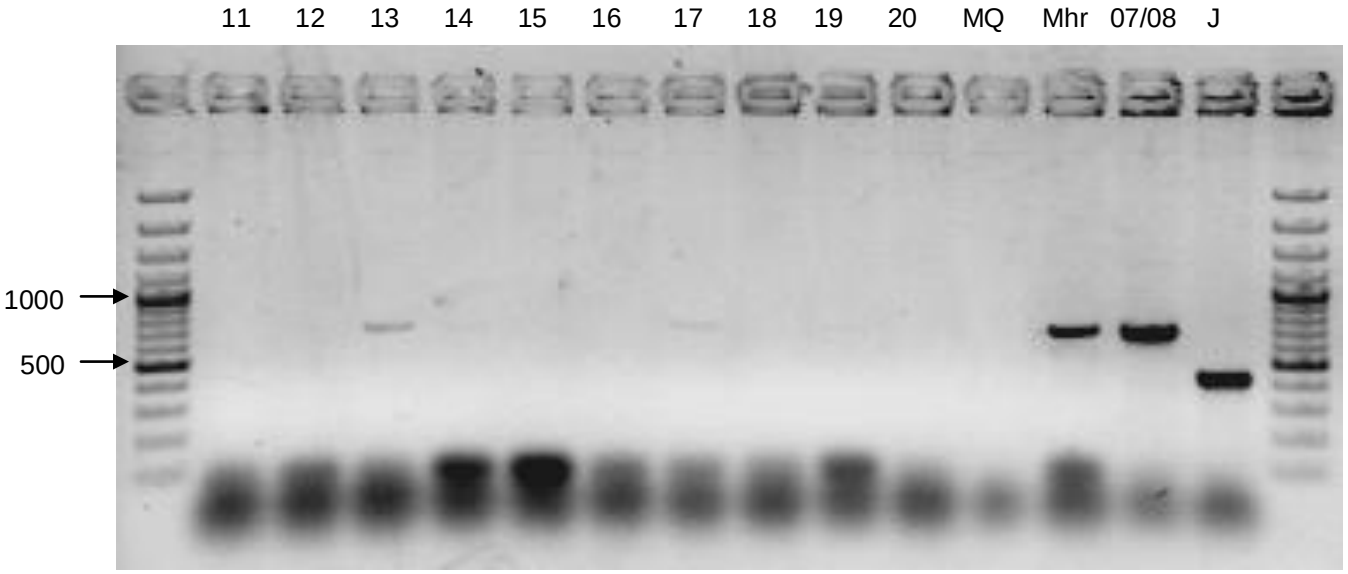


3.2.6 Lin Duplex PCR

The Lin PCR detected fewer *M. hyorhinis* reactors among the 20 nasal swabs from healthy pigs than the Stakenborg PCR, but reacted with all eight Qld isolates tested (Table 3.3, Figure 3.8).

Figure 3.8: Lin PCR at the published annealing temperature of 60°C applied to 20 nasal swabs from healthy grower pig...
...and to 8 isolates of M. hyorhinis (HR1-4, HR 6-9) derived from Qld. Target product is 430 bp for M. hyopneumoniae and 751 bp for M. hyorhinis. Molecular weight markers indicate 1000 bp and 500 bp as darker gel lines.

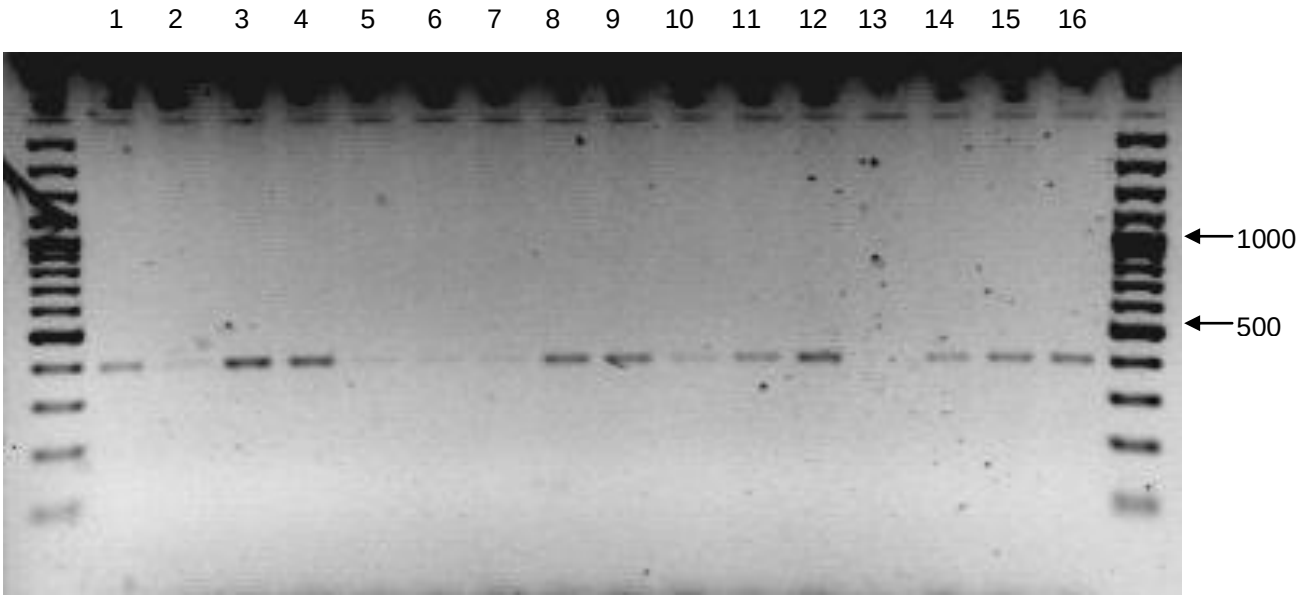




3.2.7 Stemke Duplex PCR

In the 20 nasal swabs from healthy pigs, the Stemke PCR detected a similar number of *M. flocculare* reactors as the Stakenborg PCR, with most samples giving a reaction (Table 3.3, Figure 3.9).

Figure 3.9: Stemke PCR at the published annealing temperature of 61°C applied to 20 nasal swabs from healthy grower pigs...
...Target product is 400 bp for M. flocculare and 238 bp for M. hyopneumoniae.
Molecular weight markers indicate 1000 bp and 500 bp as darker gel lines.



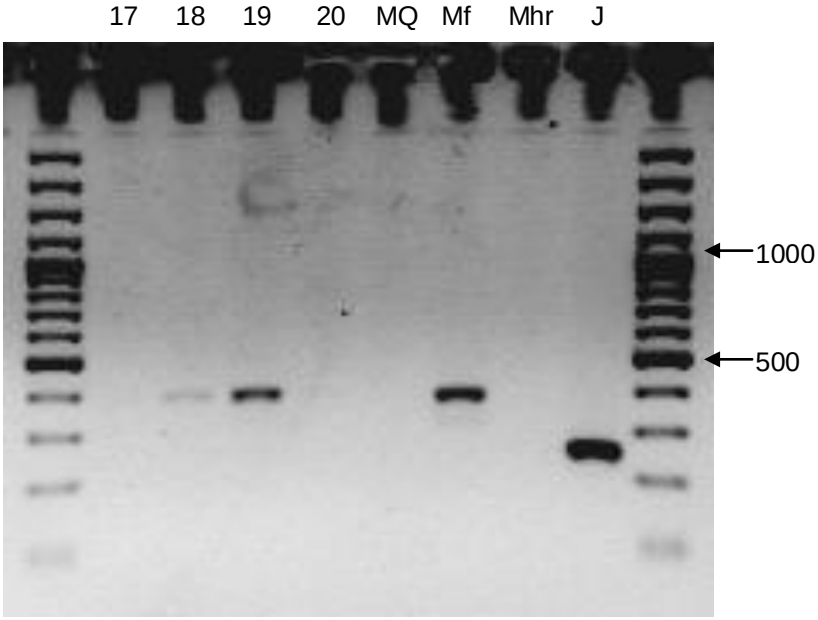


Table 3.3: Comparison of results of Lin duplex, Stakenborg multiplex and Stemke duplex PCR...
...nasal swabs from healthy pigs showing prevalence of M. hyorhinis and M. flocculare DNA. No M. hyopneumoniae was detected in any assay.

Swab	Lin	Stakenborg		Stemke
	M.hyorhinis	*M. hyorhinis*	*M. flocculare*	*M. flocculare*
1	-	+	+	+
2	-	+	-	wk
3	-	-	+	+
4	+	+	wk	+
5	+	+	-	vwk
6	-	-	+	vwk
7	+	+	-	vwk
8	-	-	+	+
9	+	+	-	+
10	-	+	-	wk
11	-	+	wk	+
12	-	+	+	+
13	+	+	-	-
14	wk	+	+	+
15	-	-	+	+
16	-	-	+	+
17	wk	+	-	-
18	-	+	+	wk
19	wk	-	+	+
20	-	+	wk	-
Total of + and wk	8	14	13	14

+: positive; wk: weak positive; vwk: very weak line (barely evident)

3.2.8 Calsamiglia PCR

The optimised Calsamiglia PCR reacted strongly with all 11 *M. hyopneumoniae* isolates of HP1-HP7 plus Beaufort, Sue, OMZ407 and C1735/2 (Figure 3.10). However, when tested against the full panel of *M. flocculare*, *M. hyorhinis* and *M. hyopneumoniae* isolates from Table 3.1, cross-reactivity was still evident against all *M. flocculare* (7/7) and several *M. hyorhinis* strains (5/11) (Figure 3.11).

Figure 3.10: Calsamiglia nested PCR at optimised annealing temperatures of 64°C and 62°C...
... first and second generation reactions respectively for 11 M. hyopneumoniae isolates. These are equivalent to HP1-HP7 (Table 3. 1) plus strains Beaufort, Sue, OMZ407 and C1735/2 (equivalent to HP5 but obtained several years earlier). Molecular weight markers indicate 1000 bp and 500 bp as darker gel lines. The M. hyopneumoniae second generation target is 352 bp; residual first generation product is consistent with a band at 649 bp.

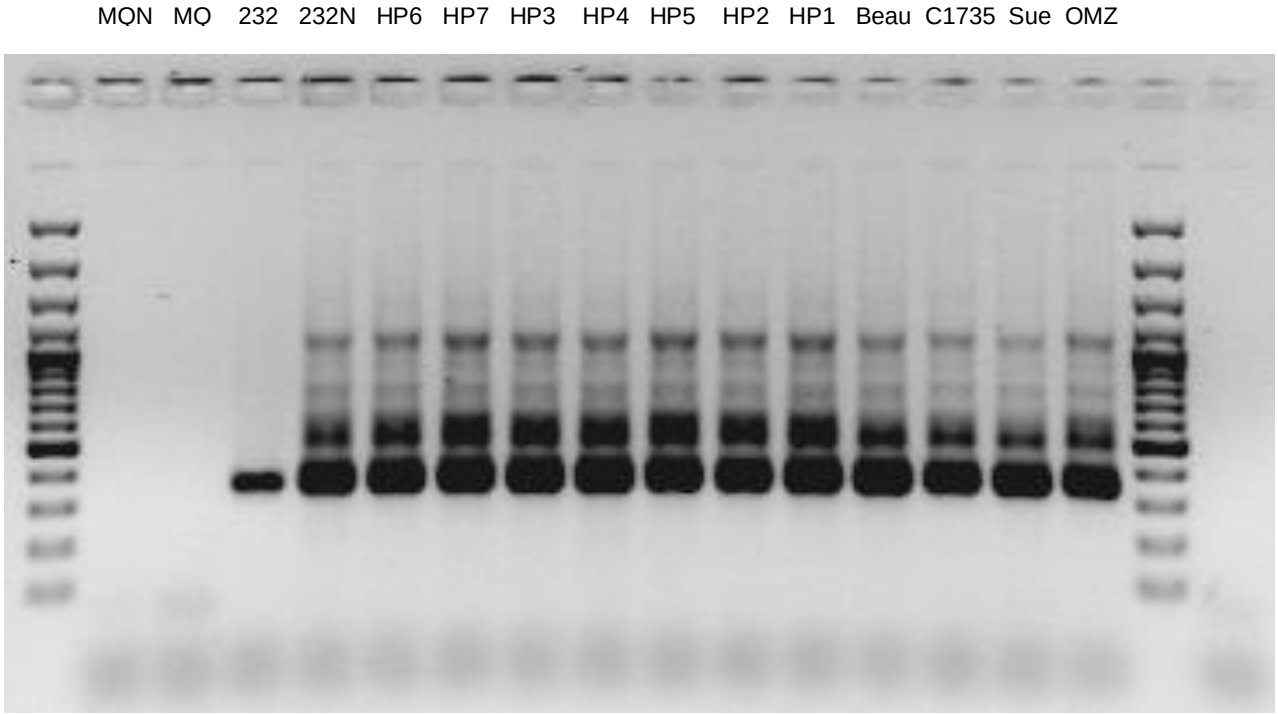
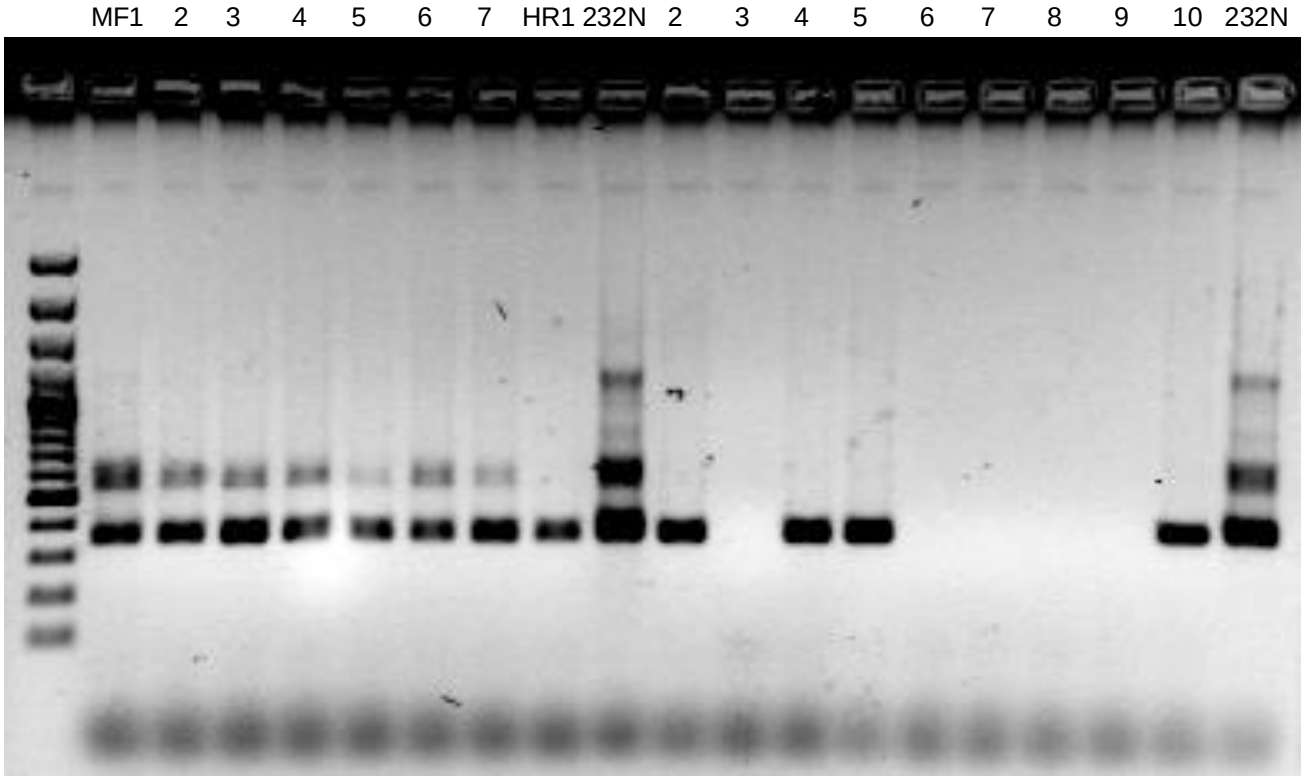


Figure 3.11: Calsamiglia nested PCR at optimised annealing temperatures of 64°C and 62°C in the first and second generation reactions...
...tively for 7 M.flocculare isolates (MF1-MF7), 11 M. hyorhinis isolates (HR1-HR11) and 7 M. hyopneumoniae isolates (HP1-HP7) as described in Table 3.1. Molecular weight markers indicate 1000 bp and 500 bp as darker gel lines. The M. hyopneumoniae second generation target is 352 bp; residual first generation product is consistent with a band at 649 bp.



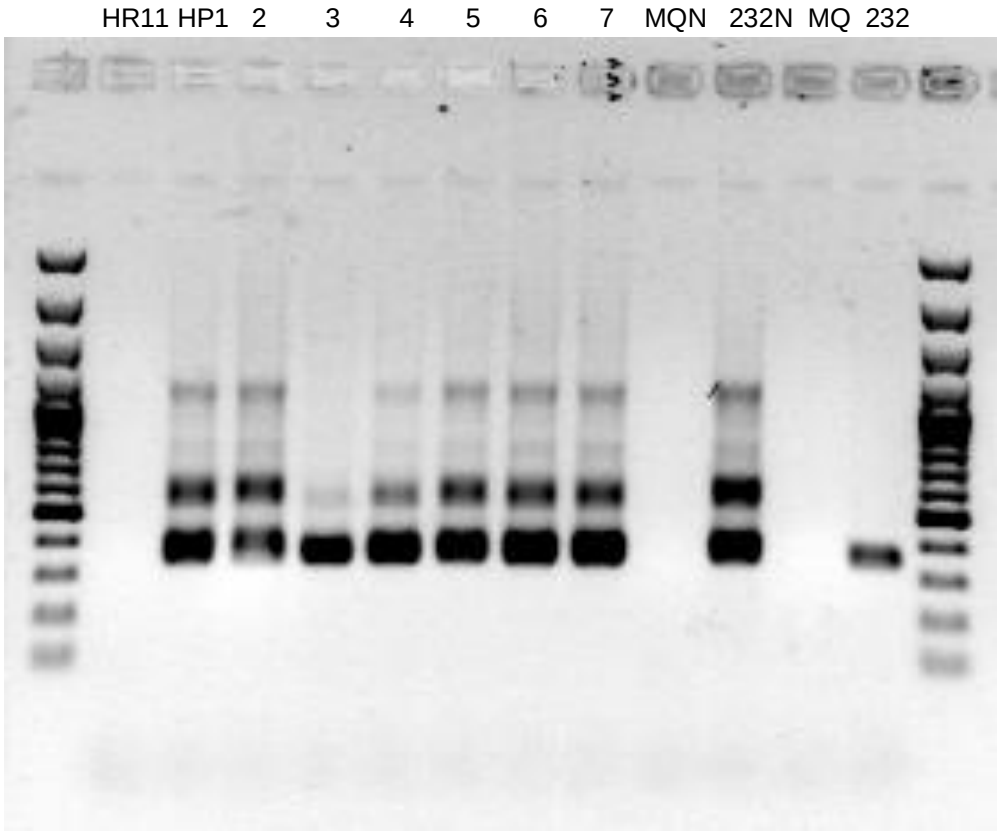


Table 3.4: Final selection of reaction times and temperatures for original and optimised versions of PCR assays designed...
...ect M. hyopneumoniae. Optimisation was not demonstrated for the Stark PCR, and specificity was not 100% for the optimised Baumeister, Kurth or Calsamiglia assays.

Assay	Step	Original T (°C)	Optimised T (°C)	Time	Cycles	Product size for 1st generation/ 2nd generation PCR (*M. hyopneumoniae*)
Stark nested		94	NA	3 min	x 1	913/ 808 bp
	Denature	94	NA	30 s		
	Anneal	**52**	NA	30 s	x 35	
	Extend	72	NA	1 min		
		25	NA	1 min	x 1	
Baumeister		94	94	5 min	x 1	853 bp
	Denature	93	93	30 s		
	Anneal	**55**	**64**	30 s	x 50	
	Extend	72	72	1.5 min		
		72	72	10 min	x 1	
Calsamiglia nested	*1st generation*					649/ 352 bp
		95	95	2 min	x 1	
	Denature	95	95	30 s		
	Anneal	**60**	**64**	45 s	x 30	
	Extend	72	72	30 s		
		72	72	30 s	x 1	
	2nd generation					
		95	95	2 min	x 1	
	Denature	95	95	30 s		
	Anneal	**60**	**62**	45 s	x 30	
	Extend	72	72	30 s		
		72	72	30 s	x 1	
Kurth nested	*1st generation*					1040/ 456 bp
		94	94	4 min	x 1	
	Denature	94	94	1 min		
	Anneal	**49**	**60**	1 min	x 30	
	Extend	72	72	1 min		
		72	72	7 min	x 1	
	2nd generation					
		92	92	2 min	x 1	
	Denature	92	92	1 min		
	Anneal	**56**	**64**	1 min	x 30	
	Extend	72	72	1 min		
		72	72	3 min	x 1	
Verdin nested	*1st generation*					1561/ 706 bp
	Denature	92	92	45 s		
	Anneal	**57**	**62**	45 s	x 20	
	Extend	72	72	1.5 min		

	2nd generation					
	Denature	92	92	45 s		
	Anneal	**58**	**62**	45 s	x 40	
	Extend	72	72	45 s		

Table 3.5: Results obtained for type strains of M. flocculare (MS42) and M. hyorhinis (BTS-7) when assessed...
...CRs under optimised conditions for the Verdin, Kurth, Calsamiglia and Baumeister assays (Table 3.4) or under original conditions for the Stark and Stakenborg PCRs, and as applied to field samples in Part 6 of this study.

Organism	Type strain	PCR					
		Verdin	Kurth	Calsamiglia	Baumeister	Stark	Stakenborg
		optimised	optimised	optimised	optimised	original	original
M. flocculare	MS42	-	+/-*	-/+ #	-	-	-
M. hyorhinis	BTS-7	-	-	-	-	-	-

* Inconsistent results when assessed during similar time period (refer Table 3.2)
\# Inconsistent results when assessed in initial (-ve) vs subsequent (+ve) testing (refer Table 1.8 and Figure 3.11)

3.3 Discussion

The final parameters for the optimised PCRs, together with the original published annealing temperatures are described in Table 3.4. These were applied to field samples, together with the Stakenborg PCR which was applied to field lung samples, as described in Part 6.

These studies enabled optimised annealing temperatures to be selected for the Verdin assay, and improved (but not specific) assays for the Baumeister, Kurth and Calsamiglia PCRs.
While the optimised Verdin nested PCR showed little cross-reactivity, the other nested PCRs reacted with several isolates of *M. flocculare* or *M. hyorhinis* when adjusted to annealing temperatures sufficiently sensitive to detect infected lung samples. The Kurth assay, for example, could be made more specific by increasing its annealing temperature, but the accompanying loss of sensitivity in infected lung samples was found to be so significant that a compromise was required.

The optimised Baumeister PCR still demonstrated strong reactivity with one *M. flocculare* strain (MF2, a subclone of MF1) and weaker bands with three other isolates of *M. flocculare* or *M. hyorhinis* among the 25 mycoplasmal isolates examined. Such a false positive rate among 18 non-*M. hyopneumoniae* cultures suggests this assay could be problematic in applications in clinical samples such as lung specimens.

The Calsamiglia nPCR has been applied in several studies in the U.S.A. (Ruiz *et al* 2002a, 2002b), Spain (Calsamiglia *et al* 2002; Sibila *et al* 2002a, 2002b, 2004), and Japan (Otagiri *et al* 2005), and is built on first generation primers developed in Sweden by Mattsson *et al* (1995). The results shown in Part 1 indicated the first generation primers are not specific, and will amplify other mycoplasmal species, and the first generation product was found to appear in assays where DNA concentrations are not limiting. This was also true of samples tested in Part 3, where bands of 649 bp, as well as higher molecular weight bands, were found with assays on DNA from *M. hyopneumoniae* isolates. However, the same bands were still evident in several strains of *M. flocculare* and *M. hyorhinis* in later testing of the Calsamiglia nested PCR, despite early indications in Part 1 that a number of these strains (MF1, MF3, MF7) failed to cross-react under the same test conditions. It would therefore appear that the Calsamigila PCR shows variable specificity and cannot reliably and repeatedly differentiate *M. hyopneumoniae* DNA from that of *M. hyorhinis* or *M. flocculare*.

A key limitation of specificity testing of *M. hyopneumoniae* PCRs is the number of available strains of other mycoplasmas that are assessed. In many published assays, only the type strain of *M. flocculare* and *M. hyorhinis* are assessed in such testing, and numerous publications list a large number of unrelated organisms that have been assessed with no cross-reactivity evident. It is clear from the present study that evaluation of mycoplasmal type strains alone can provide a misleading indication of specificity. As Table 3.5 suggests, if we choose only the type strains of *M. flocculare* and *M. hyorhinis*, all PCRs would be able to pass the specificity barrier on at least one occasion, with only the Kurth and Calsamiglia assays showing occasional positive results.

The study also confirmed the applicability of the Stakenborg PCR, and its comparative sensitivity to two other duplex PCRs (Lin, Stemke) when applied to nasal swabs for detection of *M. flocculare* and *M. hyorhinis.* The Stakenborg PCR was apparently more sensitive for *M. hyorhinis* than the Lin PCR and similar to Stemke in detection of *M.flocculare* but with the added feature of detecting an additional mycoplasmal species.

Part 4 - Quantitative Sensitivity of PCRs

4.1 Materials and Methods

DNA from *M. hyopneumoniae* strain 232 extracted with chloroform/ethanol was assessed for concentration by nanotechnology and then diluted in sterile water according to Tables 4.1 and 4.2 starting at 10 ng/uL. Initially a basic set of 6 dilutions between 10 ng/uL and 1 fg/uL were used, and then an extended set of 14 dilutions between 10 ng/uL and 0.5 fg/uL. The original and optimised versions of the Verdin, Kurth, Baumeister and Calsamiglia PCRs and the original version of the Stark and Stakenborg PCR were assessed for quantitative sensitivity.

The Stakenborg PCR was also tested in the extended dilution series for quantitative sensitivity against *M. hyorhinis* and *M. flocculare* DNA.

4.2 Results

Gels depicting typical results of the basic dilution series for the Kurth, Verdin, Calsamiglia nd Baumeister PCRs are shown in Figures 4.1 and 4.2. In some assays in the extended series, PCR bands were detected at dilutions where a prior dilution tested negative (Figures 4.3, 4.4), so the final result was determined by the result of the basic and extended set and by repeat testing where indicated. The most sensitive assay was the Stark PCR, followed by the Kurth and Calsamiglia PCRs. The Verdin PCR was the least sensitive of the nested PCRs, while both the non-nested PCRs (modified Baumeister, Stakenborg) were the least sensitive of all assays, requiring 1-10 pg/uL of DNA for a detectable signal (Table 4.3; Figure 4.5). The Stakenborg PCR similarly required 1-10 pg/uL of DNA to detect *M. hyorhinis* and *M. flocculare* (Table 4.2).

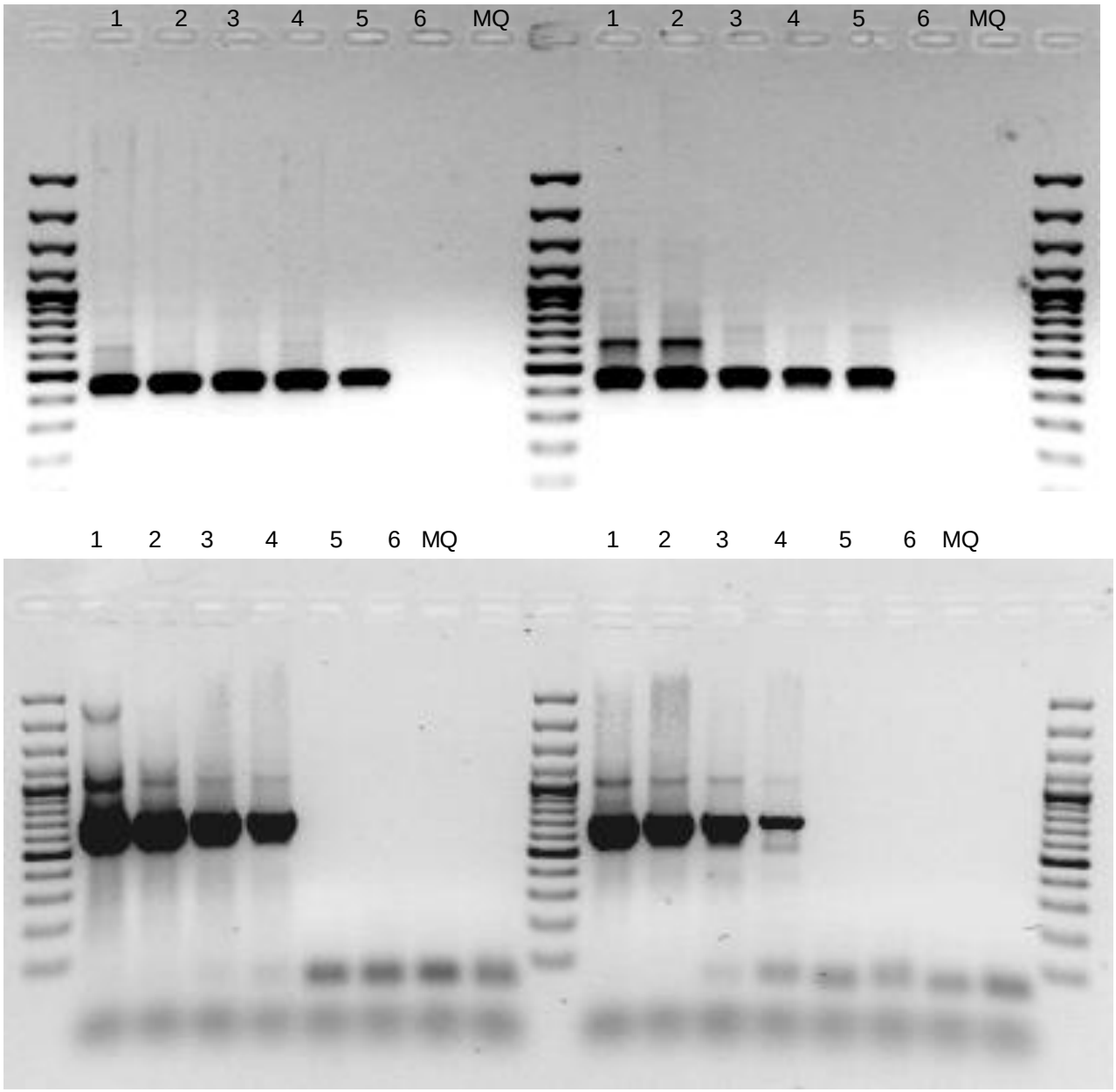
The modified annealing temperatures applied to the optimised versions of the Kurth, Baumeister and Calsamiglia assays had no adverse effect on quantitative sensitivity. Initial extended testing suggested the Verdin PCR may have lost some sensitivity in the optimised format, but repeat testing indicated no significant loss of sensitivity (Table 4.2).

Table 4.1: Basic dilution series used in quantitative sensitivity estimates with specific gel band intensities...
...scale of 0 (negative), 0.5 (weak), 1(low strength), 2 (moderate) and 3 (strong line).
Variable band intensities indicate result of repeat testing

Assay	Version	Score	10 ng/uL	1 ng/uL	10 pg/uL	1 pg/uL	10 fg/uL	1 fg/uL
Kurth	Original	14	3	3	3	3	2	0
Kurth	Optimised	14	3	3	3	3	2	0
Verdin	Original	12	3	3	3	3	0	0
Verdin	Optimised	11	3	3	3	2	0	0
Calsamiglia	Original	12-12.5	3	3	3	3	0-0.5	0
Calsamiglia	Optimised	15	3	3	3	3	1-3	0-2
Baumeister	Original	8.5	3	3	2	0.5	0	0
Baumeister	Optimised	9	3	3	2	1	0	0
Stark	Original	12	3	3	3	3	0	0

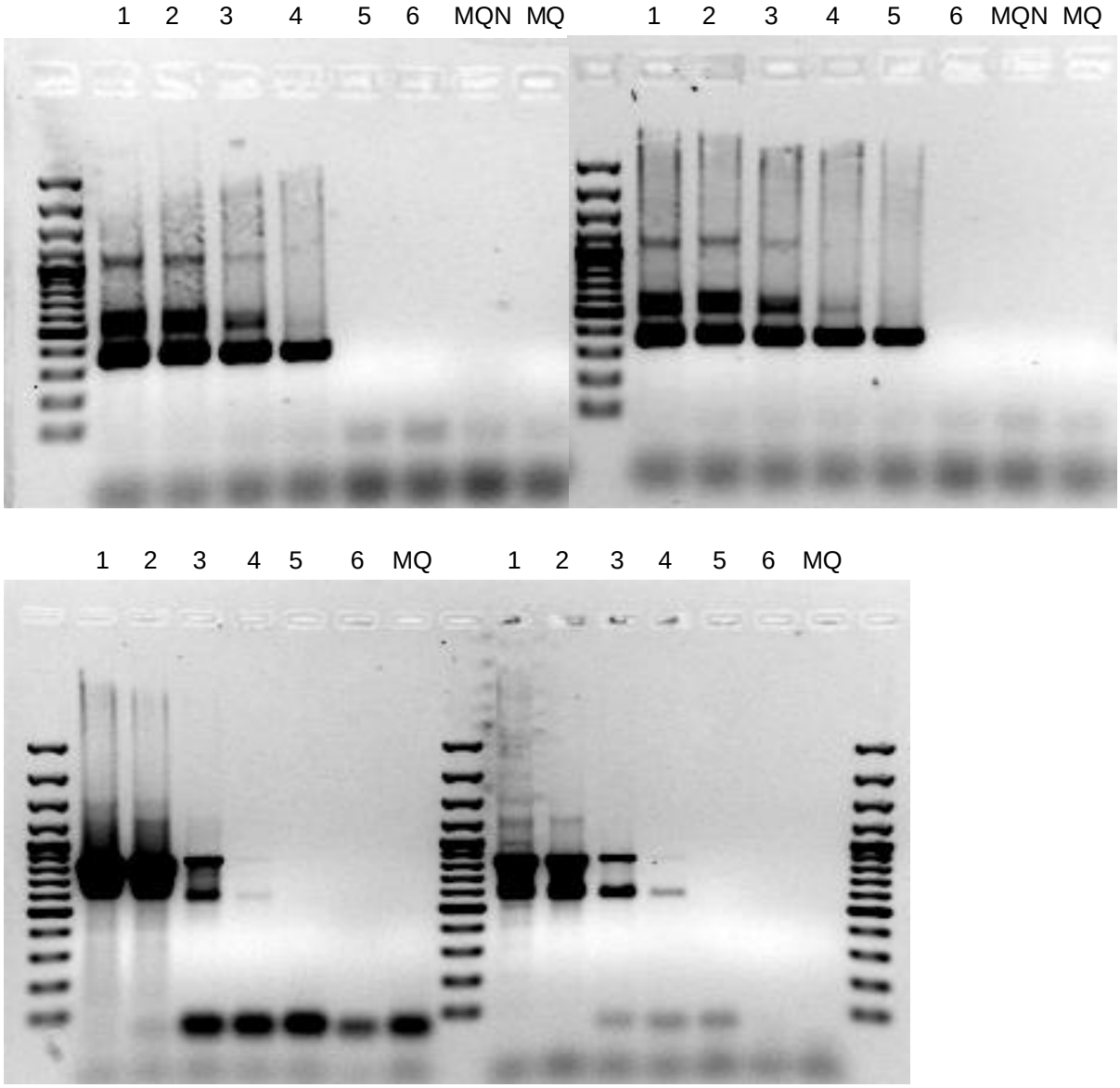
Note: 1 ng = 10^{-9} g; 1 pg = 10^{-12} g; 1 fg = 10^{-15} g

Figure 4.1: Quantitative sensitivity testing (basic dilution series) for the Kurth PCR (top; target 456 bp)...
... Verdin PCR (bottom; target 706 bp), with dilutions described in the table at the foot of the figure.
Left: Original parameters Right: Optimised parameters



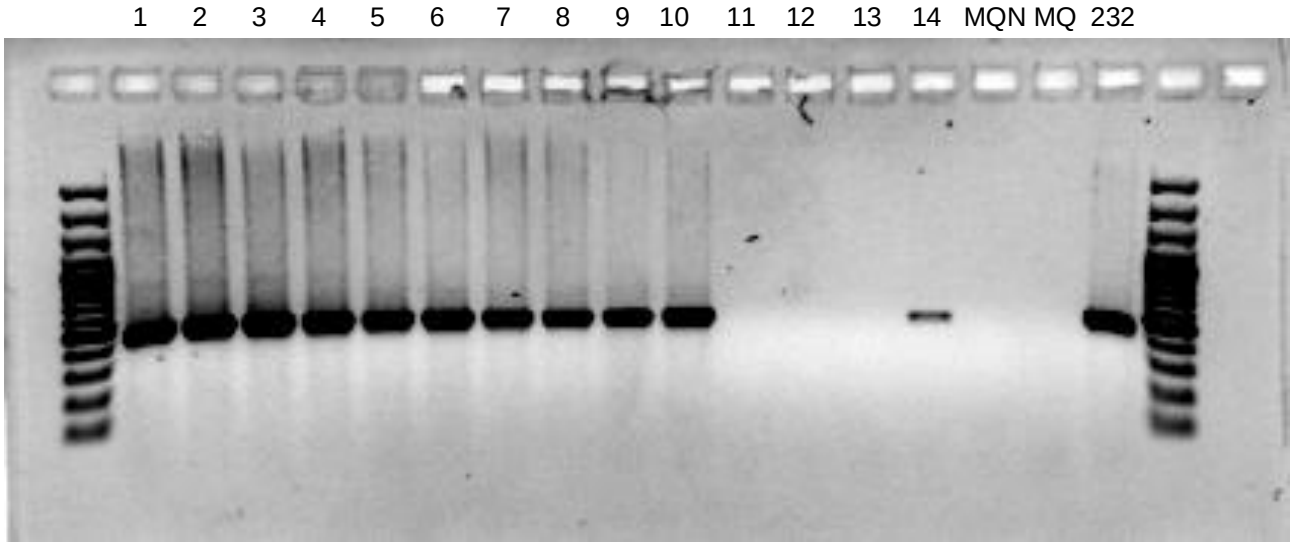
Lane	Conc	Lane	Conc
1	10 ng/uL	5	10 fg/uL
2	1 ng/uL	6	1 fg/uL
3	10 pg/uL	MQ	MilliQ water control
4	1 pg/uL		

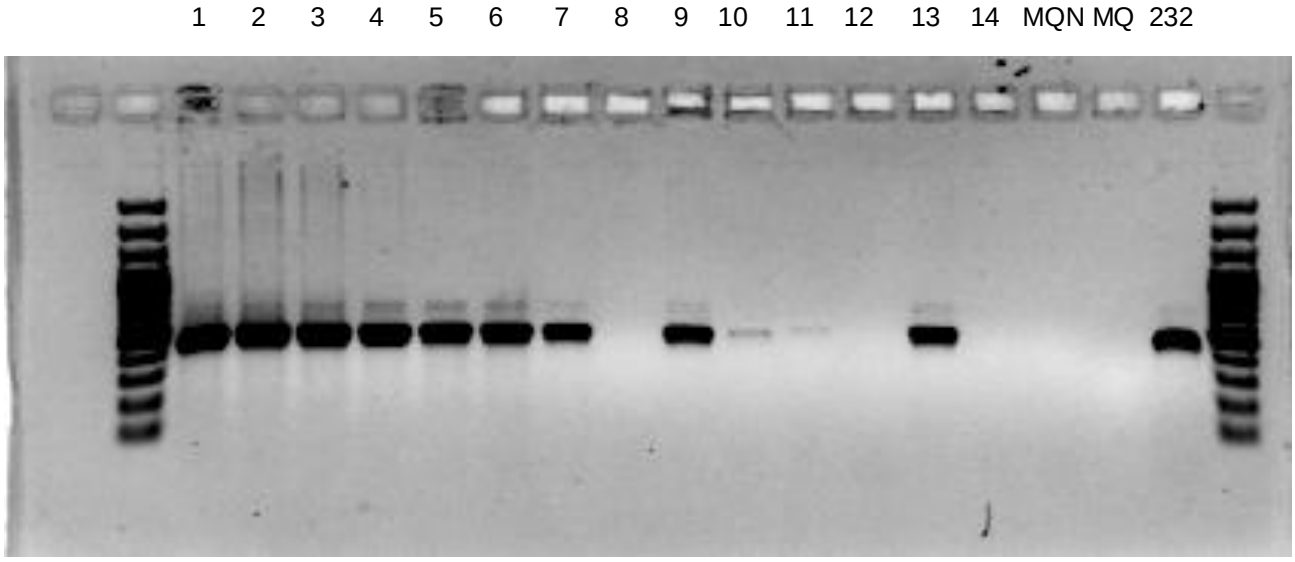
Figure 4.2: Quantitative sensitivity testing (basic dilution series) for the Calsamiglia PCR (top; target 352 bp...
...he Baumeister PCR (bottom; target 853 bp), with dilutions described in the table at the foot of the figure.
Left: Original parameters Right: Optimised parameters



Lane	Conc	Lane	Conc
1	10 ng/uL	5	10 fg/uL
2	1 ng/uL	6	1 fg/uL
3	10 pg/uL	MQN	MilliQ water control (nested)
4	1 pg/uL	MQ	MilliQ water control

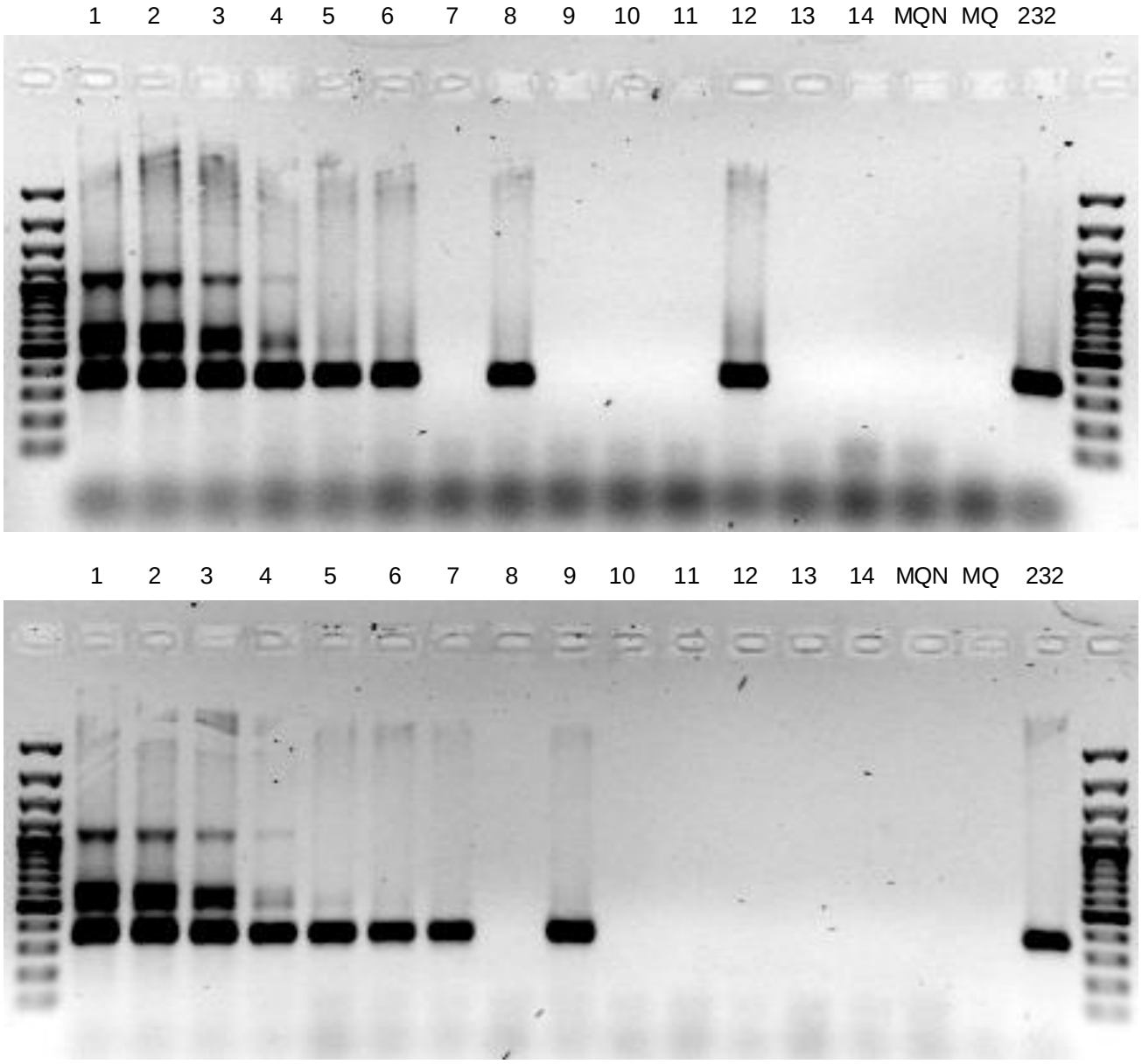
Figure 4.3: Quantitative sensitivity testing (extended dilution series) for the Kurth PCR, showing carryover of DNA....
...at some high dilutions was a problem in interpretation of the data. The dilutions are described in the table at the foot of the figures.
Top: Original parameters Bottom: Optimised parameters Target: 456 bp





Lane	Conc	Lane	Conc
1	10 ng/uL	10	10 fg/uL
2	1 ng/uL	11	5 fg/uL
3	100 pg/uL	12	2.5 fg/uL
4	10 pg/uL	13	1 fg/uL
5	1 pg/uL	14	0.5 fg/uL
6	500 fg/uL	MQN	MilliQ water nested
7	100 fg/uL	MQ	MilliQ water non-nested
8	50 fg/uL	232	232 Control
9	25 fg/uL		

Figure 4.4: Quantitative sensitivity testing (extended dilution series) for the Calsamiglia PCR,...
...also showing carryover of DNA at some high dilutions was a problem in interpretation of the data. The dilutions are described in the table at the foot of the figures.
Top: Original parameters Bottom: Optimised parameters Target: 352 bp



Lane	Conc	Lane	Conc
1	10 ng/uL	10	10 fg/uL
2	1 ng/uL	11	5 fg/uL
3	100 pg/uL	12	2.5 fg/uL
4	10 pg/uL	13	1 fg/uL
5	1 pg/uL	14	0.5 fg/uL
6	500 fg/uL	MQN	MilliQ water nested
7	100 fg/uL	MQ	MilliQ water non-nested
8	50 fg/uL	232	232 Control
9	25 fg/uL		

Table 4.2: Extended dilution series used in quantitative sensitivity estimates with specific gel band intensities on a scale of 0 (negative),...
...0.5 (weak), 1(low strength), 2 (moderate) and 3 (strong line).

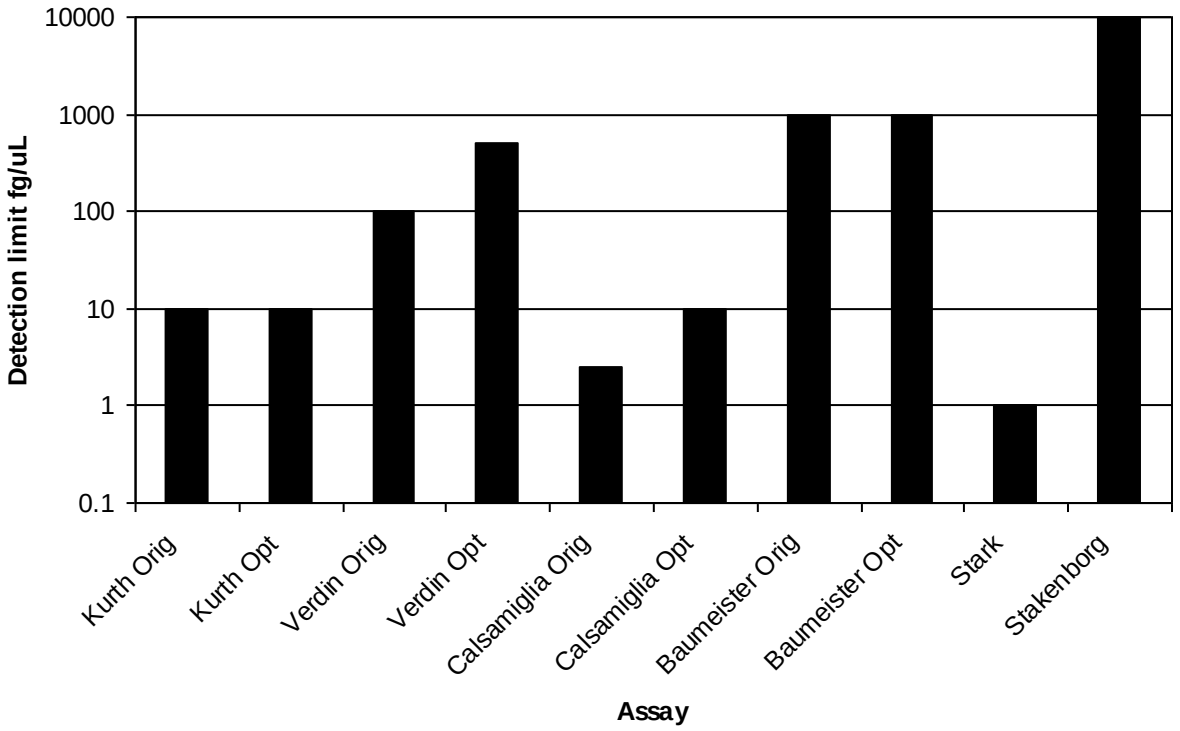
Assay	Version	Score	10 ng/uL	1 ng/uL	100 pg/uL	10 pg/uL	1 pg/uL	500 fg/uL	100 fg/uL	50 fg/uL	25 fg/uL	10 fg/uL	5 fg/uL	2.5 fg/uL	1 fg/uL	0.5 fg/uL
Kurth	Original	32	3	3	3	3	3	3	3	3	3	3	0	0	0	1
Kurth	Optimised	29	3	3	3	3	3	3	3	0	3	1	0.5	0	3	0
Verdin	Original	24	3	3	3	3	3	3	3	0	3	0	0	0	0	0
Verdin (repeat)	Original	18	3	3	3	3	3	3	0	0	0	0	0	0	0	0
Verdin	Optimised	17	3	3	3	3	3	2	0	0	0	0	0	0	0	0
Calsamiglia	Original	24	3	3	3	3	3	3	0	3	0	0	0	3	0	0
Calsamiglia	Optimised	24	3	3	3	3	3	3	3	0	3	0	0	0	0	0
Stark	Original	39	3	3	3	3	3	3	3	3	3	3	3	3	3	0
Stakenborg Mhp	Original	9	3	3	2	1	0	0	0	0	0	0	0	0	0	0
Stakenborg Mhp	Original	11	3	3	3	2	0	0	0	0	0	0	0	0	0	0
Stakenborg Mhr	Original	7	3	0	2	1	1	0	0	0	0	0	0	0	0	0
Stakenborg Mfloc	Original	7	3	2	1	1	0	0	0	0	0	0	0	0	0	0

Table 4.3: Quantitative sensitivity testing of M. hyopneumoniae PCRs using DNA from M. hyopneumoniae strain 232,...
...showing estimated detection limit from at least two assays: a basic set of dilutions and a more comprehensive extended set of dilutions. These are compared against published sensitivities as described earlier in Table 1.2.

Assay	Version	BASIC fg/uL	EXTENDED fg/uL	FINAL fg/uL	PUBLISHED fg*
Kurth	Original	>1-10	0.5-10	≤10	0.5-1
Kurth	Optimised	>1-10	1-10	≤10	NT
Verdin	Original	>10-1000	25 & 500	100	1
Verdin	Optimised	>10-1000	500	500	NT
Calsamiglia	Original	>10 -1000	2.5	2.5	1-96
Calsamiglia	Optimised	>1-10	25	≤10	NT
Baumeister	Original	1000	NT	1000	NT
Baumeister	Optimised	1000	NT	1000	5-18.5#
Stark	Original	25	1	1	1.2–2.5
Stakenborg	Original	NT	10000	10000	1000

* published data refers to amount per reaction tube
# at an annealing temperature of 62◦C

Figure 4.5: Summary of minimum detection limits (fg DNA/uL) for M. hyopneumoniae strain 232 among various PCRs for M. hyopneumoniae



4.3 Discussion

The published quantitive sensitivities for the various PCRs tested suggest that the values obtained in our study were lower, but the relative order of sensitivities can be used as a comparison. Kurth *et al* (2002) found approximately similar high sensitivity for the Verdin, Kurth, Stark and Calsamiglia PCRs, which is in marked contrast to the present findings where the Verdin PCR appeared ten fold less sensitive. The Calsamiglia PCR was possibly marginally less sensitive than its original counterpart, but the test data suggested there may be no significant difference as variation was evident in the results of the basic and extended evaluations. The Verdin PCR appears the only assay where optimisation may have reduced quantitative sensitivity, but further testing would be needed to confirm that this reduction was significant.

As expected, the non-nested Baumeister and Stakenborg PCRs were the least sensitive, with the Baumeister being the more sensitive of the two by a factor of approximately 10 fold. As there appeared no evidence of any reduction in sensitivity by increasing the annealing temperature in the Baumeister optimised PCR assay, then these findings indicate that if the optimised Baumeister PCR was insensitive on nasal swab samples then the original Baumeister would be expected to have a similar performance and the Stakenborg would be less sensitive again. Thus in testing of field samples, nasal swabs analyses could be preferentially assessed by the Baumeister assay, and if found insensitive there would be little reason to evaluate the Stakenborg assay. This approach was adopted in Part 6 of this study.

The Stakenborg PCR appeared slightly more sensitive in detecting *M. hyorhinis* than *M. hyopneumoniae* or *M. flocculare* (Table 4.2). This assay was assessed on nasal swabs from normal pigs in a Part 3 in which *M. hyorhinis* reactivity appeared stronger than *M. flocculare* reactivity (Figure 3.7). These findings are consistent with a higher sensitivity for *M. hyorhinis* (500 fg genomic DNA) compared to *M. flocculare* (1 pg DNA) and *M. hyopneumoniae* (1 pg DNA) reported by Stakenborg et al (2006), and indicates that a higher threshold of *M. hyopneumoniae* is likely to be required in this assay compared to *M. hyorhinis*. However, the relative abundance of the various mycoplasmas in the anterior nasal cavity is unknown, so equivalence in ability to detect *M. hyopneumoniae* and *M. flocculare* may not be relevant if *M. flocculare* exists in much higher concentrations at the sampling site.

Part 5 - Development of Assays to Confirm Absence of PCR Inhibitors (Amplification Controls)

5.1 Introduction

PCR inhibitors usually affect PCR through interaction with DNA or interference with the DNA polymerase. Inhibitors may be present in clinical samples such as blood and tissues or be added during sample processing or DNA extraction, via excess salts, ionic detergents, alcohols or phenolics (Bessetti 2007). Inhibitors can escape removal during the DNA purification procedure by binding directly to DNA, or may inhibit PCR by reducing the availability of cofactors (such as Mg^{2+}) or interfere with their interaction with DNA polymerase (Bessetti 2007). The method of sample acquisition can reduce the collection of inhibitors, and techniques and commercial kits can enable extraction of DNA to the exclusion of some inhibitors. Additionally, some DNA polymerases offer varying resistance to different inhibitors.

In order to try to assess the extent of inhibition that occurs in a reaction, a control can be performed by adding a known amount of a template to the investigated reaction mixture, and comparing the amplification of this template to that of the same template used in the absence of inhibitors. Alternatively, the sample itself can be a source of a template unrelated to the original microbial target. Such a target in pig tissues or tissue swabs would be DNA present in all pig tissues, and the successful amplification of such DNA would be a measure of a lack of PCR inhibitors in the sample. This second approach was used in the current study.

DNA of pig origin can be determined by detection of cell components such as beta actin and beta 2-microglobulin. Lung samples and nasal swabs from pigs are likely to contain measurable quantities of beta actin and beta 2-microglobulin. In Genbank, the porcine (*Sus scrofa*) beta actin ACTB gene is described in Genbank accession DQ45269 (Lin et al. 2006a) while exons 1-4 for the *Sus scrofa* beta 2-microgloblin gene are described in AF452448 (Keene et al 2001, direct submission to Genbank) and for beta 2-microglobulin mRNA in L13854 (Milland et al. 1993).

5.2 Materials and Methods

5.2.1 Selection of Primers

The three Genbank sources described in 5.1 were used to construct 19mer to 22mer primers as shown in Table 5.1, using Bioedit software (Ibis Biosciences, Carlsbad CA) to align sequences in the two beta 2-microglobulin Genbank accessions for common mRNA sequences. The latter represented a 205 bp sequence commencing at nucleotide 183 of Genbank L13854 and revealing a single nucleotide difference at nucleotide 311.

Table 5.1: Primer sequences and their characteristics used in detection of genes for beta actin and beta 2-microglobulin in pig samples

Gene	Primer and sequence		Melt T (°C)	Expected products
Beta actin (ACTB gene)	P1	5'-ACC GTG AGA AGA TGA CAT CAG G-3'	65	P1/P2: 1065 bp P2/P3: 180 bp
	P2	5'-GAC GTC GCA CTT CAT GAT GG-3'	67	
	P3	5'-CTC TTC CAG CCC TCC TTC C-3'	65	
Beta 2- microglobulin (mRNA gene)	P4	5'-CCT GAA CTG CTA TGT ATC TGG G-3'	62	P4/P5: 205 bp
	P5	5'-CTT GGG CTT ATC GAG AGT CAC G-3'	67	

5.2.2 Reactivity with Pig DNA in Conditions of Kurth PCR

The three sets of primers were initially tested on DNA extracted from single pig nasal swab, lung, liver and ileum samples and tested in a one step PCR using conditions and reagents as per the Kurth *M. hyopneumoniae* PCR assay, at annealing temperatures of both 62°C and 64°C.

Subsequently, nasal swabs from 20 healthy pigs housed at EMAI for an unrelated nutrition trial were assessed in the 1st generation of the Kurth PCR at 64°C with B2M primers added.

5.2.3 Evaluation of B2M on Nasal Swabs in Conditions of Stark PCR

Instagene nasal swab extracts from 16 pigs previously tested in the Stark PCR were tested under the same conditions in a one step PCR using conditions and reagents of the Stark 1st generation assay, including an annealing temperature of 52°C. These samples had been stored at -20°C for over 12 months prior to testing. Assays were completed using the primer set P4/P5 with and without 1st generation Stark primers.

5.2.4 Evaluation of B2M on Lung Extracts and Nasal Swabs Concurrently with Kurth PCR

Primers for P4/P5 for beta 2-microglobulin (B2M) were assessed in a one step PCR under the conditions of the 1st generation Kurth PCR using DNA extract from a positive lung sample (CM 04/101/1) and a negative lung sample (CM 07/112) and 1st generation primers of Kurth and/or P4/P5 on the following range of samples:

(a) 100 uL of lung extract
(b) 10 uL lung extract and 90 uL J strain control, and
(c) 50 uL lung extract and 50 uL J strain control.

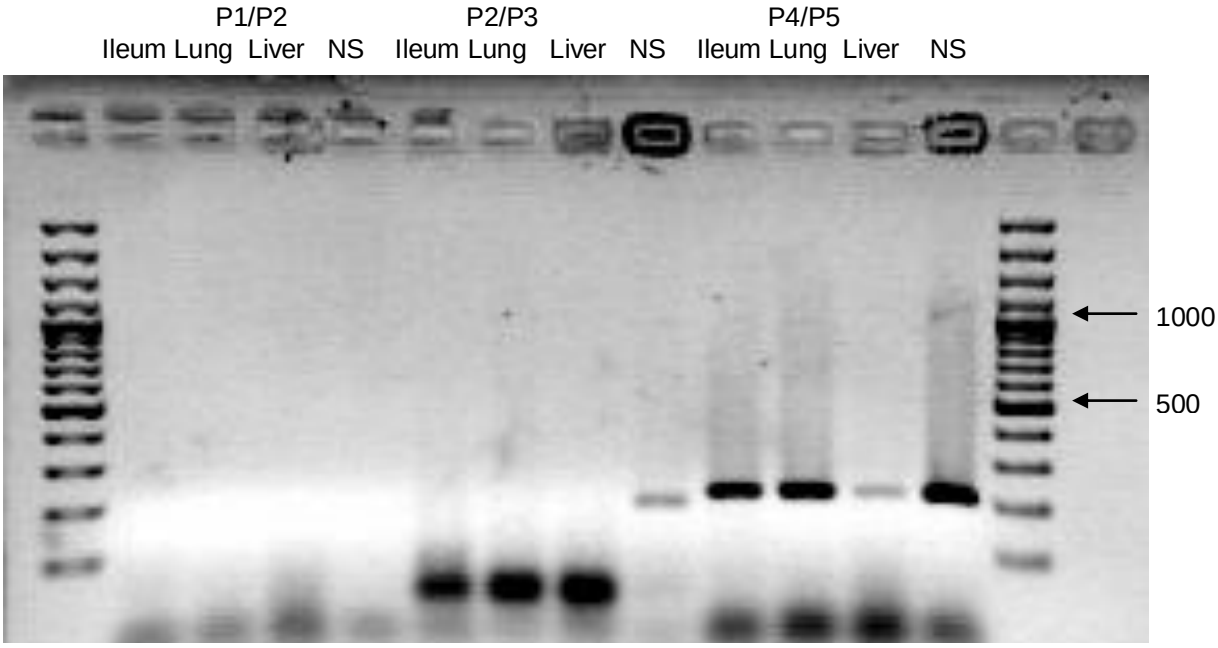
The above samples and 20 nasal swab samples from normal healthy pigs were assessed in a nested PCR with primers P4/P5 for B2M, using the B2M primers in each generation of the nested assay. All testing was carried out at annealing temperatures of 64°C in the first and second generation reactions, as these were the suggested optimised temperature for the Kurth PCR at that time. The primer concentrations for B2M were kept the same as those for each of the Kurth primers in the first (50 pmoles) and second generation (5.5 pmoles) and the Taq and magnesium concentrations, as with all other reactants, were as specified for the normal Kurth PCR.

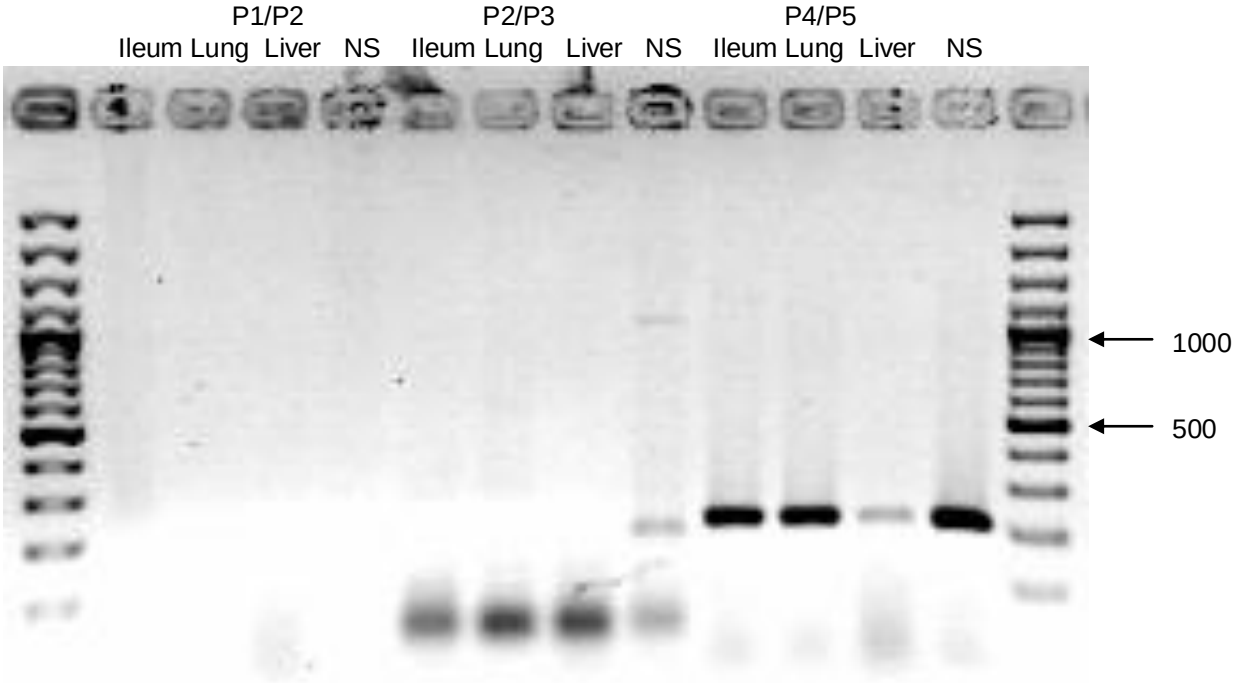
5.3 Results

5.3.1 Reactivity of Beta Actin and B2M Primers with DNA from Pig Tissues and Swabs

Neither of the beta actin primer sets (p1/P2, P2/P3) reacted with DNA derived from pig lung, liver or ileum, and set P2/P3 showed a weak reaction with nasal swab DNA (Figure 5.1) at annealing temperatures of 62°C and 64°C. In contrast, the beta 2-microglobulin (B2M) primers P4/P5 produced the expected 205 bp product with all samples (Figure 5.1) at both annealing temperatures.

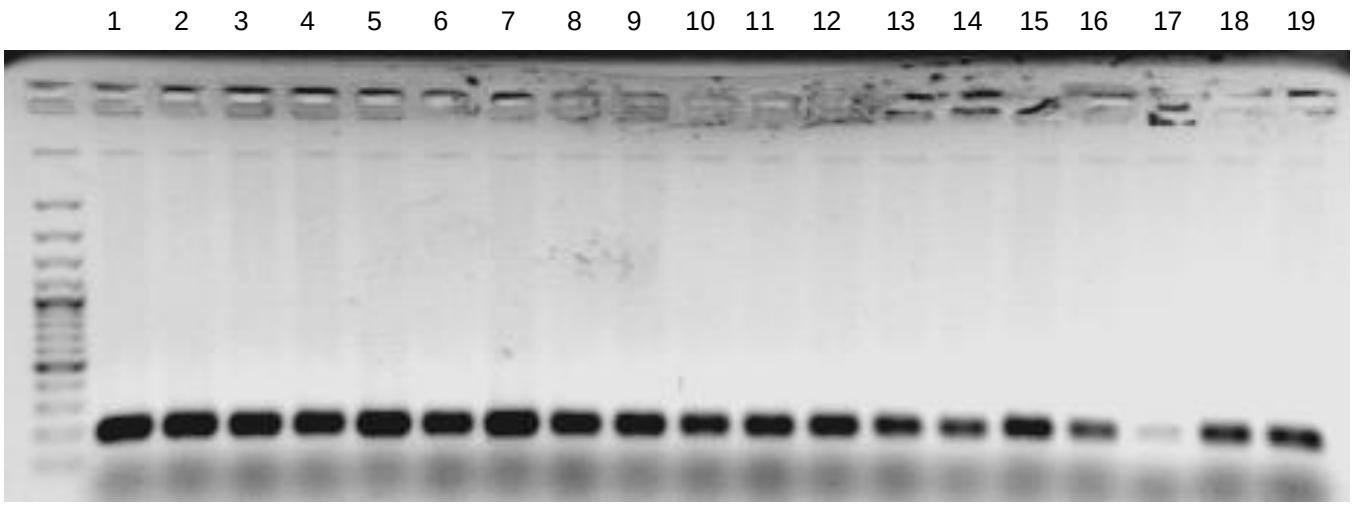
Figure 5.1: Detection of beta actin and beta 2-microglobulin DNA in pig tissues and nasal swab...
...extracts under conditions of the Kurth M. hyopneumoniae PCR with annealing temperatures of 62°C (top) and 64°C (bottom). Target products are: Beta actin primers P1/P2 1065 bp; P2/P3 180 bp; Beta 2-microglobulin primers P4/P5 205 bp. MW markers at 1000 and 500 bp are darker bands.





Subsequent testing of 20 nasal swabs under Kurth 1st generation PCR conditions and including Kurth 1st generation primers indicated that all 20 samples were positive for B2M DNA. Figure 5.2 depicts results for samples 1-19 of that evaluation.

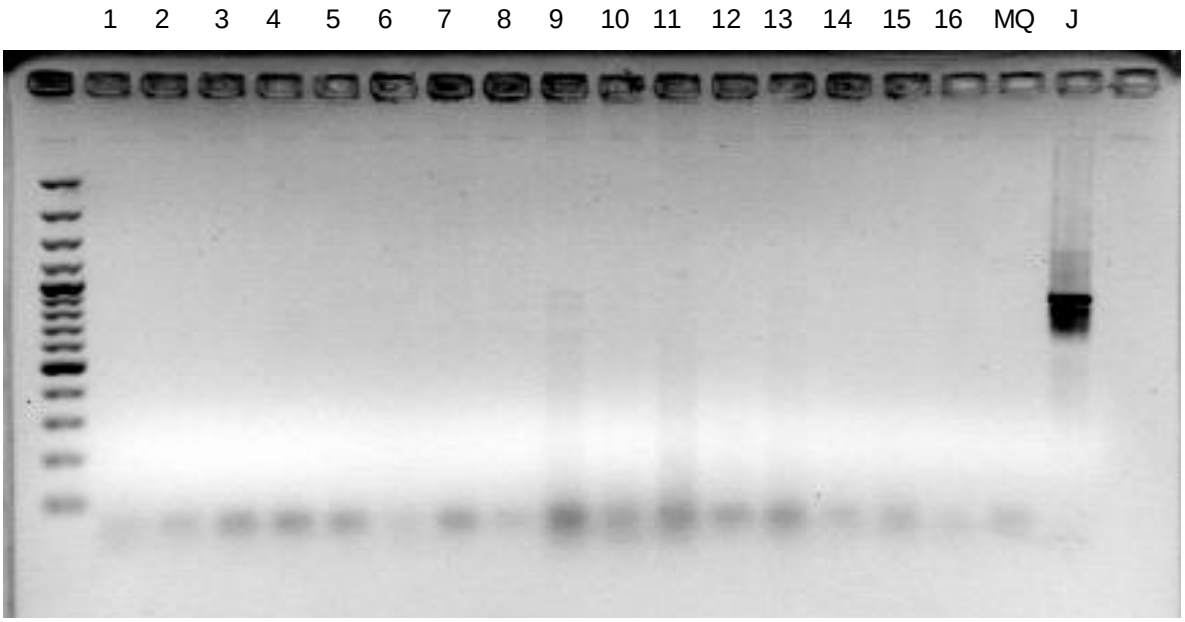
Figure 5.2: Detection of beta 2-microglobulin (B2M) DNA in fresh nasal swab extracts from...
...healthy pigs under conditions of the 1st generation Kurth M. hyopneumoniae PCR with an annealing temperature of 64°C. Target product for B2M primers P4/P5 is 205 bp, while that for the 1st generation Kurth primers is 1040 bp. MW markers with 1000 and 500 bp as darker bands are shown.

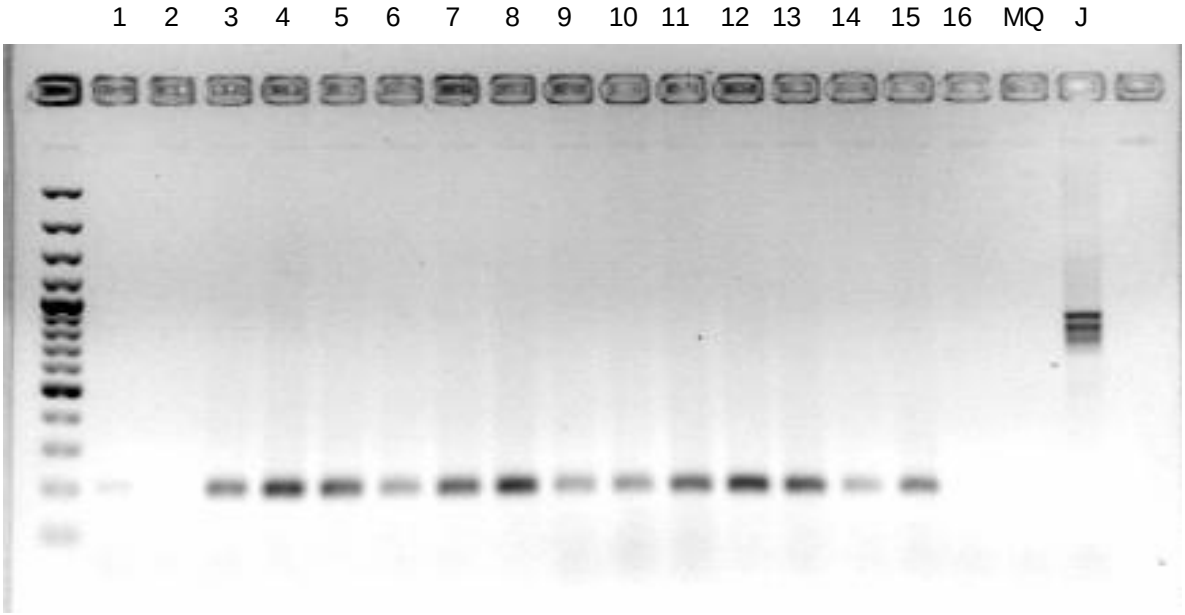


5.3.2 Reactivity of B2M Primers on Nasal Swabs under Stark PCR 1st Generation Conditions

The B2M PCR readily detected pig DNA in Instagene nasal swab extracts under the conditions of the first generation assay of Stark et al, and the control J strain extract signal was partly impaired by the presence of the B2M primers (Figure 5.3).

Figure 5.3: Detection of beta 2-microglobulin (B2M) DNA in 16 stored nasal swab extracts under conditions of the Stark M. hyopneumoniae PCR...
...with an annealing temperature of 52°C. While the Stark 1st generation PCR failed to deliver a positive signal in the absence of B2M primers (top), the B2M primers together with Stark primers indicated 13/16 samples still had detectable B2M DNA (bottom). Target products are: B2M primers P4/P5 205 bp; Stark primers 913 bp. MW markers with 1000 and 500 bp as darker bands are shown.

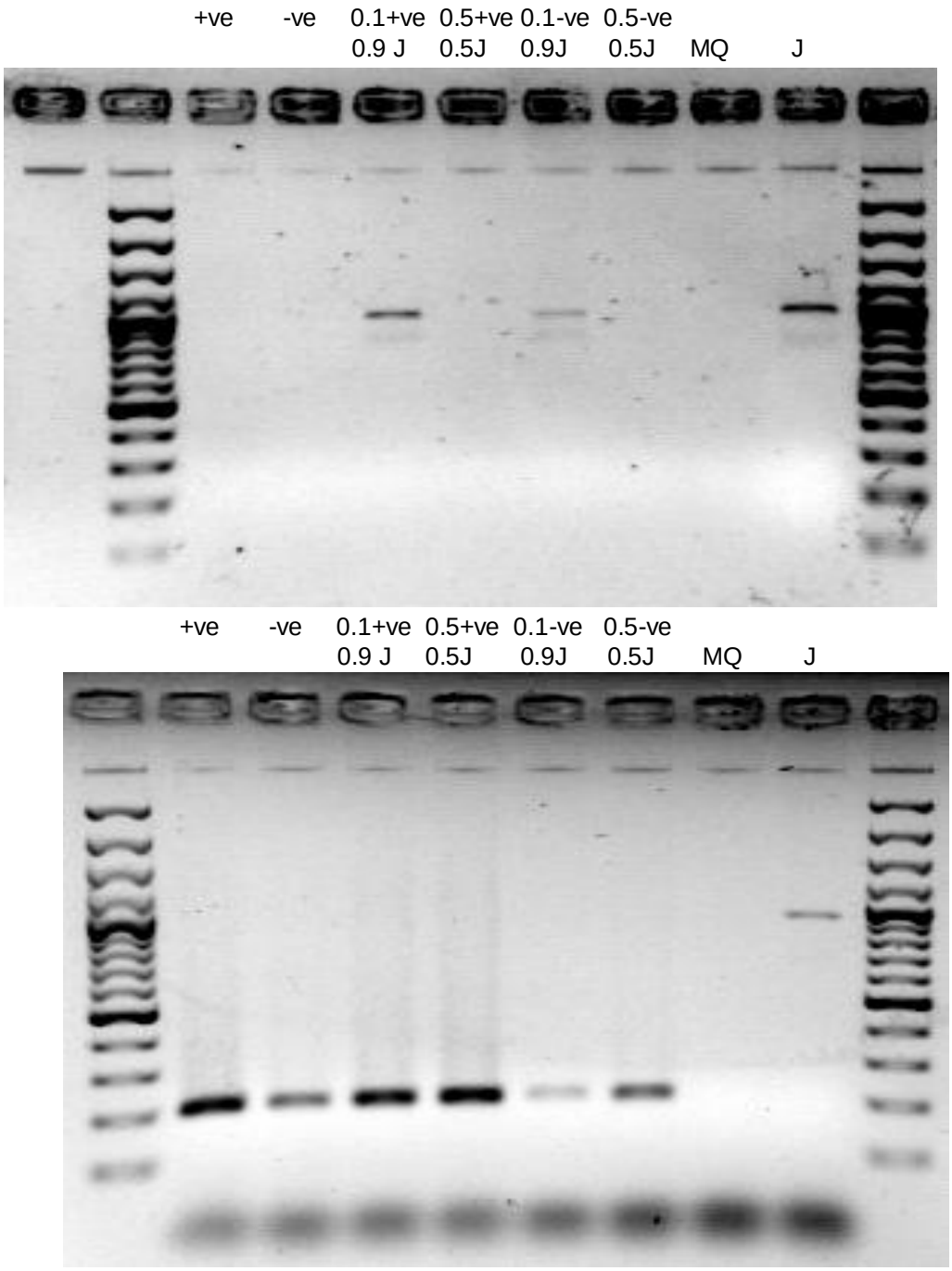




5.3.3 Reactivity of B2M Primers on Lung Samples under Kurth PCR 1st Generation and Nested PCR Conditions

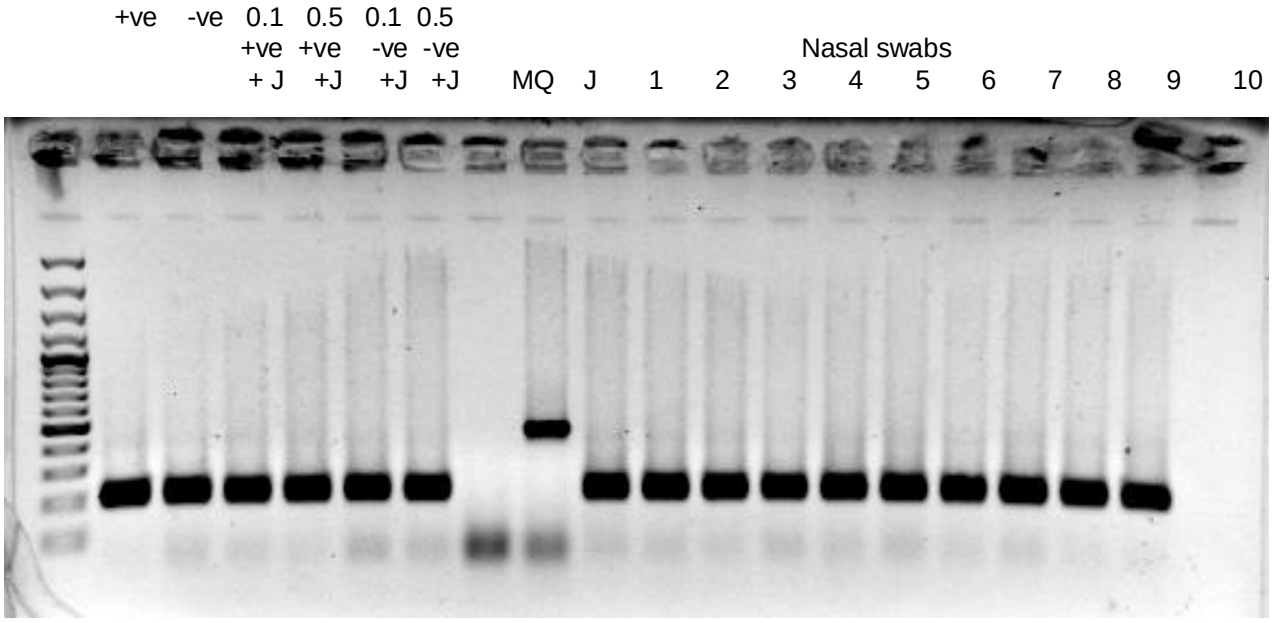
The B2M primers were found to reduce the signal from the 1st generation Kurth primers when applied concurrently. The addition of *M. hyopneumoniae* (J strain) DNA was unable to overcome this inhibition in the first generation reaction (Figure 5.4)

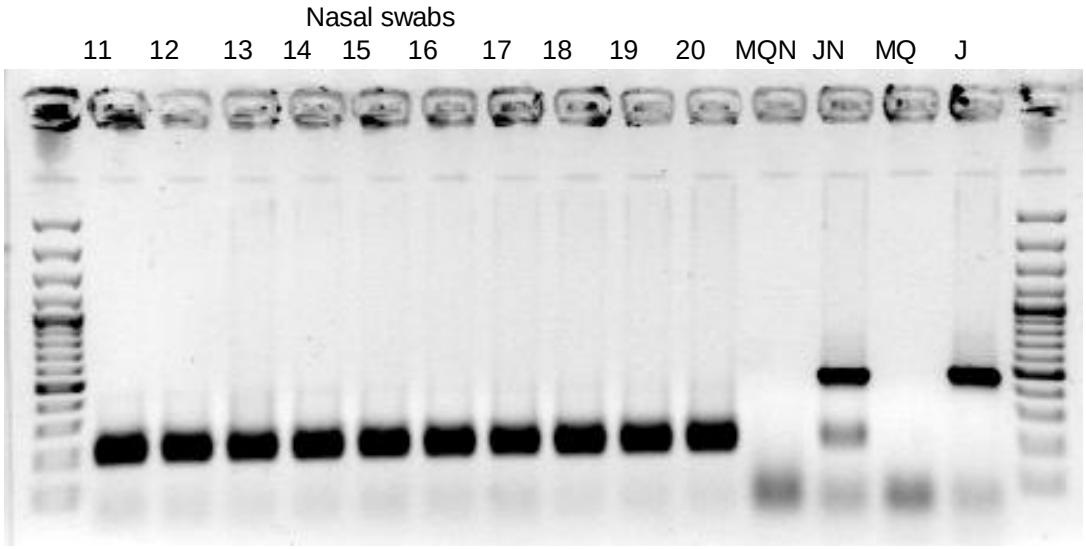
Figure 5.4: Detection of beta 2-microglobulin (B2M) DNA in lung extracts under conditions of the Kurth 1st generation...
...M. hyopneumoniae PCR with an annealing temperature of 64°C. Known positive (+ve) and negative (-ve) lung extracts were assessed. The Kurth 1st generation PCR delivered a positive signal in the absence of B2M primers when strain J made up 90% of the extract but not 50% (top), while in testing using the B2M primers together with Kurth primers in the same reaction all lung samples had detectable B2M DNA only (bottom). Target products are: B2M primers P4/P5 205 bp; Kurth 1st generation primers 1040 bp. MW markers with 1000 and 500 bp as darker bands are shown.



As shown in Figure 5.5, the Kurth nested PCR did not deliver a positive *M. hyopneumoniae* signal with lung material or lung material supplemented with J strain DNA in the presence of B2M primers at identical amounts for the Kurth and B2M primers (1st generation 50 pmol; 2nd generation 5.5 pmol). Instead, only a strong B2M signal was detected in the lung samples and lung samples supplemented with strain J. All swabs showed a strong B2M signal. These results indicated that the B2M reaction was effectively inhibiting the Kurth reaction in both the 1st and 2nd generation test. It should be noted that subsequent evaluation of the Kurth PCR adjusted the temperatures for the 1st and 2nd generations to 60/64°C to improve test sensitivity on lung samples. However, the lack of reactivity with samples supplemented with strain J DNA confirm the inhibitory nature of the B2M reactants.

Figure 5.5: Detection of beta 2-microglobulin (B2M) DNA in lung extracts and nasal swabs...
...under conditions of the nested Kurth M. hyopneumoniae PCR with annealing temperatures of 64°C/64°C and including B2M primers in all reactions. Known positive (+ve) and negative (-ve) lung extracts were assessed, together with 20 nasal swabs from pigs involved in a nutrition trial at EMAI. Target products: B2M 205 bp; M. hyopneumoniae 456 bp. MW markers with 1000 and 500 bp as darker bands are shown.





5.4 Discussion

Further studies of the Kurth PCR at 60/64◦C (and alternate assays) with increased Taq and/or reduced concentrations of B2M primers in the first and second generation PCR may be needed to overcome the problem encountered when primers for B2M are present, if the assays are to be run within the same reaction rather than as separate tests. At present, only the latter can be recommended to assess inhibitors.

In a concurrent application of Kurth primers and B2M primers, product for each has been reported to be detectable after increasing the Taq concentration in each step and the magnesium concentration in the second PCR reaction, and increasing the concentration of first generation product as template for the second generation assay (Kurth *et al.* 2002). As those workers also reduced the B2M primer concentrations to approximately 3% of the *M. hyopneumoniae* primer concentration in the first generation assay and to approximately 25% of the *M. hyopneumoniae* primer in the second generation assay to achieve a satisfactory result, a similar approach could be adopted in examining any recommended *M. hyopneumoniae* PCR assay. Kurth *et al* (2002) utilised a primer set that targeted a 270 bp product of B2M, which compares with a target size of 205 bp used in our assays, but the primers were not published so the precise target is unknown, although it was based on the same Genbank accession (L13854) as used in our study.

Part 6 - PCR Test Application to Field Samples

6.1. Materials and Methods

6.1.1 Field Samples

On-farm collection was undertaken in six NSW herds (WW, MU, HF, WE, HT, BB) and abattoir lung sampling in seven herds (previous six and an additional herd DHG). Herds were chosen on the basis of evaluation by their consulting veterinarian, based on recent slaughter examinations and on-farm history. Herd MU had undertaken a Swiss depopulation 18-24 months prior to sampling. The on-farm collections involved nasal swabs from 40 x 8 week old weaners and 40 x 20-24 week old finishers per herd, plus blood from the finisher group. This was followed up in each herd by lung inspection of 72-100 lungs and sampling of 11-12 suspect lesions for histopathology and *M. hyopneumoniae* PCR. The sampling included four high health status herds that killed pigs at Young abattoir (herds WW, MU, HF, WE) and two herds considered to have endemic *M. hyopneumoniae* infection based on prior history and that killed pigs at either Young or Picton, NSW (herds HT and BB). The additional herd (DHG) was sampled at Young abattoir only, and sourced pigs from within the same production group as herd WW.

The possibility of abattoir contamination of lung samples was able to be examined, as the samples from herd WW were the first to be killed at the abattoir on a working day. This significantly reduced if not precluded potential prior contamination of *M. hyopneumoniae* in scalding tank water from pigs sourced from another herd at slaughter.

Whole lungs were collected off the chain and at the end of a slaughter run, portions of 11-12 lung lesions of consolidation from different animals were removed with separate sterile instruments into two 70 mL sterile plastic jars, one plain and one containing buffered formalin. The samples were stored in an esky on cool bricks during immediate transport to the laboratory. Fresh samples were stored at -20°C for later DNA extraction and PCR testing.

Nasal swabs were collected using sterile cotton tipped swabs inserted into each nostril to a depth of approximately 2 cm. After rotation of the swab in the nares, the collected material was transferred to a 1 mL aliquot of sterile phosphate buffered saline (pH 7.5) and briskly rotated before discarding the swab. The PBS/swab material was stored on cool bricks during immediate transport to the laboratory, then stored at -20°C for later DNA extraction and PCR testing.

Plain blood samples were collected on farm by vacutainer sampling from the precava. Samples were allowed to clot at room temperature, then stored on cool bricks during immediate transport to the laboratory, where the serum was removed and stored at -20°C for ELISA testing.

6.1.2 Additional Lung Samples Sourced from Abattoir Collection from Low Health Status Herds

Samples of 30 lungs with typical mycoplasmal gross pathology were collected from slaughter age pigs from eight herds at Picton abattoir on 24/5/06, 29/5/06 and 30/5/06 as indicated in Table 6.1. Lesion materials from all lungs were processed and stored at -80°C for PCR analyses and samples were also cultured in Friis broth for *M. hyopneumoniae* on a maximum of four lungs per herd.

Table 6.1: Endemic herd lung lesions collected in May 2006 for PCR testing

Herd ID	Pig lung lesions collected from separate animals
759	4
496	4
604	4
216	1
4G0	3
67G	5
877	5
283	4
Total	30

6.1.3 DNA Extraction Systems

The PCR assays were assessed on lung and nasal swab material using two commercial DNA extraction kits, Instagene (BioRad) and DNeasy (Qiagen). Both had been previously applied to the Stark PCR in NSW and overseas (Kobisch and Frey 2003; Meyns *et al.* 2004), while the DNeasy kit has been applied to the Baumeister PCR in Victoria (Moore and Turni 2005). An alternate Qiagen kit (QIAamp tissue kit) was reported for lung sample applications of the Kurth PCR (Kurth *et al.* 2002), while the Calsamiglia PCR has only been assessed using other kits (Otagiri *et al.* 2005; Ruiz *et al.* 2002).

To evaluate the two DNA extraction methods on lung samples, the 30 lung samples from suspect infected herds collected at Picton (Wollondilly) abattoir in May 2006 were tested using each DNA extraction procedure with the Stark nested PCR.

6.1.4 ELISA Testing

Sera were tested in two ELISA for the detection of antibodies to *M. hyopneumoniae*. The first was an in-house ELISA based on a 43kDa purified membrane component of *M. hyopneumoniae* (Djordjevic et al. 1994), and the second was a commercial monoclonal blocking ELISA initially described in 1992 (Feld *et al.* 1992; Sorensen *et al.* 1992a; b) and produced by DakoCytomation Ltd, UK, but currently marketed through Oxoid Australia.

6.1.5 Restriction Endonuclease Analysis (REA) of PCR Products from Field Testing

REA was used to evaluate and compare PCR products between high health and endemically-infected herds. PCRs were repeated on a random selection of 50 nasal swab samples that yielded positive PCR products in the Kurth, Calsamiglia and Verdin optimised PCRs. The DNA extracts from samples used to generate sufficient product for further investigation by REA are listed in Table 6.2a-c.

Based on the target product for these three nested PCRs, a commercial software program (Nebcutter, New England Biolabs) was used to select a suitable enzyme for each PCR product. The various PCRs and their selected enzyme, digestion temperature and expected enzyme products are shown in Table 6.3. using a 20 uL reaction volume in an Eppendorf tube, the amount of amplified product subjected to enzymic digestion was varied according to the strength of the gel bands they had produced. For amplicon solutions producing weak bands, 17.75 uL of PCR product was added to the tube, followed by 2 uL of 10x restriction enzyme buffer and 0.25 uL of enzyme (approx 1-2 units, New England Biolabs, Genesearch, Qld). For amplicon solutions producing normal or strong bands,

7 uL of PCR product was added, then 2 uL of 10x restriction enzyme buffer, 10.75 uL of water and 0.25 uL of enzyme. Samples were digested overnight in a heating block and run on 3% agarose.

Table 6.2a: Calsamiglia samples reamplified for REA analyses

Sample ID	Herd	Extraction type	Sample no.
1	MU	Ig	24
2		Ig	54
3	HF	Q	74
4		Ig	3
5		Ig	62
6	WW	Ig	29
7		Ig	49
8	WE	Q	21
9		Ig	38
10		Ig	88
11	BB	Q	57
12		Ig	21
13		Ig	65
14	HT	Ig	20
15		Ig	66
31	MU	Ig	60
32		Ig	74
33	HF	Q	56
34		Q	80
35		Ig	7
36		Ig	28
37	WW	Ig	39
38		Ig	75
39		Ig	77
40	WE	Q	38
41	BB	Q	63
42		Q	75
43		Ig	25
44		Ig	75
45	HT	Ig	21
46		Ig	80

Table 6.2b: Kurth samples reamplified for REA analyses

Sample ID	Herd	Extraction type	Sample no.
16	MU	Ig	28
17		Ig	59
18	HF	Ig	74
19	WW	Ig	32
20		Ig	78
21	WE	Ig	38
22		Ig	88
23	BB	Q	57
24		Ig	63
25	HT	Ig	20
47	MU	Ig	30
48	HF	Ig	22
49	WW	Ig	39
50	BB	Q	63
51		Q	75
52		Ig	75

Table 6.2c: Verdin samples reamplified for REA analyses

Sample ID	Herd	Extraction type	Sample no.
53	WE	Q	38
54	HT	Ig	20
55		Ig	80

Table 6.3: Enzymes used in REA analyses

PCR	Target size bp	Enzyme	T °C	Enzyme products bp
Calsamiglia	352	*Tsp*5091	65	131 107 95 20
Kurth	456	*Mse*I	37	227 111 72 46
Verdin	706	*Taq*I	65	215 203 171 113

6.2 *Results*

6.2.1 Initial Comparison of Qiagen vs Instagene Extractions on 30 Lungs ex Picton Abattoir

A similar number of positives were found with each procedure, but much stronger gel bands with the Instagene procedure (Figs 6.1, 6.2).

Figure 6.1: Comparison of Qiagen DNeasy (top) and Instagene (bottom) extractions on stored lung samples 1-15 with suspect mycoplasmal lesions tested in the Stark nested PCR.

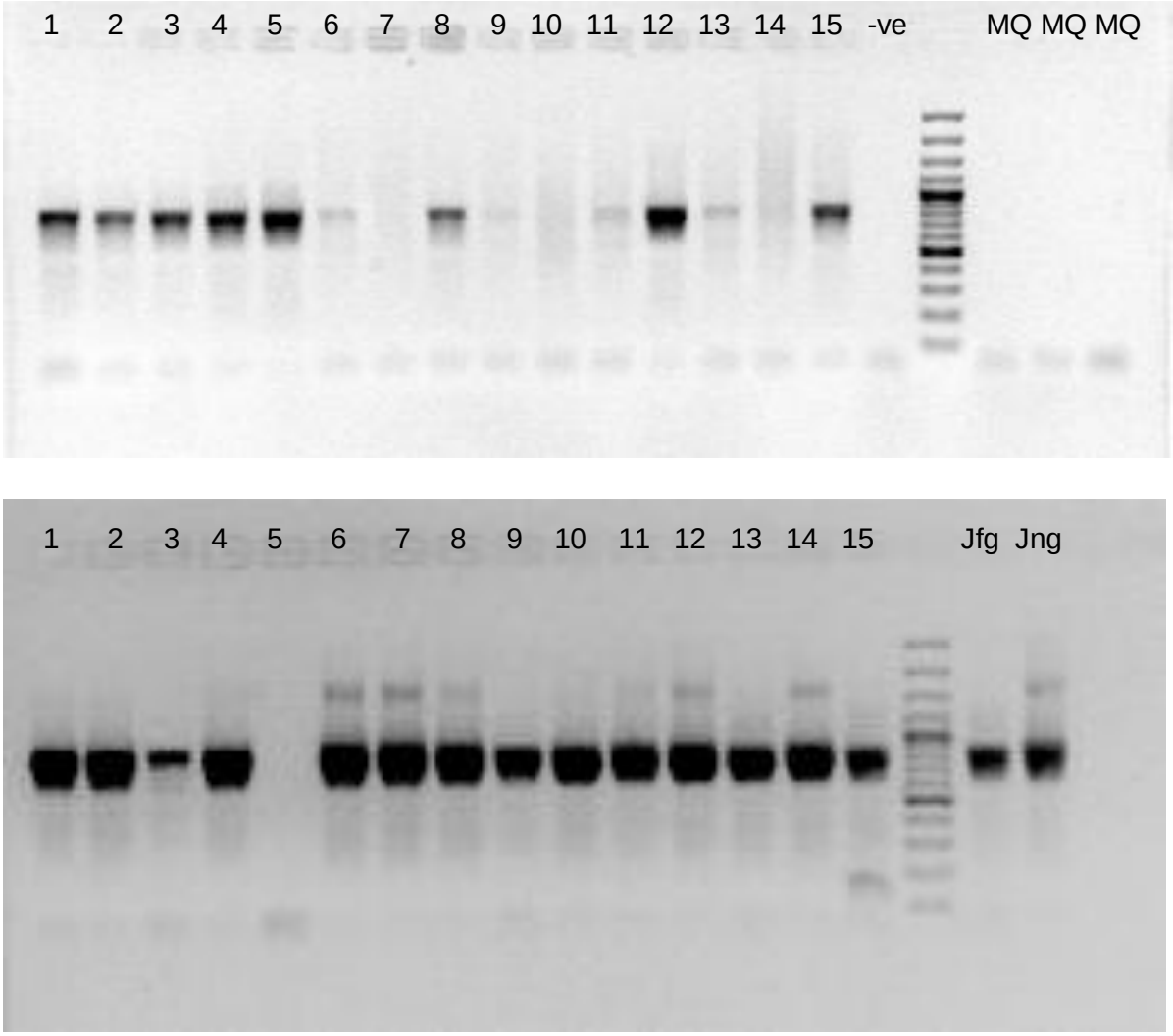
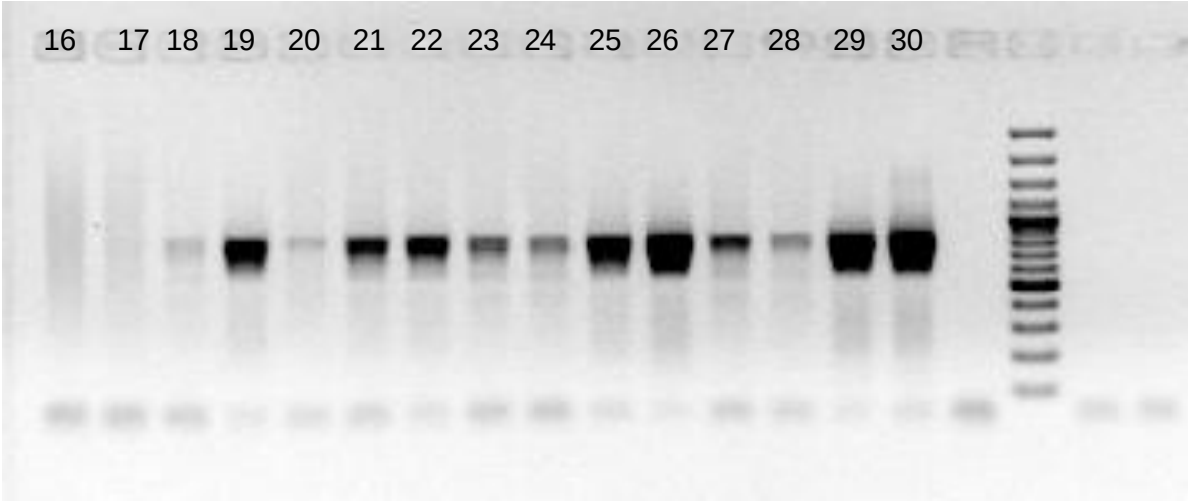
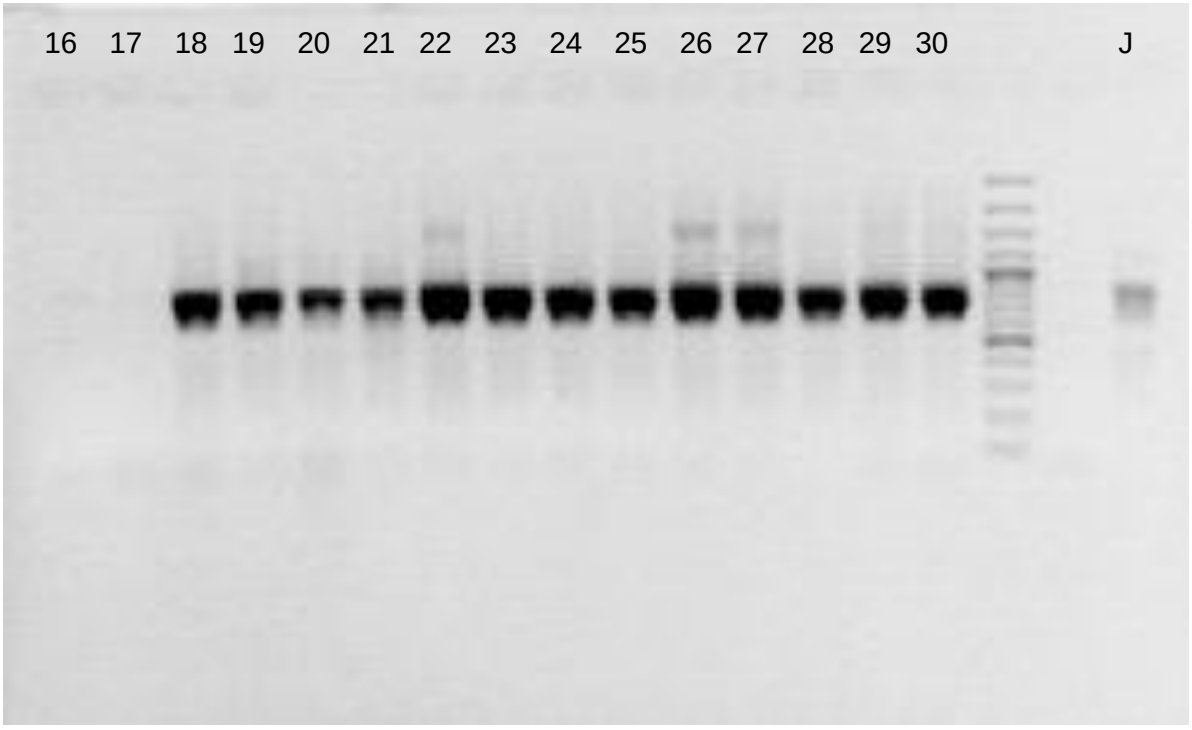


Figure 6.2: Comparison of Qiagen DNeasy (top) and Instagene (bottom) extractions on stored lung samples 16-30 with suspect mycoplasmal lesions tested in the Stark nested PCR.





6.4.2 Status of Herds Based on Lung Scores, Histopathology and Serology

In selection of lung samples from the study herds, the gross lung findings in the batch from which the samples were selected at slaughter for PCR testing is shown for the six key herds (WW, MU, HF, WE, HT and BB) in Table 6.4, confirming that the two herds considered to have endemic disease (HT and BB) had typical gross findings at slaughter, and high lungs scores, with average lung scores (ALS) of 9.76 and 3.29 compared to <2 in the four high health herds WW, MU, WE and HF. The histopathology for the suspect lesions collected from all seven herds studied (Table 6.4) indicated mycoplasmal pneumonia and/or enzootic pneumonia was highly prevalent among samples from herds HT and BB, whereas consolidated lesions in the four high health herds WW, MU, WE and HF had mainly slaughter artefacts associated with localised areas of lung collapse (atelectasis). The exceptions were two pigs in herds WW and WE that had evidence of a bacterial bronchopneumonia, and another in herd WW with mild lesions that could be consistent with early mycoplasmal infection. A further two pigs in herd WW had non-specific mild bronchitis. Herd DHG showed no lesions typical of mycoplasmal pneumonia, but most pigs with consolidated lesions appeared to be affected with inhalation of foreign matter. Subsequent investigation indicated these animals were kept on straw-based deep litter systems prior to slaughter, and could explain the pathological changes that were found.

Serology of the six key herds (Table 6.5) indicated that herds HT and BB were currently infected with *M. hyopneumoniae*, with 100% of 40 finishers giving positive reactions in the Dako ELISA, and 20 and 58% respectively showing ELISA reactivity in the 43 kDa ELISA. The status of herds MU, HF and WE appeared negative in the Dako ELISA, and none gave ELISA responses in the 43 kDa ELISA except for an inconclusive reaction in one pig in herd WE. On the basis of the Dako ELISA, herd WW would be classified as suspect infected, while this herd returned only a single inconclusive animal in the 43 kDa ELISA, and which tested negative in the Dako assay.

Table 6.4: Goodwin lung scores, average lung score (ALS) and histopathological findings in selected lungs collected from seven NSW herds

Herd	No. lungs examined (ALS)	0	1-5	6-10	11-20	>20	Severe pleurisy	Lung no.	H/P findings (summary)
DHG	NA							1, 5, 7, 9	Foreign body/inhalation pneumonia
								2, 3, 11	Mild inhalation bronchiolitis/pneumonia
								4, 6	Non-specific consolidation, probable inhalation pneumonia
								7	Mild to moderate inhalation pneumonia
								8, 10	Non-specific consolidation
WW	100 (1.26)	70 70%	25 25%	4 4%	1 1%	0 0%		1, 3-6, 8-9, 11-12	Atelectasis
								2	Possible early/limited mycoplasmosis
								4, 7	Mild atelectasis and mild bronchitis
								10	Suppurative bronchopneumonia - possibly pasteurellosis
MU	75 (1.73)	36 48%	29 39%	10 13%	0 0%	0 0%		1-12	Atelectasis
HF	93 (1.30)	59 63%	30 32%	4 4%	0 0%	0 0%		1-12	Atelectasis - slaughter artefacts
WE	100 (1.09)	75 75%	20 20%	2 2%	3 3%	0 0%		1-8,10-12	Atelectasis - slaughter artefacts *NB: No. 6 not examined by PCR*
								9	Purulent bronchopneumonia - possibly pasteurellosis
HT	77 (9.76)	17 22%	19 25%	17 22%	13 17%	11 14%		1	Mycoplasmosis
								2-7,10	Enzootic pneumonia (Mhp + bacteria)
								8	Chronic mycoplasmosis with some bacterial bronchitis
								9	Enzootic pneumonia with marked secondary bacterial infection
								11	Mainly bacterial bronchopneumonia
								12	Mild mycoplasmosis
BB	72 (3.29)	34 47%	22 31%	6 8%	5 7%	1 1%	4 6% (not able	1, 3	Predominantly bacterial bronchopneumonia with little mycoplasmal involvement
								2, 5-6, 8-9,	Enzootic pneumonia

							to be scored)	11-12	
								4	Subacute to chronic enzootic pneumonia
								7	Predominantly bacterial bronchopneumonia and mild mycoplasmosis
								10	Enzootic pneumonia with marked secondary bacterial infection

Table 6.5: Serology of finisher pigs in six study herds, using an M. hyopneumoniae competitive ELISA (Dako)...
...and an in-house indirect ELISA based on the M. hyopneumoniae-specific 43 kDa subunit. The latter provides results classified by ELISA ratio that are interpreted as negative, inconclusive or positive.

Herd	Sera	Dako pos	% positive	43kDa incl	43kDa pos	% incl or pos
WW	40	6	15	1	0	2.5
MU	40	0	0	0	0	0
HF	40	0	0	0	0	0
WE	40	0	0	1	0	2.5
HT	40	40	100	0	8	20
BB	40	40	100	2	21	57.5

6.4.3 Evaluation of Lung Samples Collected from High and Low Health Herds for this Project

Since neither extraction procedure proved more accurate in preliminary testing, both were applied to lungs sampled from the six herds. Summaries of PCR findings in lung samples are given in Figures 6.3 – 6.7, with a differentiation of reactivity on the basis of the proportion of all reactors in each herd [PCR score 1 (weak) or score 2 (strong)], and the proportion of only strong positive reactions in each herd. Similar breakdowns are provided for 21 lungs from herds HT and BB with histopathology indicative of mycoplasmal pneumonia (i.e. excluding lung 11 from herd HT and lungs 1 and 3 from herd BB) (Figure 6.8). The latter three lungs with evidence of bacterial bronchopneumonia are compared in Figure 6.9. In order to investigate whether some PCRs showed a high predominance of strong or weak PCR reactions, the rate of weak reactions compared to all reactions is given is Figure 6.10 for all tests.

The results indicated that all lungs in herds BB and HT gave a PCR positive reaction in at least one test. The least sensitive assays appeared to be the two non-nested assays - Baumeister and Stakenborg PCRs. There was also a lower sensitivity in herd HT for the Kurth assay and particularly for Qiagen extracts in herd HT samples for the Kurth, Baumeister and Stark assays (Figure 6.3). The Verdin and Calsamiglia assays were the most sensitive for lung samples across these two herds, with the optimised parameters giving good results in each.

Herd DHG appeared to have suspicion of infection based on PCR positives in 10-20% of samples in the most specific assays (Stakenborg, Verdin optimised)

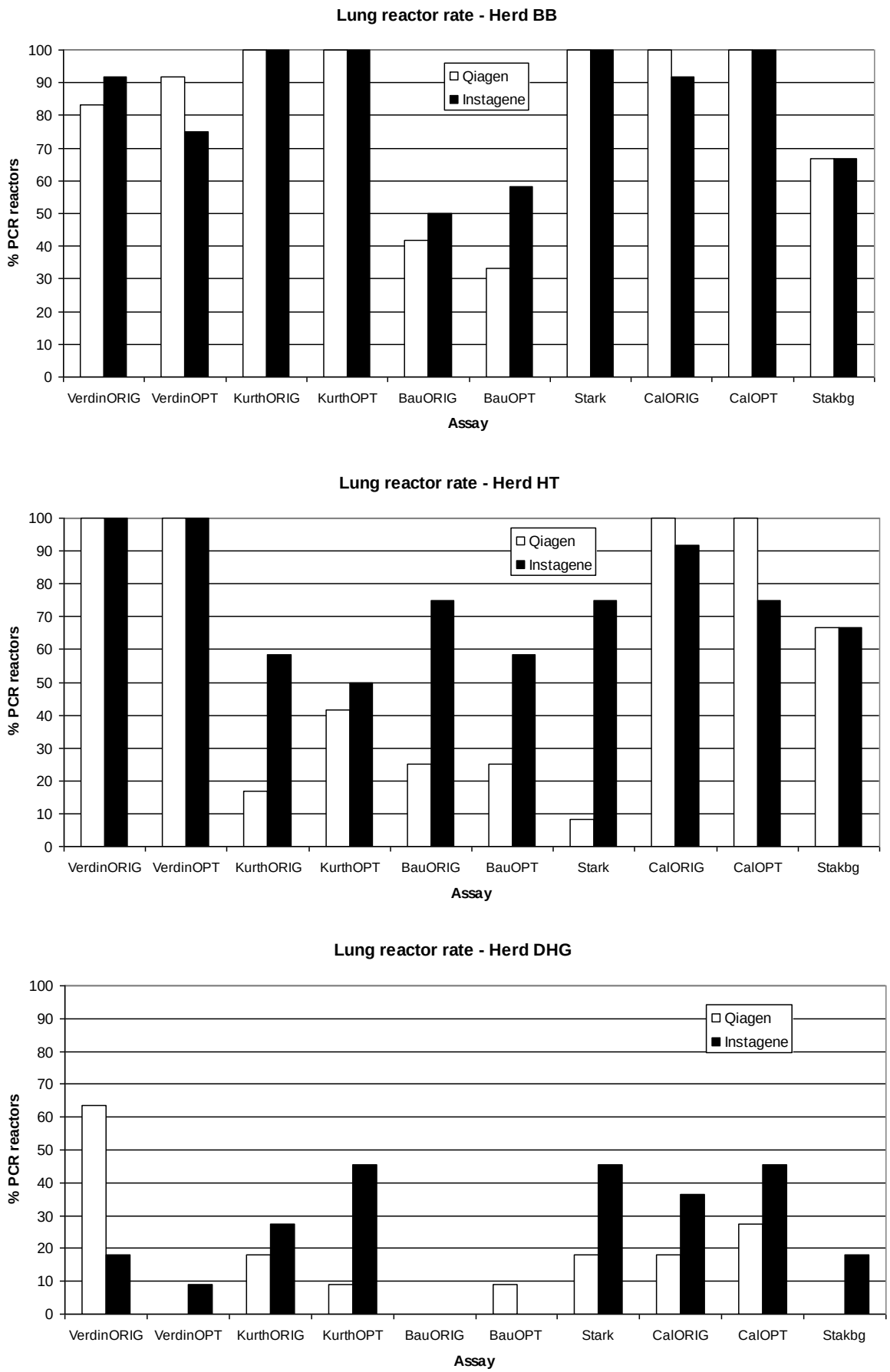


Figure 6.3: PCR reactor rates for lung lesions at commercial slaughter in two herds with endemic...
...M. hyopneumoniae infection (herds BB, HT) and in a third herd DHG of uncertain

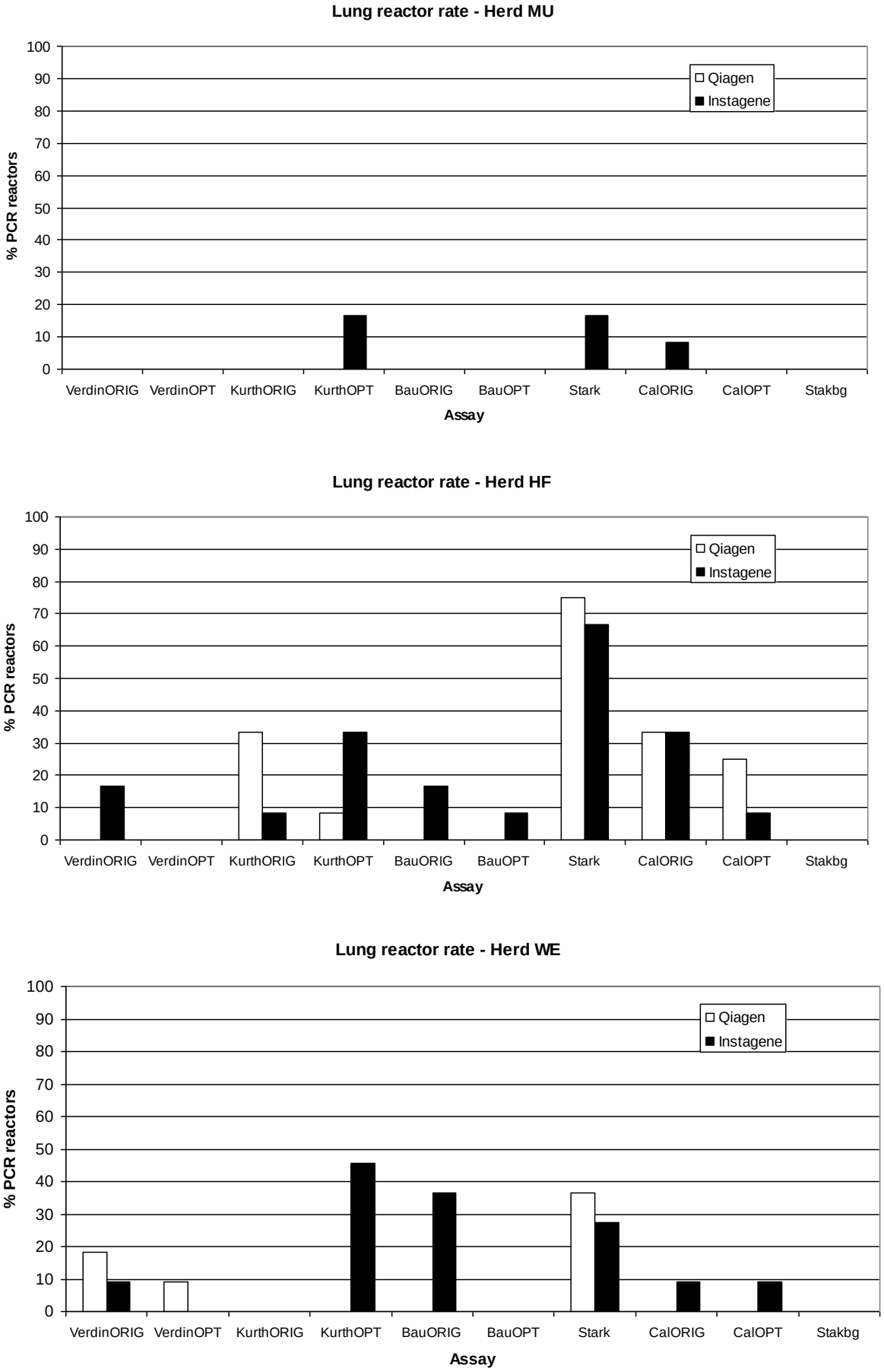
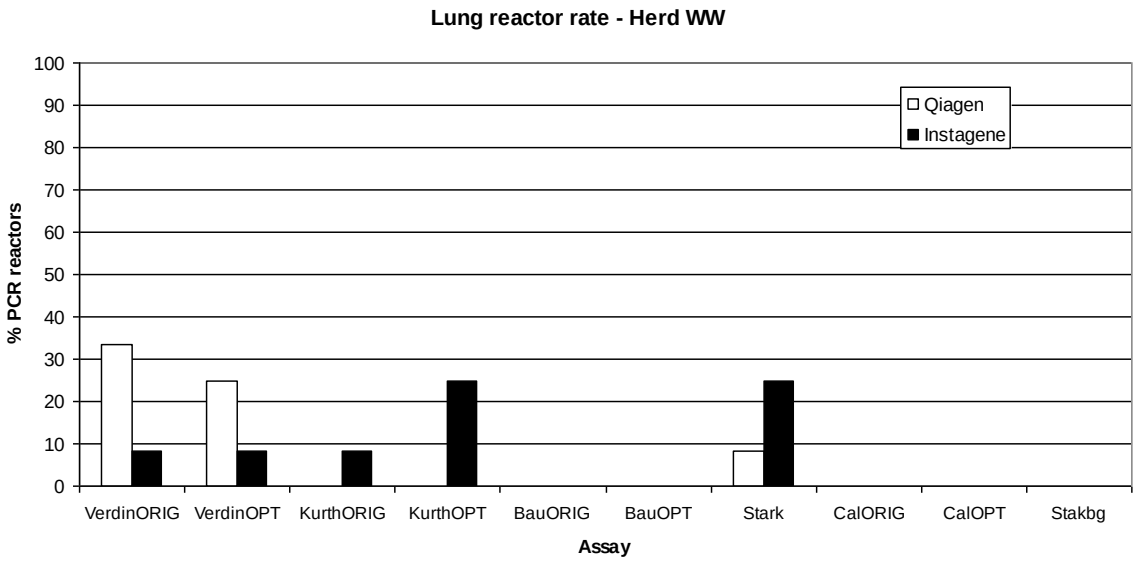


Figure 6.4. PCR reactor rates for lung lesions at commercial slaughter in three herds considered high health status (low risk) for M. hyopneumoniae infection (ELISA and histopathology negative)



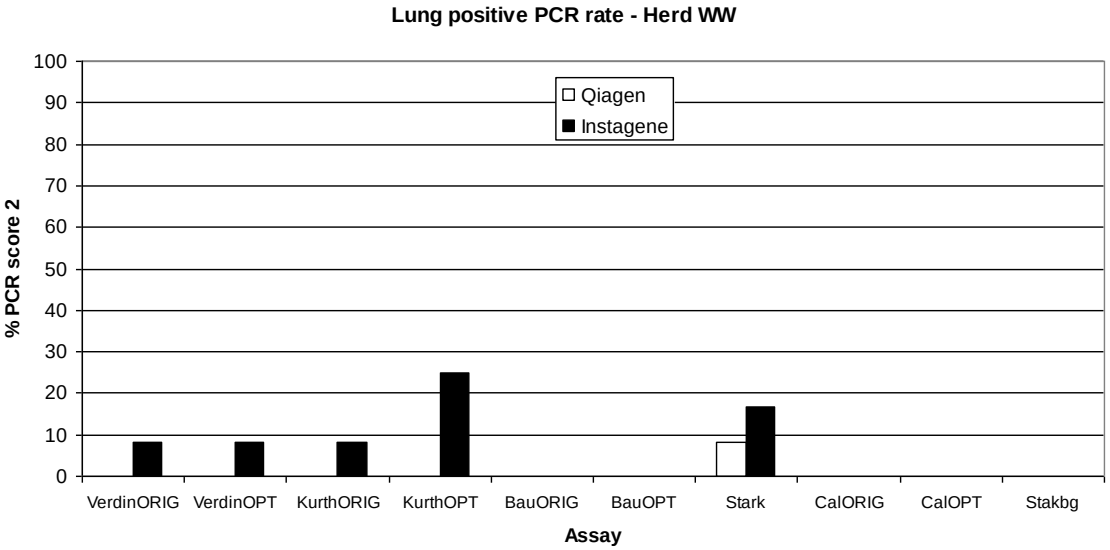
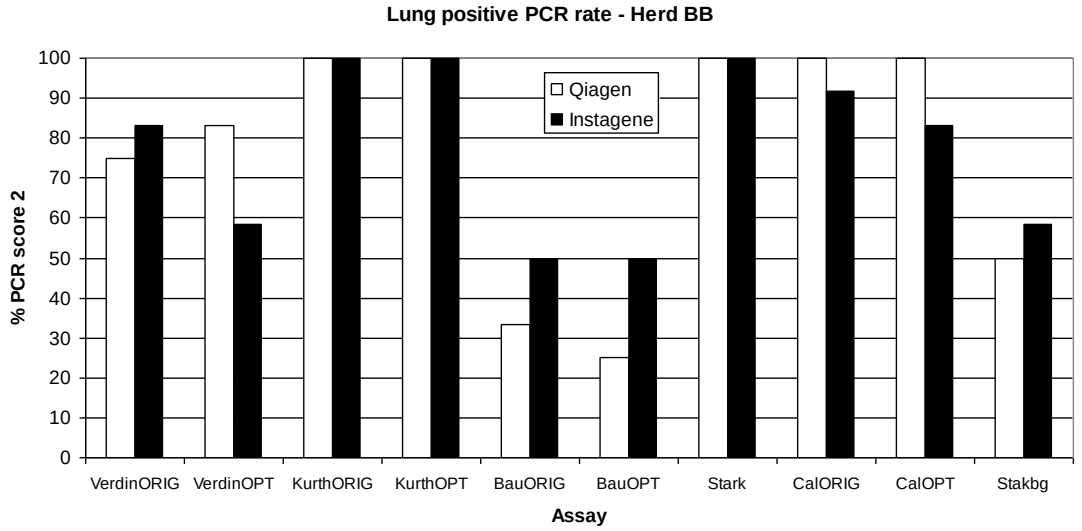
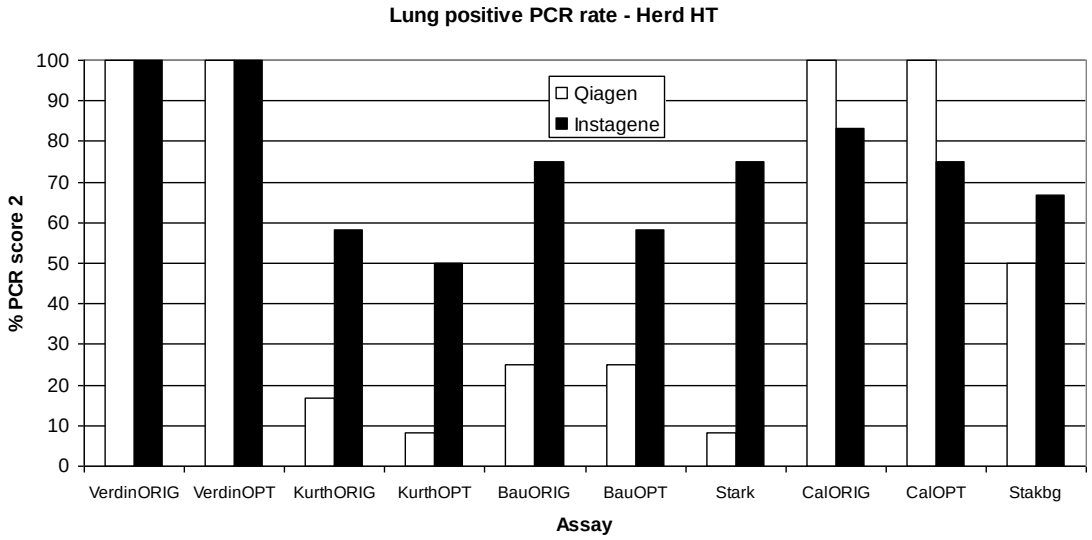


Figure 6.5: PCR reactor and positive reactor rates for lung lesions at commercial slaughter in one herd considered high health for M. hyopneumoniae infection but histopathology doubtful and a small number of ELISA reactors on herd testing.


Lung positive PCR rate - Herd BB
% PCR score 2
100
90
80
70
60
50
40
30
20
10
0
Qiagen
Instagene
VerdinORIG
VerdinOPT
KurthORIG
KurthOPT
BauORIG
BauOPT
Stark
CalORIG
CalOPT
Stakbg
Assay


Lung positive PCR rate - Herd HT
% PCR score 2
100
90
80
70
60
50
40
30
20
10
0
Qiagen
Instagene
VerdinORIG
VerdinOPT
KurthORIG
KurthOPT
BauORIG
BauOPT
Stark
CalORIG
CalOPT
Stakbg
Assay

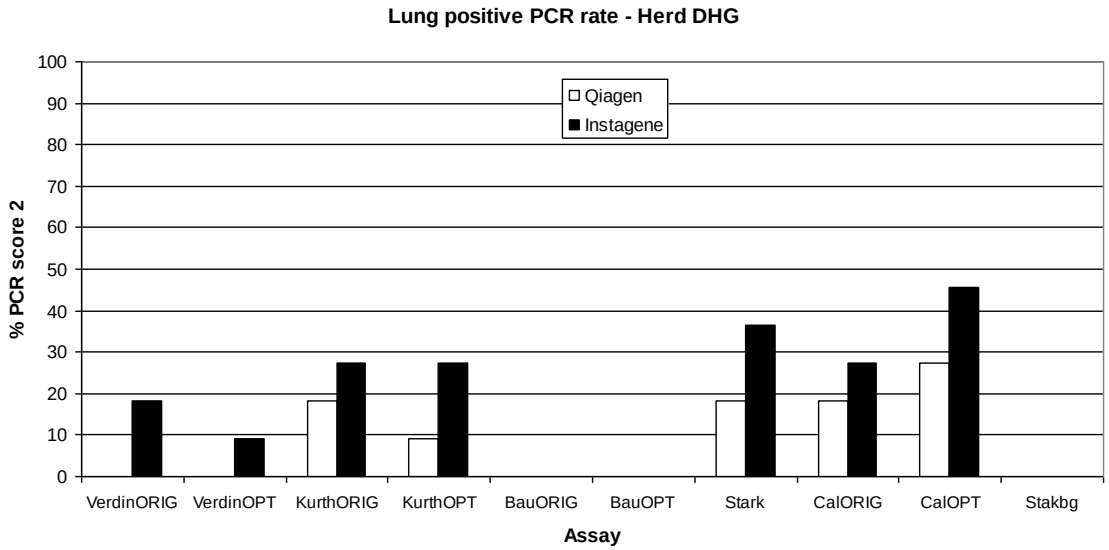
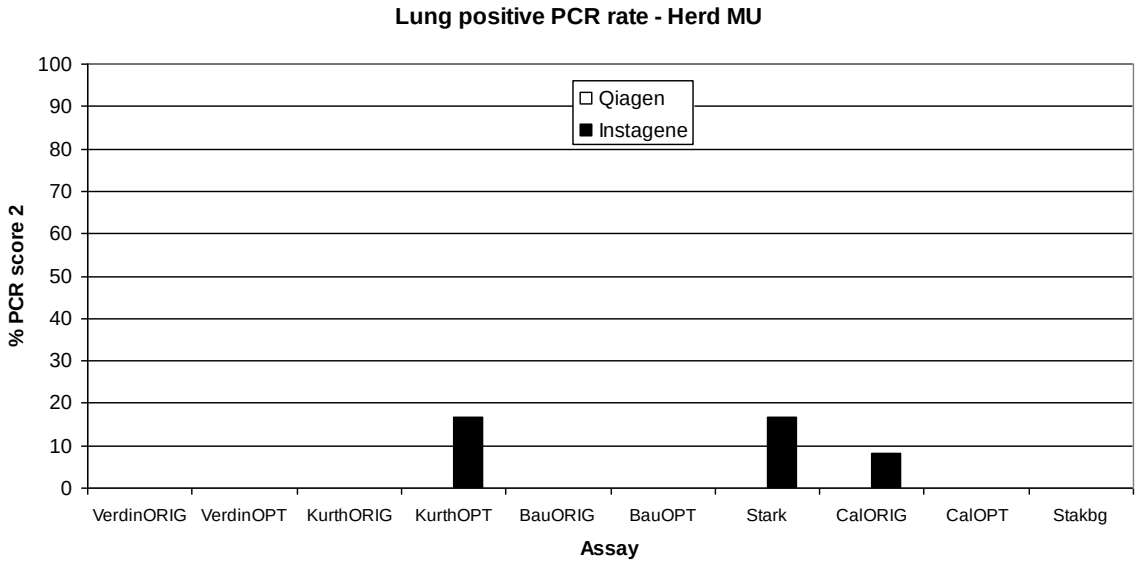
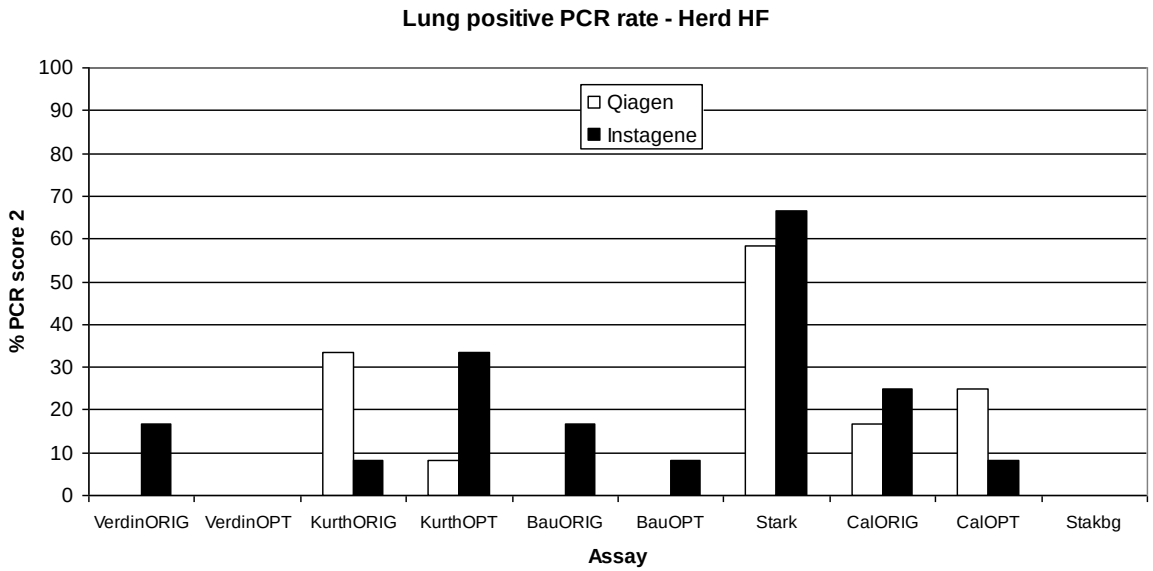

Lung positive PCR rate - Herd DHG
% PCR score 2
100
90
80
70
60
50
40
30
20
10
0
Qiagen
Instagene
VerdinORIG
VerdinOPT
KurthORIG
KurthOPT
BauORIG
BauOPT
Stark
CalORIG
CalOPT
Stakbg
Assay

Figure 6.6: Positive PCR reactor rate (score 2) for lung lesions at commercial slaughter..
....in two herds with endemic M. hyopneumoniae infection (herds BB, HT) and in a third herd DHG of uncertain status (histopathology negative).


Lung positive PCR rate - Herd MU
% PCR score 2
100
90
80
70
60
50
40
30
20
10
0
Qiagen
Instagene
VerdinORIG
VerdinOPT
KurthORIG
KurthOPT
BauORIG
BauOPT
Stark
CalORIG
CalOPT
Stakbg
Assay


Lung positive PCR rate - Herd HF
% PCR score 2
100
90
80
70
60
50
40
30
20
10
0
Qiagen
Instagene
VerdinORIG
VerdinOPT
KurthORIG
KurthOPT
BauORIG
BauOPT
Stark
CalORIG
CalOPT
Stakbg
Assay

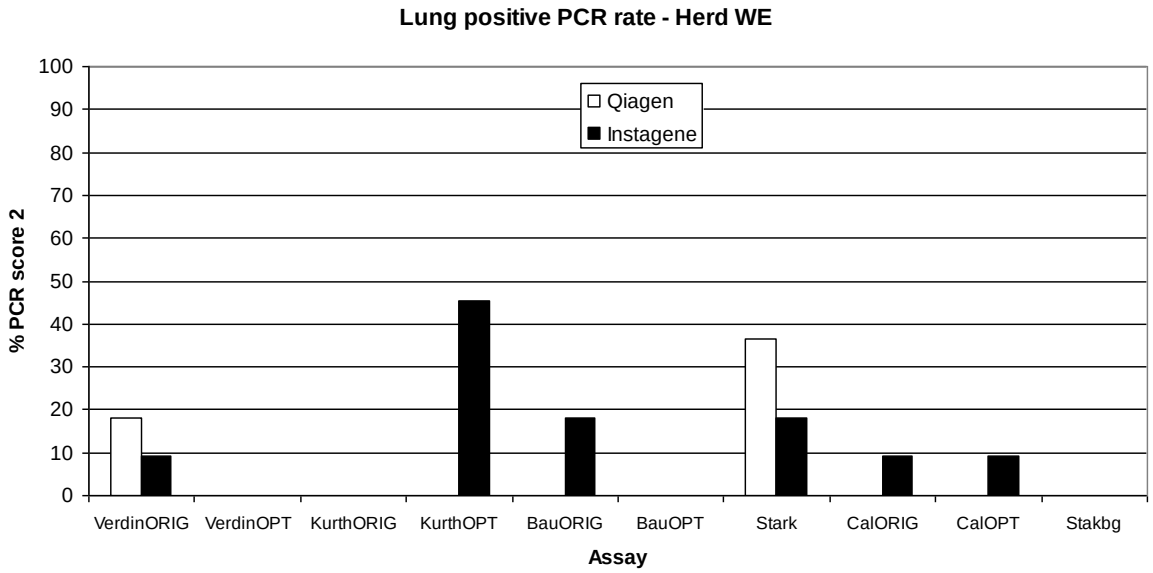
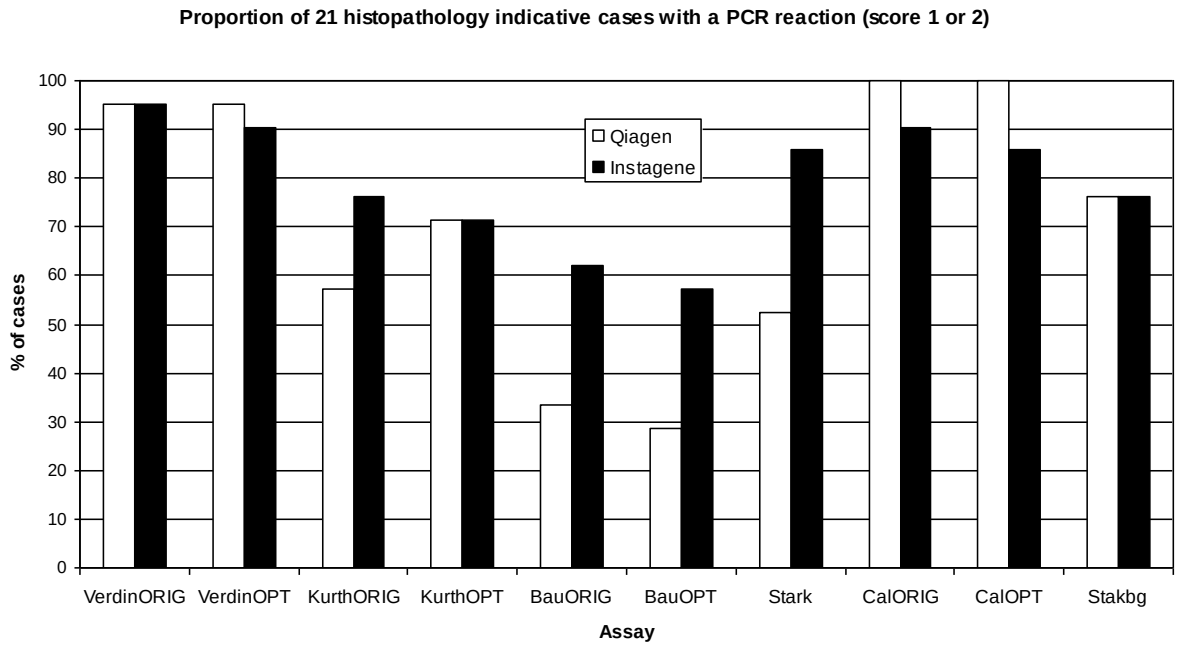


Figure 6.7: Positive PCR reactor rates (score 2) for lung lesions at commercial slaughter among three herds..
... considered high health status (low risk)for M. hyopneumoniae infection (all ELISA and histopathology negative).



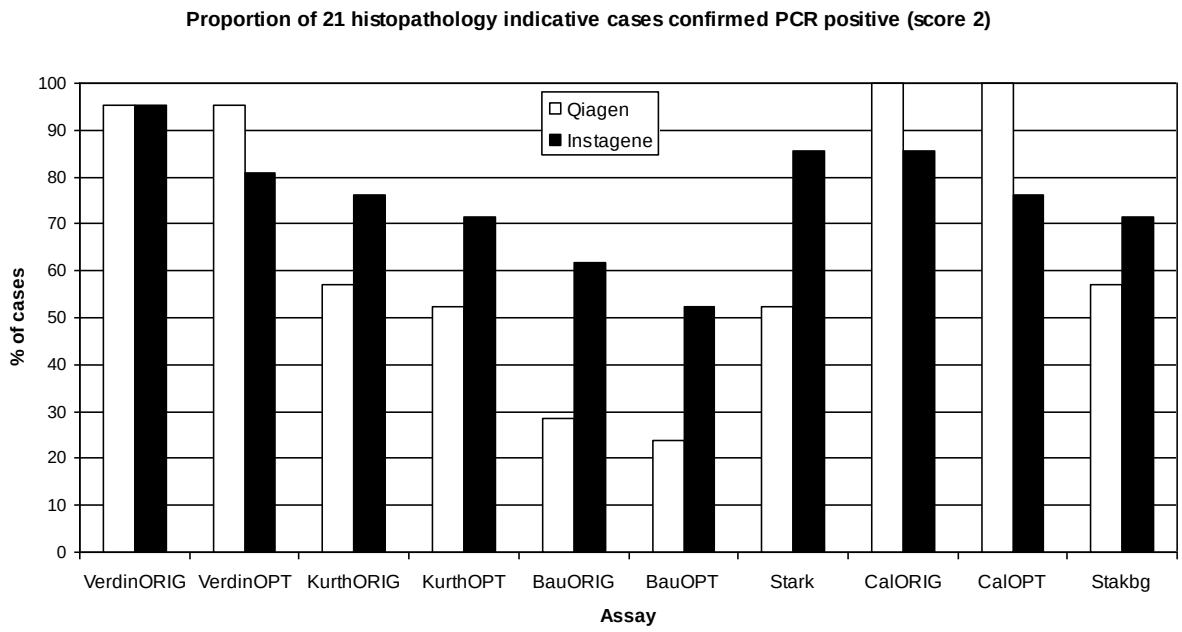
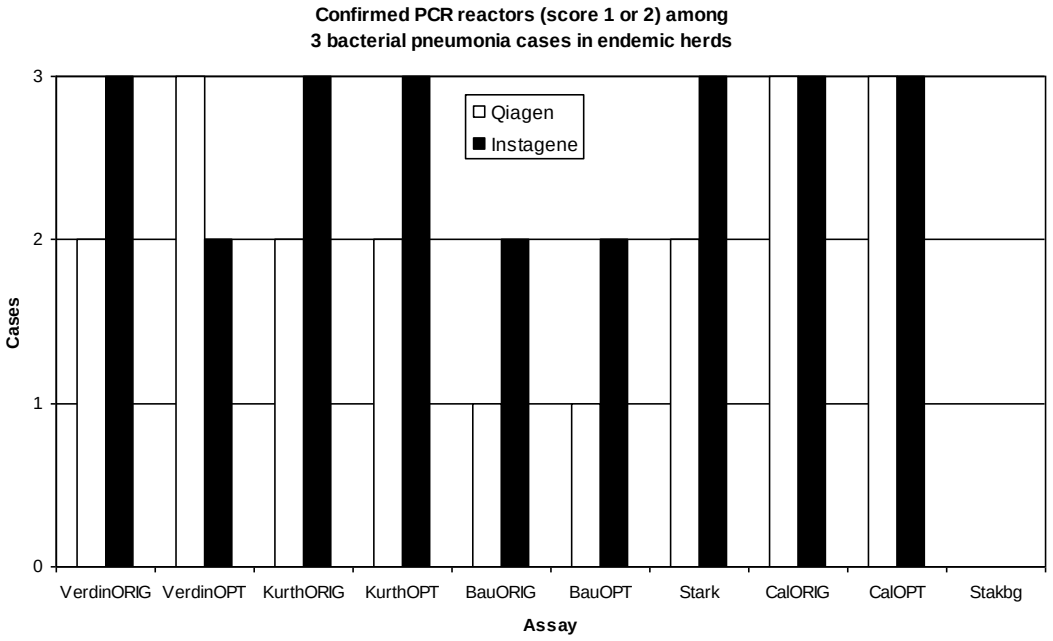


Figure 6.8: PCR results for 21 cases with lung histopathology indicative of mycoplasmal pneumonia...
... from herds BB (n = 10) and HT (n = 11). Top: lung samples with a PCR positive reaction (score weak or strong); bottom: lung samples with a strong PCR reaction (score 2).



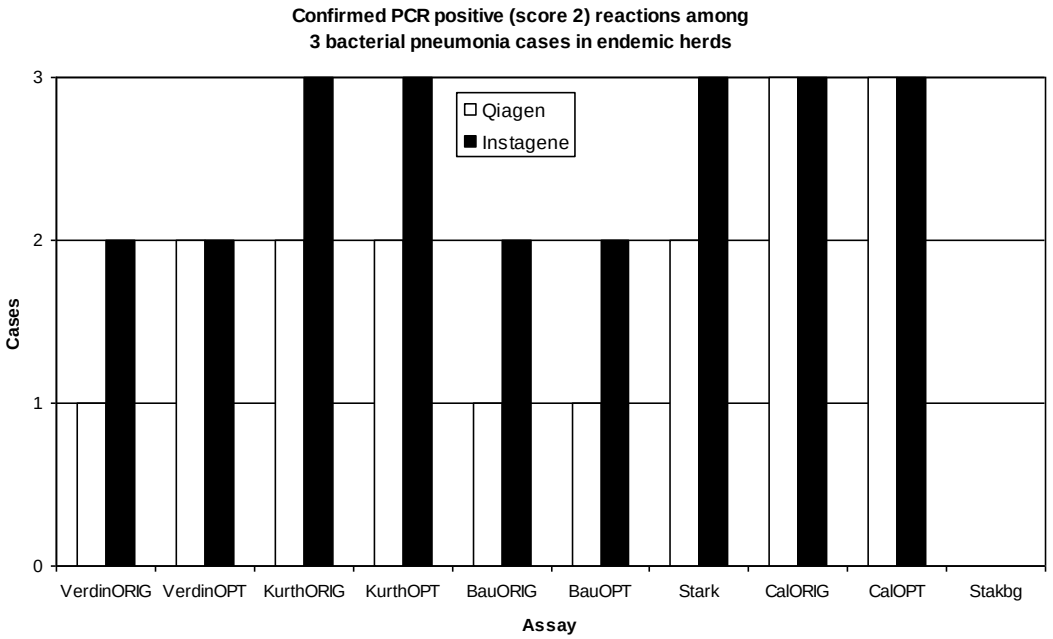


Figure 6.9: Positive PCR reactions were detected in three lungs from endemic herds BB (n = 2) and HT (n = 1)...
....that were classified histologically as bacterial bronchopneumonia, except in the Stakenborg assay. Top: any positive reaction (weak score 1 or strong score 2); bottom: strong positive reactions (score 2).

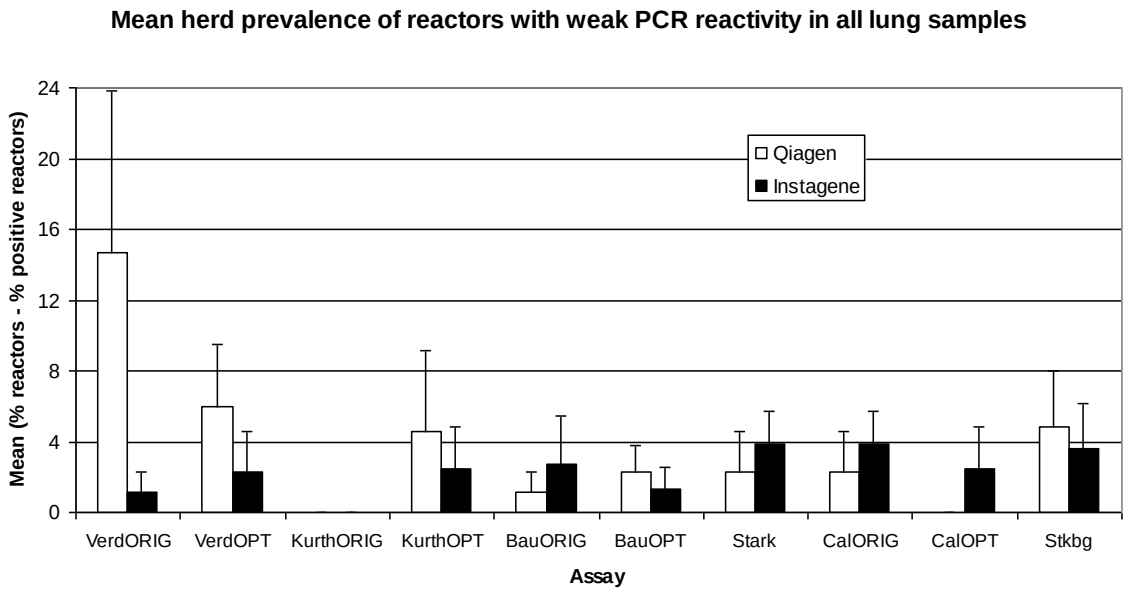


Figure 6.10: Mean proportion of all reactors per herd that gave weak positive results for lung samples...
...with SEM shown.

6.4.4 Evaluation of Nasal Swab Samples Collected from High and Low Health Herds for this Project

The results of nasal swab PCRs are given in graphic form per herd for each test in Figures 6.11-6.18. The results are grouped into those for herds with endemic infection (herds BB, HT) or suspect infection (WW) compared to the three herds considered of high health status (low risk) for mycoplasmal pneumonia (Herds MU, HF and WE). The proportion of samples giving a PCR reaction, either weak (score1) or strong (score 2) is shown in Figures 6.12-6.12, and this data is broken down into two age classes (weaner vs finisher) in Figures 6.13-6.14. Figures 6.15-6.18 provide similar information relevant to strong PCR responses (score 2) only.

PCR reactivity of nasal swabs was not found to be different between the endemic and the high health herds for any test, but some PCRs gave markedly more positive reactions. Most reactors were detected among the Stark and Calsamiglia PCRs, and with Instagene extracts compared to Qiagen extracts. Adjusting the annealing temperature did not appear to greatly reduce the reactivity rate among nasal swabs in any herd except for the Verdin assay, where fewer reactors were detected in herds MU, HF and WW, but not high health herd WE.

While the optimised Baumeister assay detected no reactors in the higher health herds MU, HF, WE and WW, it also found only 1-2.5% positives among swabs from the two endemically infected herds. In contrast the Verdin PCR detected approximately twice as many reactors in the endemic herds, but detected many more reactors among the high health herd WE.

Qiagen extracts from weaner pigs tended to contain fewer positive reactors in most tests in five of the six study herds. The exception was the high health status herd WE which showed a high rate of PCR reactions among Qiagen extracts from weaner pigs for most assays (Figure 6.14). This herd was also the only one with a high rate of Verdin PCR reactors, of which all were among weaner age pigs. No other herd or test showed any particular age predominance, with similar proportions of weaners and finishers providing positive results.

In examining only those samples yielding a strong positive PCR result, there was no apparent age predominance. With respect to strong positive results from nasal swabs, the optimised Verdin assay performed poorly and was found to detect no strong positives in herd BB and few in herd HT or WW. In contrast it was found that most reactions detected in the Stark, Calsamiglia and Kurth assays were classified as strong (score 2) (Figures 6.11 and 6.12 vs Figures 6.15 and 6.16).

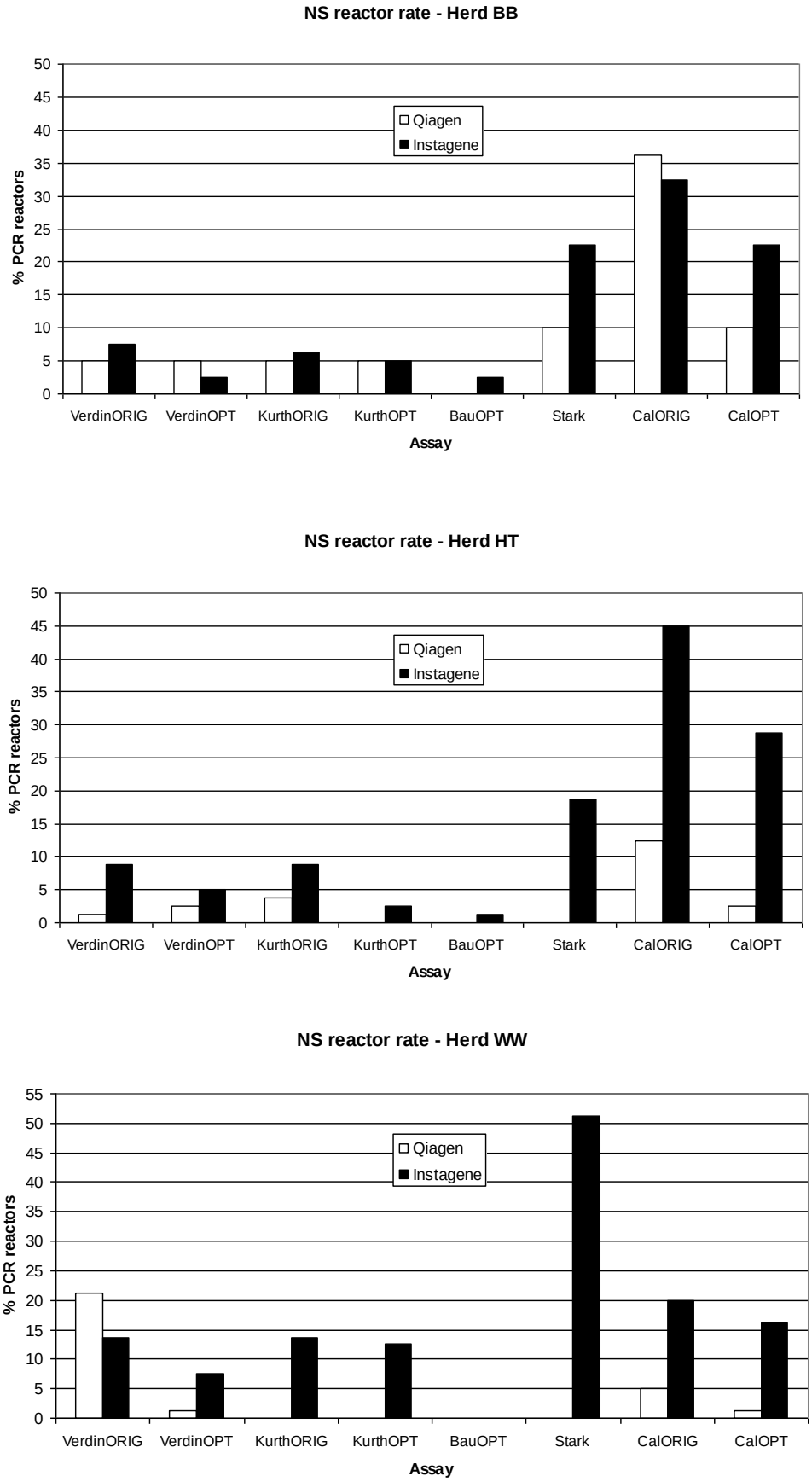
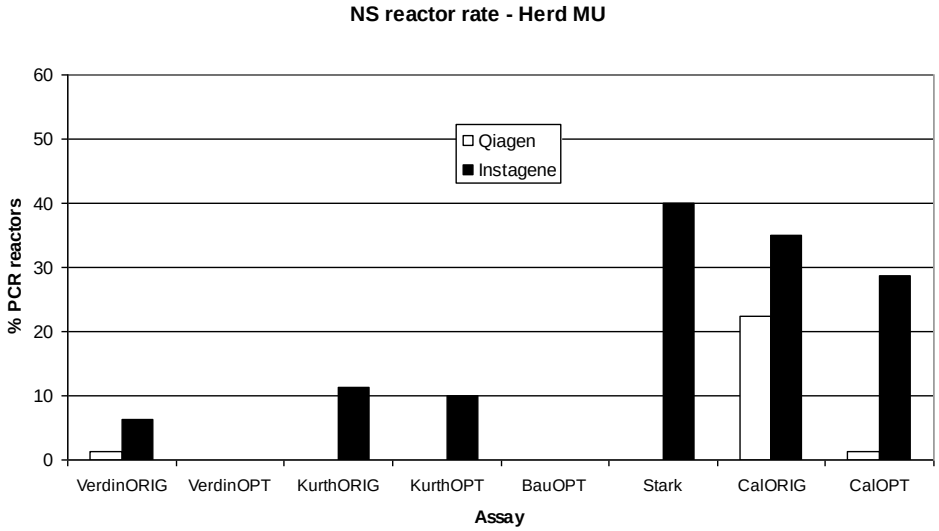
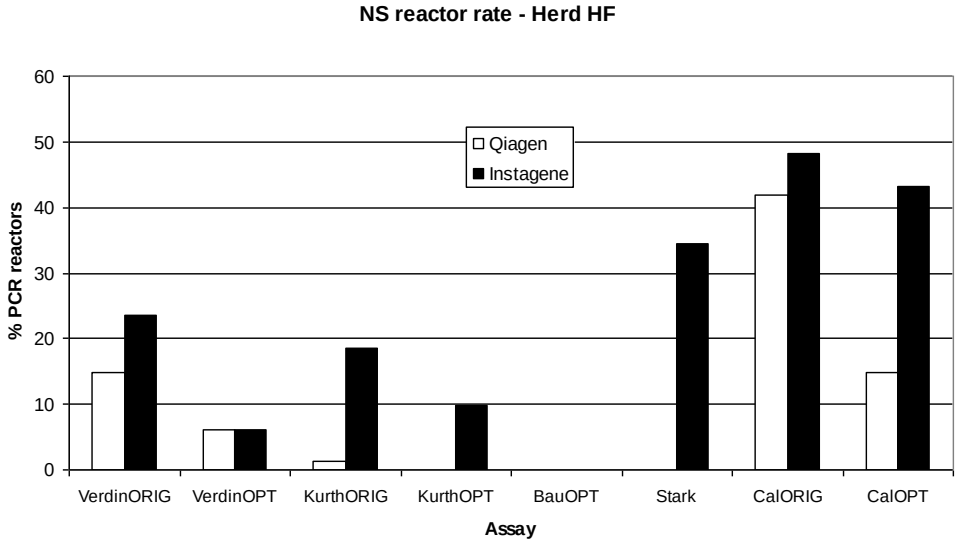


Figure 6.11: Nasal swab PCR reactor rate in two herds with endemic M. hyopneumoniae infection (herds BB, HT), and one higher health herd with suspicious history on serology (herd WW).





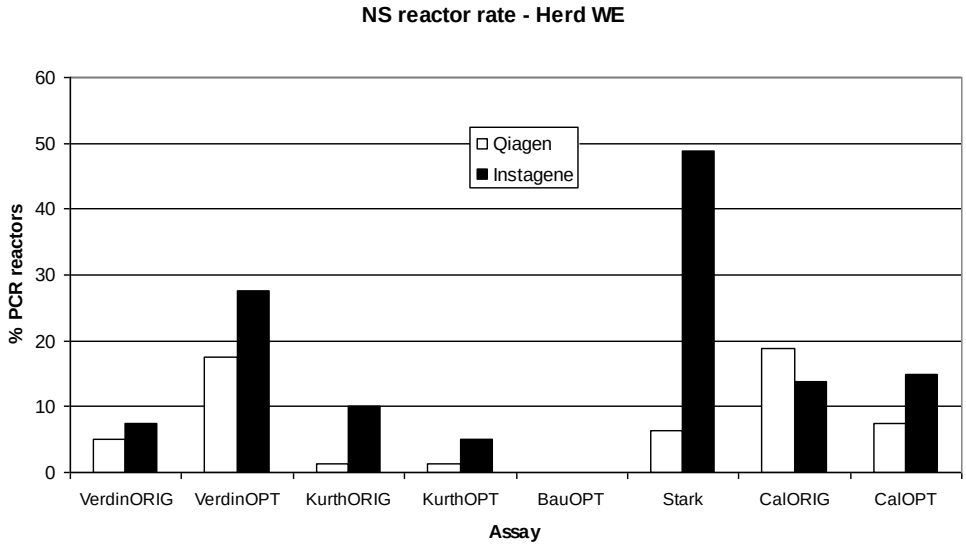


Figure 6.12: Nasal swab PCR reactor rates in three herds considered high health status (low risk)...

... for M. hyopneumoniae infection (ELISA and histopathology negative).

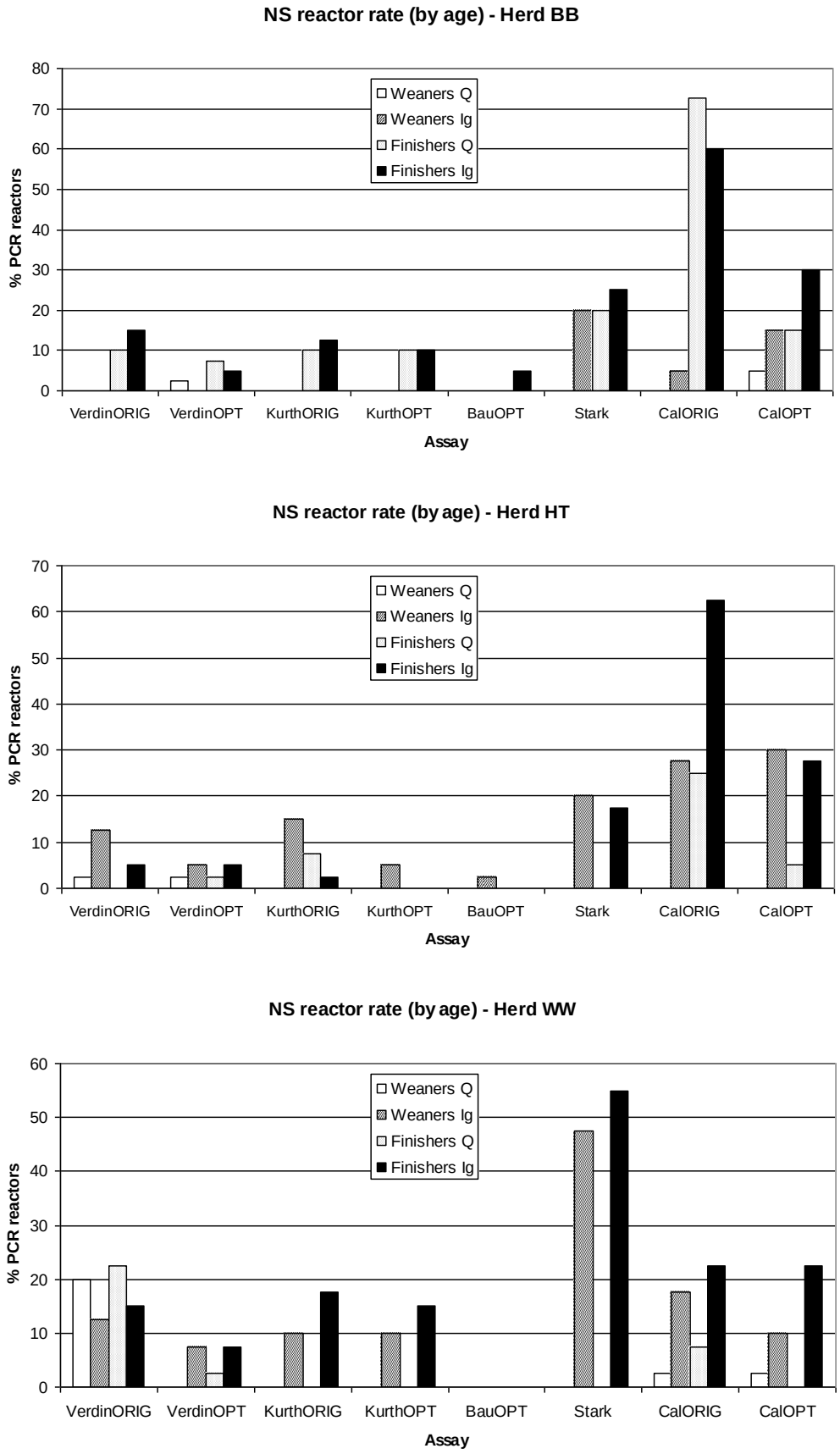


Figure 6.13: Nasal swab PCR reactor rate by age in two herds with endemic M. hyopneumoniae infection..

...(herds BB, HT), and one higher health herd with suspicious history on serology (herd WW).

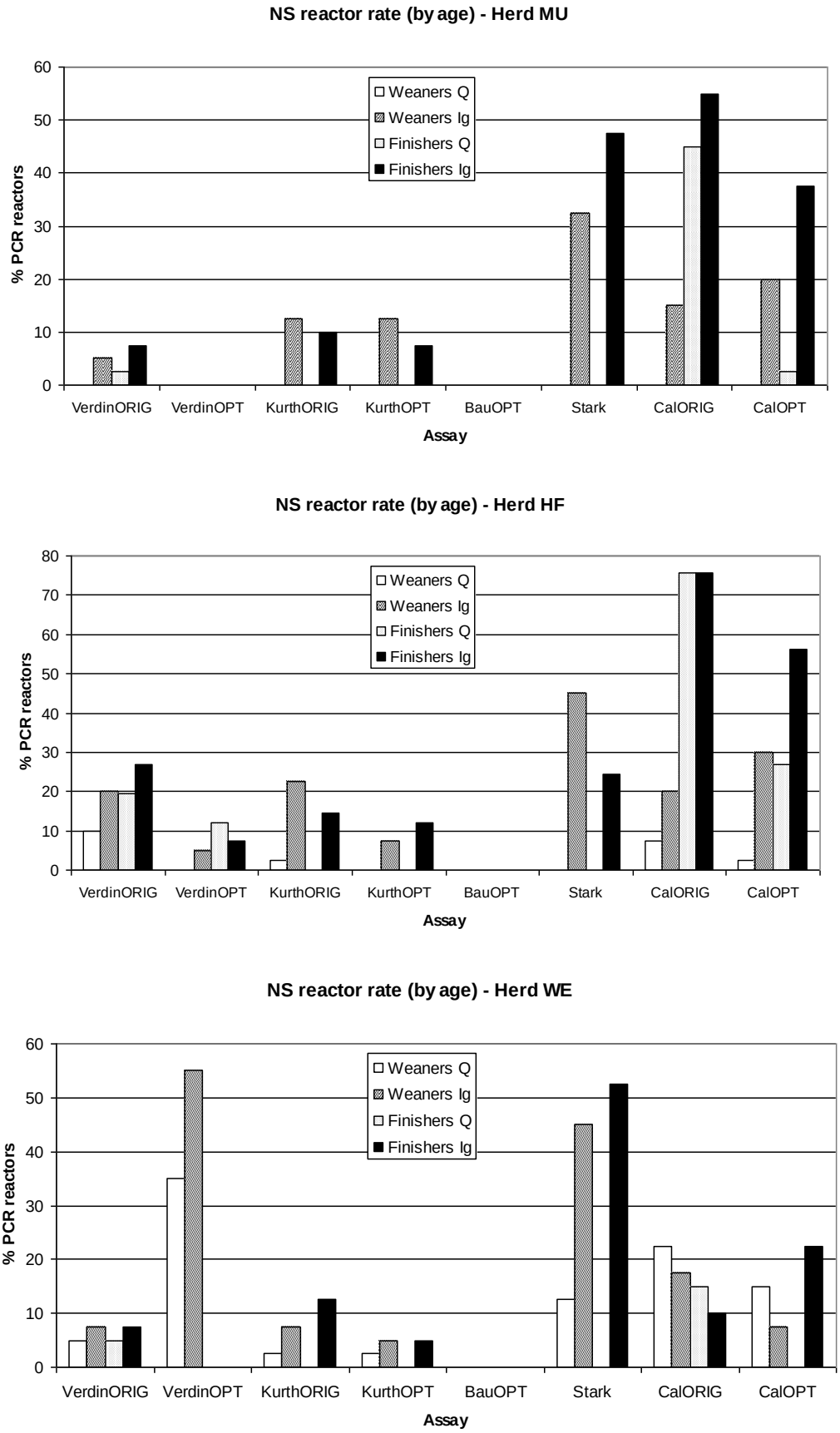


Figure 6.14: Nasal swab PCR reactor rates by age in three herds considered high health status (low risk)...

... for *M.* hyopneumoniae infection (ELISA and histopathology negative).

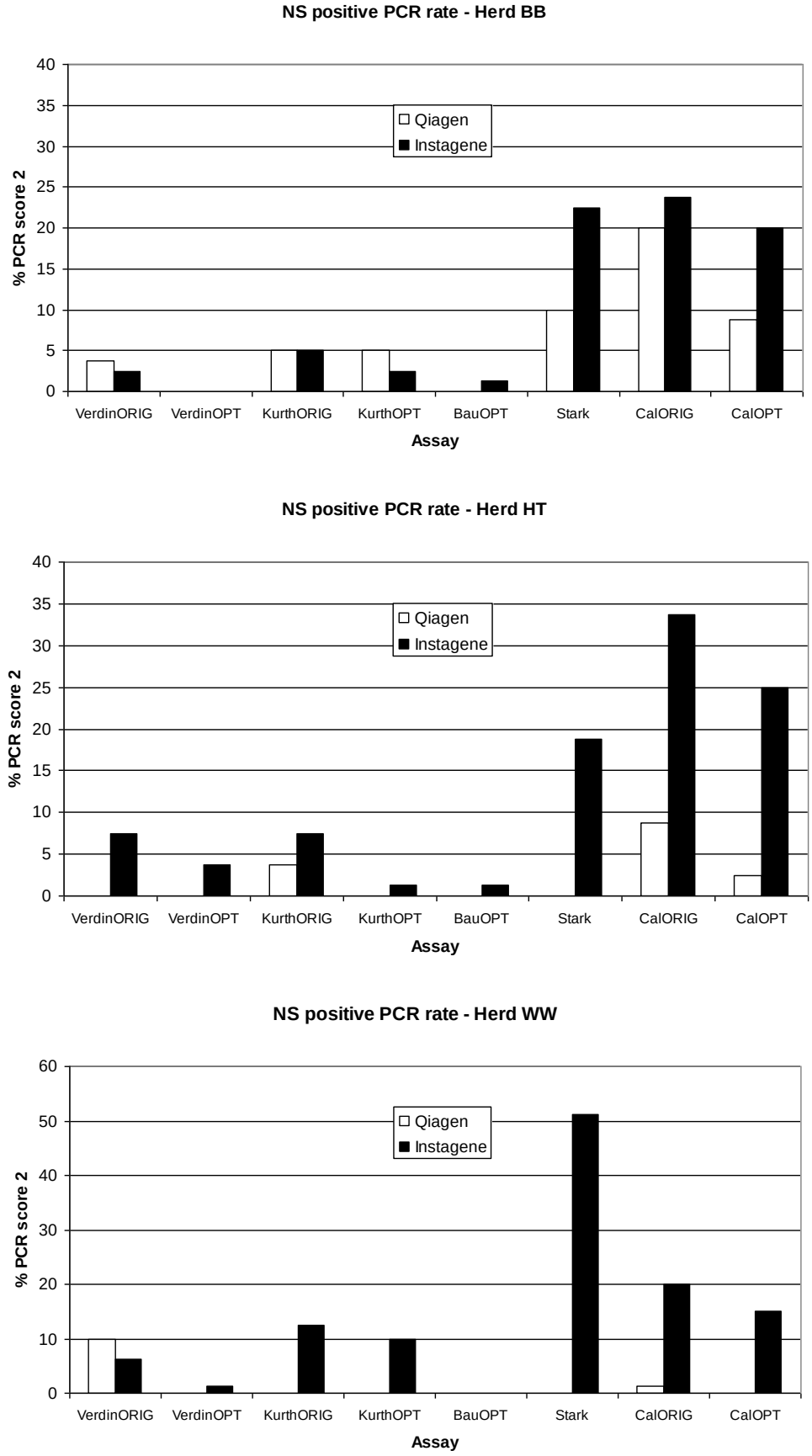


Figure 6.15: Strong positive PCR rates from nasal swabs in two herds with endemic M. hyopneumoniae...

... infection (herds BB, HT), and one higher health herd with suspicious history on serology (herd WW).

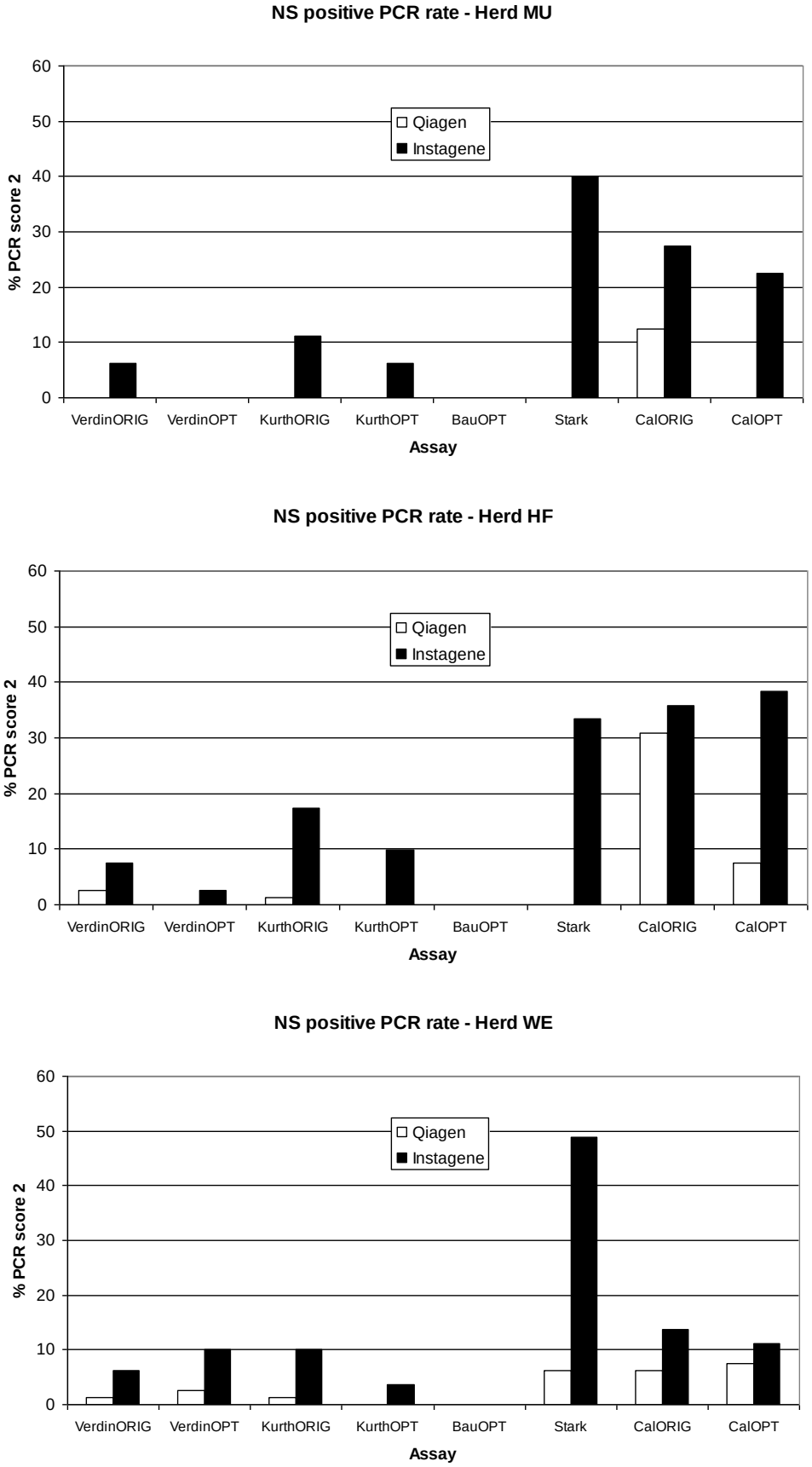


Figure 6.16: Strong positive PCR rates from nasal swabs in three herds considered high health status (low risk)...

... for M. hyopneumoniae infection (ELISA and histopathology negative).

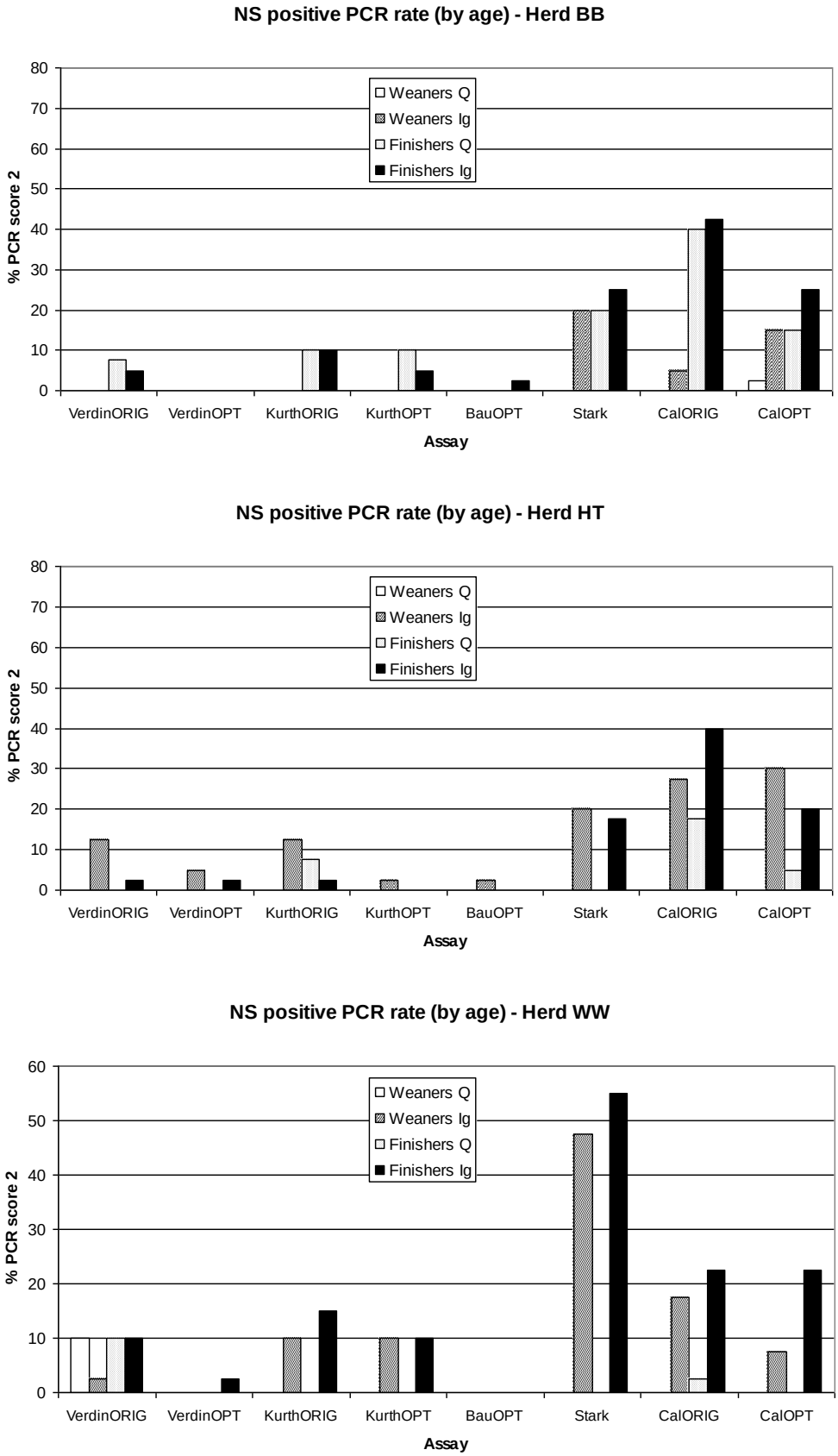


Figure 6.17: Strong positive PCR rates from nasal swabs in two herds with endemic M. hyopneumoniae infection (herds BB, HT), and one higher health herd with suspicious history on serology (herd WW).

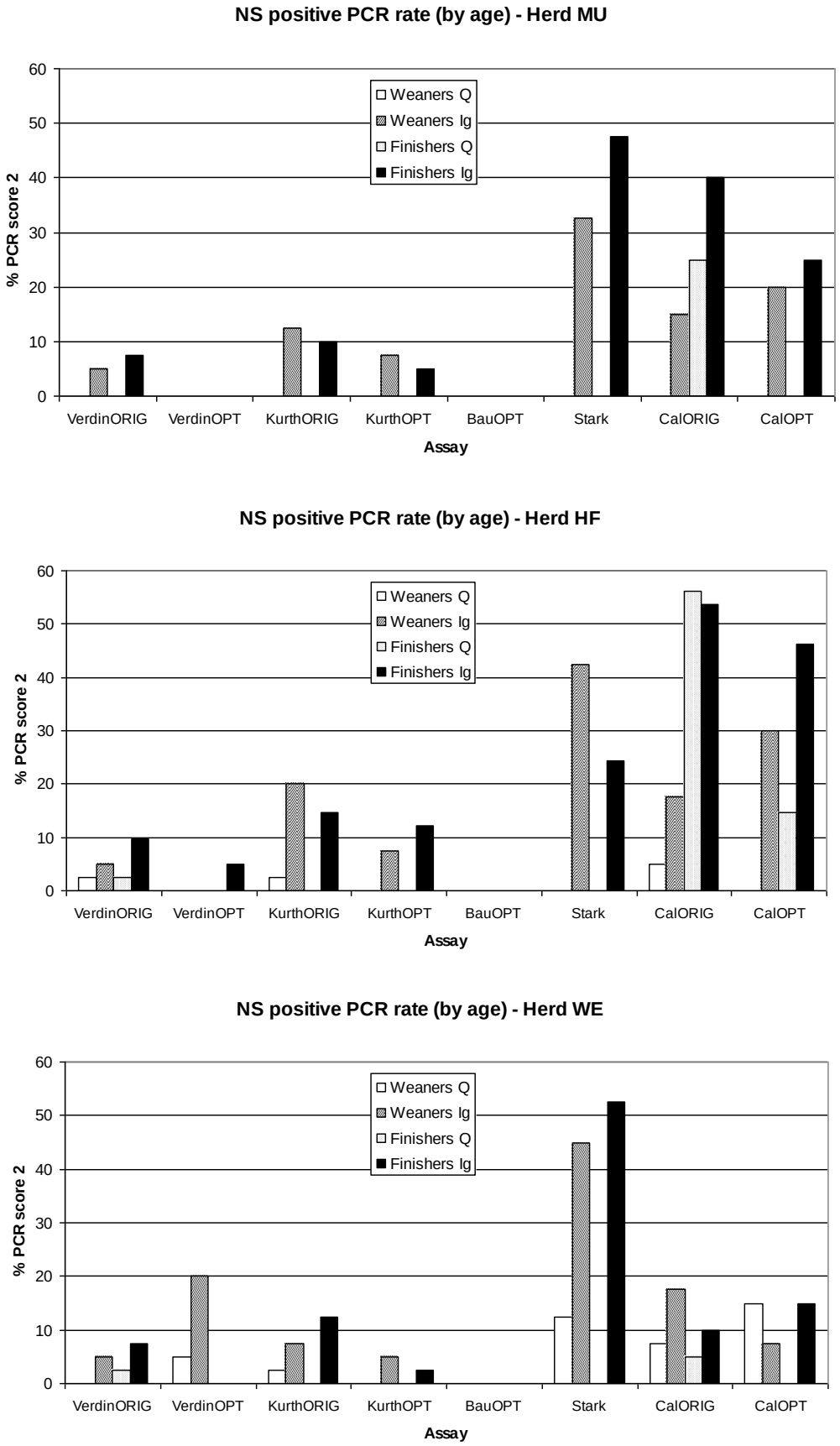


Figure 6.18: Strong positive PCR rates by age from nasal swabs in three herds...

...considered high health status (low risk) for M. hyopneumoniae infection (ELISA and histopathology negative).

Figure 6.19. Typical positive PCR results for the optimised Verdin PCR on lung (top) and nasal swabs (bottom)...
...Target: 706 bp. Lanes containing DNA from M. hyopneumoniae strain 232 (positive control) are designated 232N.

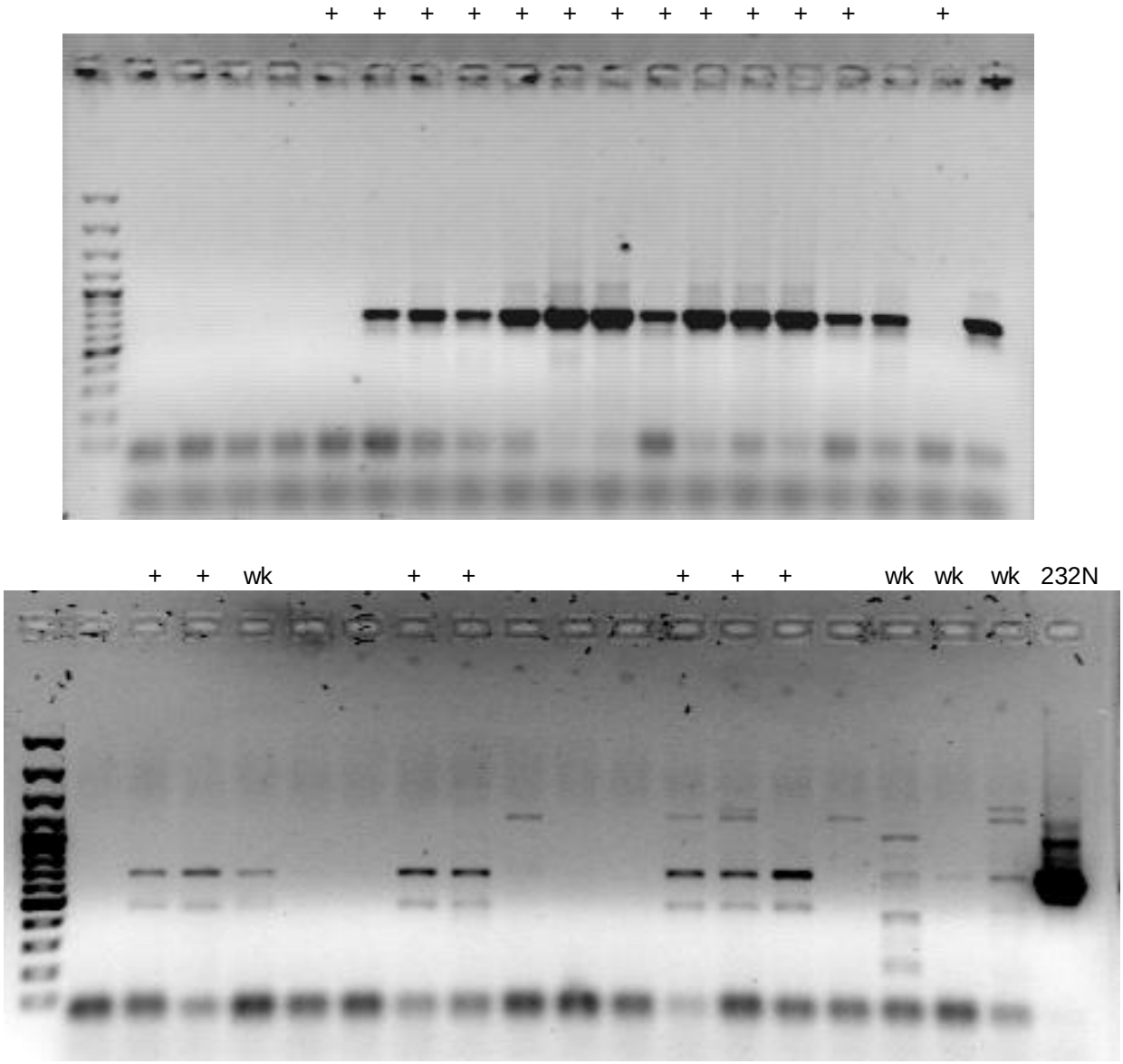
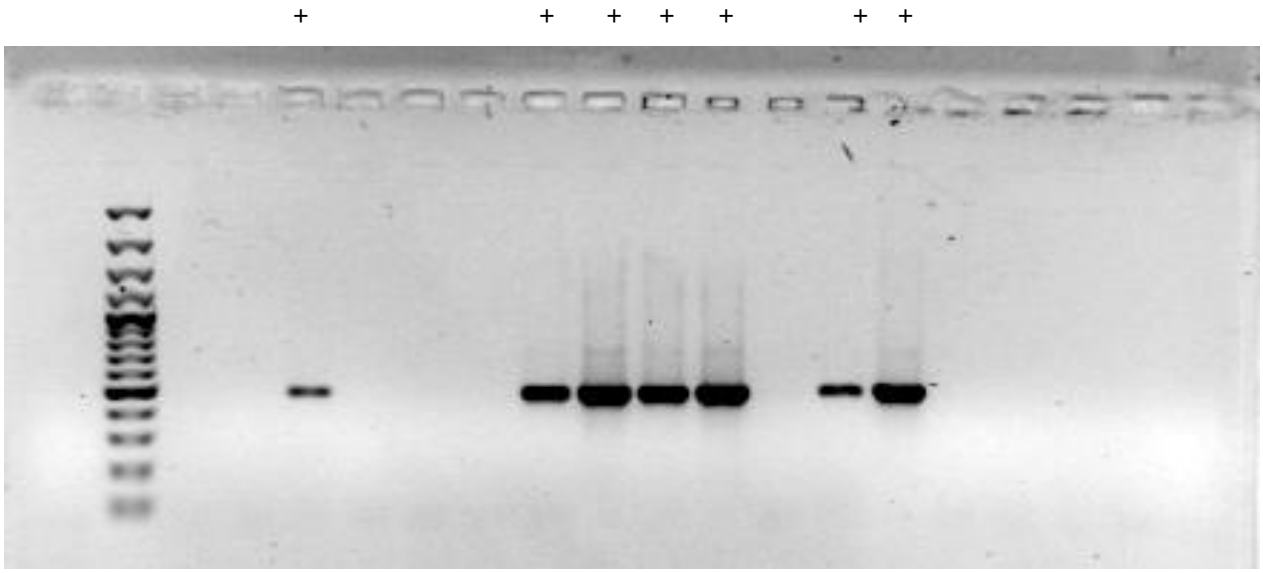


Figure 6.20: Typical positive PCR results for the optimised Kurth PCR on lung (top) and nasal swabs (bottom)...
... Target: 456 bp. Lanes containing DNA from M. hyopneumoniae strain 232 (positive control) are designated 232N.



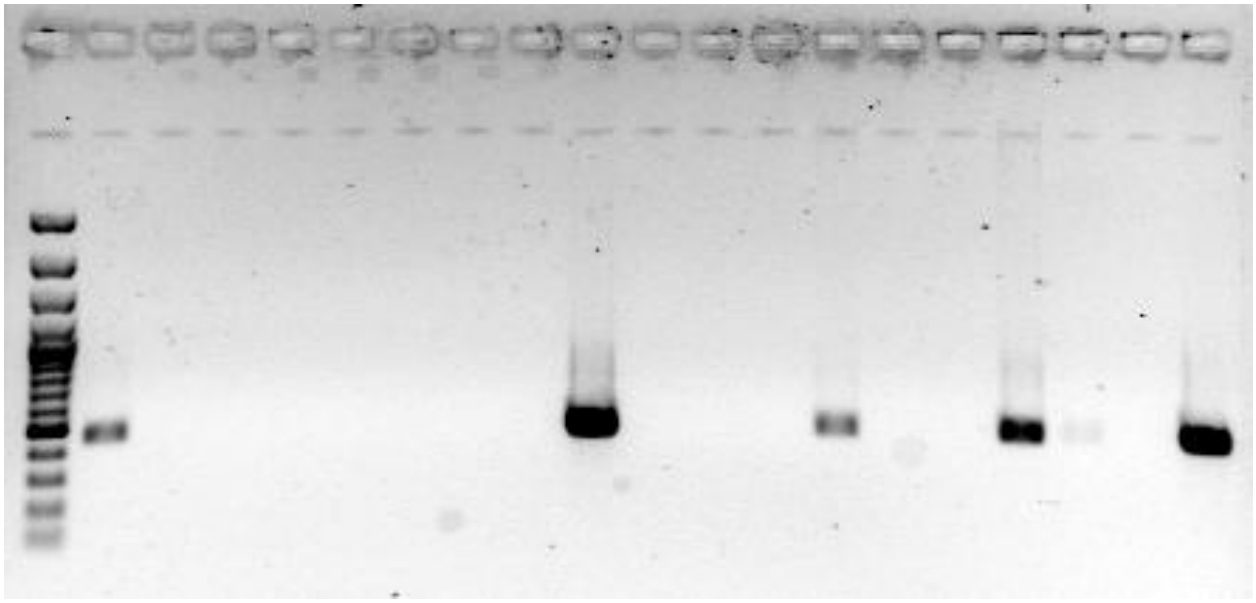


Figure 6.21: Typical positive PCR results for the optimised Baumeister PCR on lung (top) and nasal swabs (bottom)..
... Target: 853 bp. Lanes containing DNA from M. hyopneumoniae strain 232 (positive control) are designated 232.

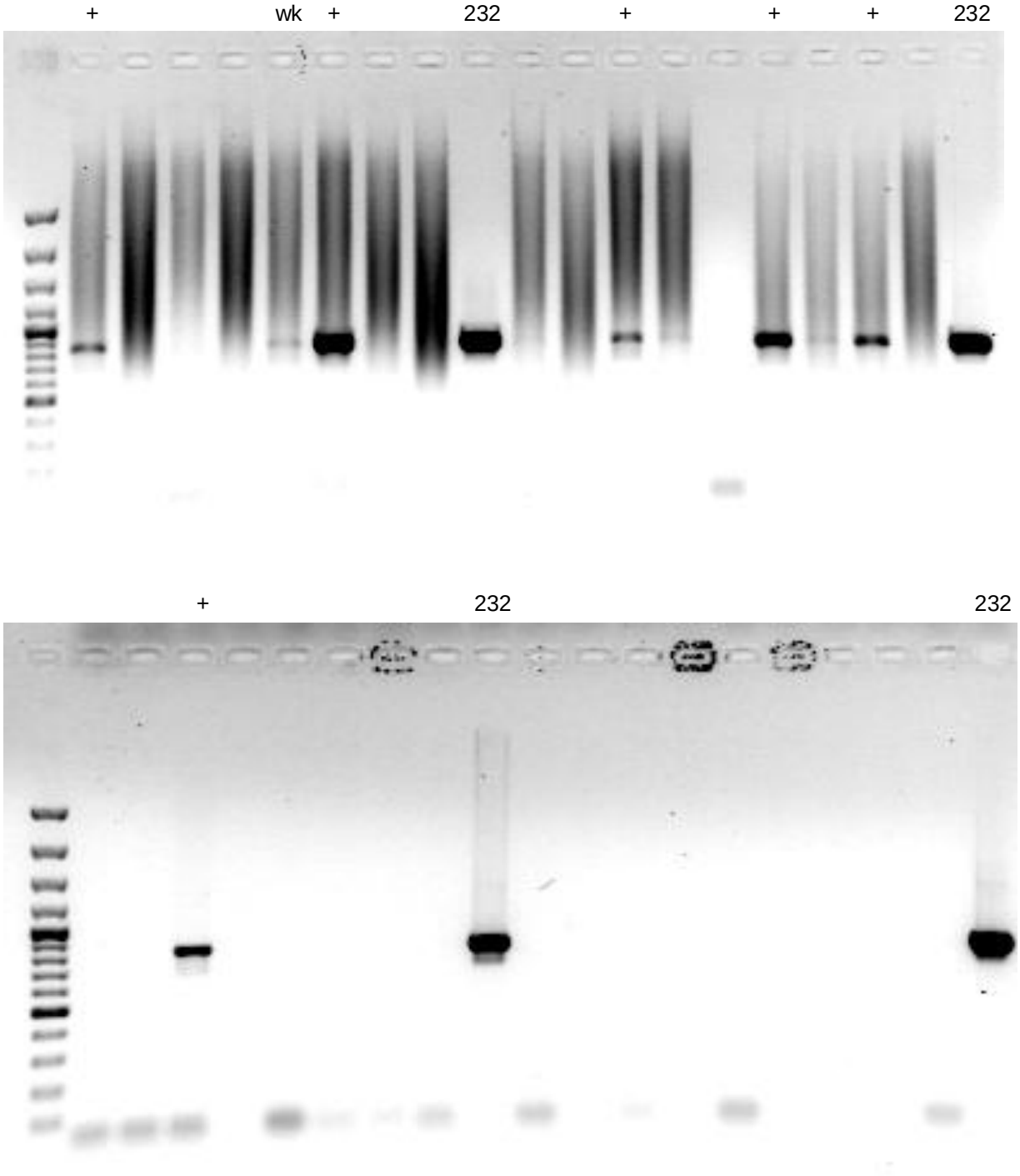
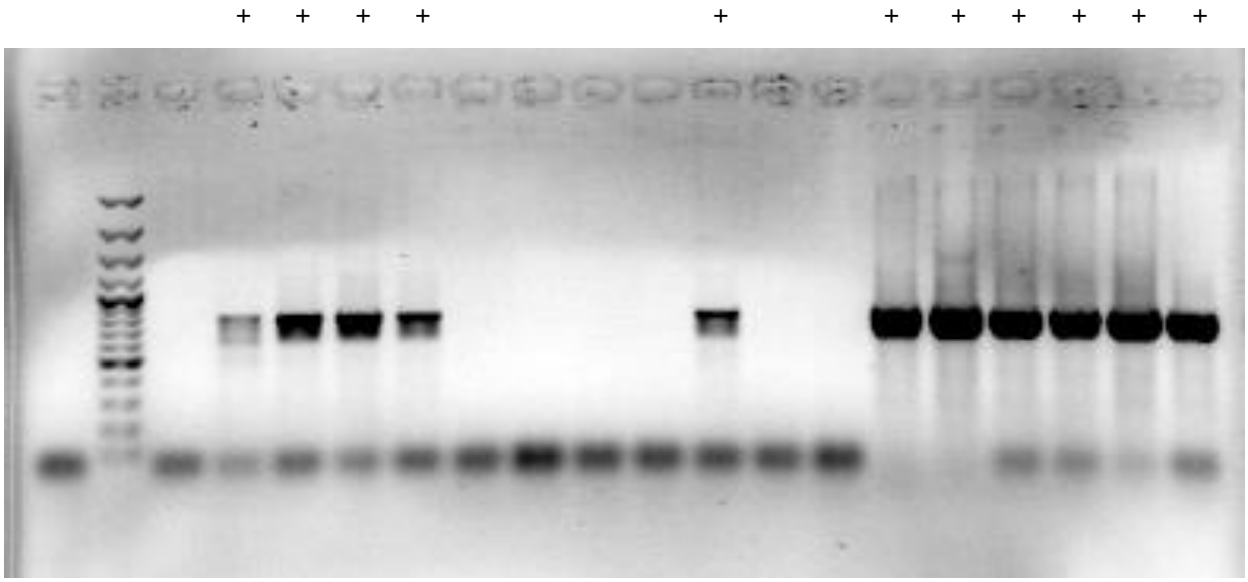


Figure 6.22: Typical positive PCR results for the Stark PCR on lung (top) and nasal swabs (bottom)...
...Target: 808 bp. Lanes containing DNA from M. hyopneumoniae strain 232 (positive control) are designated 232N.



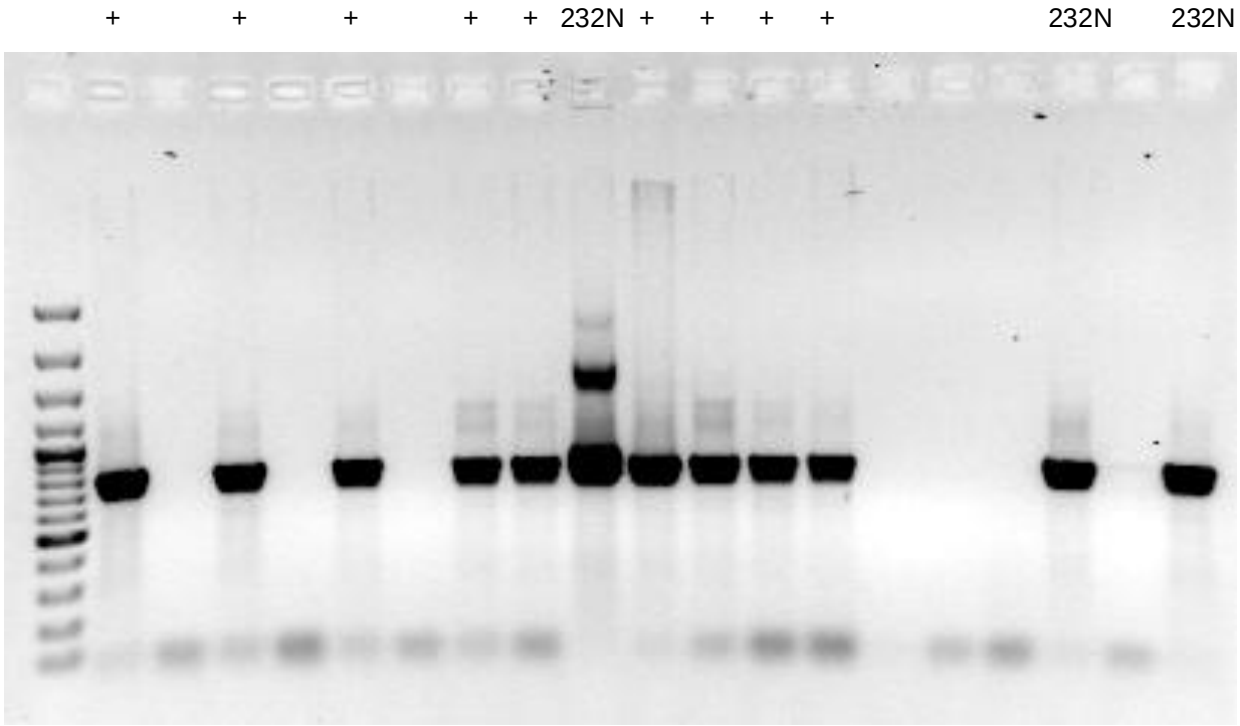


Figure 6.23: Typical positive PCR results for the optimised Calsamiglia PCR on lung (top) and nasal swabs (bottom)..
...Target: 352 bp. Lanes containing DNA from M. hyopneumoniae strain 232 (positive control) are designated 232N (nested) or 232 (2nd gen only).

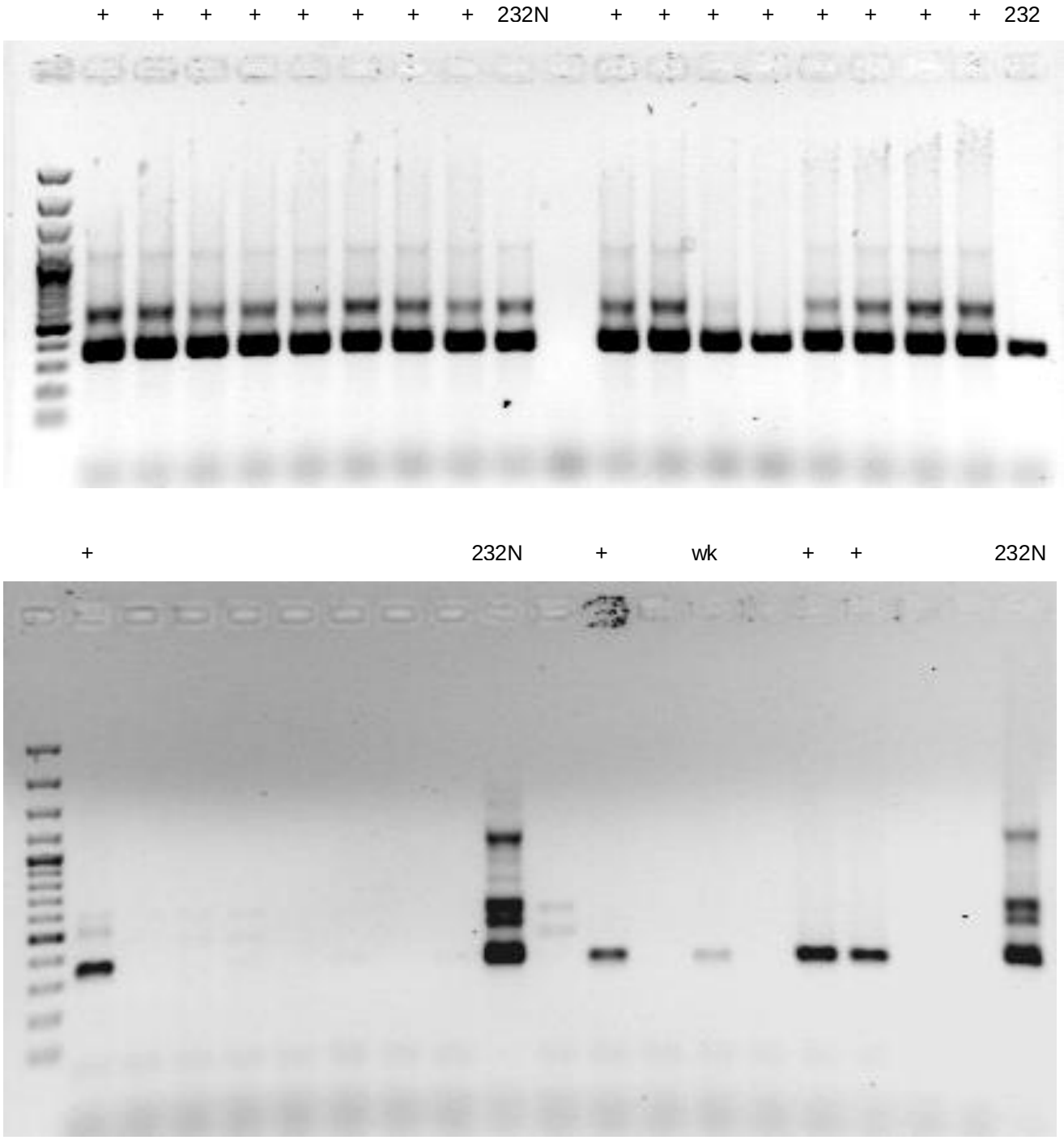
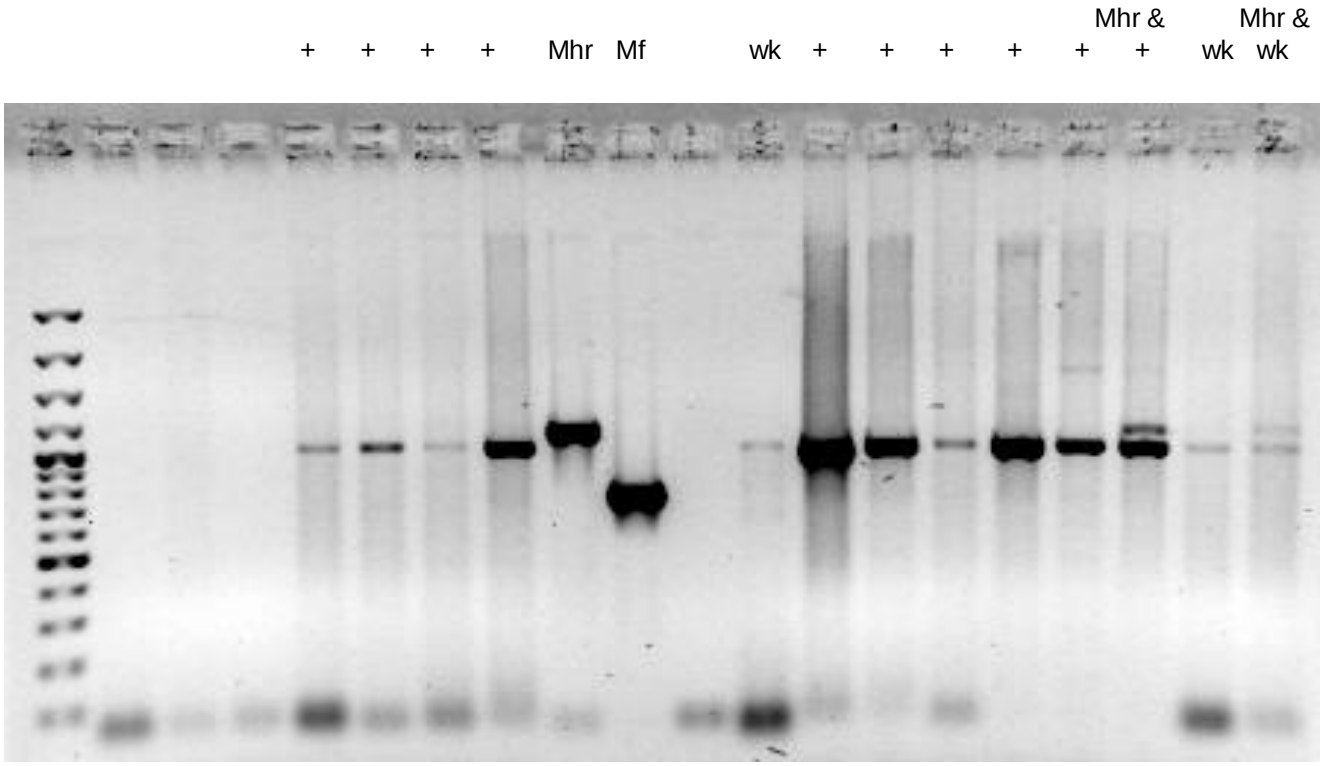


Figure 6.24: Typical positive PCR results for M. hyopneumoniae in the Stakenborg PCR on lungs...
... Lanes 18 and 20 also contain Mhr signal. Targets: Mhr 1129 bp, Mhp 1000 bp, Mfloc 754 bp. Lanes containing DNA from M. hyorhinis and M. flocculare positive controls are designated Mhr and Mf.



6.4.5 REA Analyses

REA on nasal swab sample products amplified in the Calsamiglia PCR showed a common pattern equivalent to the expected cut sites for *Tsp*5091 of 131, 107, 95 and 20 bp (Figure 6.25). However those for the Kurth PCR showed a marked variation between samples derived from the infected herd BB and samples derived from all other herds, including the second infected herd HT (Figure 6.26). While samples from five herds including herd HT yielded the expected size product for *Mse*I REA on the nested Kurth PCR product, all herd BB samples showed a weak band at 227 kb and additional distinct bands at approximately 150 and 80 kb, indicative of a distinct strain difference in that herd. The nasal swab products from the Verdin PCR were generally weak and gave poor REA lines in 2 samples from herd HT and none in the third, with no variation in sizes detected (Figure 6.27).

Figure 6.25: Tsp5091 REA on Calsamiglia product from nasal swabs from various herds according to Table 6.2a....
... Herd identities are indicated above each lane. Expected products are 131, 107, 95 and 20 bp. Sizes of molecular weight markers are indicated by arrows.

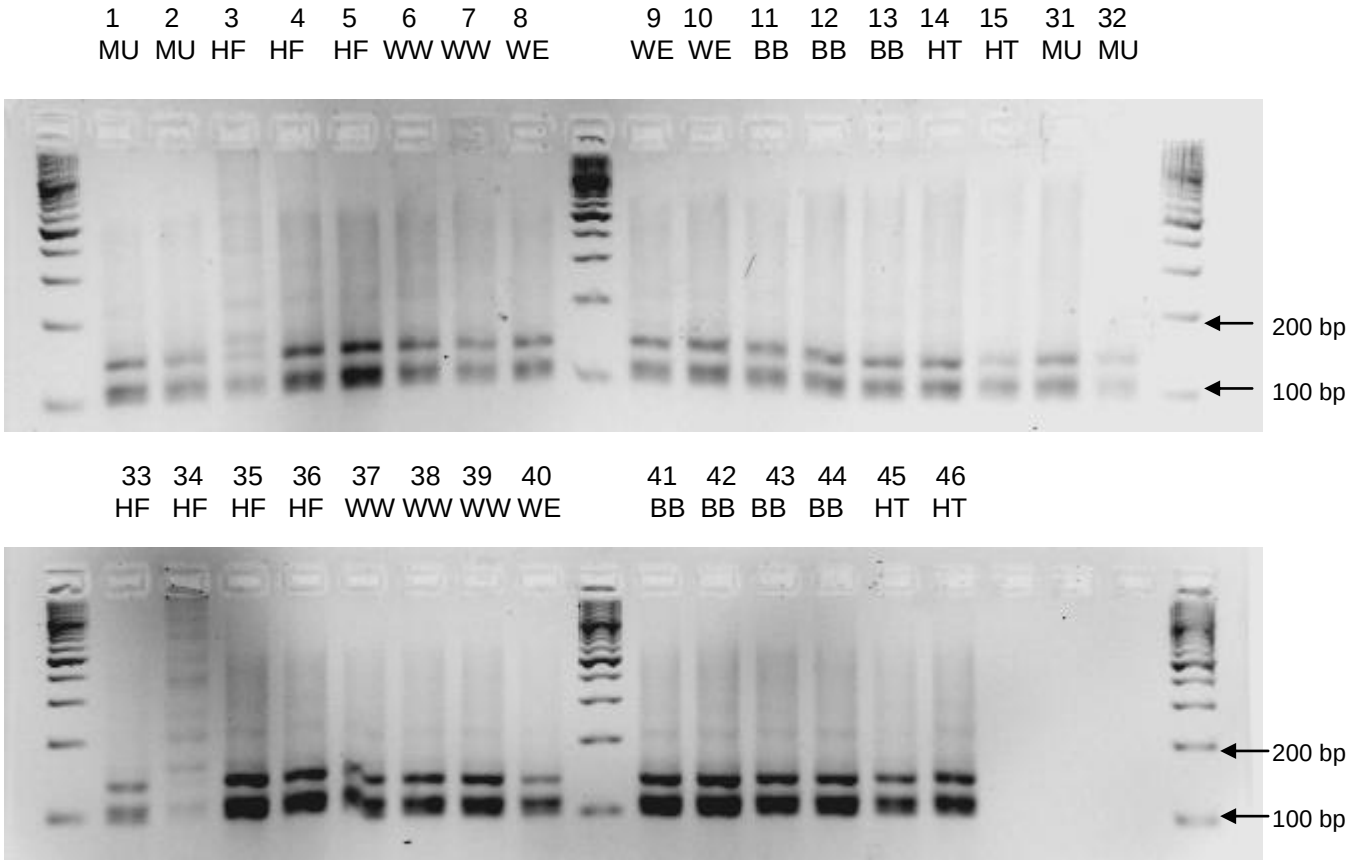


Figure 6.26: Mse1 REA on Kurth product from nasal swabs from various herds according to Table 6.2b...
... Herd identities are indicated above each lane. Expected products are 227, 111, 72 and 46 bp. Sizes of molecular weight markers are indicated by arrows. Note weak band at 227 kb and additional bands at approximately 150 and 80 kb in all herd BB samples.

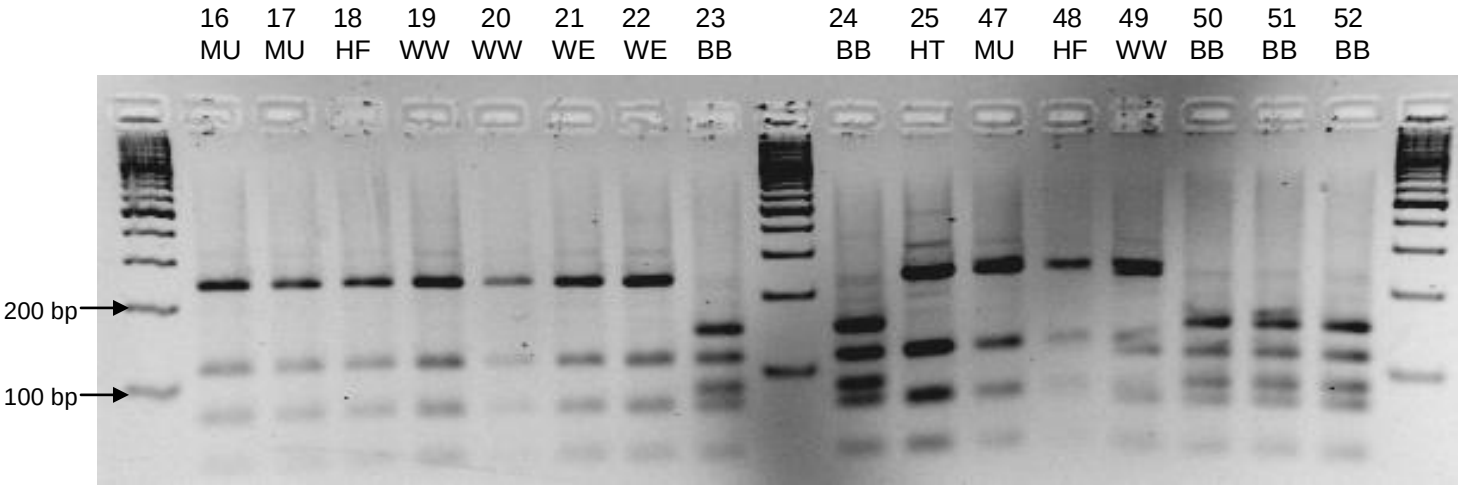
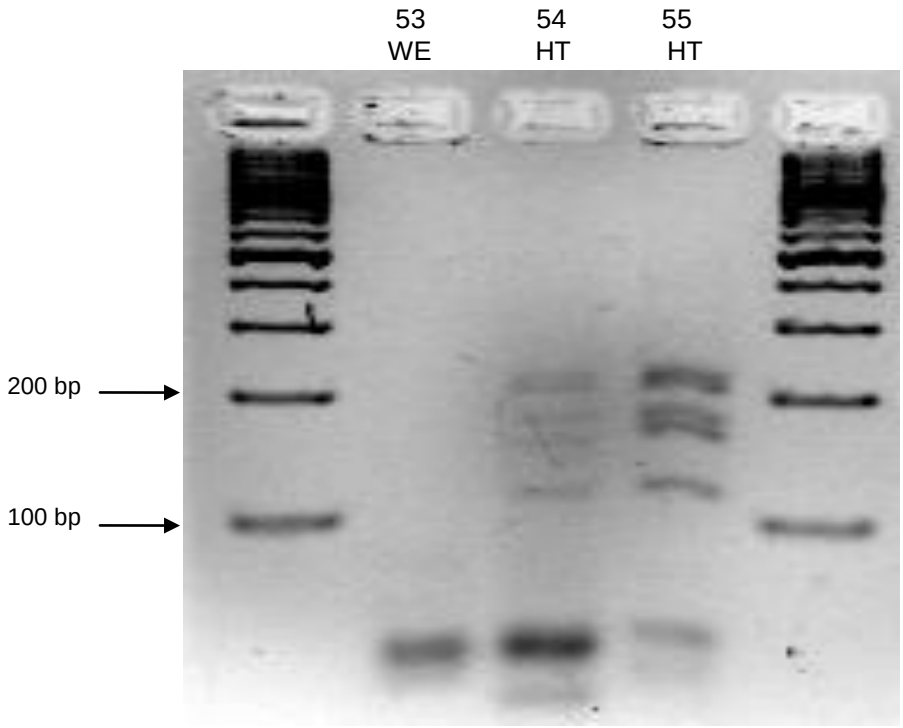


Figure 6.27: Taq1 REA on Verdin product from nasal swabs from herds according to Table 6.2a....
...Herd identities are indicated above each lane, with sample 53 generating only a weak product insufficient to characterise by REA. Expected products are 216, 203, 171 and 113 bp. Sizes of molecular weight markers are indicated by arrows.



6.3 Discussion of Overall Results

The serological results among the six study herds indicated a greater sensitivity for the Dako competitive ELISA compared with the indirect 43 kDa ELISA, although herd classification by either test was similar. While the serological status of herds HT and BB clearly suggested these were both endemically infected herds, the lower (15%) prevalence of Dako ELISA positives in herd WW is still consistent with findings in some infected UK herds early in a screening program (Sorensen *et al.* 1992b).

For lung and nasal swab PCRs, the two commercial DNA extraction kits used in this study were based on differing technologies. The Qiagen DNeasy kit is far more expensive and requires significantly higher labour inputs. This kit employs an extraction method is based on DNA-binding to spin columns with a silica-gel membrane, and has been applied in detection of several bacteria in foods, including salmonellae, *Yersinia spp., Staphylococcus aureus* and *Listeria monocytogenes* (Alarcon *et al.* 2006; Aznar and Alarcon 2003; Jenikova *et al.* 2001; Lambertz *et al.* 2007; Park *et al.* 2001), as well as for *Chlamydia* in human tissue (Mygind *et al.* 2003).

In contrast, the Biorad Instagene kit uses rapid extraction of DNA by a chelex (iminodiacetid acid) ionic resin. Chelex-based kits such as the Instagene kit are considered potentially applicable to a vast array of organisms and/or biological materials (Giraffa *et al.* 2000), and have been reported as useful for detection of salmonellae and *E. coli* in cultures from contaminated food (Grant 2003; Lim *et al.* 2008), for salmonellae from animal environmental swabs (Soumet *et al.* 1999), for tuberculosis and *Legionella* in lung or bronchiolar lavage samples in humans (Chan *et al.* 1996; Hayden *et al.* 2001), but not for detecting *Burkholderia cepacia* after artificial seeding of lung tissue (Merk *et al.* 2006).

Comparisons between the two kits are not frequently reported. The Qiagen DNeasy tissue kit was found more sensitive than the Instagene kit for detection of *Listeria, Salmonella, E. coli* and *Staphylococcus aureus* in vegetables (Elizaquivel and Aznar 2008), while another study found them equally sensitive for *Clostridium botulinum* detection in tissues (Fenicia *et al.* 2007). Our study suggested that the Instagene kit was generally more sensitive, and can be recommended for routine applications on pig lung and swabs for *M. hyopneumoniae* detection.

The PCR assays themselves varied in quantitative sensitivity (Part 4) and the results on field samples were consistent with the assessed test sensitivity. Improving test specificity by increased annealing temperatures in the first or second generation assay was not accompanied by any significant fall in quantitative sensitivity (Part 4), so the results for the optimised assays should be comparable in sensitivity to those reported in the literature with lesser annealing temperature. Increasing the annealing temperature to maximise specificity did however affect sensitivity in the Kurth assay, which yielded its highest specificity (removed cross-reactivity) at the expense of practical sensitivity. Thus the Kurth nested assay at 64°C/64°C was more specific, but lacked sensitivity for infected lung samples compared to a 60°C/64°C regime, so the latter was adopted as the "optimised" version of the assay on field samples to compare with results obtained at parameters of the original published assay (49 °C /56 °C).

For the Stark assay, no increase in annealing temperature was found suitable for reducing cross-reactivity. While this assay proved to be the most sensitive of those evaluated, it cannot be recommended as a laboratory diagnostic assay as it appears to lack specificity for *M. hyopneumoniae*. This assay targets a repeat segment of DNA found in *M. hyopneumoniae* but this segment appears to resemble an insertion sequence that can move to other mycoplasmas, based on results of sequencing the Stark PCR product from *M. flocculare* and *M. hyorhinis* strains. Thus, the repeat sequence that

forms part of the ISHp1 transposase gene (and target of the Stark assay) is not specific for *M. hyopneumoniae*. This gene appears to have gained insertion into some strains of *M. flocculare* and *M. hyorhinis,* explaining the cross-reactivity encountered in the Stark PCR.

Since *M. hyorhinis* and *M. flocculare* are regarded as common inhabitants of the respiratory tract of healthy pigs, and both are considered to reside in the nasal cavity (Kobisch and Friis 1996), these mycoplasmas would be frequent contaminants of nasal swabs. However, few assays have been thoroughly evaluated for specificity across a range of mycoplasmal isolates with some being tested on only a single species of *M. hyorhinis* or *M. flocculare,* and typically only against the type strain for that species. Our study found that some isolates of *M. flocculare* and *M. hyorhinis* are more cross-reactive than others. The three isolates received from the U.S.A., identified in our study as CM 07/06 (*M. flocculare*), CM 07/07 (*M. flocculare*) and CM 07/08 (*M. hyorhinis*), proved to be very cross-reactive with most *M. hyopneumoniae* PCR assays in their original format/annealing temperature. In contrast we found the type strains of *M. flocculare* (MS42, equivalent to MF7 in Table 3.1) and *M. hyorhinis* (BTS-7, equivalent to HR11 in Table 3.1) to be unreactive in several assays (Table 3.5). In order to ensure that the three U.S. mycoplasmal isolates were not contaminated with *M. hyopneumoniae*, we undertook triple cloning procedures on them at EMAI and additionally forwarded the *M. flocculare* isolates CM 07/06 and CM 07/07 to another laboratory for confirmation of their status. Those results confirmed their correct identity.

Additional isolates were received from QDPI and identified as *M. hyopneumoniae* by them based on application of an older one step (Mattsson) PCR to uncloned cultures derived from pig clinical lung materials. With these cultures, we undertook triple cloning before testing and then were able to apply a battery of assays to confirm their identity as either *M. hyorhinis* (9 isolates) or *M. hyopneumoniae* (2 isolates). As the Mattson PCR (Mattsson *et al.* 1995) is similar to the first stage of the original Calsamiglia PCR (Calsamiglia *et al.* 1999), it is therefore not surprising that this assay may have misidentified the *M. hyorhinis* isolates as *M. hyopneumoniae*.

Neither the Kurth nor Calsamiglia assays were found to be sufficiently specific for diagnostic application, based on reactivity with several strains of *M. flocculare* or *M. hyorhinis*. Thus gene segments targeted by the Kurth PCR (unique hypothetical gene segment) and Calsamiglia PCR (16S rRNA gene segment) may be present in other mycoplasmal species. The nasal swab results obtained in the high health herds, particularly herds MU, HF and WE tend to indicate that these were likely to have been due to carriage of *M. flocculare* or *M. hyorhinis* in the nasal cavity. Since REA profiles of amplified Kurth and Calsamiglia PCR products from nasal swabs from low and high health herds were similar, the organisms responsible for the reactivity appear to be carrying the same gene target. However, one infected herd (BB) did show a different fingerpint in its amplified Kurth product from other herds of varying (low and high) health status, suggesting a strain variation in herd BB. Testing of lung samples with these two PCRs was consistent with a high sensitivity in herd BB but the Kurth PCR performed poorly in herd HT, and may be restricted by failure to amplify some strains of *M. hyopneumoniae* as reported elsewhere (Strait *et al.* 2008).

Since the Verdin assay showed no similar lack of response among lung samples from herds BB and HT, it is unlikely that the lack of nasal swab PCR positives in these herds can be attributed to an *M. hyopneumoniae* strain that was undetected by the assay. The low response in nasal swabs for the Verdin assay however is in agreement with the moderate quantitative sensitivity of this assay found in Part 4 (500 fg/uL), and in contrast to the claimed sensitivity for the original assay of 1 fg per reaction (Kurth *et al.* 2002; Verdin *et al.* 2000), since we found a value of 25-500 fg/uL in the current study, which would equate to a one hundred fold higher threshold (≥ 125 fg per reaction). The high

number of reactors detected in the Verdin assay in nasal swabs from weaner pigs in herd WE, but lacking from lung samples, is of interest. Since most of these (>50%) were classified as weak reactors, and the Verdin assay did yield a weak positive reaction with one strain of *M. hyorhinis*, it is possible that the reactions were attributable to a strain of this organism. Further, it is possible that *M. hyorhinis* may show higher carriage rates in weaner pigs, as nasal carriage and disease due to this organism is considered most common among young pigs (Ross 1992). However, of the other original or optimised assays known to cross-react with *M. hyorhinis*, only the Stark PCR gave reactor rates > 20% among Instagene extracts, and the Verdin PCR in its original format yielded far fewer reactors among weaner pigs. Alternatively, the possible presence of low virulent strains of *M. hyopneumoniae* in high health herds such as WE cannot be discounted. But if the reactions in weaner pigs in herd WE were due to the presence of *M. hyopneumoniae* strains of low virulence, it is surprising that few other assays with a higher quantitative sensitivity for *M. hyopneumoniae* did not detect a high proportion of these samples as positive.

The Stakenborg PCR detected a high proportion of affected lung samples and was the only assay to demonstrate 100% specificity with culture isolates in Part 3 and give negative lung results in all the high health herds as well as suspect herd WW (which also tested negative in the Calsamiglia PCR). Both these PCRs, as well as the Stemke duplex PCR, target the 16S rRNA gene of *M. hyopneumoniae*, for which a 1537 nucleotide sequence is described in Genbank accession Y00149. The Stakenborg PCR targets a 1000 bp segment between nucleotides 205-1204, compared to the Calsamiglia PCR whose target is smaller and lies within this segment. The Calsamiglia PCR outer primers (based on those of Mattsson *et al* 1995) target a 649 bp segment between nucleotides 212 and 860 while the inner primers are designed to amplify a 352 bp segment between nucleotides 464-815 of Genbank accession Y00149. Since the Stakenborg PCR was not cross-reactive with *M. flocculare* or *M. hyorhinis* whereas the Calsamiglia inner primers showed amplification from both organisms, the differing 5' primers between the Stakenborg and Calsamiglia PCR is the likely reason for the improved specificity. At present, the published Genbank database identifies some organisms other than *M. hyopneumoniae*, including *M. flocculare* and *M. hyorhinis*, that closely match the inner segment amplified by the inner pair of Calsamiglia primers, so the ability of the outer (Mattsson) primers to exclude these organisms appears to be very important. Since the Mattsson primers appear to be unable to exclude *M. hyorhinis* (based on prior evaluation of the Qld isolates), and the Calsamiglia nested PCR using those primers (or primers very close to that target location) in the first generation reaction definitely amplifies *M. flocculare* and *M. hyorhinis* (from our data), then it is apparent that these similarly lack specificity.

Of interest is that the forward primers for *M. hyopneumoniae* in the Stakenborg multiplex and Stemke duplex PCRs target a single nucleotide sequence within the 16S rRNA gene of *M. hyopneumoniae*. The Stemke PCR amplifies a 254 bp element that lies within the larger segment amplified by the Stakenborg PCR, involving nucleotides 204-458 described in Genbank accession Y00149. While both PCRs identify separate 3' targets that amplify multiple mycoplasmas, their 5' specific *M. hyopneumoniae* targets are nucleotides 204-227 (Stemke) or nucleotides 205-226 (Stakenborg). The results of the current study suggest this sequence appears to be a stable *M. hyopneumoniae*-specific primer target within the 16S rRNA gene.

Conclusions and Recommendations

This study investigated a number of conventional PCRs for *M. hyopneumoniae* for application to cultures and clinical samples such as lung tissue and nasal swabs. The Stark nested PCR and the optimised Kurth PCR could not achieve maximum specificity of the other assays, and both have been reported recently as failing to detect some U.S. strains (Kurth – 25%, Stark 11%) (Strait *et al.* 2008). The optimised Baumeister PCR lacked sensitivity in applications in nasal swabs and lung samples, while the optimised Calsamiglia PCR was sensitive but lacked specificity, with reactivity against several strains of other mycoplasmas in this study. Thus none of these four assays can be recommended for diagnostic application.

In contrast, the optimised Verdin PCR and the Stakenborg multiplex PCR were found the most reliable in examining cultures and lung samples. The Verdin nested PCR was likely to be the better assay for samples containing low concentrations of *M. hyopneumoniae* DNA, as would be expected in nasal swabs, but the current study suggests that either nasal swabs are a poor source of *M. hyopneumoniae* in infected herds, or the Verdin assay is too insensitive to detect *M. hyopneumoniae* from nasal swabs. Further, a recent report that found the internal primer set used in the Verdin PCR fails to detect a significant proportion (25%) of 36 U.S. strains of *M. hyopneumoniae* (Strait *et al.* 2008), indicates this assay could have some limitations in diagnostic applications in Australia. Similarly, a real time PCR based on the ABC transporter gene that is targeted by the Verdin PCR found one U.S. field strain and the reference strain J to be unreactive after multiple passages (Dubosson *et al.* 2004). However, we found little evidence of a lack of reactivity with the limited number of *M. hyopneumoniae* strains available in the current study, except strain J, a highly passaged strain, was often poorly reactive in the Verdin assay. Others have indicated the loss of genetic material from this strain in some culture collections due to high passage is expected to give poor reactivity in the Verdin PCR (Blanchard *et al.* 1996), and thus strain 232 is recommended as the *M. hyopneumoniae* control strain for this PCR.

In order to assess PCR inhibitors in clinical samples, a PCR targeting normal pig DNA (via a portion of the pig beta 2 microglobulin gene) was developed. This was found to be reactive with lung tissue samples and nasal swabs even in nested PCR reactions and under the higher annealing temperature conditions used elsewhere in the study. Thus an assay to assess PCR inhibitory substances in either lung or nasal swab samples can be based on primers developed in the present study to detect porcine beta 2-microglobulin (B2M). However, further optimisation would be required to run this as a duplex assay to demonstrate lack of PCR inhibitors in a given *M. hyopneumoniae* PCR, as the PCR tended to overwhelm components directed against *M. hyopneumoniae* in two assays tested (Kurth and Stark).

In cultures, distinction of *M. hyopneumoniae* (Mhp) from the closely related mycoplasmas *M. hyorhinis* (Mhr) and *M. flocculare* (Mfloc) was readily achieved using the Stakenborg PCR (Mhp vs Mhr vs Mfloc), the Lin duplex PCR (Mhp vs Mhr) and the Stemke duplex PCR (Mhp vs Mfloc) with their original test parameters. A single PCR to detect *M. hyosynoviae* (Assuncao/Ahrens PCR) required a modification to its annealing temperature for improved specificity, while a second single PCR to detect *M. hyorhinis* (Caron PCR) was useful but showed mild cross-reactivity. Since purified strains of *M. hyopneumoniae* were able to be isolated and confirmed in this project, together with a range of *M. flocculare* and *M. hyorhinis* isolates, these can be applied to new investigations such as vaccine studies or evaluation of new diagnostic tests.

This study highlights the unreliability of existing specificity data for *M. hyopneumoniae* PCRs based on studies of few strains of closely related mycoplasmas. Assays with improved sensitivity (for low concentration samples such as nasal swabs) but with concurrent high specificity may depend on further assessment of newer real-time PCR technologies.

Communication of Results

Aspects of the literature review and preliminary design of the study were reported in:

Eamens GJ (2007). Detection of *Mycoplasma hyopneumoniae* in pig samples using polymerase chain reaction tests. In: *Proc World Association of Veterinary Laboratory Diagnosticians* – 13th International Symposium, Melbourne, Australia, 12-14 November 2007 www.csiro.org/files/files/pm04.pdf. Accessed 2/2/09.

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