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Cost-effective testing of beef herds for Johne's disease

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Abstract

The Johne's Beef Assurance Scheme (J-BAS) score is used to determine the risk of Johne's disease (JD) on a beef property by testing faecal samples in pools of size 5. This project was conducted to identify alternative cost-effective pooling strategies for J-BAS testing. Laboratory experiments were conducted to estimate the HT-J sensitivity and specificity for a range of pool sizes. Additionally, simulation models were built to evaluate herd-sensitivity and specificity of various sampling strategies for Sample and Check tests for testing herds of various sizes with a range of prevalence levels. The test sensitivity was estimated of to be 63% for pools of size 5, 73% for pools of size 10, 57% for pools of size 15 and 63% for pools of size 20. Pools of size 10 achieved a higher herd-sensitivity than pools of size 5 for both Sample and Check testing for all simulation scenarios, except when the HT-J specificity was assumed to be imperfect. The results suggest that costs of Sample test can be halved by testing 30 pools of size 10 instead of the current approach of testing 60 pools of size 5 while maintaining the same level of accuracy. Testing 10 pools of size 10 instead of testing 10 pools of size 5 will increase the herd-sensitivity of Check test without increasing testing costs. The new testing regime should be validated in the field and then used to replace the current J-BAS testing regime to save millions of dollars to the beef industry in J-BAS testing costs.

Executive summary

Background

Bovine Johne's disease (JD) is a chronic wasting disease of cattle caused by *Mycobacterium avium* subsp. *paratuberculosis* (Mptb). JD was managed in the past using disease zoning, but the onus to maintain a disease-free herd has shifted to producers in the revised National BJD Strategic Plan 2012-20. The Johne's Beef Assurance Scheme (J-BAS) score is used by the beef industry to determine the risk of JD on a property: the higher the score, the lower the probability of buying a JD-infected cow from a farm. To achieve and maintain a high J-BAS score, producers must demonstrate no history of JD on the property at least in the past five years, prepare and implement a biosecurity plan and get their cows regularly tested. The current testing regime requires conducting a *Sample test* to screen a representative sample (up to 300) of the adult herd (or the whole herd for properties with <300 cattle) followed by a *Check test* every three years to confirm continued freedom of the herd from the infection by testing 50 adult animals in the herd. Both tests are performed using a pooled high throughput Johne's (HT- J) PCR test by pooling faeces from five cattle, i.e. testing 60 pools of five cattle for the Sample test and 10 pools of five cattle for the Check test. Both of these tests combined could cost up to $8400 for a typical beef property with 300 or more animals.

Objectives

This project was conducted to identify alternative, more cost-effective options to achieve the same or better confidence of disease freedom as achieved by the current strategy. We hypothesised that increasing pool size would reduce the costs without impacting the test sensitivity and specificity. Specifically, the project aimed to (a) estimate the effect of pooling on pooled HT-J sensitivity and specificity in a laboratory setting; (b) model the HT-J herd-sensitivity and specificity achieved in real-life scenarios for farms of various sizes, with a range of disease prevalence levels; and to (c) evaluate if herd environmental culture (HEC) currently recommended as equivalent to a 'Check test' for dairy herds could be used in a beef enterprise.

Methods

Archived faecal samples with low, medium and high quantities of Mptb (n= 30) were pooled into pool sizes of 5, 10, 15 and 20 with confirmed negative JD faeces to evaluate the impact of increasing pool size on diagnostic sensitivity. Similarly, samples from JD-free cattle (n = 10) were pooled with faeces from other negative cattle to evaluate diagnostic specificity. Each pool contained faeces from individual positive or negative samples and bulk negative faeces, in ratios of 1:4, 1:9. 1:14 and 1:19, by weight. Pooled faecal samples were then tested using HT-J quantitative (q)PCR. Test sensitivity, relative to individual faecal qPCR results, was calculated as the proportion of the tested pools for a particular pool size within a shedding group that tested positive. The test specificity was estimated as the proportion of the negative pools of a particular size that tested negative.

A simulation model was then developed to simulate herds with a range of sizes (50 to 2000) and prevalence levels (0.5% to 3.0%). Random samples of cattle without replacement were selected from the simulated herds to create pools of sizes 5, 10, 15 and 20 for Sample (300 animals) and Check (50 or 100 animals) testing. A pool including ≥1 infected animal was considered as a true positive pool. The test status of the pool was determined based on the HT-J sensitivity for a pool of a given size as determined in the laboratory experiment. Herd-sensitivity and specificity estimates were calculated to describe the ability of the HT-J test to correctly determine the JD herd status. Sensitivity analyses were conducted to evaluate the impact of input values on simulation results. Similarly, the HEC test was performed on samples collected from simulated herds to determine herd-sensitivity.

Results

The pooled HT-J test sensitivity, compared to an individual HT-J test, was estimated to be 63% for pools of size 5, 73% for pools of size 10, 57% for pools of size 15 and 63% for pools of size 20, although it was not significantly different between pool sizes in the laboratory study. The specificity was perfect for all pool sizes. PCR inhibition was observed particularly in pools constituted with faeces from high Mptb shedding cattle and larger pool sizes but was successfully relieved by 5-fold dilution of the DNA extract. However, 10- to 25-fold dilutions of PCR extract do not appear to provide any additional benefit than the 5-fold dilution.

Simulation modelling results indicated that the herd-sensitivity achieved for pools of size 5 was similar to or better than that achieved for pools of size 10 for all herd sizes and prevalence levels for both the Sample and Check tests scenarios. This pattern was consistent in all scenarios as long as the HT-J specificity was assumed to be perfect. The herd-sensitivity for the Check test involving testing 10 pools, each of size 10 (i.e. n= 100) was 70% higher than the current approach of testing 10 pools, each of size 5. The herd-sensitivity for the HEC test was estimated to be 35.2% based on the limited information available to us.

The herd-sensitivity increased when the results were not adjusted for individual sample HT-J sensitivity or if an imperfect test specificity was assumed. In contrast, the herd-sensitivity decreased when varying proportions of infected cattle were assumed to shed low, medium and high concentrations of Mptb (50%, 40% and 10%, respectively), instead of assuming that these were present in equal proportions. As expected, the herd-sensitivity increased with an increase in disease prevalence and the herd-sensitivity achieved with the Sample test was higher than that achieved with the Check test.

Conclusions and recommendations

The results of this project suggest that the cost of Sample testing can be halved from $7200 to $3600 by switching from a pool size of 5 to 10, without any loss in test or herd-level sensitivity, i.e. by testing 30 pools of size 10 instead of the current approach of testing 60 pools of size 5. The results also suggest that substantial gains in herd sensitivity can be achieved for Check testing by testing 10 pools of size 10 instead of 10 pools of size 5, without increasing testing costs. The sensitivity analyses indicate that it is very important to maintain a perfect HT-J specificity. The laboratory results indicate that diagnostic laboratories should consider PCR inhibition when employing the pooled HT-J, with a current recommendation to test both neat (undiluted) and 5-fold diluted DNA extracts to check and relieve PCR inhibition.

We recommend that 30 pools of size 10 should be used instead of 60 pools of size 5 to reduce the costs for Sample testing while maintaining herd-sensitivity. We also recommend using 10 pools of size 10 for Check testing instead of 10 pools of size 5 to increase herd-sensitivity without increasing costs. A workshop of the stakeholders should be organised to discuss the results of the project and to decide upon approaches for modifying the J-BAS testing protocol in the future. Stakeholders would include representatives of the beef industry, Animal Health Australia as well as scientists and technicians from various State diagnostic laboratories using the HT-J for Sample and Check testing. Further research should be conducted for field validation of the new pooling strategy recommended in this project and to find long-term solutions for PCR inhibition.

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1 Background

Bovine Johne's disease (BJD) is a chronic wasting disease of cattle caused by *Mycobacterium avium paratuberculosis* (Mptb). The bacteria primarily infect the intestines and are shed by the infected animal in faeces leading to contamination of the pasture and the environment. Although the bacteria cannot grow outside the animal body, they have been shown to survive for long periods in the outside environment (Whittington *et al.* 2004; Eppleston *et al.* 2014). Susceptible animals become infected after consuming pasture contaminated with the bacteria, but the disease is usually expressed many months or years after the initial infection. By that time, the animal would have already contaminated the pasture and passed on the infection to other susceptible animals. As a result, the infection is difficult to manage and eradicate.

BJD was previously managed in Australia using the concepts of disease zoning. Animal Health Australia (AHA) undertook a review of the National BJD Strategic Plan 2012-20 during 2015 and prepared a revised plan in consultation with Australian cattle industries and other stakeholders. The revised approach recognises the role of the producer as a decision-maker, requires no State or Territory regulation to manage the disease and imposes no regulated transactional or movement restrictions on producers (Animal Health Australia 2015).

However, the deregulation of BJD has put the onus on beef producers to maintain a disease-free herd or to improve the Johne's Beef Assurance Scheme (J-BAS) score of their herd (Animal Health Australia 2019). J-BAS score provides information about the risk of BJD being present on a property and can be used by the sellers to indicate their property status and by the buyers to assess the risk of purchasing a BJD infected animal. To achieve and maintain a high J-BAS score, producers must demonstrate no history of BJD on the property at least in the past five years, prepare and implement a biosecurity plan, and get their animals regularly tested.

The current testing protocol requires conducting two tests: (a) a *Sample test* to screen a representative sample (up to 300) of the adult herd (or the whole herd for properties with <300 cattle) and (b) a *Check test* to test 50 adult animals in the herd (Animal Health Australia 2019). Although a number of tests including ELISA and pooled faecal culture have been approved for use in both test procedures, pooled High-Throughput – Johne's (HT-J) faecal quantitative PCR (qPCR) is considered to be a test of choice as it has been thoroughly tested and validated (Plain *et al.* 2014b) and detects the bacterial DNA rather than antibodies as in ELISA. Moreover, it has a higher sensitivity and specificity than the ELISA and a relatively fast turnaround compared to faecal culture (Whittington 2010; Whittington *et al.* 2013; Plain *et al.* 2014a). Faeces from five cows are usually pooled before testing.

However, the costs for producers for following the testing protocol are substantial. For example, for a typical beef property with 300 or more animals, using a pool size of 5 would require testing of 60 pools for initial testing and 10 pools for follow-up testing. Assuming the cost of the pooled HT-J test to be $120 per test, a Sample and a Check test would cost $7200 and $1200 to the producer, respectively. Due to the high costs involved in testing to achieve or maintain a good J-BAS score, this project was conducted to investigate if the cost of testing could be reduced without sacrificing accuracy (diagnostic sensitivity and specificity) or compromising the J-BAS status of a herd.

We hypothesised that the testing cost can be reduced by increasing pool sizes. For example, the testing cost will nearly halve if the pool size is increased to 10, by keeping the sample size constant, assuming that the costs for sample collection and testing for a pool of size 10 are almost the same as the costs for a pool of size 5. We are not aware of any previous research conducted to specifically determine the sensitivity and specificity of the HT-J test at different pooling rates for bovine faeces. However, we had conducted a study to investigate the effect of pooling on pooled faecal culture sensitivity in sheep (Dhand *et al.* 2010), which identified that laboratory sensitivity at different pool sizes for multibacillary (high shedding) sheep was not significantly different, but the sensitivity

decreased significantly with pool size in paucibacillary (low shedding) sheep. Similar work done by Eamens *et al.* (2007c) in cattle found that, with an increase in pool size from 5 to 10, the sensitivity of pooled faecal culture marginally reduced from 100% to 97% and that of direct PCR from 87% to 84%. A similar study conducted for cattle shedding low concentrations of Mptb showed that a pool size of 5 was able to detect 6 of the 8 low shedding cattle, but the numbers reduced as pool size increased (Eamens *et al.* 2007a). It is worth mentioning here that all this work was done for pooled faecal culture or direct PCR and not the HT-J, the current test of choice for BJD testing. While these results are relevant for HT-J testing and probably the basis of defining a pool size of 5 for the current guidelines, to our knowledge, the effect of pooling has not been investigated for the HT-J. This project was conducted to address this gap in our understanding of the effect of pooling.

2 Project objectives

The overall aim of the project was to investigate ways in which the cost to beef producers of herd screening tests for BJD can be reduced without sacrificing accuracy or compromising the J-BAS status of a herd. Specifically, the project was conducted to:

(1) estimate the effect of pooling on pooled HT-J sensitivity and specificity (accuracy) in a laboratory setting;

(2) model HT-J sensitivity and specificity achieved in real-life scenarios for farms of various sizes, with a range of disease prevalence levels and by selecting a varying number of pools and pool sizes for both the 'Sample' and 'Check' testing;

(3) evaluate if herd environmental culture (HEC) currently recommended as equivalent to a 'Check test' for dairy herds could be used in a beef enterprise without compromising the sensitivity and specificity achieved in real life scenarios.

It was expected that successful completion of these objectives will enable us to identify alternative cheaper options for BJD testing that provide at least the same level of confidence as the currently used 'Sample test' and 'Check test' protocols.

3 Methodology

The project was undertaken in two phases: a laboratory investigation was initially conducted to achieve objective (1) followed by simulation modelling to investigate objectives (2) and (3).

3.1 Laboratory investigation

3.1.1 Selection and characteristics of cattle faecal samples

We used archived faecal samples collected from naturally infected cattle or previous cattle trials conducted by the Farm Animal Health group at the University of Sydney, Camden (Plain *et al.* 2014a). The samples were stored at -80^{o}C prior to processing for this study.

The *negative* samples were from those collected from control-unexposed cattle during longitudinal animal trials performed at the University of Sydney, Camden (Begg *et al.* 2018). The individual negative faecal samples (n=10) were used for the testing of specificity, with additional bulk negative faecal samples (n=2) used for the creation of pools. All negative faecal samples were negative in the individual Mptb faecal qPCR (HT-J test) and faecal culture tests (Plain *et al.* 2014a; Begg *et al.* 2018).

The *positive* samples were from naturally infected beef cattle from a herd in Tasmania, Australia. The samples had previously tested positive in an individual HT-J qPCR test. These were selected to

provide a representation of how the pooled HT-J qPCR test may perform with field samples. This was informed from the range of HT-J qPCR test results identified during the validation of the HT-J qPCR method on Australian cattle (Plain *et al.* 2014a). The positive faecal samples were categorised into Low, Medium and High faecal shedding groups based on the DNA quantity detected in individual HT-J qPCR results. The Mptb DNA quantity detected in the qPCR test was between 0.0005 – 0.005 pg in the low shedding group (n=10), between 0.005 – 0.1 pg in the medium shedding group (n = 10), and between 0.1 - 10 pg in the high shedding group (n=10). However, not all samples selected were Mptb faecal culture positive; as per the validation of the HT-J test, more samples tested positive using the HT-J test than in faecal culture. This was particularly evident at the lower DNA quantity ranges, as the low, medium and high group, had 3, 8 and 10 samples culture positive, respectively (Plain *et al.* 2014a).

3.1.2 Pooling of samples

Individual positive faecal samples (n=30) were pooled into pool sizes of 5, 10, 15, and 20 so that each pool contained faeces from individual positive and bulk negative faeces in the ratios of 1:4, 1:9. 1:14 and 1:19, by weight, respectively (Table 1). Similar pools were created from individual negative samples (n =10). In total, 120 pools were created from positive samples for the estimation of sensitivity (30*4 pool sizes) and 40 pools from negative samples for the estimation of specificity (10*4 pool sizes). The pooled faecal samples were blended with saline to create a homogenised mixture. The faecal samples were homogenised with an Interscience MiniMix blender for 60 s, before the homogenised faecal samples were transferred to a sterile 25 mL tube.

Table 1. Creation of pools by pooling positive and negative archived faecal samples.

Pool size	Pool creation			Test input
	Positive faeces (g)	Negative faeces (g)	Saline (ml)	Amount (g)
5	2	8	10	4 +/- 0.3
10	1	9	5	3 +/- 0.3
15	1	14	7.5	4 +/- 0.3
20	1	19	10	3 +/- 0.3

3.1.3 Pooled HT-J test

DNA extraction

Following blending of the pooled faeces, 3 or 4 ± 0.3 g of homogenised faeces (Table 1) was added to 10 mL of sterile saline in a 15 mL sterile tube. The faecal suspension was mixed for 5 s and allowed to settle for 5 min, before inverting the tube to dislodge floating debris. The suspension was allowed to settle for 30 min, followed by the transfer of 3 - 5 mL of the supernatant to a 15 mL sterile centrifuge tube. The supernatant was centrifuged at 900 x *g* for 30 min to obtain a pellet. The supernatant was then discarded, and 600 µL of lysis buffer (BioSprint 96 one-for-all vet kit; Qiagen – 597.2 µL buffer RLT and 2.8 µL carrier RNA reconstituted in buffer AVE) was added to the pellet. The lysis and pellet suspension were then transferred into 2.0 mL screw cap bead tubes, containing 0.3 g of 0.1 mm diameter zirconia/silica beads for mechanical bead beating.

Bead beating and magnetic bead-based DNA purification were performed as previously described by Plain et al. (2014). Briefly, the suspension was mechanically disrupted with a TissueLyser II (Qiagen), centrifuged at 16,000 x *g* for 3 min in a microfuge, and 500 µL of the supernatant transferred to a sterile 1.5 mL tube and the centrifugation repeated. The DNA purification was conducted with the

BioSprint 96 One-for-all Vet kit (Qiagen) using 96-well plates and an automated magnetic particle processor (MagMax Express 96; Life Technologies). A process control (buffers alone) and Mptb positive control (5.67 x 10^3 Mptb/well) were included during the DNA purification step.

Dilution of DNA extracts

The eluted DNA extracts were further diluted 5- and 25-fold. This was based on the recommendations from a previous study, which investigated PCR inhibition in cattle faecal samples, in which the dilution of the DNA extract increased the overall sensitivity of the individual HT-J qPCR from 55 to 80% relative to faecal culture (Acharya *et al.* 2017). Dilutions were performed automatically with AVE buffer (RNase free water with 0.04% sodium-azide, Qiagen) using the QIAgility (Qiagen). In addition, we included a 10-, 15- and 20-fold dilution of the DNA extracts for the faecal pool size 10 samples to determine the optimal dilution to relieve PCR inhibitors for this pool size.

qPCR

The undiluted and diluted extracts were assessed using qPCR. The undiluted and 5-fold DNA dilutions were tested in duplicate qPCR reactions, while the 25-fold DNA dilution was tested in a single qPCR replicate.

An IS*900* qPCR was performed to detect Mptb DNA using Mx3000P real-time PCR instrument (Stategene, Agilent) (Kawaji *et al.* 2007; Plain *et al.* 2014a). Each qPCR reaction had a total volume of 25 µL; 5 µL of DNA template, 250 nM each of forward and reverse primers (MP10-1 5'-ATGCGCC ACGACTTGCAGCCT-3' and MP11-1 5'-GGCACGG CTCTTGTTGTAGTCG-3'), 12.5 µL of SensiMix SYBR Low ROX qPCR master mix (Bioline) and 7.4 µl of nuclease-free water. The reaction parameters were: initial denaturation at 95˚C for 8 ½ min, 40 cycles of denaturation at 95˚C for 30 s and annealing/extension at 68˚C for 60 s, followed by melt curve analysis from 65 to 95˚C. Mptb DNA quantification was conducted with reference to a standard curve included in every qPCR experiment, comprising a 10-fold serial dilution of Mptb genomic DNA ranging from 10 – 0.001 pg/reaction.

Classification criteria

The criteria for a positive IS*900* qPCR amplification was an amplification curve with Tm range of 89.1 ± 1.5^{o}C. The acceptance criteria for each IS*900* qPCR experiment were: (i) an amplification efficiency for the Mptb genomic DNA standard curve of between 90 – 110% for at least 4 of the 5 standards, with at least one of the standard 5 (0.001pg) IS*900* qPCR replicates having positive amplification, and (ii) a negative result (no positive amplification curve) for the no template control. The acceptance criteria for each 96 well qPCR extraction plate was: (i) the positive faecal control was positive in the IS*900* qPCR (replicates with positive amplification curves), and (ii) the negative process control had a negative result in the IS*900* qPCR (no positive amplification curve).

To be classified as a HT-J positive result, a sample had to meet the following criteria: (i) the HT-J extraction plate and IS*900* qPCR experiments including this sample passed the above acceptance criteria (ii) the average DNA quantity of the positive IS*900* qPCR sample replicates exceeded the ≥ 0.001 pg Mptb genomic DNA cut point. A sample was considered positive if either of the undiluted or diluted DNA extracts was HT-J positive.

3.1.4 Estimation of sensitivity and specificity

Test sensitivity, relative to individual faecal qPCR results was calculated as the proportion of the tested pools for a particular pool size within a shedding group that tested positive. Sensitivity was compared between pool sizes using generalised linear mixed models using the positive/negative status of the pools as an outcome, the pool size as a fixed effect and the animal ID as a random effect. The test specificity was estimated as the proportion of the negative pools of a particular size that tested negative.

3.2 Simulation modelling

3.2.1 Evaluation of HT-J herd-sensitivity and specificity in real-life scenarios

Model

A simulation model was developed in the software environment R (version 3.5.3, © 2019 The R foundation for statistical computing), to simulate sampling of cattle herds and testing with a pooled HT-J assay, to estimate the herd-level sensitivity of various sampling strategies for the detection of Mptb in the herd. Briefly, the model simulated herds with a range of herd sizes and disease prevalence levels and sampling with pool sizes of 5, 10, 15 and 20 for both Sample Test (300 animals) and Check Test (50 animals). An additional scenario for Check test with 100 animals was also evaluated (Table 2). Sampling was also simulated for uninfected herds to estimate herd-level specificity for the same sampling strategies.

Herds were simulated for herd sizes of 50, 100, 200, 500, 1000 and 2000 eligible animals. Within each herd, infected animals were identified at random to yield the relevant prevalence, determined as the product of prevalence and herd size (rounded up to the next integer).

For each combination of herd size, prevalence and testing type (Check or Sample), the appropriate number of animals, not exceeding the entire herd where appropriate, were sampled at random from the simulated herd, without replacement. Selected animals were then allocated to pools of the relevant size for each of the simulated pool sizes, with one pool having fewer animals where the sample size was not perfectly divisible by pool size. For example, for a herd size of 50 and pool size of 20, there were two simulated pools of 20 and one of 10. Infection status was determined for each pool, with any pool including ≥1 infected animal considered infected and all other pools as uninfected.

Herd sensitivity was estimated for each iteration as:

$$SeH = 1 - (1 - Se_p)^{ip} \times Sp_p^{up}$$

Where Se_p and Sp_p are pool level sensitivity and specificity estimates, respectively, for that iteration and *ip* and *up* are the numbers of infected and uninfected pools, respectively.

Herd-specificity was estimated similarly as

$$SpH = Sp_p^{np}$$

Where np is the total number of pools sampled for that pool size and testing strategy, assuming a prevalence of 0.

The model was run for 10,000 iterations and herd-sensitivity was estimated for each combination of testing type, herd size, prevalence and pool size.

Input values

Input values used in simulation models are described in Table 2.

Table 2. Input values used in simulation models described in the report.

Description	Values
Number of iterations	10,000
Herd size	50, 100, 200, 500, 1000 and 2000
BJD prevalence	0.5%, 1%, 1.5%, 2%, 2.5% and 3%
Pool sizes	5, 10, 15 and 20
HT-J testing cost per pool	$120 per pool for a pool size of 5
Sample size	Sample test: n = 300
	Check test: n = 50 or 100

Pool-level specificity was considered to be perfect based on the laboratory results. However, further sensitivity analyses were conducted to evaluate the impact of imperfect specificity (see below).

Pool-level sensitivity input values were based on the results of the laboratory experiment for each of the simulated pool sizes (see Section 4.1 below). Estimates were incorporated in the model as Beta probability distributions, with α and β parameters:

$$\alpha = x + 1$$

$$\beta = n - x + 1$$

where n is the total number of pools of the specified size tested and x is the number that was positive (see Table 3). For each pool size, values were generated at random from the relevant distribution for each iteration.

Pool sensitivity estimates are relative to the sensitivity of HT-J in individual faecal samples because pool-sensitivity estimates were based on laboratory pooling of known positive samples with known negative faeces. Reliable estimates of the individual sensitivity of HT-J are not available, although it has been shown to be comparable to that of individual faecal culture (Plain *et al.* 2014a). Accordingly, the sensitivity estimates were adjusted using published estimates of the sensitivity of individual faecal culture (Nielsen and Toft 2008; Meyer *et al.* 2019; Sergeant *et al.* 2019) using a mean of 26% and a 95th percentile of 35% (α = 16.9, β = 48.6) to evaluate their influence on model outputs.

Cost

Cost for testing was evaluated assuming the testing cost of $120 per pool, regardless of the pool size. Costs incurred in collecting faecal samples were not included in modelling.

Sensitivity analysis

Three sensitivity analyses were conducted to evaluate the influence of some input values used in the simulation model:

(1) The main simulation model was built after taking into account the HT-J sensitivity for both individual faecal samples (based on previous research) and pooled faecal samples (based on results from this project). This approach could be a bit too stringent, and therefore, the models were refitted without adjusting for the HT-J sensitivity of individual faecal samples.

(2) The specificity of a pooled test was assumed to be perfect in the main model. This assumption was by modified by modelling specificity as a pert distribution with the minimum, mode and maximum of 0.975, 0.995 and 1, respectively, based on expert opinion to evaluate the influence of imperfect specificity on our results.

(3) In the main model, we assumed equal proportions of low, medium and high Mptb shedding animals in a herd. This assumption was changed by including different proportions of low, medium and high shedding animals in a herd to evaluate their impact on the herd sensitivity achieved in various pool sizes. This simulation scenario assumed that 50% of the infected animals in a herd are low shedders, 40% are medium shedders and 10% are heavy shedders.

Table 3. HT-J testing results and resulting probability distributions for sensitivity for each pool size. HEC sensitivity estimates were based on (Champness 2011).

Pool size	n	x	Distribution	Median	95% CI
5	30	19	Beta (20,12)		
10	30	22	Beta (23,9)		
15	30	17	Beta (18,14)		
20	30	19	Beta (20,12)		
Sensitivity of individual culture	–	–	Beta (16.9,48.6)[1]		
HEC sensitivity	–	–	Pert (0.23, 0.36, 0.46)		

[1](Nielsen and Toft 2008; Meyer *et al.* 2019); Sergeant *et al.* (2019)

3.2.2 Herd environmental culture (HEC) test sensitivity

The HEC test was simulated based on publicly available estimates of herd-sensitivity for this test (Champness 2011). The HEC test was estimated to have an overall sensitivity of 45.8% in dairy herds that were tested. However, the sensitivity was 23% in herds with a previous history of JD but seronegative at the time of sampling (low prevalence herds), 36.1% in herds with a seroprevalence between 0 to 1.5% and 46.6% in herds with a seroprevalence of 1.51% to 3.0%. Accordingly, HEC test was modelled using a Pert distribution with the minimum, mode and maximum of 0.23, 0.36 and 0.46, respectively, as the herds in our study were simulated with prevalence ranging from 0.5% to 3%. Because the HEC estimate used is independent of herd size, a single estimate was generated.

4 Results

4.1 Laboratory investigation

4.1.1 HT-J Sensitivity and specificity

The numbers of positive pools and the sensitivity achieved for different pool sizes are shown in Table 4. All of the positive pools were detected in all pool sizes in the high Mptb DNA group but only 20 – 30% of the positive pools were detected in the low Mptb group. The pool size of 10 had the greatest sensitivity compared to both pool sizes 5 and 20, although the differences were not statistically significant (p = 0.27).

The pooled testing demonstrated 100% specificity, with all negative pools having negative test results, regardless of the pool size.

Table 4. Number and proportion of HT-J positive results detected at each pooling rate and for each dilution of the DNA extract.

Mptb *quantity*	Proportion of positive pools[a] for various pool sizes (Number of HT-J positive pools/number of tested pools)			
	5	**10**	**15**	**20**
Low	2/10	3/10	2/10	2/10
Medium	7/10	9/10	5/10	7/10
High	10/10	10/10	10/10	10/10
Overall	19/30	22/30	17/30	19/30
Sensitivity (95% CI)	0.63 (0.44–0.80)	0.73 (0.54–0.88)	0.57 (0.37–0.80)	0.63 (0.44–0.80)

[a]HT-J positive in any of the DNA dilutions tested for a particular pool size; CI: Confidence interval.

4.1.2 PCR inhibition

In this project, the eluted DNA extracts were diluted 5- and 25-fold and then tested by qPCR in addition to testing the neat (undiluted) DNA extract based on the recommendations from a previous study (Acharya *et al.* 2017). For the results presented in Table 4, a pool was considered positive if the qPCR was positive based on the neat or the diluted DNA extract. The detailed results for the neat and diluted DNA extracts are presented in Table 5 and Fig 1.

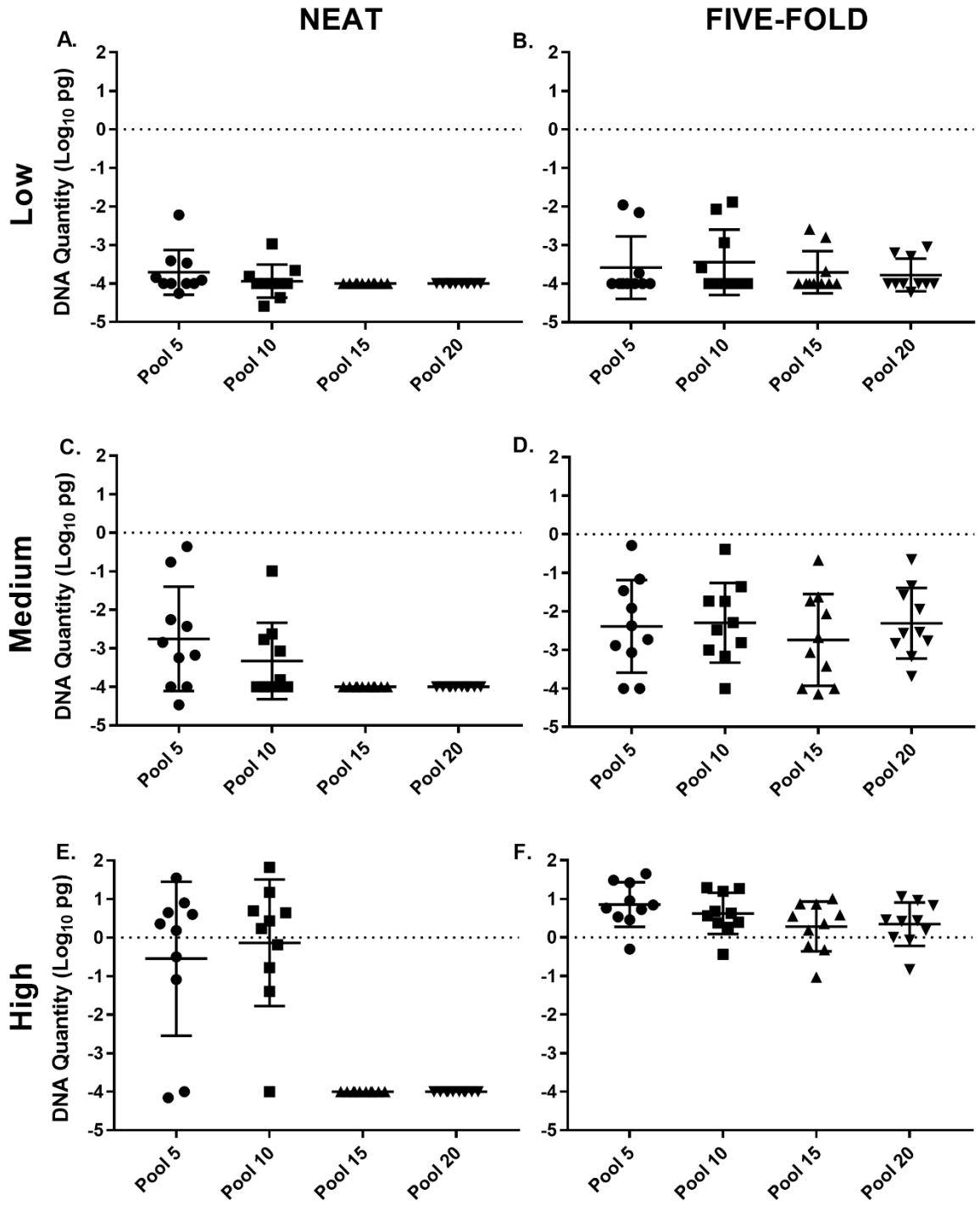
The process of dilution led to greater detection of Mptb for some pools. This effect was more obvious for the high Mptb group in which only pool sizes 5 and 10 had any positive results detected in the undiluted DNA samples (Table 5) but a five-fold dilution led to the detection of all (100%) of the samples for all pool sizes confirming the presence of PCR inhibition and suggesting that it can be relieved with the process of dilution. No additional positive samples were detected in the 25-fold dilution compared to the undiluted and five-fold DNA dilution for the majority of the pool sizes in the different groups. Rather, a dilution effect occurred, as more results were detected positive with the five-fold dilution, compared to the 25-fold dilution. However, for pool size 20 in the low prevalence group, the 25-fold dilution unexpectedly led to the detection of two positive results that were not detected in the undiluted and five-fold dilution (Table 5).

Table 5. Number and proportion of HT-J positive results detected at each pooling rate and for each dilution of the DNA extract.

Mptb *quantity*	DNA Dilution	Proportion of positive pools for various pool sizes (Number of HT-J positive pools/Number of tested pools)			
		5	10	15	20
Low	Undiluted	1/10	1/10	0/10[a]	0/10[a]
	1 in 5	2/10	3/10	2/10	0/10
	1 in 25	1/10	2/10	2/10	2/10
Medium					
	Undiluted	5/10	3/10	0/10[a]	0/10[a]
	1 in 5	6/10	7/10	5/10	7/10
	1 in 25	3/10	4/10	4/10	4/10
High					
	Undiluted	8/10	8/10	0/10[a]	0/10[a]
	1 in 5	10/10	10/10	10/10	10/10
	1 in 25	10/10	10/10	10/10	10/10

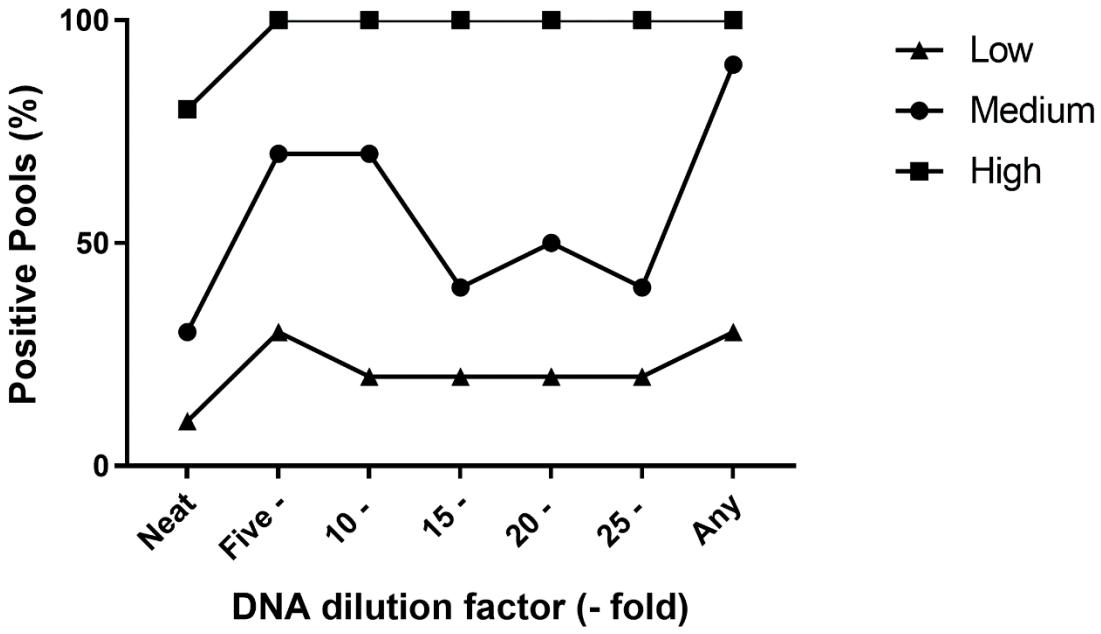
[a.]No positives detected due to PCR inhibition; [b]Any is HT-J positive in any of the DNA dilutions tested for a particular pool size.

*Fig 1. Box-and-whiskers plots of the log-transformed Mycobacterium avium subsp. paratuberculosis (*Mptb*) DNA quantities detected in qPCR.* A, C, and E: The undiluted DNA extract of the Low, Medium, and High Mptb shedding group; B, D, and F: The five-fold dilution of the undiluted DNA extract of the Low, Medium and High Mptb shedding group.



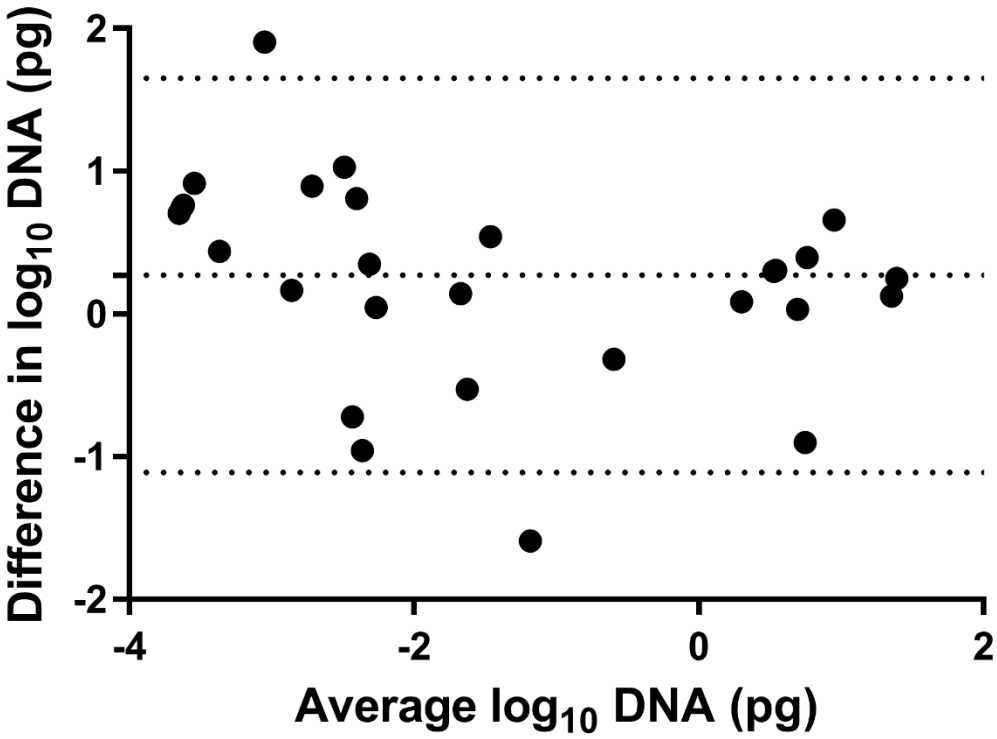
The DNA extracts for samples prepared at a pool size of 10 were further diluted by 10-, 15- and 20-folds to determine the optimal dilution rate to relieve PCR inhibitors. For the low shedding group, the five-fold dilution had the most positive results at 30% (Fig 2). For the medium level shedding group, both the five-and ten-fold DNA dilution enabled 70% of the samples to be detected positive. The dilution of the undiluted DNA extract for the high shedding group resulted in all (100%) samples testing positive, regardless of the dilution factor.

Fig 2. The DNA dilution factors applied to the pool size of 10 for different levels of Mptb *faecal shedding.*



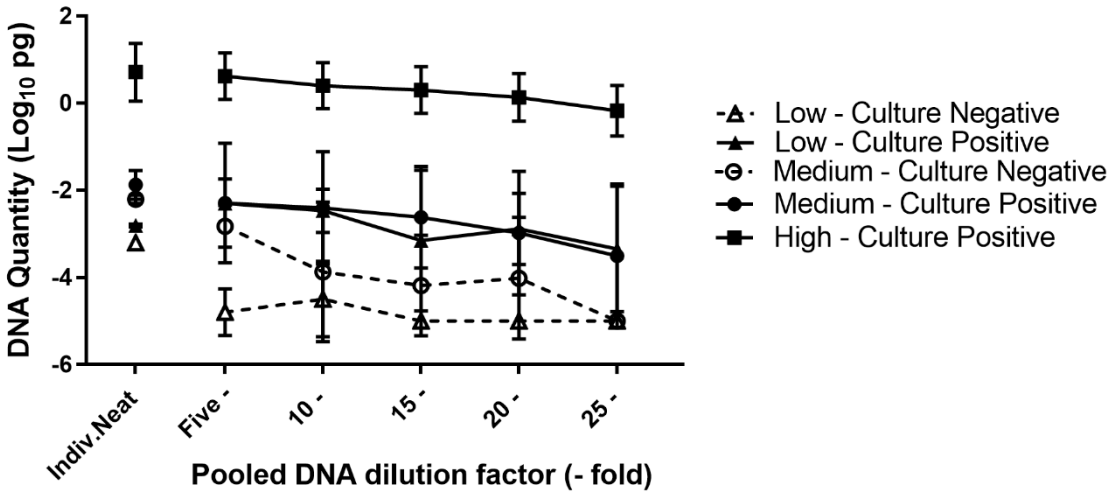
The Bland Altman plot (Fig 3) demonstrates the log_{10} difference between the original DNA quantities against the average DNA quantity of pool size 10 in a five-fold dilution. The overall difference is positive (0.273) with most of the DNA samples within the 95% CI (-1.108, 1.653). The positive mean difference suggests that there was a lower DNA quantity detected in the five-fold dilution of pool size 10, than in the original DNA quantity of individual animals, as would be expected. From the graph, it is evident that the differences are evenly scattered, with negligible pattern or trend that follows an increase in average DNA quantity.

Fig 3. The Bland Altman plot comparing the average log-transformed original DNA quantities of individual animal against the five-fold dilution of the pool size of 10. The dotted middle line and two outer dotted lines represents the region of agreement (95% confidence interval).



The results of the faecal qPCR for the pool size of 10 were comparable to the individual qPCR results, relative to faecal culture outcomes (Fig 4). The decreased DNA quantity detectable from the process of diluting the pool size of 10 was most prominent in the low and medium groups for the samples that were culture-negative (n = 7 low and n = 2 medium samples). This contrasts to the culture-positive samples within these groups; as all of these samples (n = 3 low and n = 8 medium) were test-positive in the pooled faecal qPCR and further dilution of the DNA extract demonstrated a relatively stable decline.

Fig 4. Comparison of DNA quantities detected for the different dilutions of pool size 10 with the individual faecal qPCR. The results were sub-grouped according to the individual culture status.



4.2 Simulation modelling

4.2.1 HT-J herd-sensitivity for the Sample test

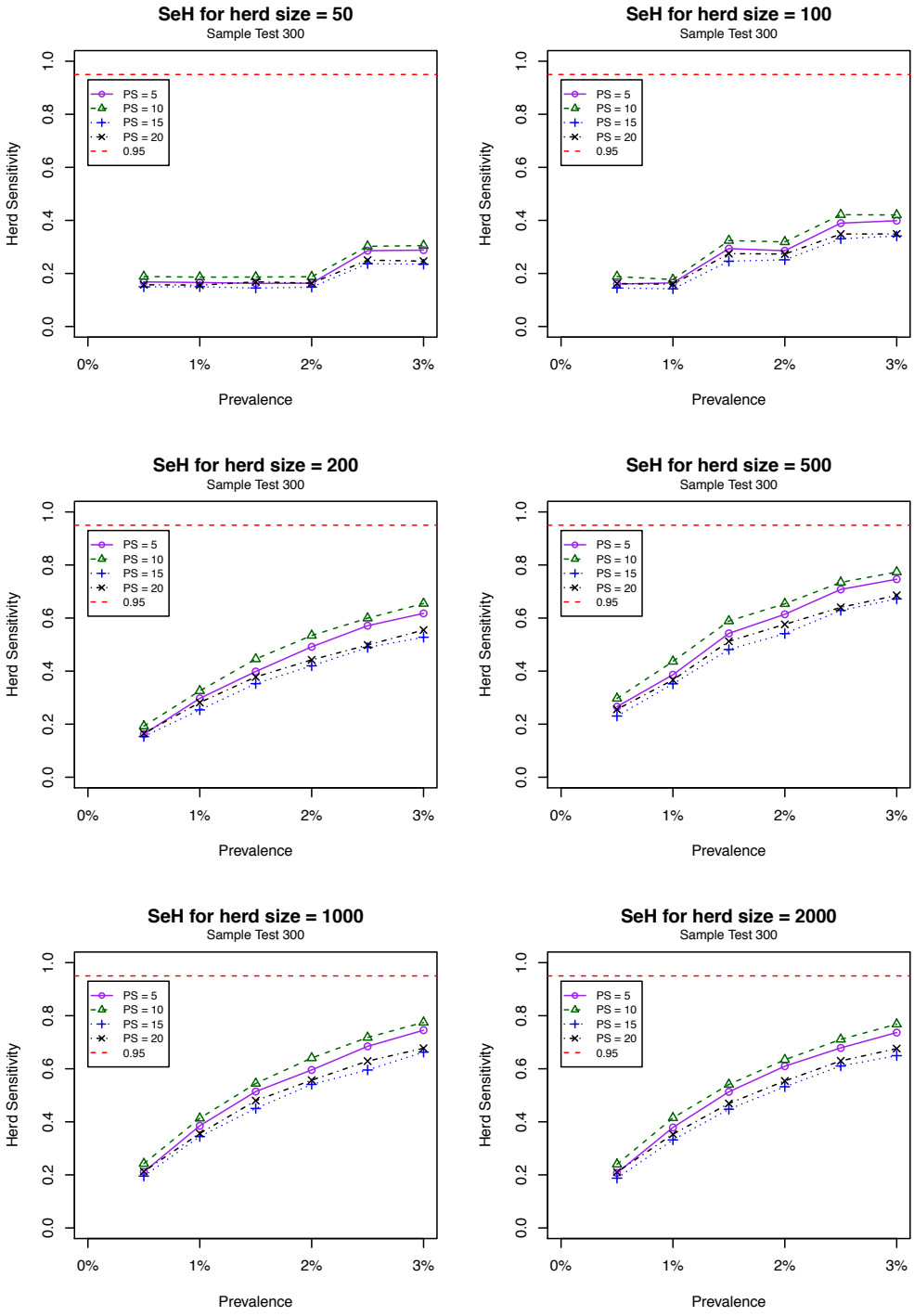
Generally, herd-sensitivity increased with herd size and prevalence, regardless of pool size (Table 6; Fig 5). Pool sizes of 5 and 10 produced similar estimates of herd-sensitivity that were consistently higher than for pools of 15 or 20 (which were also similar to each other). The difference between 5/10 and 15/20 was negligible at very low prevalence (≤1%) but increased as assumed prevalence increased. It is worth noting that herd sensitivity achieved for pool size 10 was higher than that of pool size 5 (as well as than other pool sizes) for all prevalence levels and herd sizes.

Herd-sensitivity estimates only exceeded 95% for large herds with a prevalence of 10% (results not shown). For smaller herds and lower prevalence, estimates ranged from <20% to about 80%, depending on herd size and assumed prevalence.

Table 6. Herd-sensitivity achieved for a Sample test (n = 300) using various pool sizes in a typical herd of 500 cows with a low BJD prevalence.

Herd prevalence	Herd-sensitivity at various pool sizes			
	5	**10**	**15**	**20**
0.5%	27%	30%	23%	26%
1.0%	39%	44%	35%	37%
1.5%	54%	59%	48%	51%
2.0%	61%	65%	54%	58%
2.5%	71%	73%	63%	64%
3.0%	75%	77%	67%	69%

Fig. 5: Effect of herd size, disease prevalence and pool size on herd-sensitivity (SeH) of Sample test (n= 300) in low prevalence herds (<= 3%).



4.2.2 HT-J herd-sensitivity for the Check test

The results for the Check Test had a similar pattern to the Sample test, except that estimates were substantially lower (Table 7; Fig 6). In particular, herd-sensitivity was higher for pool size 10 than for other pool sizes, similar to the Sample test.

Given the low herd -sensitivity, we also investigated a scenario of sourcing a sample size of 100 instead of 50 for the Check test. The results presented in Fig 7 indicate that the herd-sensitivity increased with increase in sample size, but the effect of pool size remained the same.

In Table 8, we compared three scenarios: the current approach of testing 10 pools of size 5, a second approach of testing 5 pools of size 10 and a third approach of testing 10 pools of size 10. The results indicate that the third approach would achieve 1.7 times the herd-sensitivity than the current approach, for the same cost.

Table 7. Herd-sensitivity and specificity achieved for a Check test (n = 50) using various pool sizes in a typical herd with 500 cows with a low BJD prevalence (ranging from 0.5% to 3.0%).

Herd prevalence	Herd sensitivity at various pool sizes			
	5	**10**	**15**	**20**
0.5%	5%	5%	4%	4%
1.0%	8%	9%	6%	8%
1.5%	12%	13%	10%	11%
2.0%	14%	16%	12%	13%
2.5%	18%	19%	15%	16%
3.0%	20%	22%	17%	18%

Table 8: Herd-sensitivity for check tests in three scenarios for an assumed prevalence level of 2%.

#	Check test scenarios	Herd size					
		50	**100**	**200**	**500**	**1000**	**2000**
1	10 pools, each of size 5 (n = 50)[a]	16%	15%	15%	15%	15%	15%
2	5 pools, each of size 10 (n = 50)	19%	18%	18%	17%	17%	17%
3	10 pools, each of size 10 (n = 100)[b]	19%	34%	32%	31%	31%	31%
	Relative increase in herd sensitivity (3 vs 1)	1.0	1.8	1.7	1.7	1.7	1.7

[a]Current approach; [b]Recommended approach.

Fig. 6: Effect of herd size, disease prevalence and pool size on herd-sensitivity (SeH) for a Check test (n = 50).

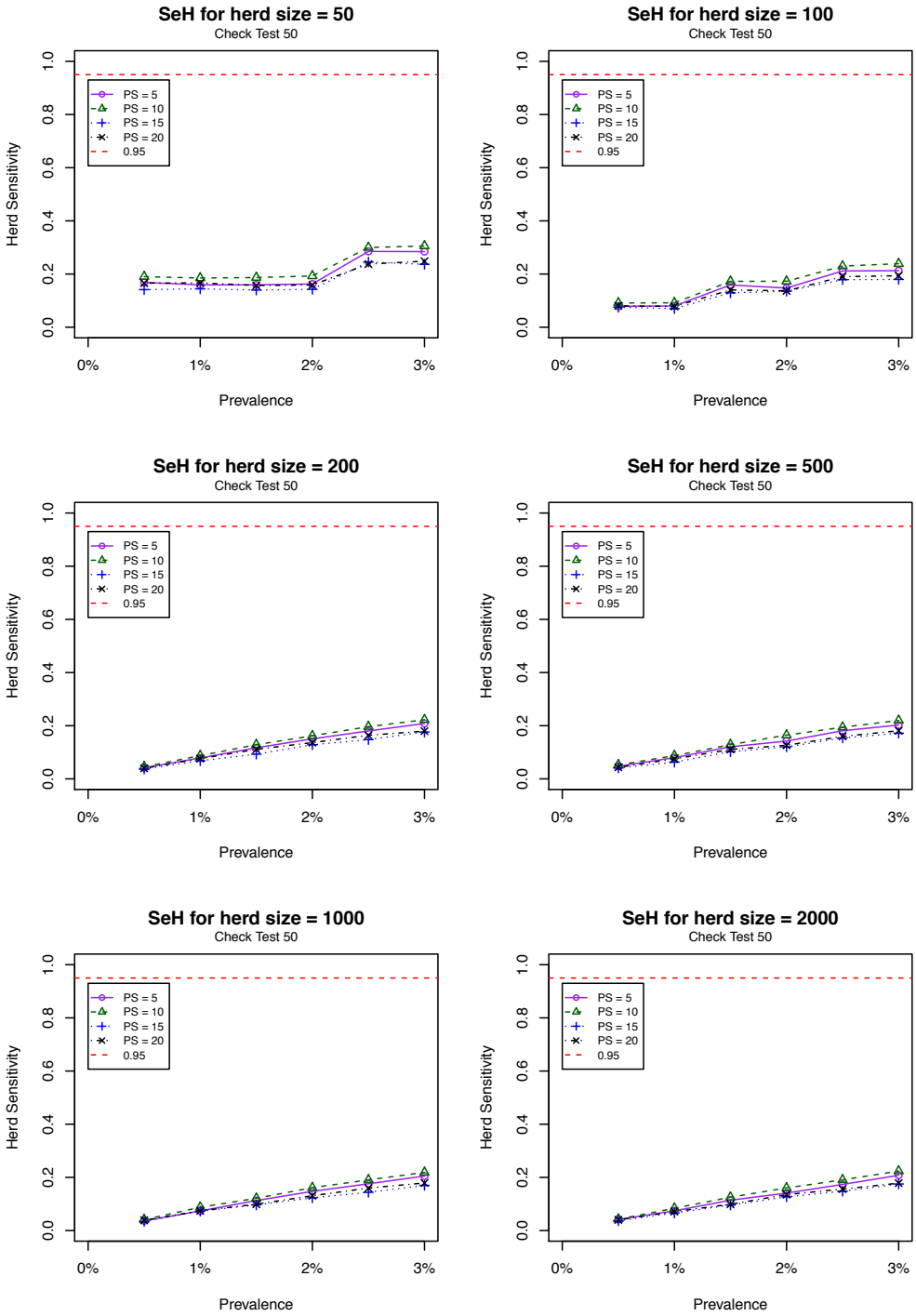
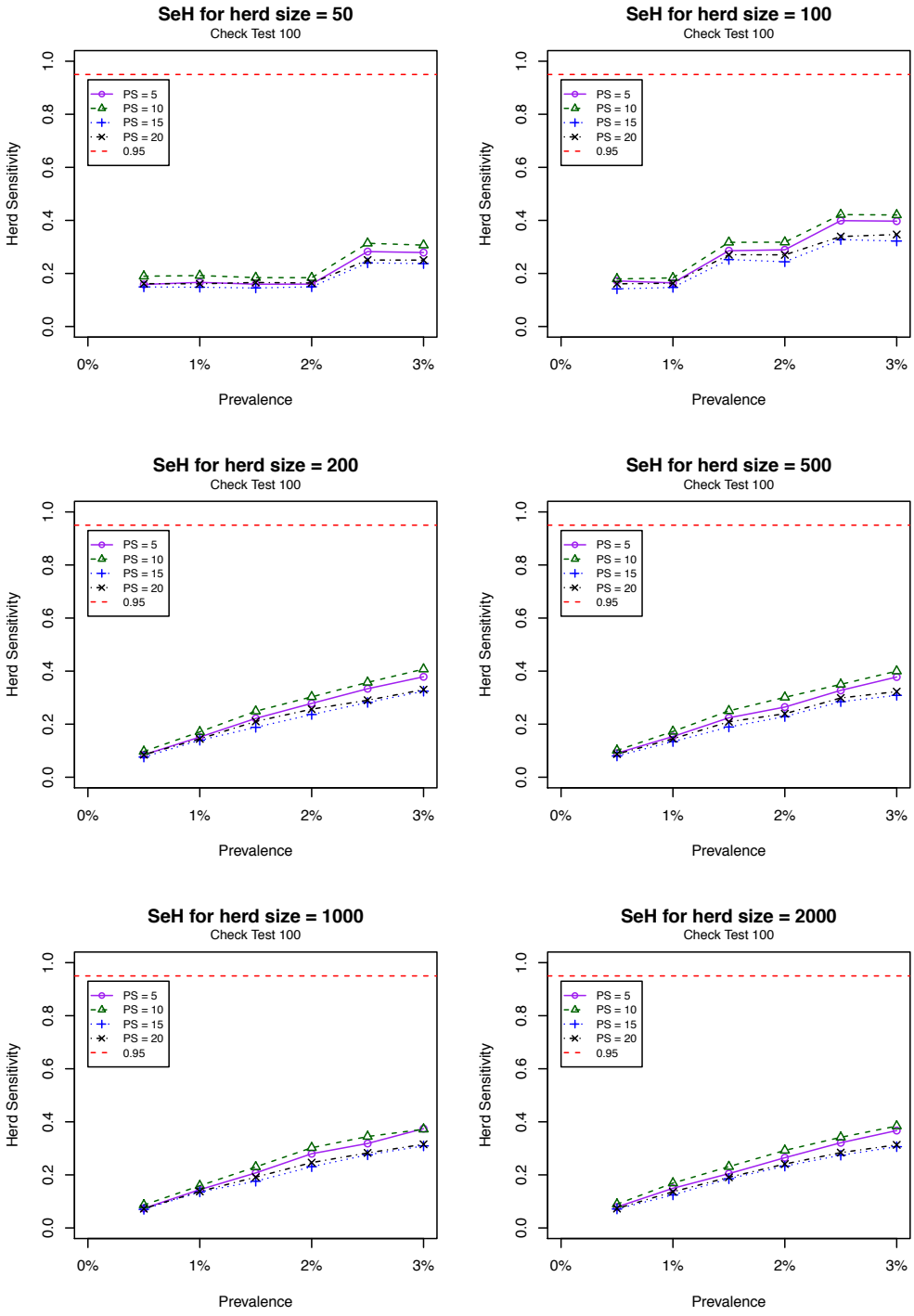


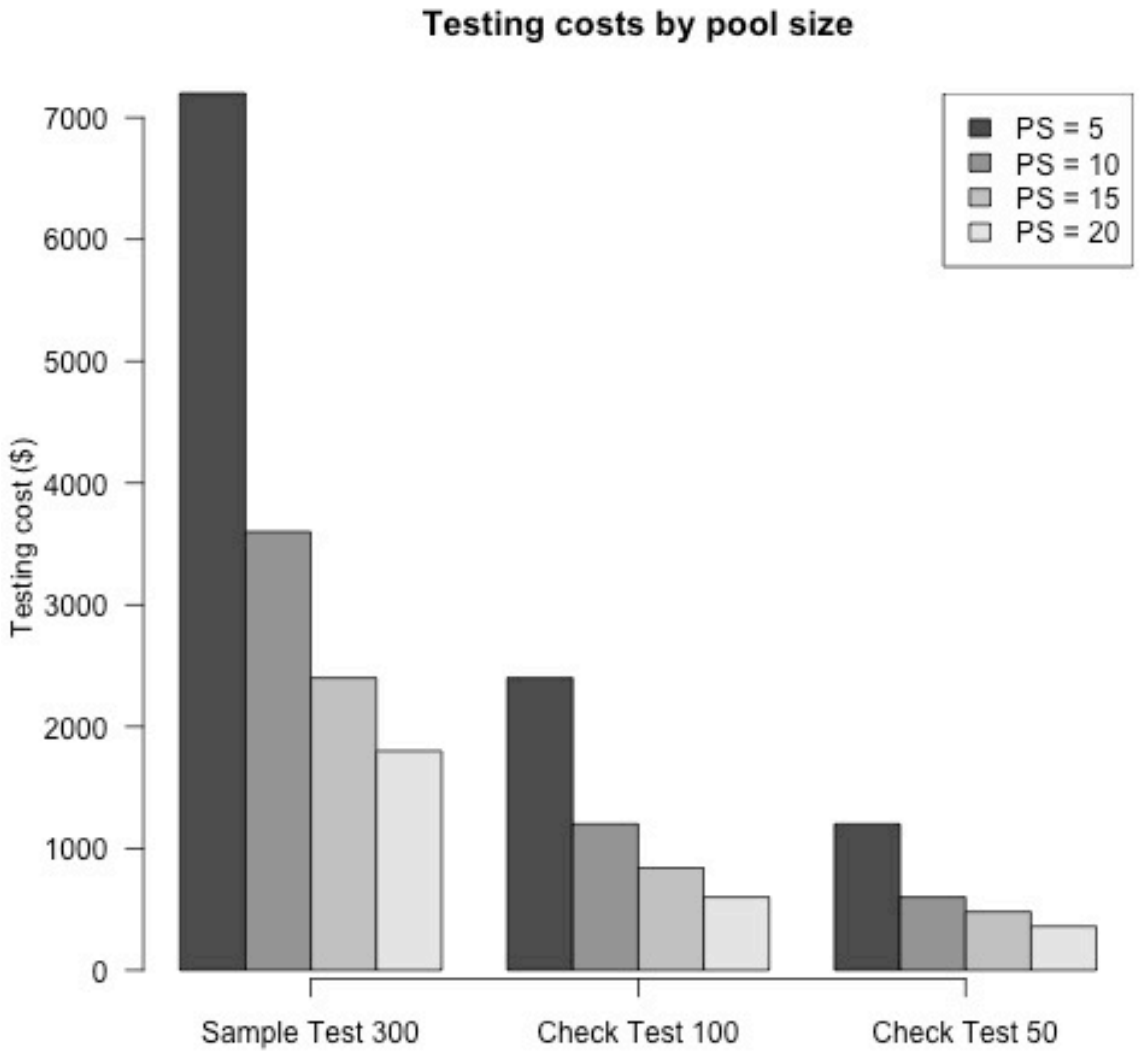
Fig. 7: Effect of herd size, disease prevalence and pool size on the herd sensitivity (SeH) for a Check test (n = 100).



4.2.3 Cost of testing

Cost of Sample testing would halve from the current $7200 to $3600 by using a pool size of 10 instead of 5, assuming that the cost of testing a pool does not increase with an increase in pool size. A similar reduction can be achieved for a Check test from the current $1200 to $600 (i.e. n = 50). Alternatively, gains in herd-sensitivity can be made by increasing the sample size to 100 by testing 10 pools of 10 by keeping the cost of testing to be the same as the current cost ($1200).

Fig. 8: Effect of pool sizes on costs of testing assuming a rate of $120 per pool.



4.2.4 Sensitivity analyses

Ignoring individual sample sensitivity

Individual sample sensitivity had a substantial effect on herd-sensitivity. Herd-sensitivity estimates were in general much higher for all prevalence levels and pool sizes when models were built without adjusting for imperfect individual animal level sensitivity. Almost perfect herd-sensitivity was achieved for some of the scenarios (Table 9). Interestingly, pool size 10 appeared to perform even better in this scenario although the pattern of herd-sensitivity for various pool sizes generally remained the same as in the main analysis (Figs 9-11).

Table 9. Herd sensitivity achieved for a Sample test (n = 300) using various pool sizes in a typical herd of 500 cows with a low BJD prevalence without considering individual sample sensitivity.

Herd prevalence	Herd sensitivity at various pool sizes			
	5	**10**	**15**	**20**
0.5%	75%	80%	70%	73%
1.0%	90%	93%	85%	89%
1.5%	97%	98%	95%	96%
2.0%	99%	99%	97%	98%
2.5%	100%	100%	99%	99%
3.0%	100%	100%	99%	99%

Fig. 9: Herd-sensitivity results for a Sample test (n = 300) without adjusting for individual sample HT-J sensitivity.

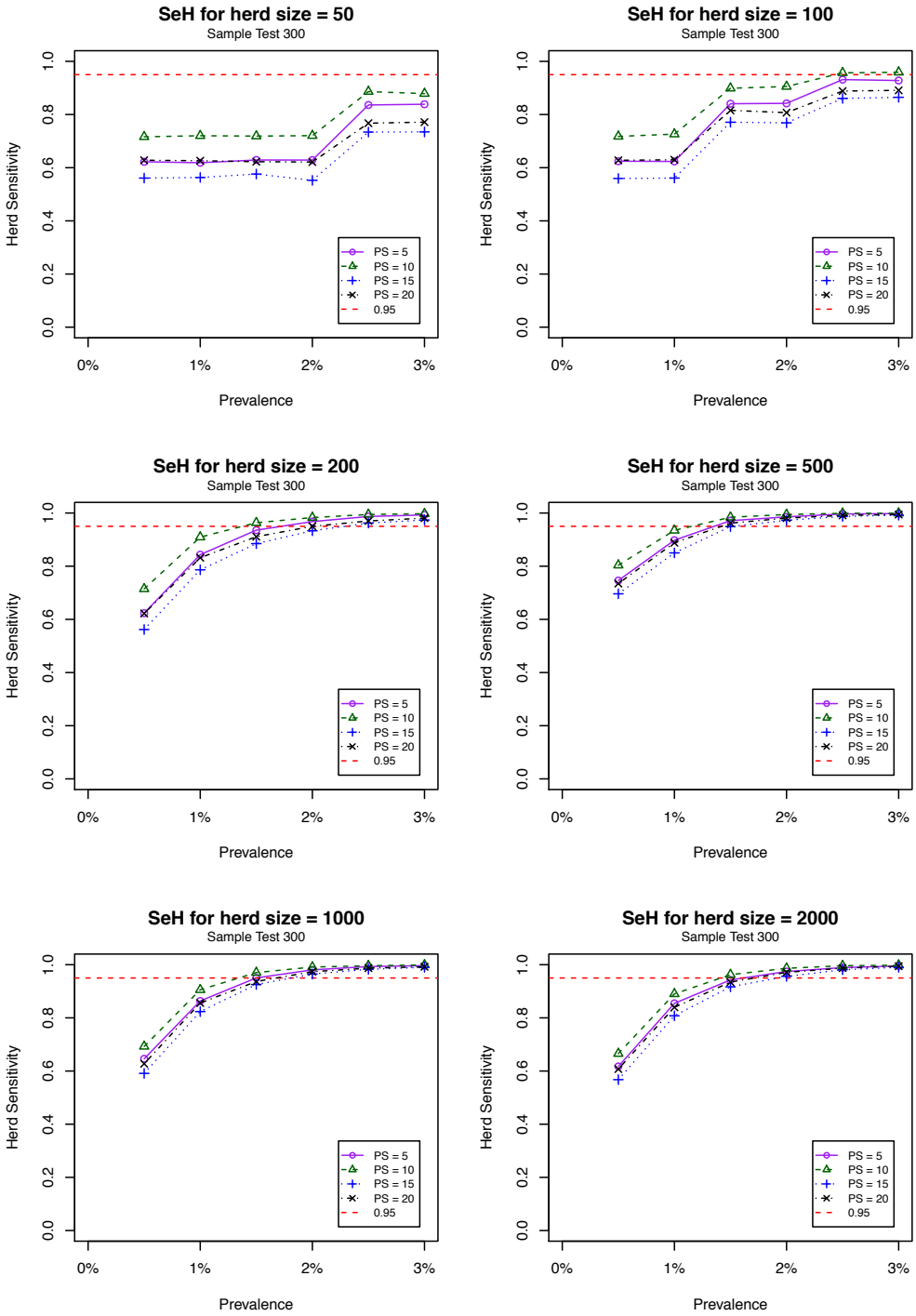


Fig. 10: Herd-sensitivity results for a Check test (n = 50) without adjusting for individual sample HT-J sensitivity.

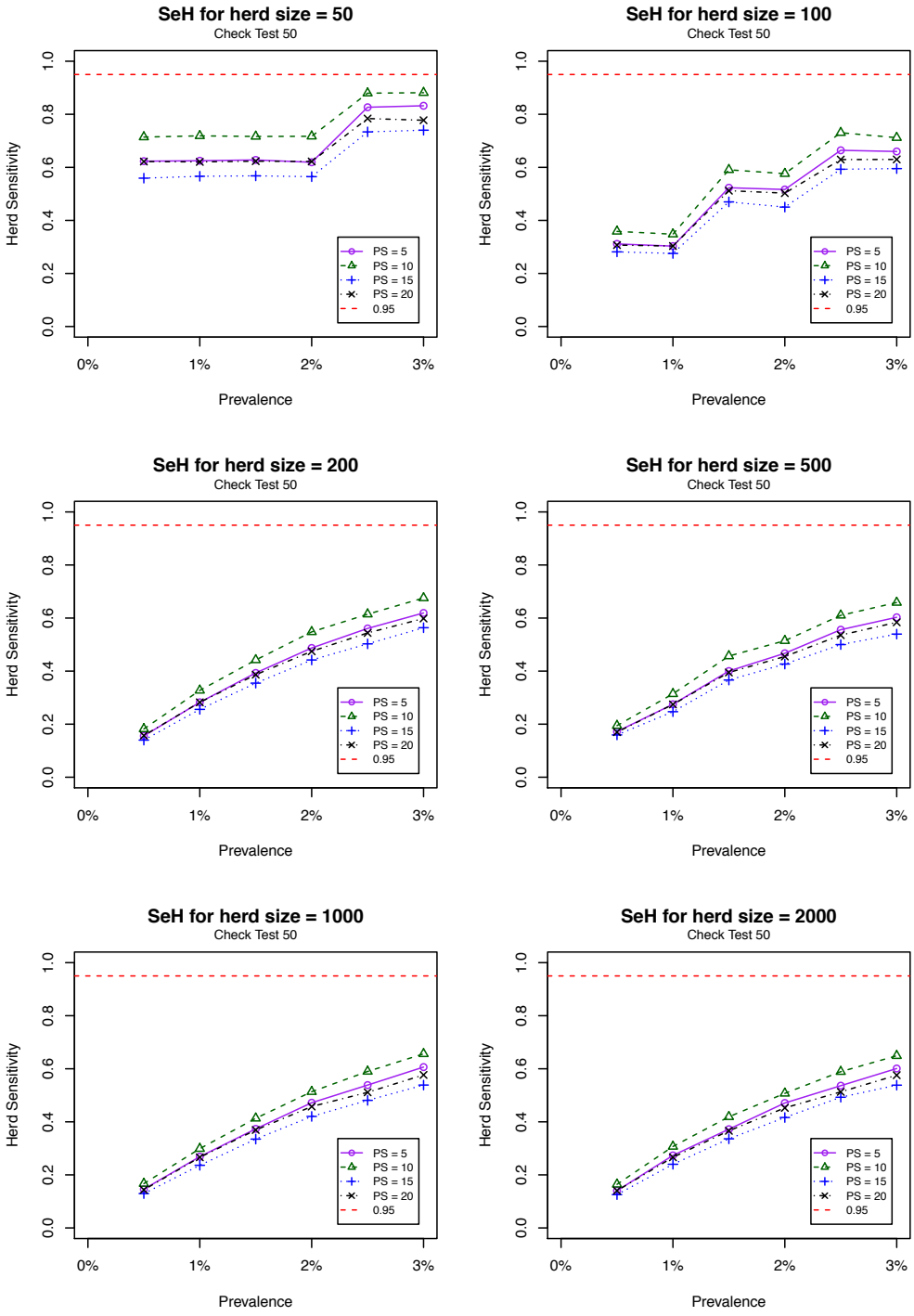
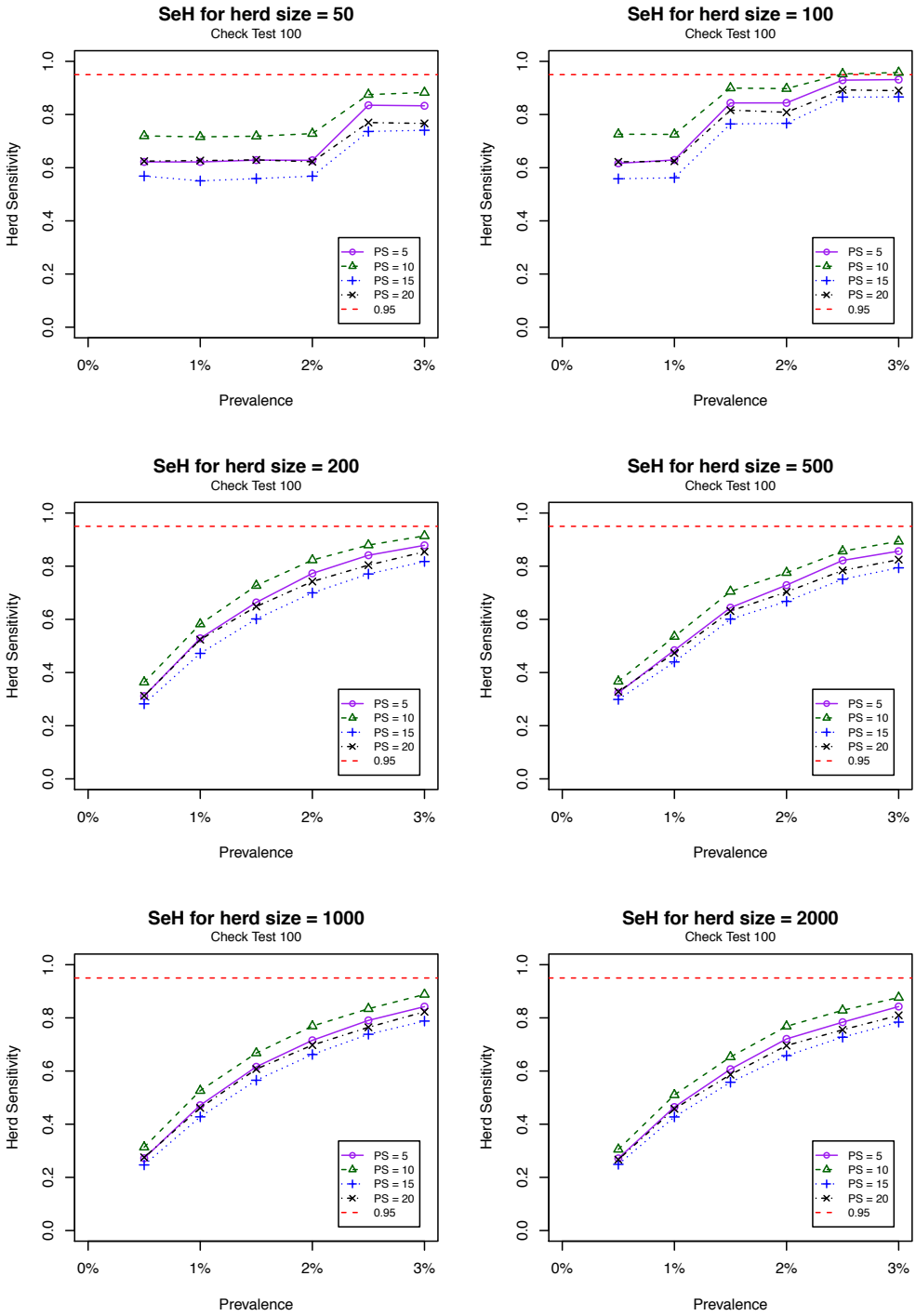


Fig. 11: Herd-sensitivity results for a Check test (n = 100) without adjusting for individual sample HT-J sensitivity.



Assuming imperfect HT-J specificity

In the main analyses, we assumed a perfect specificity. When specificity was assumed to vary between 97.5 and 99% in sensitivity analyses, it increased the achieved herd-sensitivity in most of the scenarios (Table 10 versus Table 6). Interestingly, pool size 5 performed better in this scenario and achieved a higher herd-sensitivity than other pool sizes for all prevalence levels (Fig. 12-14).

Note that this is the only scenario in which the pool size of 5 performed better than a pool size of 10. However, the pool size of 5 achieved a lower specificity than other pool sizes (Table 10, Fig. 15), which was more discernible for larger herds. In fact, the higher herd-sensitivity achieved by this pool size in the current scenario is likely to be due to its lower herd-specificity.

Table 10. Herd sensitivity and specificity achieved for a Sample test (n = 300) using various pool sizes in a typical herd of 500 cows with a low BJD prevalence assuming HT-J specificity to be imperfect.

Herd prevalence	Herd sensitivity at various pool sizes			
	5	**10**	**15**	**20**
0.5%	50%	43%	31%	32%
1.0%	60%	54%	42%	43%
1.5%	69%	66%	54%	55%
2.0%	74%	71%	60%	60%
2.5%	80%	78%	67%	67%
3.0%	83%	81%	69%	70%
Herd-specificity	66%	81%	86%	90%

Fig. 12: Herd-sensitivity results for a Sample test (n = 300) assuming imperfect specificity.

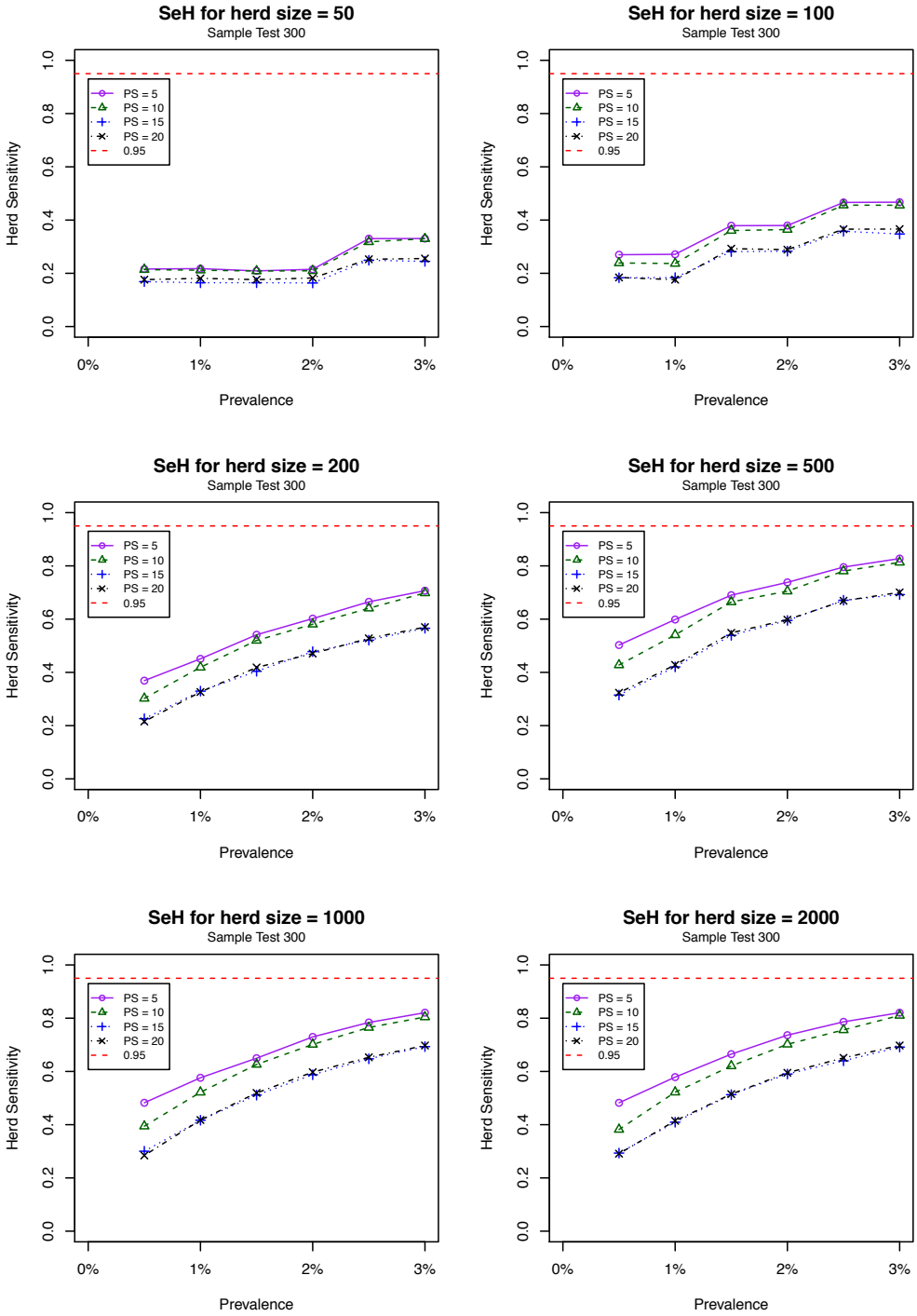


Fig. 13: Herd-sensitivity results for a Check test (n = 50) assuming imperfect specificity.

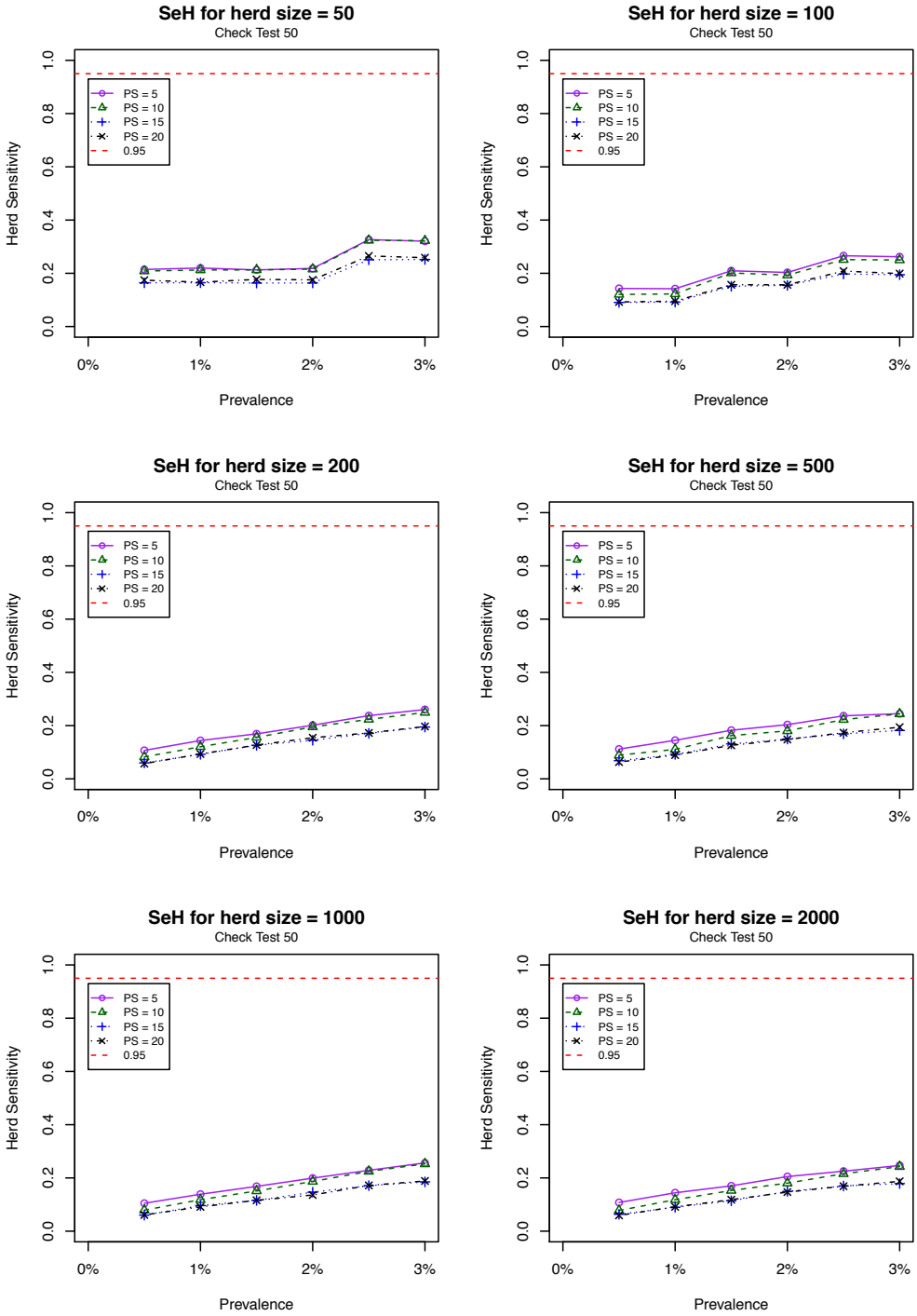


Fig. 14: Herd-sensitivity results for a Check test (n = 100) assuming imperfect specificity.

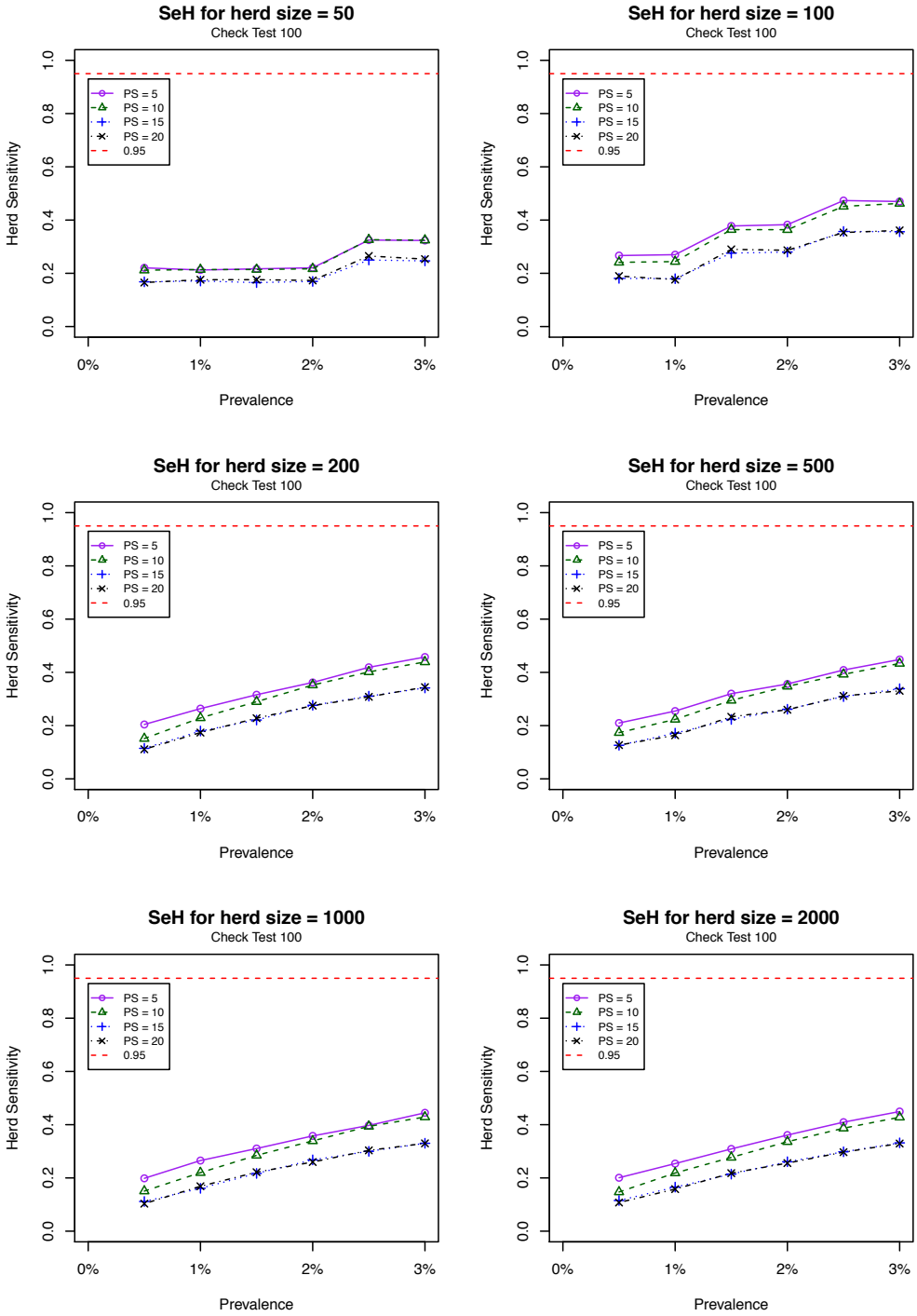
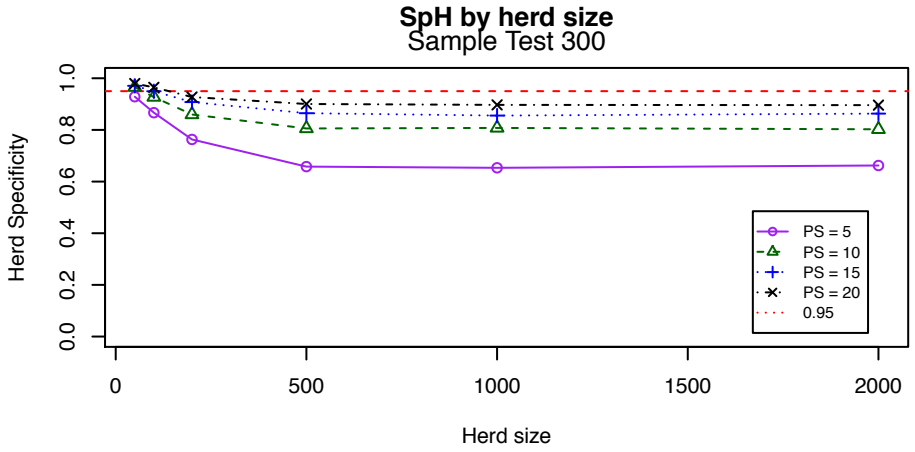
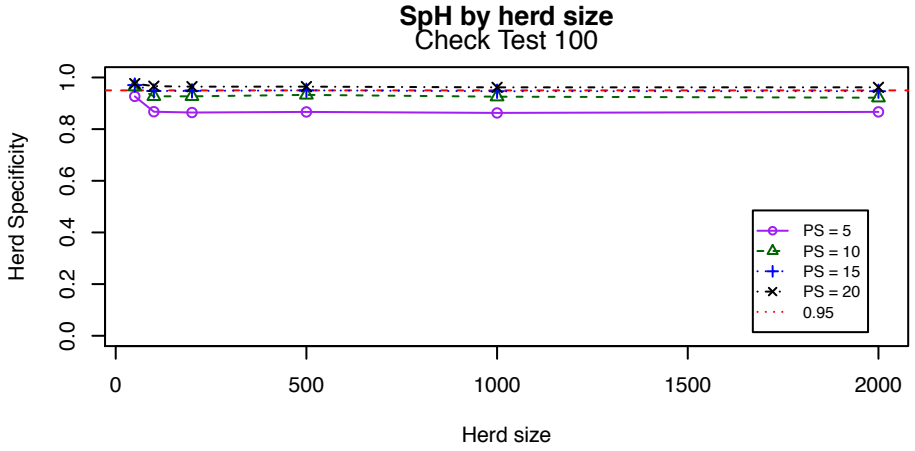
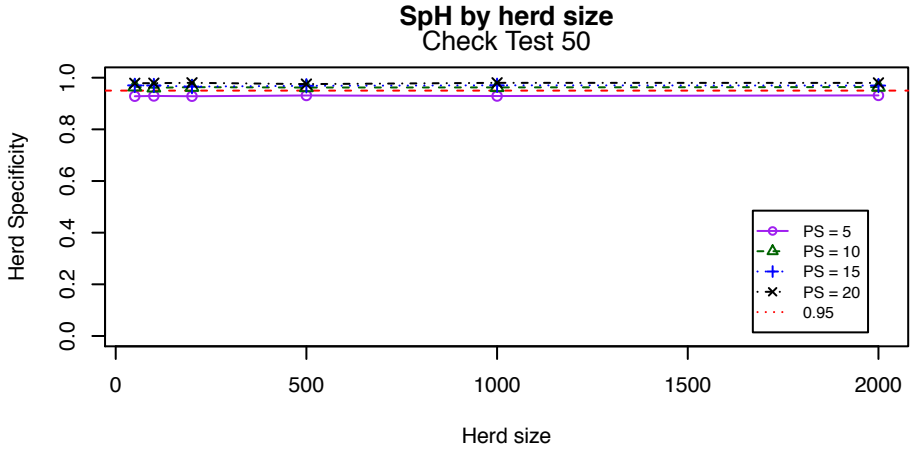


Fig. 15: Herd-specificity (SpH) achieved for a Sample test (n = 100) and a Check test (n = 50 or 100).







Assuming different proportions of high, medium and low Mptb *shedding animals in a herd*

Assuming different proportions of high, medium and low shedder animals reduced the herd-sensitivity achieved in most though not all scenarios (Tables 11-12, Fig, 16-18) but the overall pattern of the effect of pool sizes remained the same as in the main analysis.

Table 11. Herd sensitivity achieved for a Sample test (n = 300) using various pool sizes in a typical herd of 500 cows with a low BJD prevalence assuming that 50% of the infected animals in a herd are low shedders, 40% are medium shedders and 10% are heavy shedders.

Herd prevalence	Herd sensitivity at various pool sizes			
	5	**10**	**15**	**20**
0.5%	30%	35%	27%	29%
1.0%	28%	34%	23%	27%
1.5%	41%	48%	38%	40%
2.0%	48%	56%	39%	44%
2.5%	52%	60%	43%	48%
3.0%	63%	68%	53%	58%

Table 12: Comparison of herd-sensitivity for a Sample test (n=300) at a pool size of 10 and herd prevalence level of 2%, assuming different proportions of high, medium and low Mptb shedding animals in the herd.

Scenario	Proportions of animals with different shedding levels	Herd size					
		50	**100**	**200**	**500**	**1000**	**2000**
1	Equal proportions	19%	32%	53%	65%	64%	63%
2	Low shedding = 50% Medium shedding = 40% High shedding = 10%	21%	15%	53%	56%	62%	53%
	Relative change in herd sensitivity (1 vs 2)	0.9	2.1	1.0	1.2	1.0	1.2

Fig. 16: Herd-sensitivity results for a Sample test (n = 300) assuming different proportions of low, medium and high shedder animals in the herd.

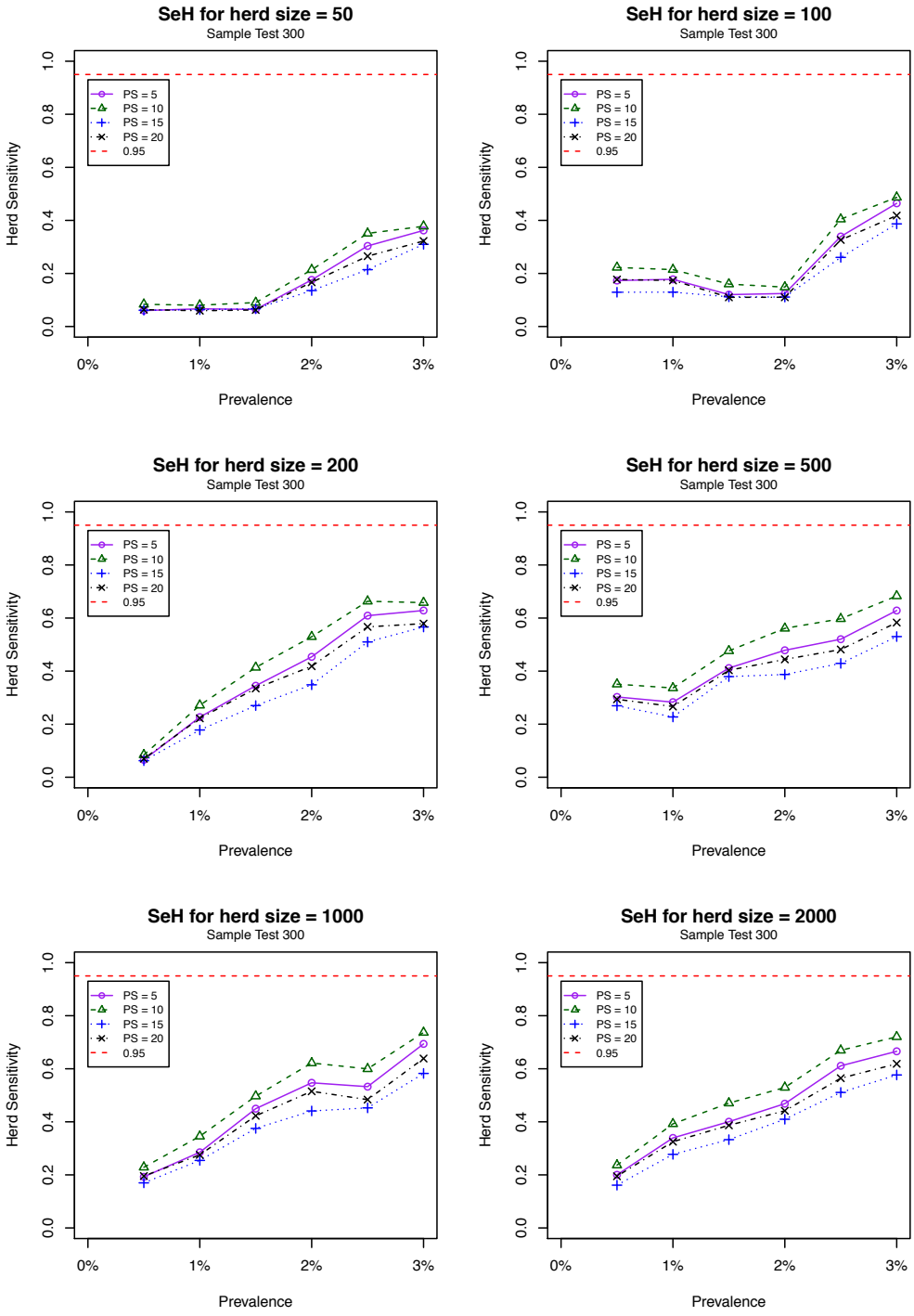


Fig. 17: Herd-sensitivity results for a Check test (n = 100) assuming different proportions of low, medium and high shedder animals in the herd.

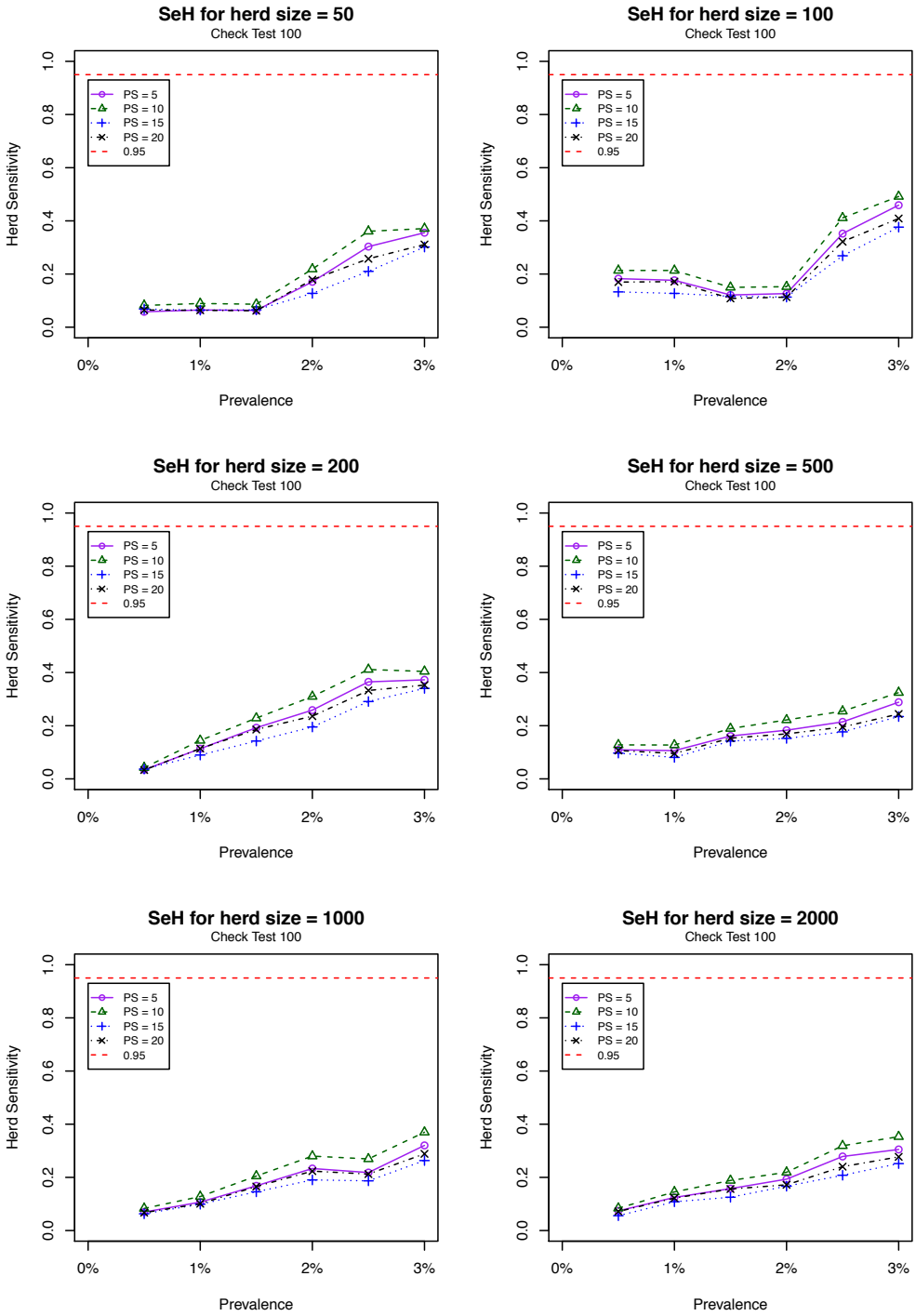
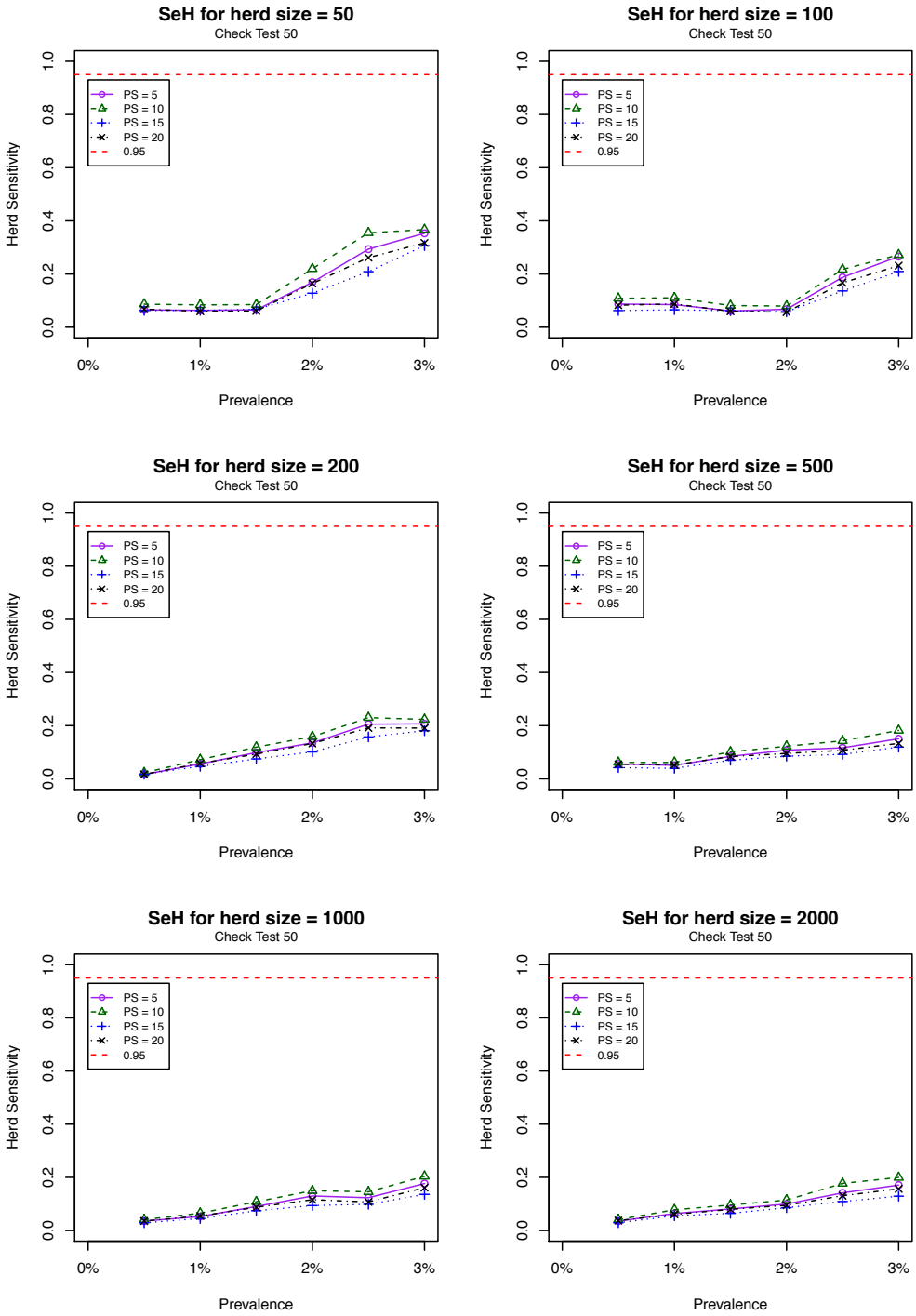


Fig. 18: Herd-sensitivity results for a Check test (n = 50) assuming different proportions of low, medium and high shedder animals in the herd.



4.2.5 HEC herd-sensitivity

The HEC test achieved a herd-sensitivity of 35.2%. This estimate is higher than the herd-sensitivity achieved for the Check test conducted using pooled HT-J but is probably an overestimate as the HEC herd-sensitivity was not modelled to vary by herd prevalence because of lack of sufficient input information for low prevalence herds simulated in this study.

5 Discussion

The overall aim of the project was to investigate ways in which the cost to beef producers of herd screening tests for BJD can be reduced without sacrificing accuracy or compromising the J-BAS status of a herd. Specifically, in this project, we compared alternative pooling strategies (pool sizes of 10, 15 and 20) to the current pool size of 5 recommended in the J-BAS screening tests. This study found that increasing the pool size from the current 5 to 10 could produce results of similar accuracy while nearly halving the costs.

5.1 HT-J sensitivity and specificity

HT-J sensitivity is similar for pool sizes of 5 and 10. We conducted a laboratory study in which confirmed HT-J positive faeces were pooled in ratios of 1:4, 1:9. 1:14 and 1:19 with confirmed HT-J negative faeces to mimic the creation of pools by combining faeces from one Mptb shedding animal with those from 4, 9, 14 and 19 non-shedding animals, respectively. These pools were then tested by pooled HT-J qPCR to estimate pool HT-J sensitivity. In this investigation, pool size 10 achieved a sensitivity of 73% (95% CI: 54 – 88%) compared to a sensitivity of 63% (95% CI: 44% – 88%) achieved for the pool size of 5. The difference in sensitivity between these two pool sizes was not statistically significant, suggesting that the HT-J sensitivity for a pool size of 10 is at least equal to the sensitivity for a pool size of 5, if not better. These results are in agreement with a recent study in dairy cattle using PFC and IS*900* PCR, which demonstrated a similar test sensitivity of 78% for the pool size of 10 (Eamens *et al.* 2007b). Contrastingly, another study on PFC demonstrated that the pool size of 5 had a higher sensitivity compared to the pool size of 10. However, similar to our study there was no significant difference in sensitivity achieved between the two pool sizes (McKenna *et al.* 2018). Thus, from previous research on PFC and the results of laboratory our study, it appears that pool size 10 can potentially replace a pool size of 5 for HT-J testing.

HT-J sensitivity also depends on the Mptb concentration in the sample. In our laboratory study pools of BJD positive faecal samples with low, medium and high levels of Mptb DNA were created to mimic the collection and testing of faecal samples from low, medium and high faecal shedding cattle. The high Mptb shedding group demonstrated 100% sensitivity with all pool sizes meaning that larger pool sizes of 15 and 20 would be more cost-effective in such herds. However, for both the low and medium Mptb faecal shedding levels, pool size 10 performed better than a lower pool size of 5 as well as higher pool sizes of 15 and 20 suggesting that a pool size of 10 can potentially be used for low and medium prevalence herds. Our results are similar to a previous study that found that increasing the pool size to 20 or 30 resulted in decreased positive results detected, which was especially evident for the low Mptb shedding animals (Eamens *et al.* 2007b). Our results are also partly in agreement with another study in which low prevalence herds were better detected in smaller pool sizes compared to larger pool sizes (van Schaik *et al.* 2003; McKenna *et al.* 2018). A Monte Carlo simulation also demonstrated that the pool size of 10 or 20 is preferred, when the DNA quantity levels ranged from 0.01 to 0.1 pg, compared to a pool size of 50 (Messam *et al.* 2010). The reason for the poor detection of low Mptb DNA quantity in larger pool sizes may be due to the 'dilution' effect (Kalis *et al.* 2000; Pedersen *et al.* 2014). This implies that the already low quantity of Mptb DNA in the low shedding group may become undetectable when pooled at a larger pool size.

HT-J has a perfect specificity regardless of the pool size. The results in this study demonstrated 100% specificity for the different pool sizes. This specificity level is in agreement with a previous study, which demonstrated that non-exposed Mptb cattle had a high specificity of 99.6% with the HT-J qPCR, relative to liquid culture (Plain *et al.* 2014a). Our results demonstrate that the direct faecal HT-J qPCR when used with pooling, is a robust test with very high specificity. The major advantage of a highly specific test is that producers would be able to confidently test their herds for JD without the concern for false-positive results. The regular occurrence of false positive results in

the past had resulted in producers 'opting out' of the control programs, due to the psychological turmoil and financial stresses associated with false positives (Herd Health Pty Ltd 2016) . Thus, knowing that false positives are unlikely, farmers can be confident in using a higher pool size than the currently used pool size of 5 in J-BAS testing.

5.2 PCR inhibition

The problem of PCR inhibition does get worse with pooling. Although pooling is economically beneficial, it does seem to exacerbate the effect of PCR inhibition. The pooling and homogenisation process of the faecal samples may result in the unexpected release of compounds from the heterogeneous distribution of microorganisms found in the diet of ruminants. These compounds may hinder PCR amplification and ultimately lead to inhibition. These potential contaminants may include polysaccharides, humic acid, phytic acid, phenols, urea and polycyclic aromatic hydrocarbons (Monteiro *et al.* 1997; Thornton and Passen 2004; Acharya *et al.* 2017). In this study, PCR inhibition was evident in pools at all Mptb shedding levels, with fewer positive test results for the undiluted DNA compared to a five-fold dilution of the DNA extract. PCR inhibition has also been reported previously for the qPCR methodology used in this investigation; a previous study identified that undiluted DNA extracts of individual cattle faecal samples had lower sensitivity (55%), compared to the five-fold dilution of the undiluted DNA extract, with a sensitivity of 76.25% (Acharya *et al.* 2017). In this study, we found that PCR inhibition occurred mainly in the larger pool sizes of 15 or 20 for all Mptb shedding levels in the undiluted DNA extract. To our knowledge, this is the first study to demonstrate that increasing the pool size of beef cattle faeces leads to an increase in the severity of PCR inhibition.

PCR inhibition can be relieved by dilution. PCR inhibition can result in false negative results but our study findings suggest that it can be relieved with the dilution of the DNA extract, a common non-specific method used to mitigate PCR inhibitors (Cao *et al.* 2012; Acharya *et al.* 2017). The five-fold dilution of the undiluted DNA extract resulted in a 91% increase in positive detection of the diluted pools, compared to the undiluted. This demonstrates that a simple non-specific five-fold dilution has the ability to relieve PCR inhibition and lead to a greater positive pool detection. However, diluting of the undiluted DNA extract too much may reduce the ability to detect the target amplicon in the sample (Morre *et al.* 2001; Cao *et al.* 2012). For instance, the 25-fold dilution conducted in this study led to a decreased number of positive samples being detected in the low and medium Mptb faecal shedding group. This decrease suggests that further dilution may have reduced the amount of Mptb DNA available in the aliquot, similar to the compromised qPCR sensitivity found when DNA dilution was used to detect *Enterococcus* (Cao *et al.* 2012).

Five-fold dilution is sufficient to relieve inhibition. Determining the optimal dilution factor is vital to successfully mitigating PCR inhibitors. Besides the 5- and 25-dilution used for all DNA extracts, we evaluated a 10-, 15- and 20-fold dilution of the DNA extracts for the faecal pool size 10 samples to determine the optimal dilution to relieve PCR inhibitors. The further dilution of a pool size of 10 for the high Mptb shedding group in this study demonstrated 100% sensitivity for all dilution factor tested. The medium shedding group had 70% of the pools detected positive for both the 5- and 10-fold dilution; however, a decrease in the number of positive pools was detected when the dilution factor exceeded 10-fold. Similarly, the 5-fold dilution was the optimal dilution factor for the low Mptb DNA group, as additional dilutions also decreased the proportion of positive pools detected. These results demonstrate that the 5-fold dilution is the optimal dilution factor, as there are no additional benefits with the further dilution of the undiluted DNA extract. Our results agree with a previous recommendation of conducting both the undiluted and five-fold dilution of DNA samples to reduce the possibility of diluting and reanalysing the sample, post inhibition (Cao *et al.* 2012). Therefore, we recommend using a 5-fold dilution to relieve inhibition in pooled samples.

5.3 Herd-sensitivity and specificity

The laboratory investigation was followed by the creation of simulation models to evaluate herd-sensitivity and specificity for various pooling strategies in a range of scenarios for both Sample and Check testing. Herd-sensitivity (SeH) is the probability of detecting a truly diseased herd, i.e. the probability of a positive herd to yield a positive Sample or Check test. Similarly, herd-specificity (SpH) is the probability that a truly negative herd yields a negative Sample or Check test result. A herd is declared positive if at least one pool from the herd tests positive.

Herd-sensitivity increases with herd prevalence. In all of our simulation models, herd-sensitivity was low for herds with very low prevalence levels but increased with an increase in prevalence. For example, in a Sample test of 300 cows using a pool size of 10, there was only 44% chance of detecting a typical BJD positive herd (herd size of 500) with a prevalence of 1%, but this probability increased to more than 75% for herds with 3% prevalence. Similar behaviour was observed for Check testing for most of the pool sizes. This is expected, as the proportion of infected (and thus Mptb shedding) animals would be higher in high prevalence herds which are obviously more likely to be detected in any testing regime. It is worth mentioning here, that we deliberately selected low prevalence levels for our simulation scenarios as J-BAS testing is more likely to be used in such herds.

Herd-sensitivity achieved with pool size 10 is comparable or better than that with pool size 5. In most of the scenarios, pool size 10 achieved similar or better herd-sensitivity that of pool size 5. This is partly due to the higher HT-J sensitivity estimated for pool size 10 in laboratory experiments but could also be due to a greater chance of inclusion of faeces from an infected animal in a pool created with faeces from 10 animals rather than from 5 animals. This pattern was evident in the main analysis for both Sample and Check tests (for both n = 50 and 100) as well as in the sensitivity analysis considering different proportions of Mptb shedding animals in a herd. This pattern became even more apparent in the sensitivity analysis conducted by ignoring individual sample HT-J sensitivity in the model. The only simulation model in which this pattern reversed, i.e. herd-sensitivity with pool size 5 was better than that of pool size 10, was the scenario assuming imperfect specificity. This higher herd-sensitivity for pool size 5 could be due to the testing of a large number of pools (60 pools for pool size 5 versus 30 pools for pool size 10), thus increasing the chance of detecting a false positive pool. This was confirmed by a lower herd-specificity achieved for this pool size compared to other pool sizes (66% for pool size 5 versus 81% for pool size 10). This suggests that pool size 10 would perform better than pool size 5 if the specificity is perfect, but pool size 5 could outperform pool size 10 if the specificity of HT-J is not perfect.

Herd-sensitivity for Check test can be increased by 70% by increasing pool size from 5 to 10. Herd-sensitivity for Check test using the current approach of testing 10 pools of size 5 (n = 50) was very low (~5% to ~20% for various pool sizes for herd-prevalence levels from 0.5 to 3%; Table 7). This sensitivity can be increased by 70-80% for various herd sizes by simply testing 10 pools of size 10 (n = 100; Table 8). Note that similar to Sample test, herd-sensitivity for Check test also increased when we ignored individual sample sensitivity or assumed an imperfect specificity and decreased when we assumed different proportions of Mptb shedding animals in a herd in sensitivity analyses, but these issues are only relevant to consider which factors influence test performance and are unlikely to be of use in increasing Check test herd-sensitivity in real-life.

Cost of Sample test can be halved by using a pool size of 10 rather than 5. Since a pool size of 10 achieved similar or better herd-sensitivity than a pool size of 5 in all scenarios when HT-J specificity was perfect, this pool size can potentially be used to replace pool size 5 in J-BAS testing. Making this change will reduce the cost of the Sample test by half while maintaining the ability of the test to detect an infected herd. This proposition is likely to hold as long as the laboratories ensure (a) testing of both neat and 5-fold diluted DNA extracts, and (b) following a rigorous protocol to ensure a perfect HT-J specificity. This reduction in cost by half assumes that the cost for sample collection and testing a pool of size 10 would be the same as that for a pool of size 5. It is not an unreasonable

assumption to make as far as laboratory testing is concerned because there is no difference in the protocol for testing a pool of size 10 or 5, if we assume that laboratories would use a 5-fold dilution of the DNA for both pool sizes. However, if the dilution protocol is only used for testing a pool size of 10, then there would be additional costs of this procedure. Collection of 30 pools of pool size 10 rather than 60 pools of pool size 5 will have similar costs. In fact, collection and transport of 30 pools could be cheaper than 60 pools due to the use of fewer sampling containers for sample collection and postage.

Cost of Check test can also be halved but it is not the recommended approach. Similar to Sample test, the cost of Check test can also be halved from the current $1200 to $600 by testing 5 pools of size 10 rather than testing 10 pools of size 5 (n = 50) if the aim is to reduce costs while maintaining the current level of herd-sensitivity. However, as the herd-sensitivity achieved in most of the Check testing scenarios in this project was low to very low, it is preferable to focus on increasing herd-sensitivity of the Check test by testing 10 pools of size 10 (i.e. n =100). Laboratory testing costs for 10 pools of size 10 would be the same as for the current approach of testing 10 pools of size 5, but sample collection costs/time is likely to increase for sampling 100 rather than 50 animals.

HT-J Herd-specificity. We assumed a perfect HT-J specificity, and thus the HT-J herd-specificity achieved in most of our scenarios was perfect. In one of the sensitivity analyses, we assumed imperfect specificity and achieved a herd-specificity ranging from 66% for pools of size 5 to 90% for pools of size 20 (Table 10; Fig. 15). For a fixed sample size (say n = 300), herd specificity increased with pool size because fewer pools were required to be tested as the pool size increased (i.e. 60 pools of size 5 versus 15 pools of size 20), thus reducing the chance of a false positive result. Imperfect specificity also had a sizeable effect on the achieved herd-sensitivity as it increased the herd-sensitivity of most pool sizes as more herds were detected as positive probably due to false positive detection of some pools. This impact was more obvious in pool size 5, potentially due to the increased likelihood of obtaining a false positive result, as discussed above. This suggests that it is very important for field validation of herd-level specificity to confirm this is similar to the individual HT-J (99.6% for cattle, Plain 2014), as modelling shows that even a minor reduction in specificity can have a substantial effect on the achieved herd-specificity and herd-sensitivity.

HEC test may be difficult to implement in the beef industry. The HEC test achieved higher herd-sensitivity than the HT-J Check test though it is likely to be an overestimate, as we could not model the herd-sensitivity to vary by prevalence because of the limited input information available to us for low prevalence herds simulated in this study.

HEC test was originally developed for the dairy industry and involves collection of faecal slurry from milking yards immediately after milking. Therefore, from a practical point of view, it is difficult to see how such a test could be implemented in beef herds, where there is no ready source of faecal slurry representing the whole herd. This issue could be further discussed in the workshop proposed below.

5.4 Achievement of project objectives

The overall aim of the project was to investigate ways in which the cost to beef producers of herd screening tests for BJD can be reduced without sacrificing accuracy or compromising the J-BAS status of a herd. Specifically, the project was conducted with the following three objectives:

- *To estimate the effect of pooling on pooled HT-J sensitivity and specificity (accuracy) in a laboratory setting.*

  This objective has been achieved as we successfully estimated the effect of pooling on pooled HT-J sensitivity and specificity in a laboratory setting. The detailed methods for this laboratory experiment are described in Section 3.1, the results are presented in Section 4.1 and discussed in Sections 5.1 and 5.2 of this report. Briefly, the results suggested that pool a

of size 10 had similar HT-J sensitivity and specificity to a pool of size 5. However, the experiment also confirmed the presence of PCR inhibition in pooled samples and revealed that this problem worsens with an increase in pool size, though it can be resolved by a 5-fold dilution of the DNA extract.

- *To model HT-J sensitivity and specificity achieved in real-life scenarios for farms of various sizes, with a range of disease prevalence levels and by selecting a varying number of pools and pool sizes for both the 'Sample' and 'Check' testing.*

  This objective was successfully achieved as we fitted simulation models as described in Section 3.2.1. The results of the models are presented in Section 4.2 and discussed in Section 5.3. These analyses indicated that a pool size of 10 achieved better/similar herd-sensitivity than a pool size of 5. The models also identified approaches to reduce costs for Sample and Check testing without sacrificing accuracy as well as to increase accuracy for Check testing without increasing costs.

- *To evaluate if herd environmental culture (HEC) currently recommended as equivalent to a 'Check test' for dairy herds could be used in a beef enterprise without compromising the sensitivity and specificity achieved in real life scenarios.*

  This objective was successfully achieved. The methods are described in Section 3.2.2 and results in 4.2.5. The herd-sensitivity achieved by the HEC test was 36%, but when faecal samples were pooled to mimic the collection of slurry from 50 or 100 cattle, the herd-sensitivity achieved ranged from 5 to 16%.

5.5 Further research

Investigate the reasons and solutions for PCR inhibition. The performance of the HT-J test was impacted by PCR inhibition. We had also observed this phenomenon in a previous study (Acharya et al., 2017). To test for and to alleviate PCR inhibition, we tested the DNA extract of all pooled samples neat (undiluted) as well as by making dilutions. The results confirm the presence of PCR inhibition in pooled HT-J testing. The impact of PCR inhibition on the HT-J test is emerging as an important issue and needs to be promptly addressed by conducting further research. Further details about the issue and the proposed experiments are provided in Appendix 1 (Section 9.1).

Investigate the sequential effect of Sample and Check testing. In this project, we built simulation models for Sample and Check testing to evaluate the influence of pooling strategies on herd sensitivity and specificity. However, it was beyond the scope of the study to investigate the effect of increased biosecurity or repeated testing results. Theoretically, the achieved herd-sensitivity is expected to be higher if two sequential Sample tests are negative or if a negative Sample test is followed by a negative Check test or by two negative Check tests. This issue can be investigated by building scenario trees or by extending the model developed in this project. This is unlikely to influence the association of pool size with herd-sensitivity observed in this project, but a higher herd-sensitivity will provide better assurance to farmers, the government and our trading partners.

Conduct field validation of the modelling results. The simulation modelling results should be validated in the field before a wider application. This can be done by enrolling properties undergoing Sample and Check tests and by collecting both individual faecal samples and pooled faecal samples (both of pool size 5 and 10). This will enable comparisons of results from different sampling strategies and enable field validation of the modelling results, before a wider implementation of the policy to increase the pool size to 10. The only scenario in which the pool size of 5 performed better than a pool size of 10 was the model assuming imperfect specificity. Since the herd-specificity depends mainly on HT-J specificity for a given number of pools tested in a herd, field validation of the herd-specificity is also important. We can prepare a complete study design for this study if required for a tender. This work can also be combined with the above two studies in a single project.

6 Conclusions/recommendations

6.1 Conclusions

J-BAS is a tool developed to determine the risk of JD on a property in the new assurance approach developed by AHA to deal with this disease in an unregulated environment. Besides considering the history of JD on the property and the implementation of a biosecurity plan, it uses a Sample test (60 pools, each of size 5, i.e. n = 300) followed by a Check test (10 pools, each of size 5, i.e. n = 50) every three years to objectively confirm the negative JD status of a cattle property. Assuming a rate of $120 for testing each pool, this testing protocol costs $7200 for the initial Sample test and $1200 for each Check test. This project was conducted to identify cost-effective strategies for determining the J-BAS status of a herd. Conclusions from this project are described below.

A pool of size 10 has the same pool-sensitivity as a pool of size 5. HT-J pool sensitivity achieved for a pool of size 10 was similar to or better than that achieved for a pool of size 5 in low, medium and high Mptb shedding animals in our laboratory investigation. This implies that a pool constituted by pooling faeces from 10 cattle is as or more likely to be detected in an HT-J test as a pool constituted with faeces from 5 cattle. As expected, HT-J sensitivity is highest in high and lowest in low shedding animals.

HT-J achieved a perfect specificity at all pool sizes. In the laboratory investigation, all 40 pools created by pooling dung from JD negative cows tested negative, confirming that the specificity of HT-J is perfect regardless of the pool size. This provides assurance that producers could use HT-J test with confidence, without worrying about false positive results even in larger pool sizes.

The problem of PCR inhibition is real. PCR inhibition for HT-J was reported previously and has been confirmed again in this study. This problem was solved by a 5-fold dilution of the PCR extract without understanding the actual reasons for PCR inhibition. Our limited experiments in this project indicated that 10- to 25-fold dilution does not provide any additional benefit and thus is not necessary but further investigations are required to fully understand this issue to improve the validity of HT-J.

Sample test with pool size 10 performs at least as well as that with pool size 5. Assuming a perfect HT-J specificity, Sample testing scenarios using a pool size of 10 achieved similar/higher herd-sensitivity than those using a pool size of 5. This implies that a property with JD is at least as likely to be detected with Sample test using a pool size of 10 than using a pool size of 5. This advantage of pool size 10 over pool size 5 holds at all prevalence levels tested in this project, at both equal or unequal proportions of low, medium and high shedding animals in a herd, and with or without adjusting for individual animal HT-J sensitivity. The only scenario in which a pool size of 5 performs better is assuming an imperfect pool HT-J specificity.

Cost of Sample testing can be halved by using a pool size of 10 while maintaining accuracy. The cost of Sample testing using a pool size of 10 would drop to $3600 for a typical property from the current $7200. Further marginal savings can be achieved in sample collection and transportation. All this can be achieved without losing the probability of detecting a truly JD positive herd, as long as a perfect HT-J specificity can be maintained.

Herd-sensitivity of Check test can be increased using a pool of size 10. Testing 10 pools of size 10 would nearly double the herd-sensitivity of Check test compared to the current scenario of testing 10 pools of 5 while maintaining costs. Alternatively, 5 pools of size 10 can be tested which will maintain herd-sensitivity while reducing costs.

Maintaining herd-specificity is crucial. Diagnostic test specificity had a substantial effect on our results. Many of the above findings would be incorrect if herd-specificity is not perfect. Therefore, it is crucial to maintain near-perfect herd-specificity. Herd-specificity depends on HT-J pool specificity

and the number of pools sampled. Assuming that the numbers of pools sampled are fixed, it solely depends on the HT-J specificity achieved in a laboratory, and therefore, it is very important to ensure that all precautions are taken to ensure specificity is maintained.

6.2 Recommendations

Based on the conclusions of the project we make the following recommendations:

Use 30 pools of size 10 for J-BAS Sample testing. Our laboratory and simulation modelling results suggest that pool- and herd-sensitivity achieved with a pool size of 10 is very similar to or higher than that achieved with a pool size of 5 for Sample testing (sample size = 300). The results hold in various simulation scenarios under various assumptions, as long as a perfect HT-J specificity can be maintained, suggesting that this finding is rigorous, and can be implemented under field conditions. Therefore, we recommend replacing the current Sample testing strategy of testing 60 pools of size 5 with a new strategy of testing 30 pools of size 10.

Use 10 pools of size 10 for J-BAS Check testing. Based on our simulation modelling results, we recommend using 10 pools of size 10 for J-BAS Check testing instead of the current approach of testing 10 pools of size 5. This would nearly double the herd-sensitivity achieved in Check testing without increasing the costs of testing. Although similar to Sample testing, costs for Check testing can also be halved by testing 5 pools of size 10 but increasing herd-sensitivity for Check test makes more sense as low or very low herd-sensitivities were achieved for Check tests in most of our scenarios.

Diagnostic laboratories should test 5-fold diluted DNA extract to relieve PCR inhibition. In addition to testing the neat DNA extract, we recommend that the diagnostic laboratories should test the 5-fold diluted DNA extract to check and relieve PCR inhibition and thus improve HT-J performance. This is more important for high prevalence herds and for larger pools, although further investigations are required fully understand this issue.

Organise a workshop to change J-BAS testing policy. MLA should organise a workshop involving representatives from the beef industry, Animal Health Australia and diagnostic laboratories to discuss the findings of this project and to decide on a way forward to change the J-BAS testing policy. The workshop is also important to sensitise diagnostic laboratories to the issue of PCR inhibition and to discuss how a change in pool size in the testing protocol can be implemented. The project team is willing to coordinate the workshop, if funding can be made available, or to present our findings at a workshop hosted by the MLA.

Further research should be conducted for field validation of new pooling strategy. Further research is required to evaluate the findings of the project by collecting and testing samples from the field. This can be undertaken during the piloting of the new testing policy to confirm findings from simulation modelling before a wider application of the strategy for using a pool of size 10. Similarly, further research is required to investigate the reasons and to identify solutions for PCR inhibition.

7 Key messages

The project has the following key messages:

For animal health policy developers and producers:

- Pool size 10 performs at least as well as pool size 5 in J-BAS testing
- Cost of the Sample test can be halved by testing 30 pools of size 10 instead of the current approach of testing 60 pools of size 5 without impacting test accuracy.
- Performance of the Check test can be increased by 70% by testing 10 pools of size 10 instead of testing 10 pools of size 5 as done currently.

For diagnostic laboratories

- It is critical to maintain the HT-J test specificity.
- PCR inhibition should be considered when testing faecal samples using the pooled HT-J.
- PCR inhibition can be relieved by dilution of PCR extracts.
- There are no additional benefits of 10- to 25-dilution over a 5-fold dilution of PCR extracts.

For the beef industry

The beef industry could save millions by switching to a pool size of 10 for Sample testing. The current J-BAS testing strategy using a pool size of 5 has been estimated to cost up to $7200 for the initial Sample test. If about half of the 43000 beef farms in Australia conduct Sample test, saving of $3600 per farm will save $77 million for the industry collectively though the actual benefits will depend on the number of Sample tests conducted.

Messages about changed recommendations for pool size should be communicated to producers, but *only after they are ratified by the workshop involving all stakeholders*. Extension education experts should be engaged to design these messages, but the project team is happy to contribute to this process.

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9 Appendix

9.1 Appendix-1: Further research to investigate and resolve PCR inhibition

9.1.1 What is PCR inhibition?

Faeces, as the primary test sample used for the molecular diagnosis of Johne's disease, is an extremely challenging sample type due to the presence of the normal faecal microbiota, undigested particulate matter and plant components, humic acid, bile, and proteins among other components. For ruminants, undigested residues of herbivorous matter such as cellulose, hemicelluloses and lignin constitute a major part of the faecal matter. Many of these substances can be inhibitory in quantitative PCR reactions, due to interactions with the components involved in the amplification of the DNA, leading to potential false-negative results. Addressing such PCR inhibition is as complex as the sample material that causes it. The summation by Dr Delphine Rapp from AgResearch NZ was apt when she stated "*In summary, the complexity and variability of the inhibition associated with faecal samples of bovine origin (evidenced by Thornton and Passen 2004) may prevent the ability to remove all the faecal components. Effective removal of faecal components has to reach a balance between obtaining a concentration of inhibitors at a level that does not affect PCR amplification, and a concentration of target DNA sufficient for PCR amplification.*" (Rapp D. 2010).

During the development of the HT-J test, there were two approaches used to address this complex issue. Firstly, debris present in the faecal sample was allowed to settle out by gravity and only the upper aqueous supernatant of the faecal-saline suspension was included in the test. Secondly, a magnetic bead-based method was used for the DNA extraction step. Rather than column-based methods, which require unbound sample lysate to be washed through the membrane in a tube, the intention of the magnetic bead method is to enable binding to the nucleic acids to the beads that and are physically removed by a magnet from the remaining sample lysate and transferred sequentially to new vessels containing wash buffers, in order to remove any contaminants. The validation of the test was based on herd/flock status. How the test would perform when applied to individual animal testing was not as well understood.

There is increasing evidence that PCR inhibition remains an issue for the HT-J test. There was some evidence that the test may not be as sensitive for individuals as it was for herd-level detection. Given the increasing use of the HT-J for individual animal testing in Australia, we conducted a study to investigate the phenomenon in more detail (Acharya et al, 2017). The aims of the study were to investigate the presence of PCR inhibition in the HT-J test within a defined sample set, partially characterise the inhibiting agent, if present, and develop a strategy to ameliorate the impact of inhibition. The faecal samples were derived from a single, high prevalence, endemically infected cattle herd. This study identified that 59/296 (approximately 20%) of samples had some evidence of inhibition, with the degree of inhibition impacting the HT-J test result in 42/296 samples (14%). A five-fold dilution of the DNA extract led to the detection of more HT-J positive individuals and an increase in test sensitivity from 55% to 80% compared to faecal culture. It was also identified that DNA extracts with higher DNA and protein content had 19.33- and 10.94-times higher odds of showing inhibition, respectively, though the inhibitory substance(s) were not specifically identified.

Further evidence from diagnostic testing performed in the Infectious Diseases Laboratory in the Farm Animal Health group suggests that pooled faecal samples may be even more prone to this phenomenon than individual samples. Based on this knowledge, a dilution of the DNA extract has been a standard practice in diagnostic testing conducted in our laboratories and this dilution step was included in the current study design, as described in this report.

9.1.2 Alternative approaches to address PCR Inhibition in pooled HT-J

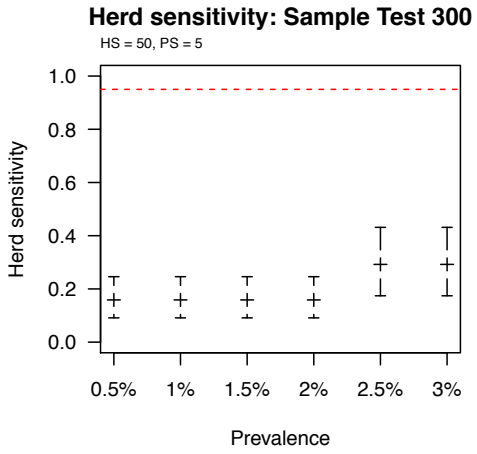
The identification of the unique inhibitory substance(s) that affect the HT-J test would be a significant advance that may allow a specific modification to the test methodology in order to address the issue. Further, advances in qPCR Taq polymerase reagents and magnetic bead technologies during and since the development of the HT-J test also offer opportunities to address the issue of PCR inhibition. PCR inhibitor-resistant enzyme mixes are available for applications ranging from forensic science, pathogen detection for clinical medicine and soil/plant biology. A new magnetic bead-based kit has also been developed that is designed to give optimal DNA purification from complex sample types such as faeces. A combination of the two is likely to reduce the impact and incidence of PCR inhibition for the HT-J test.

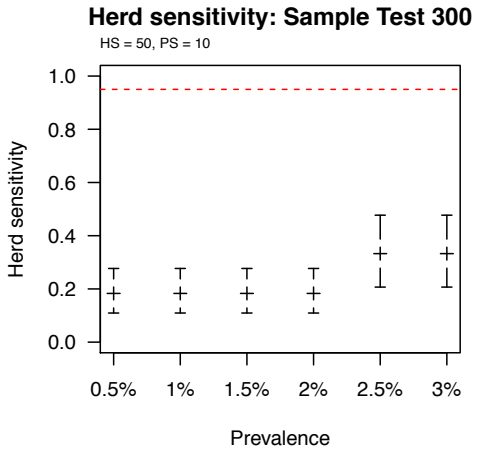
9.1.3 Proposed additional experiments

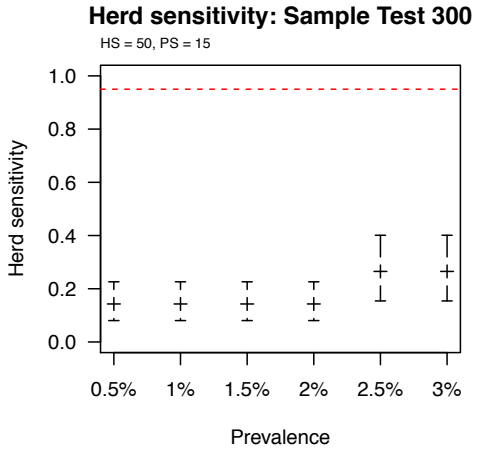
Although DNA extract dilution is proving effective, it is not optimal. A proposed study would involve parallel investigations:

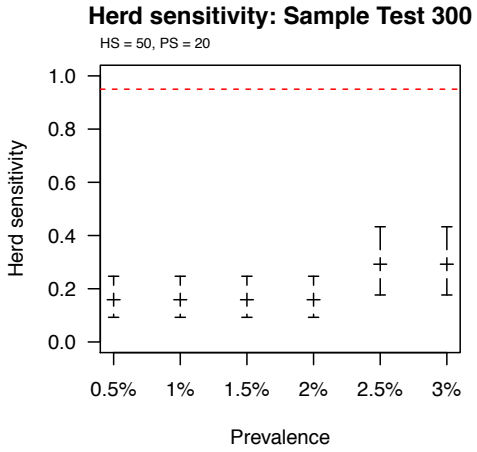
- Investigation of the unique inhibitory substance(s) that affect the HT-J, using a substantial sample archive with defined inhibitory classification and linkages to The University of Sydney NMR and mass spectrometry facilities.
- Investigation of advanced qPCR reagent and magnetic bead technologies for pooled faecal HT-J testing. An initial pilot study would be conducted using samples with known inhibition classification to investigate the potential for these technologies to reduce the presence and impact of PCR inhibitors in DNA extracts from bovine faeces.

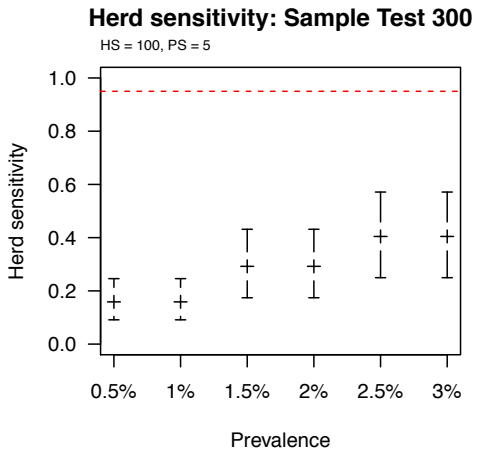
9.2 Appendix-2: Influence of prevalence on herd-sensitivity

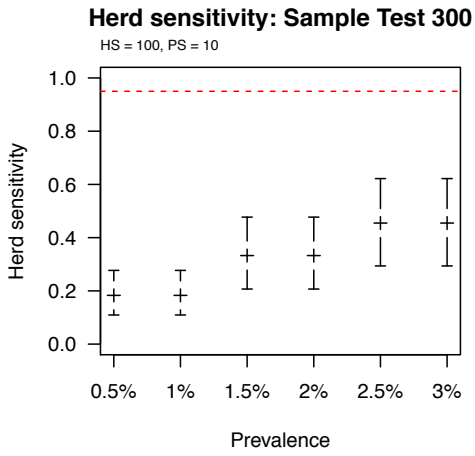


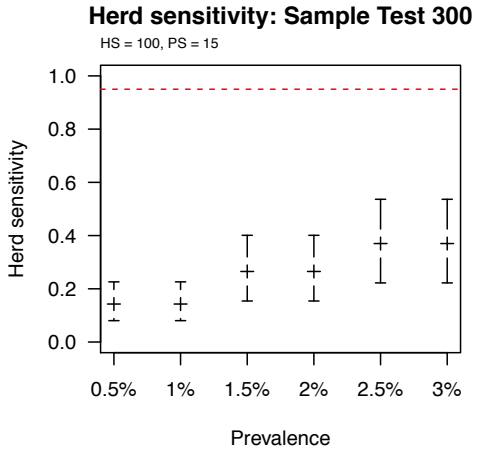


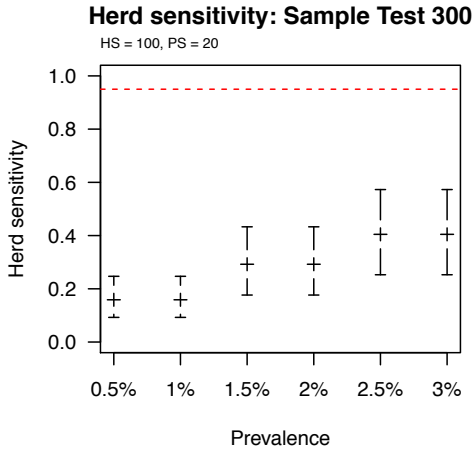


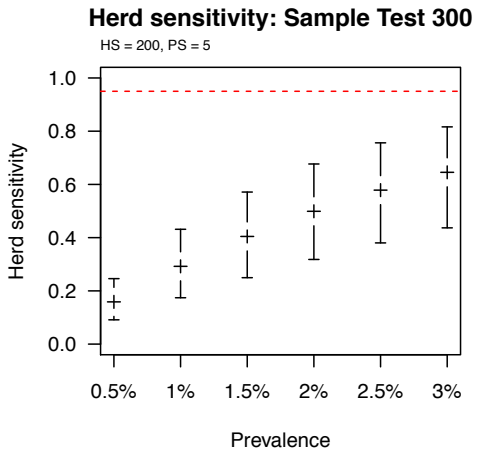


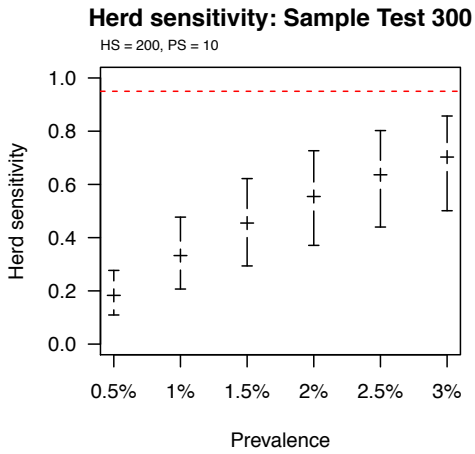

Herd sensitivity: Sample Test 300
HS = 100, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

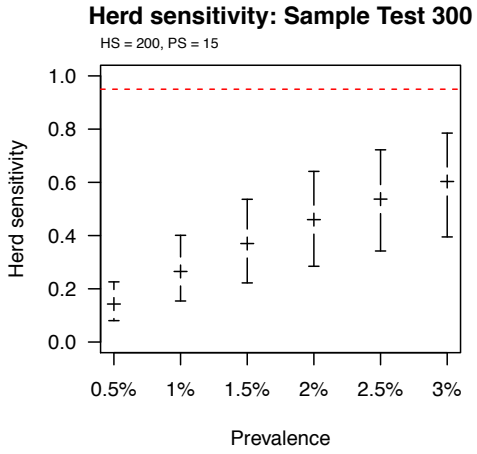

Herd sensitivity: Sample Test 300
HS = 100, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

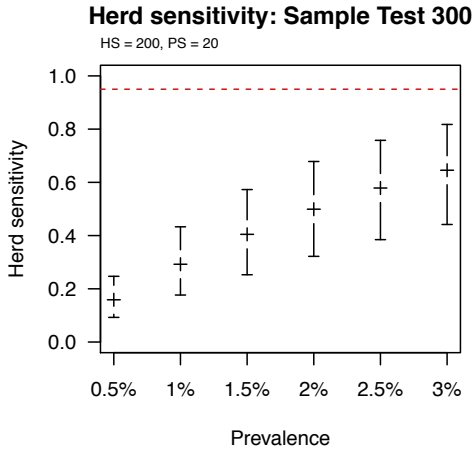

Herd sensitivity: Sample Test 300
HS = 100, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

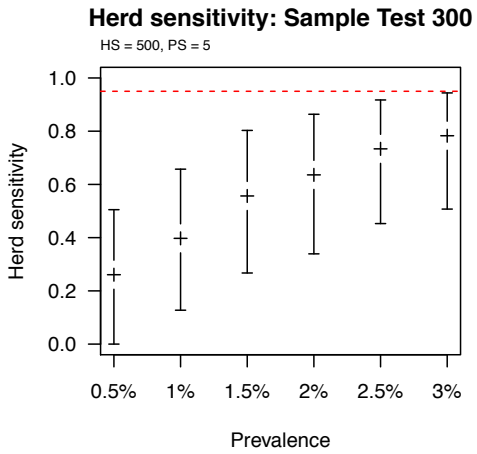

Herd sensitivity: Sample Test 300
HS = 100, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

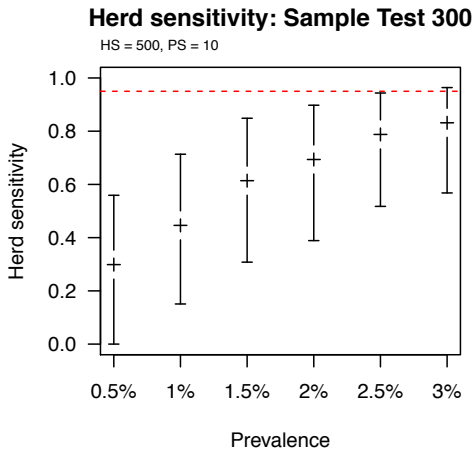

Herd sensitivity: Sample Test 300
HS = 200, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

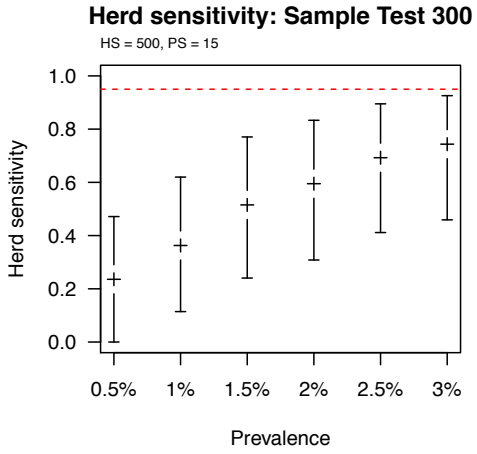

Herd sensitivity: Sample Test 300
HS = 200, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

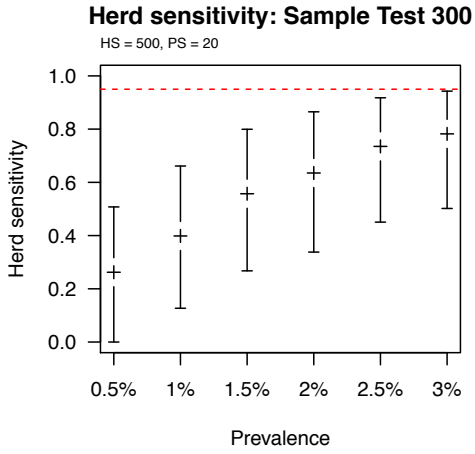

Herd sensitivity: Sample Test 300
HS = 200, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

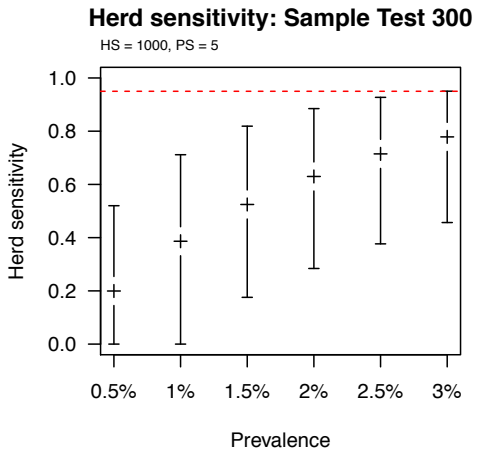

Herd sensitivity: Sample Test 300
HS = 200, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

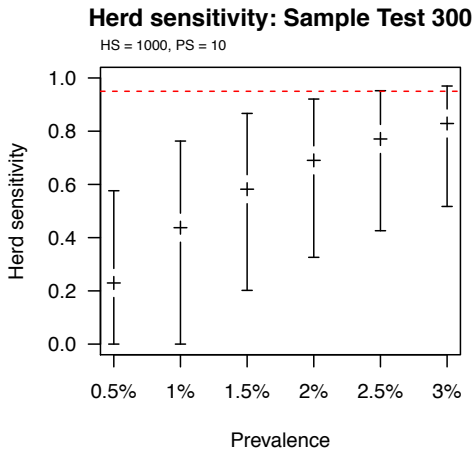

Herd sensitivity: Sample Test 300
HS = 500, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

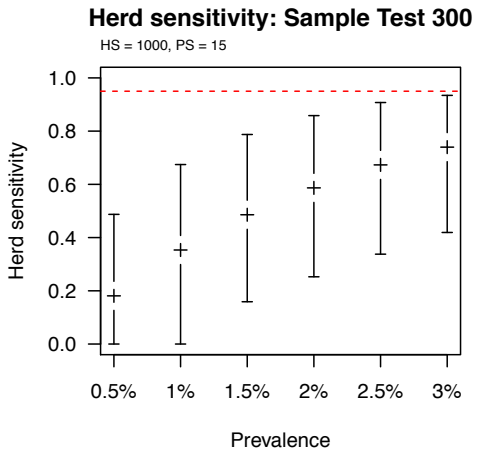

Herd sensitivity: Sample Test 300
HS = 500, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

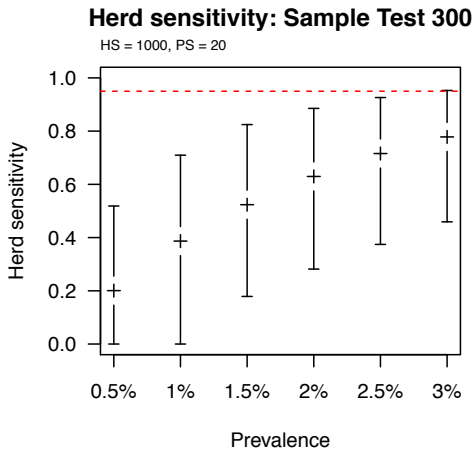

Herd sensitivity: Sample Test 300
HS = 500, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

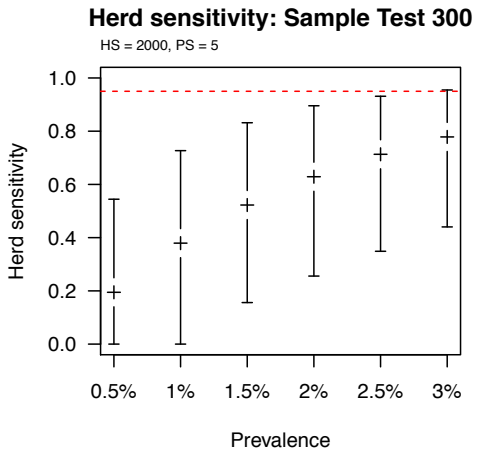

Herd sensitivity: Sample Test 300
HS = 500, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

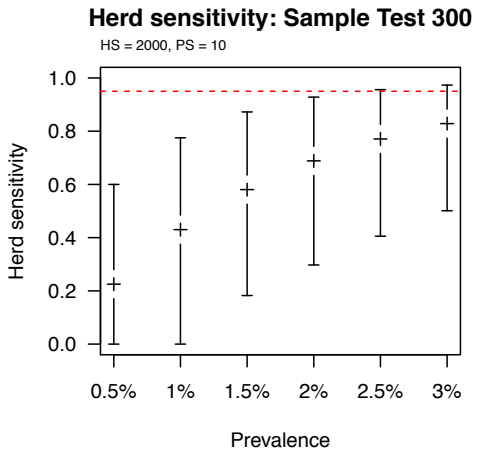

Herd sensitivity: Sample Test 300
HS = 1000, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

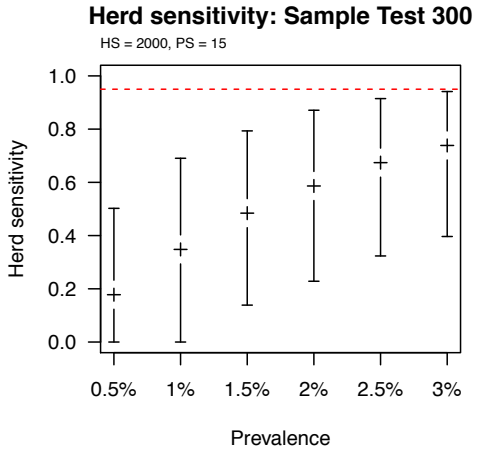

Herd sensitivity: Sample Test 300
HS = 1000, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

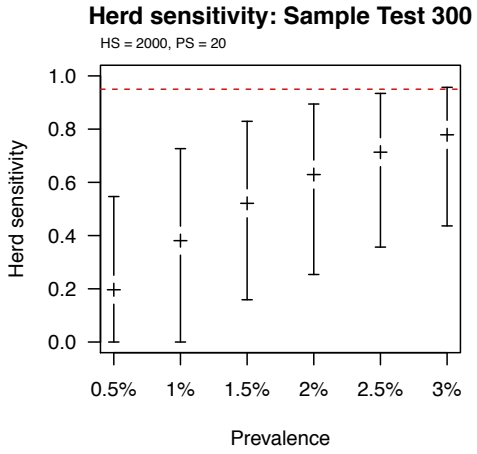

Herd sensitivity: Sample Test 300
HS = 1000, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

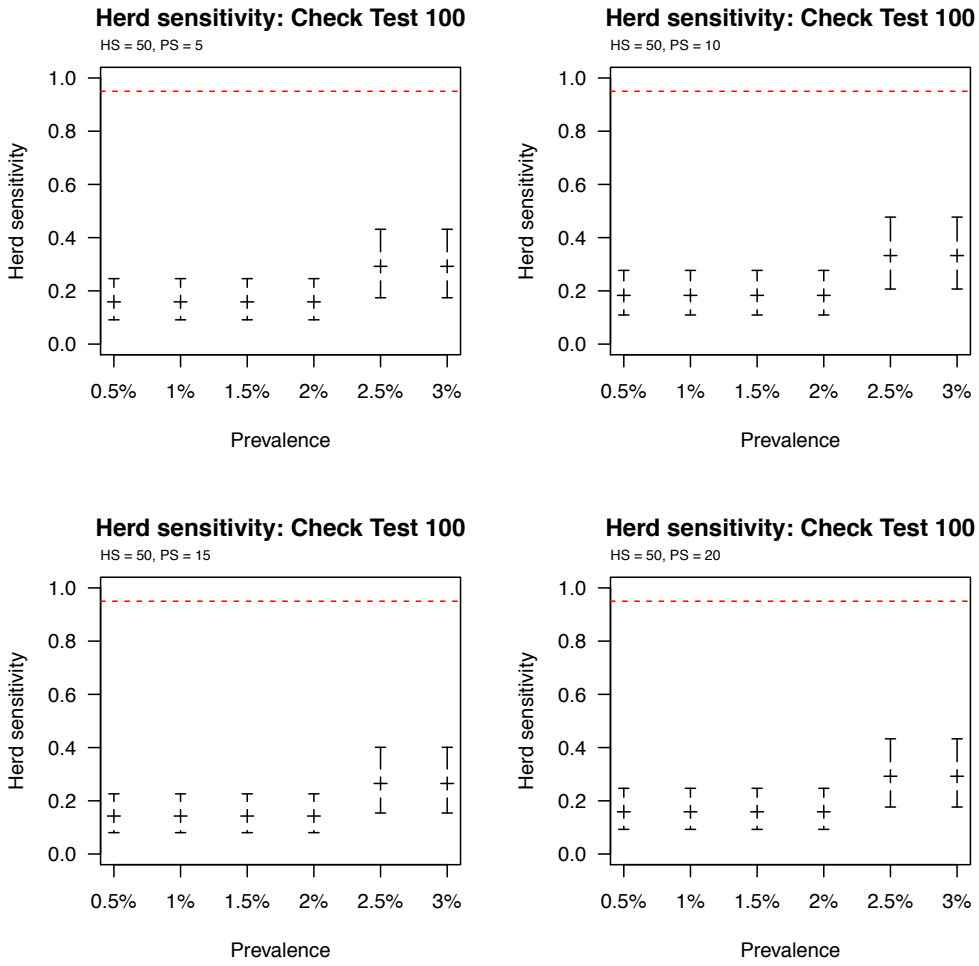

Herd sensitivity: Sample Test 300
HS = 1000, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

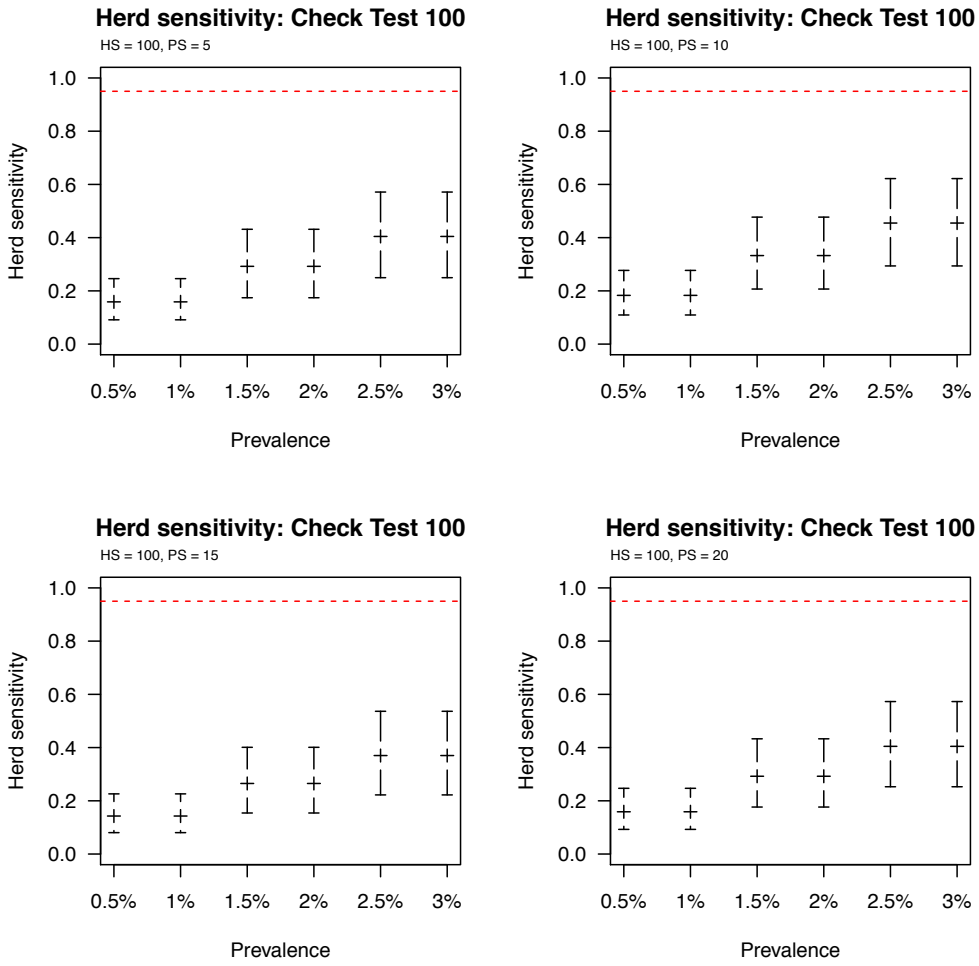

Herd sensitivity: Sample Test 300
HS = 2000, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

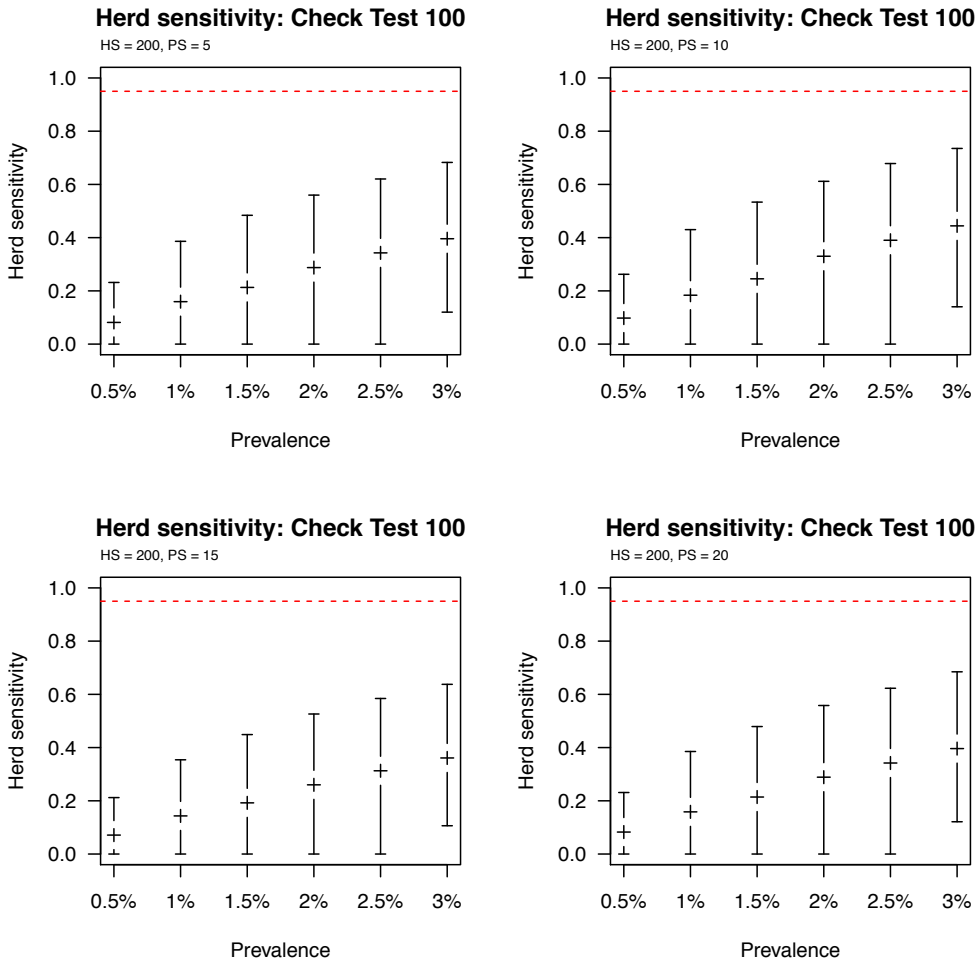

Herd sensitivity: Sample Test 300
HS = 2000, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

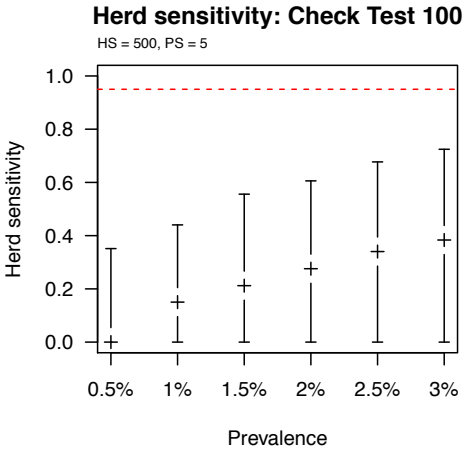

Herd sensitivity: Sample Test 300
HS = 2000, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

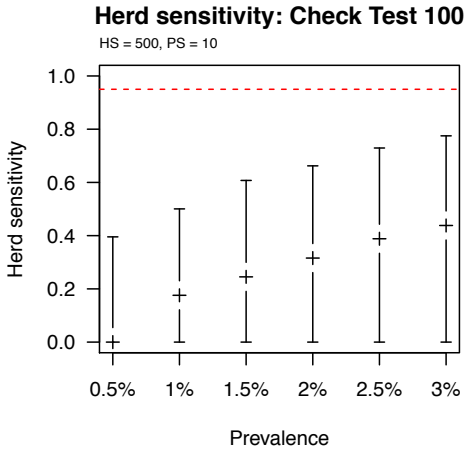

Herd sensitivity: Sample Test 300
HS = 2000, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

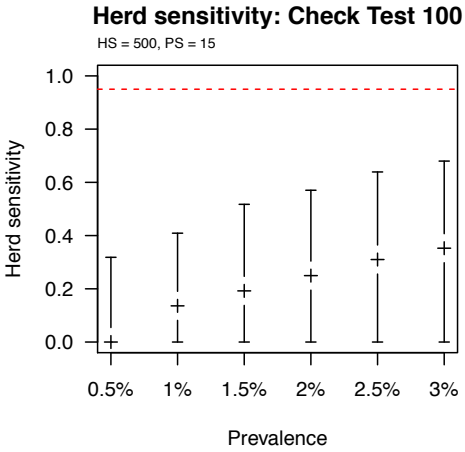

Herd sensitivity: Check Test 100
HS = 50, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 100
HS = 50, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 100
HS = 50, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 100
HS = 50, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

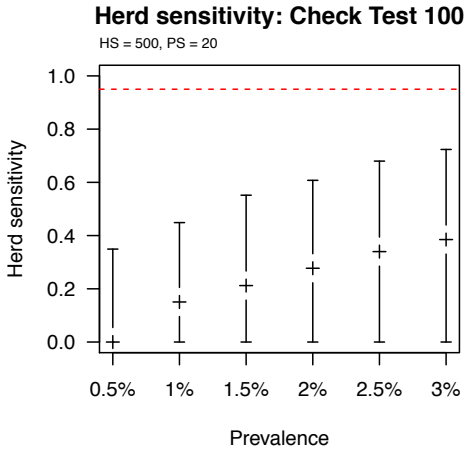

Herd sensitivity: Check Test 100
HS = 100, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 100
HS = 100, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 100
HS = 100, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 100
HS = 100, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

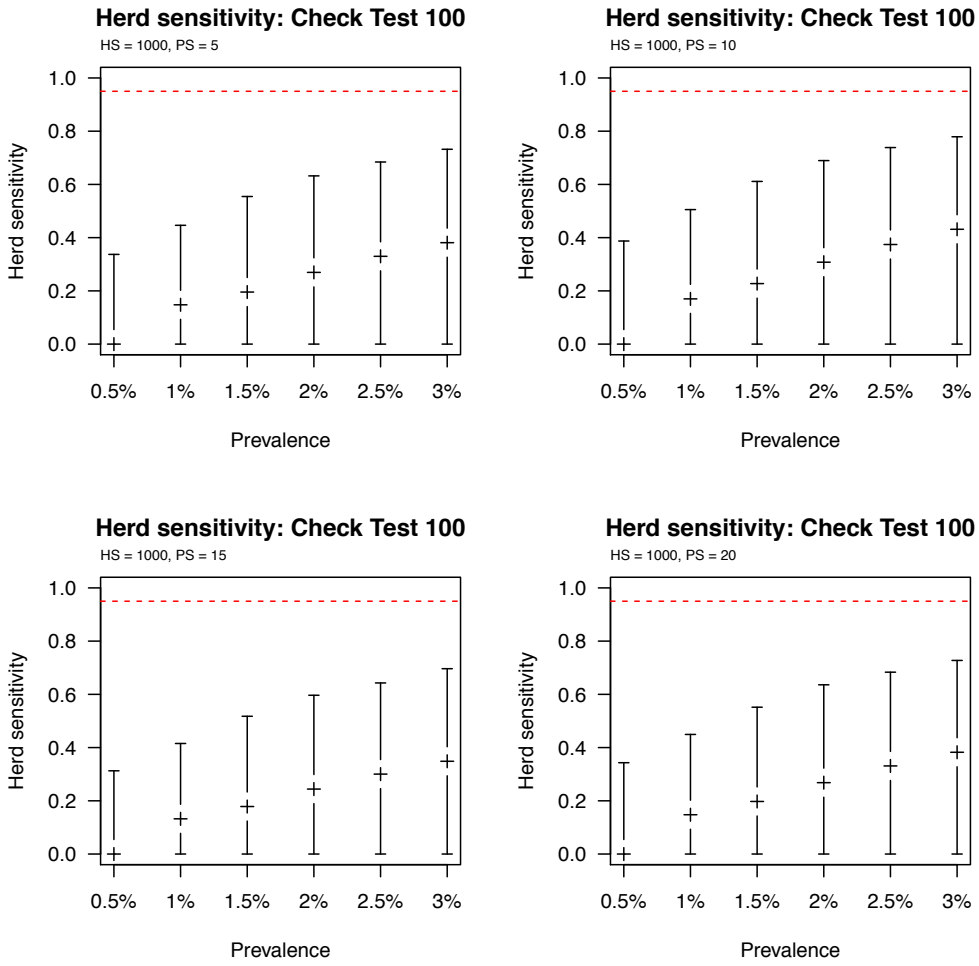

Herd sensitivity: Check Test 100
HS = 200, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 100
HS = 200, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 100
HS = 200, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 100
HS = 200, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

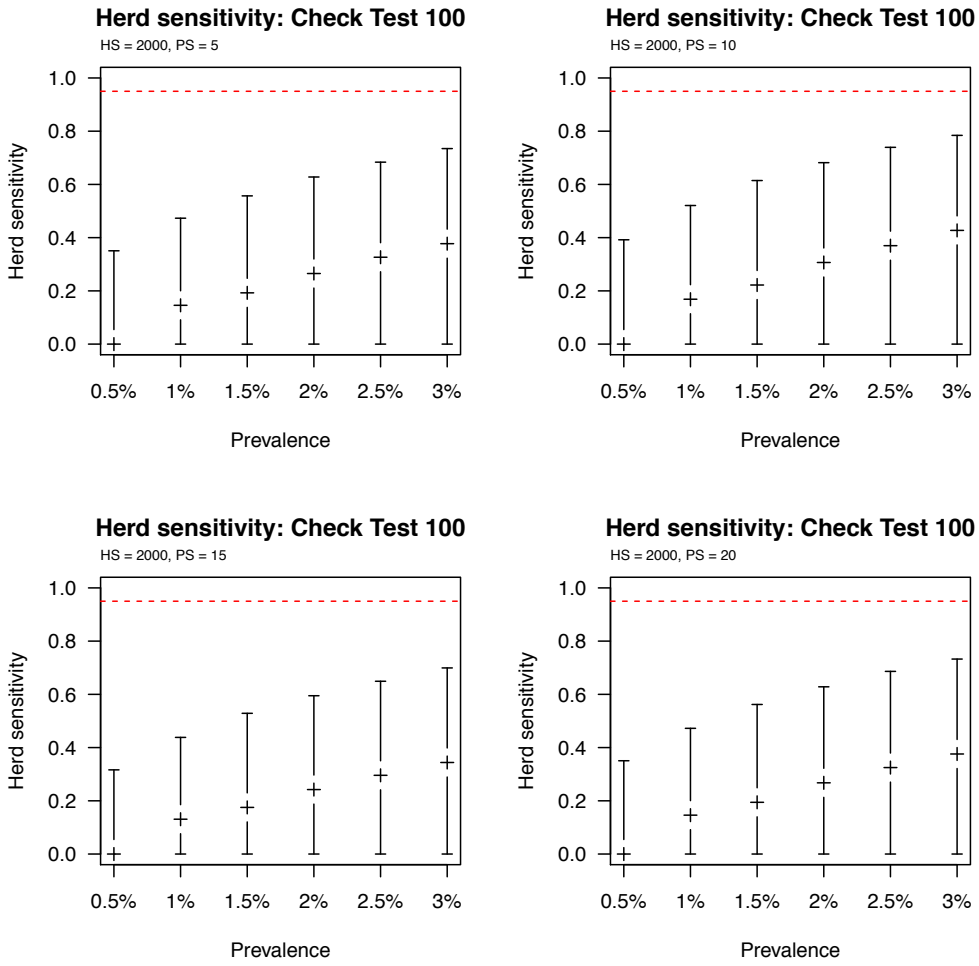

Herd sensitivity: Check Test 100
HS = 500, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

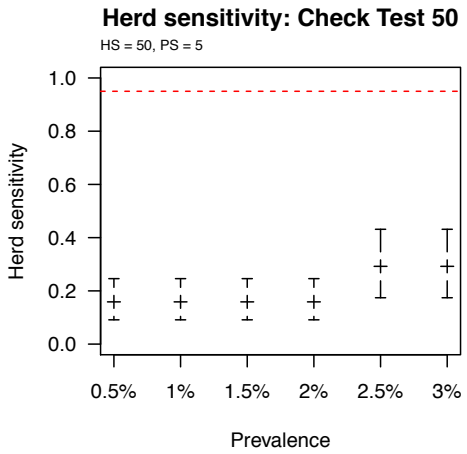

Herd sensitivity: Check Test 100
HS = 500, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

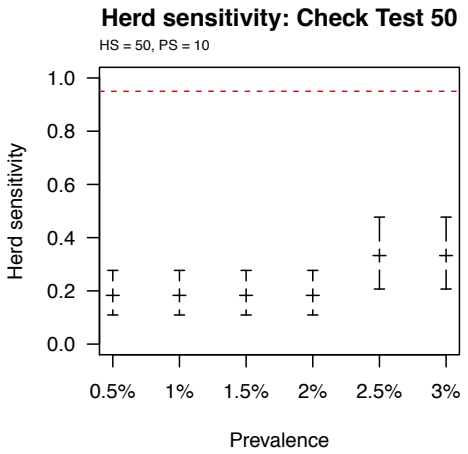

Herd sensitivity: Check Test 100
HS = 500, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

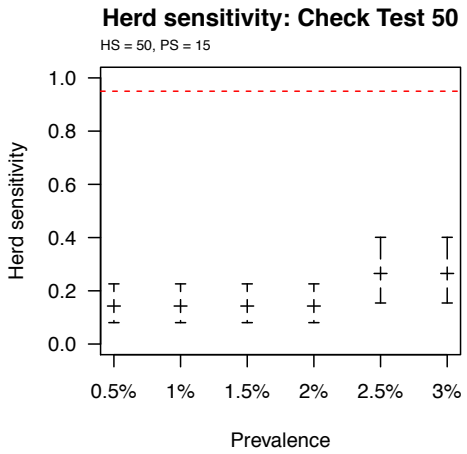

Herd sensitivity: Check Test 100
HS = 500, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

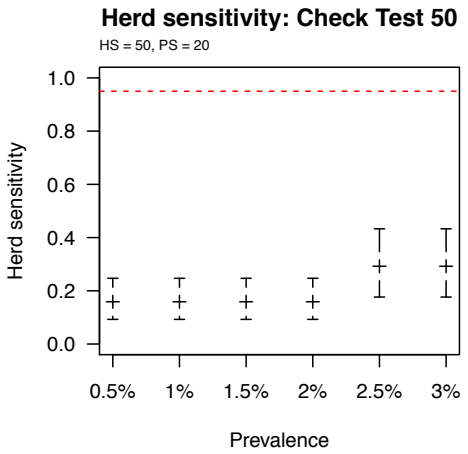

Herd sensitivity: Check Test 100
HS = 1000, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 100
HS = 1000, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 100
HS = 1000, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 100
HS = 1000, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

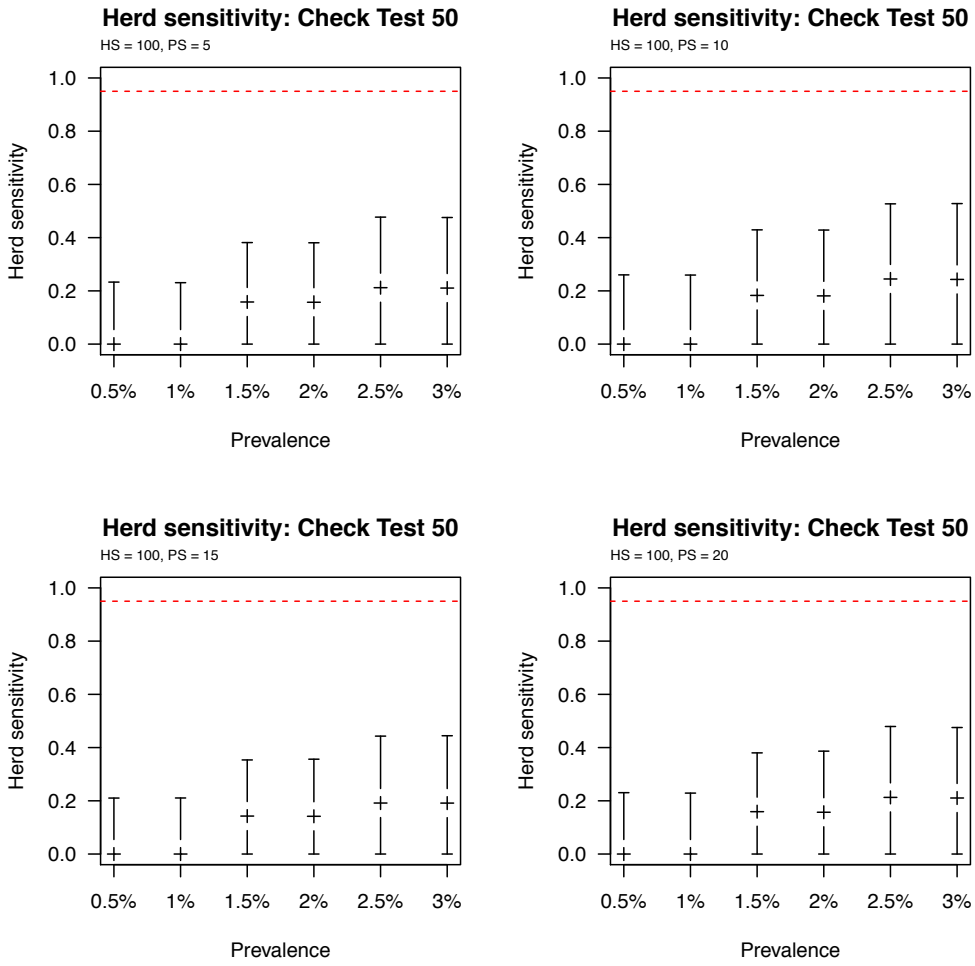

Herd sensitivity: Check Test 100
HS = 2000, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 100
HS = 2000, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 100
HS = 2000, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 100
HS = 2000, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

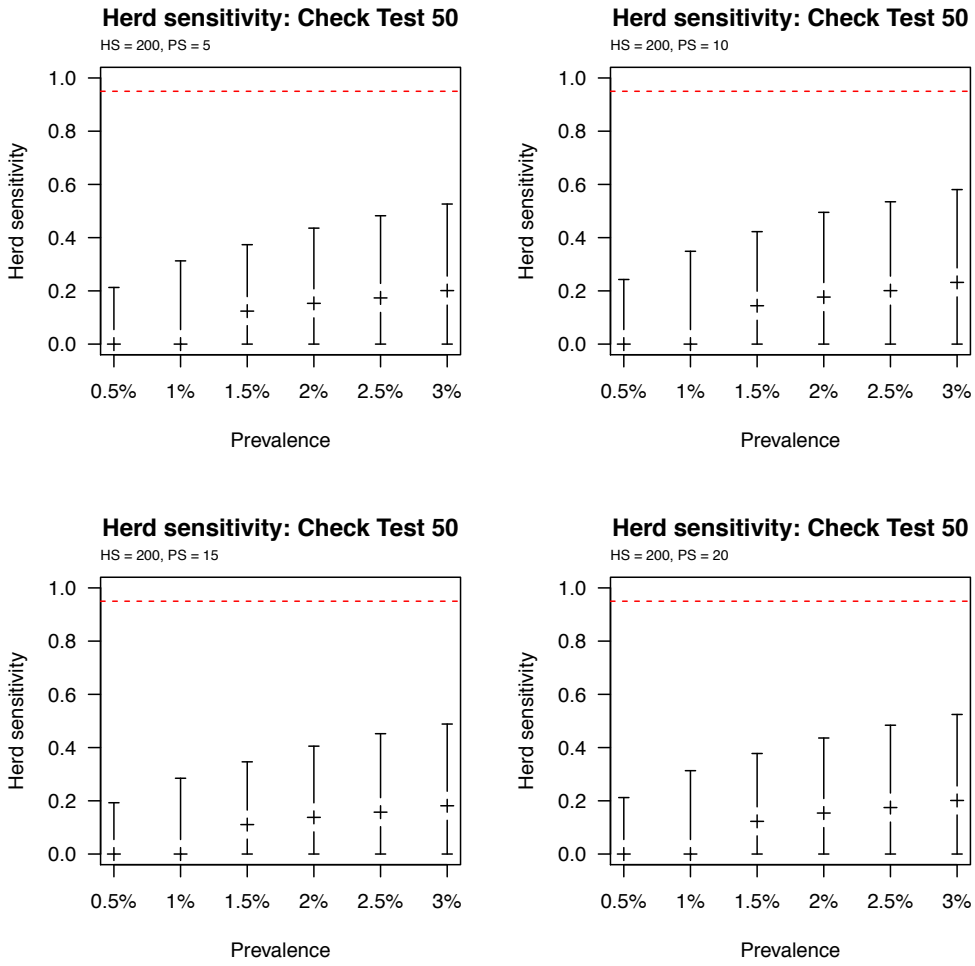

Herd sensitivity: Check Test 50
HS = 50, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

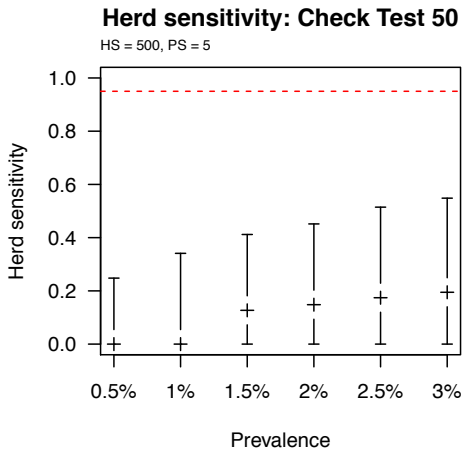

Herd sensitivity: Check Test 50
HS = 50, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

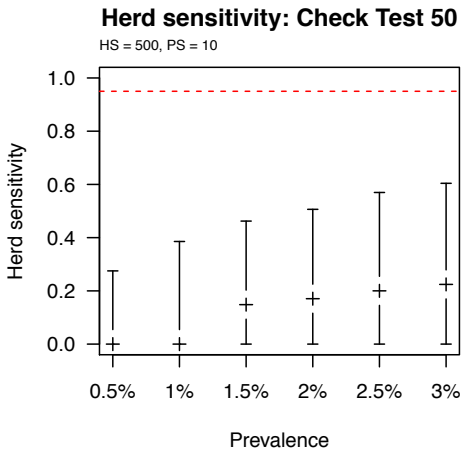

Herd sensitivity: Check Test 50
HS = 50, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

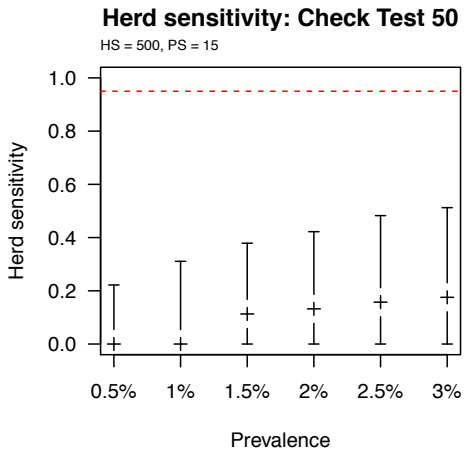

Herd sensitivity: Check Test 50
HS = 50, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

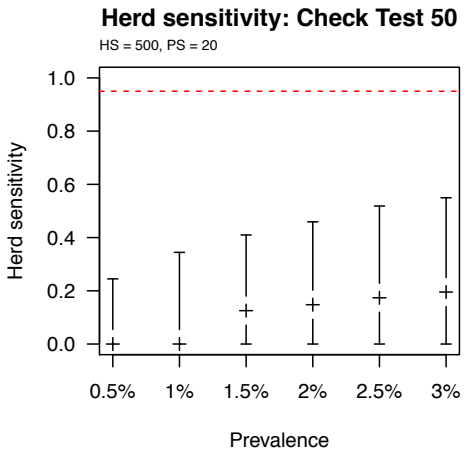

Herd sensitivity: Check Test 50
HS = 100, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 50
HS = 100, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 50
HS = 100, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 50
HS = 100, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

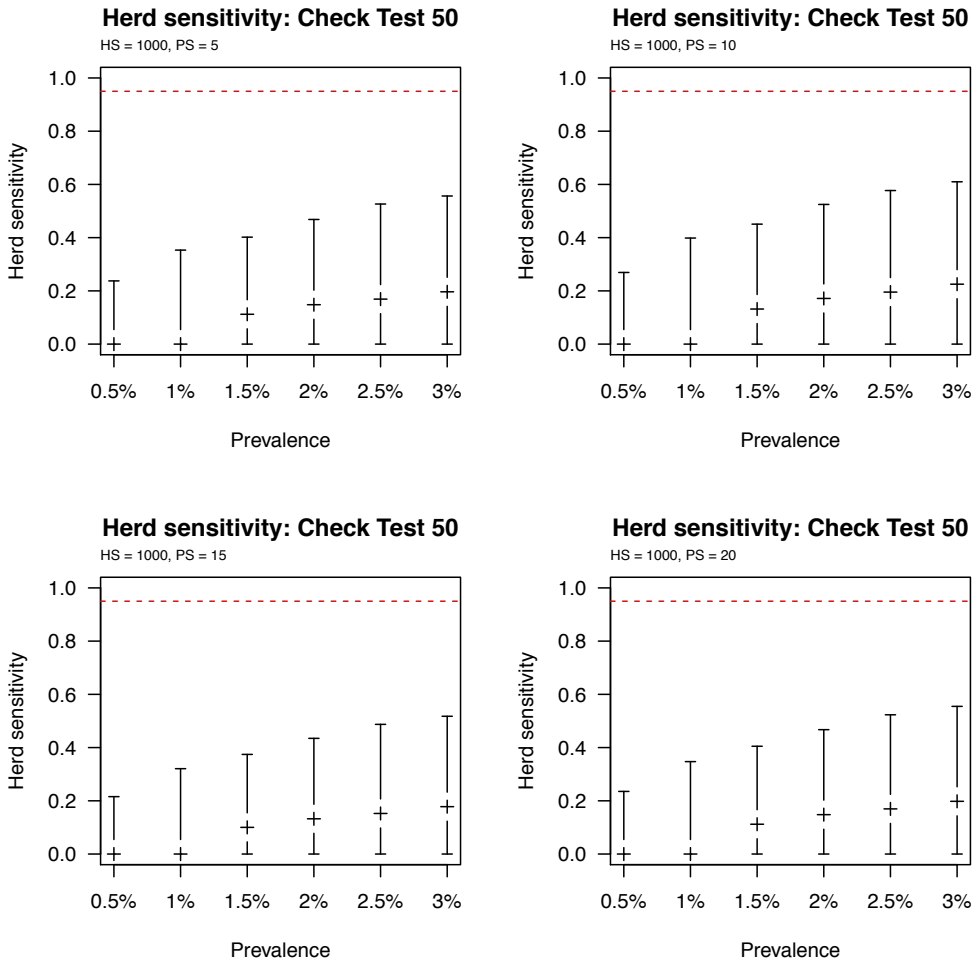

Herd sensitivity: Check Test 50
HS = 200, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 50
HS = 200, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 50
HS = 200, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 50
HS = 200, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

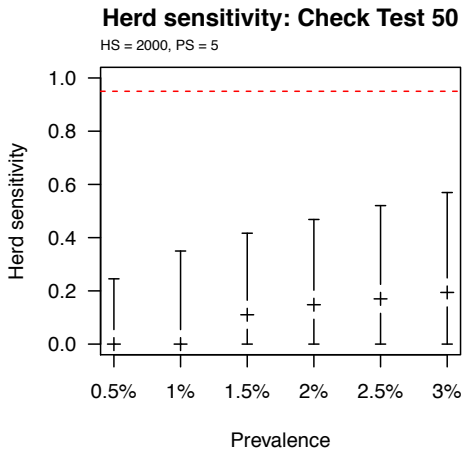

Herd sensitivity: Check Test 50
HS = 500, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

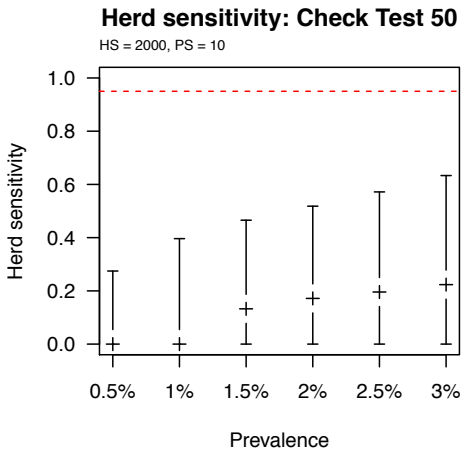

Herd sensitivity: Check Test 50
HS = 500, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

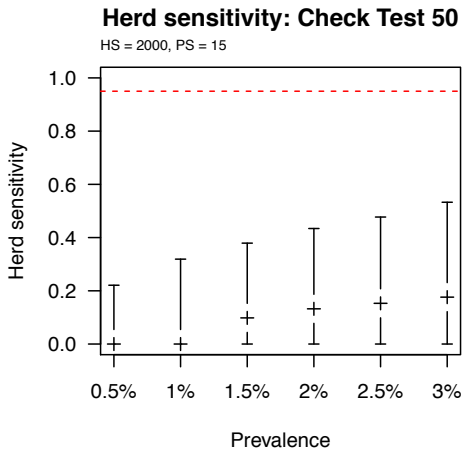

Herd sensitivity: Check Test 50
HS = 500, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

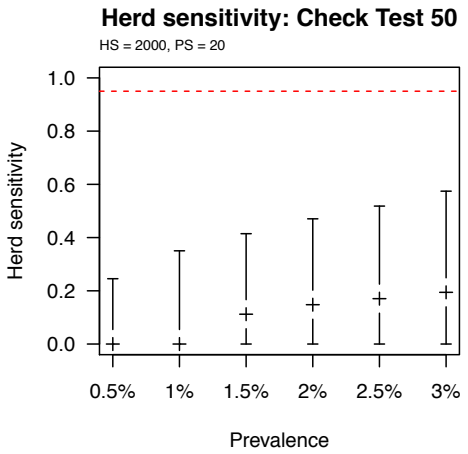

Herd sensitivity: Check Test 50
HS = 500, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence


Herd sensitivity: Check Test 50
HS = 1000, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 50
HS = 1000, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 50
HS = 1000, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence
Herd sensitivity: Check Test 50
HS = 1000, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

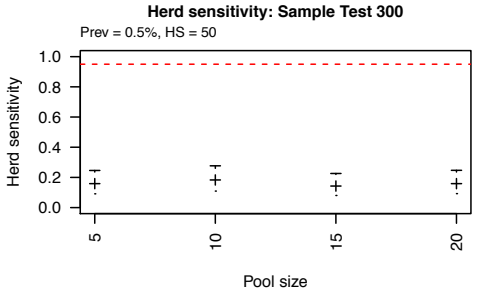

Herd sensitivity: Check Test 50
HS = 2000, PS = 5
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

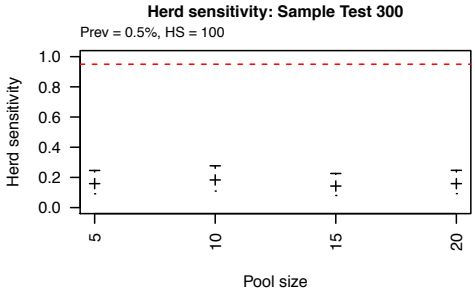

Herd sensitivity: Check Test 50
HS = 2000, PS = 10
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

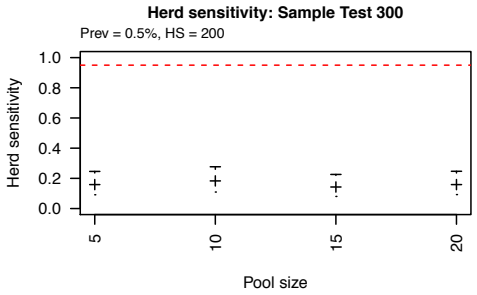

Herd sensitivity: Check Test 50
HS = 2000, PS = 15
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

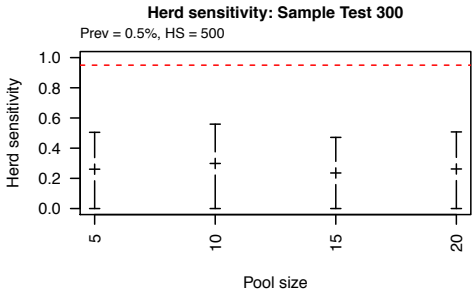

Herd sensitivity: Check Test 50
HS = 2000, PS = 20
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.5%
1%
1.5%
2%
2.5%
3%
Prevalence

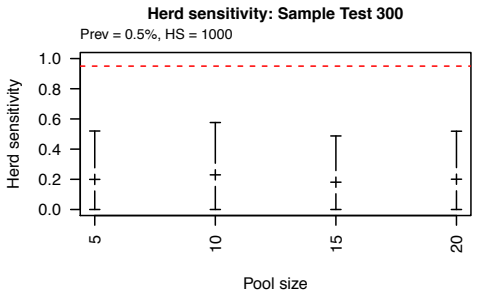
9.3 Appendix-3: Effect of pool size on herd-sensitivity

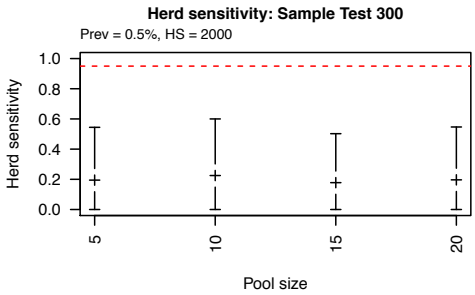


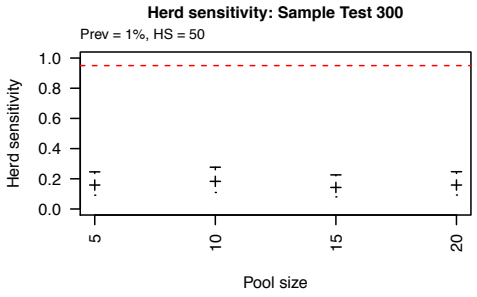


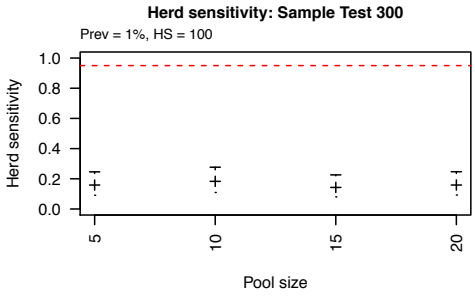


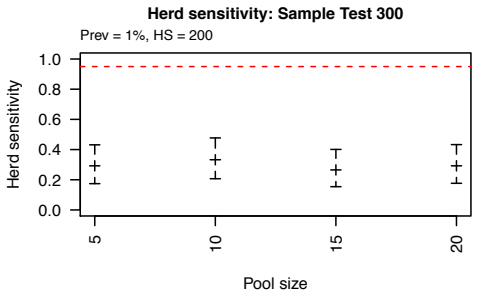


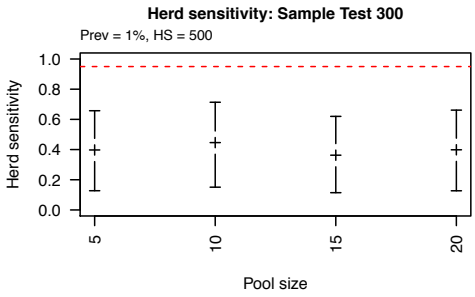


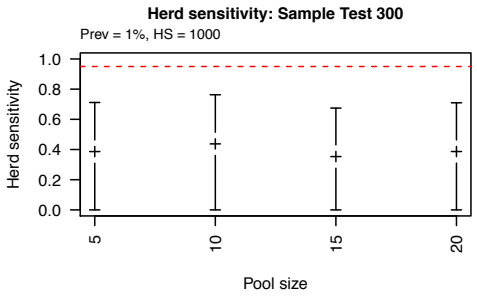


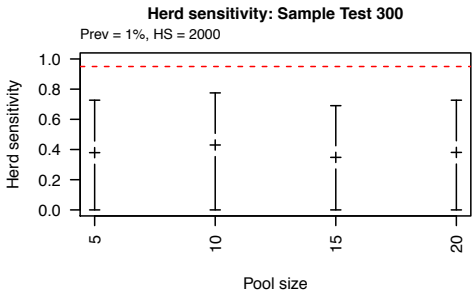

Herd sensitivity: Sample Test 300
Prev = 1%, HS = 50
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

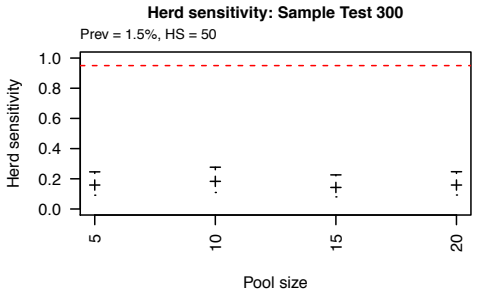

Herd sensitivity: Sample Test 300
Prev = 1%, HS = 100
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

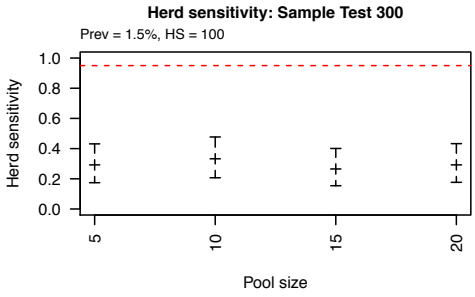

Herd sensitivity: Sample Test 300
Prev = 1%, HS = 200
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

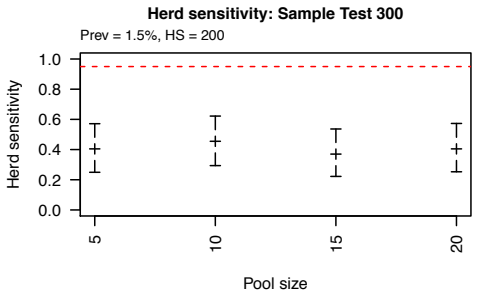

Herd sensitivity: Sample Test 300
Prev = 1%, HS = 500
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

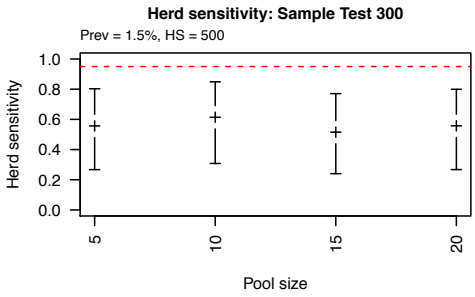

Herd sensitivity: Sample Test 300
Prev = 1%, HS = 1000
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

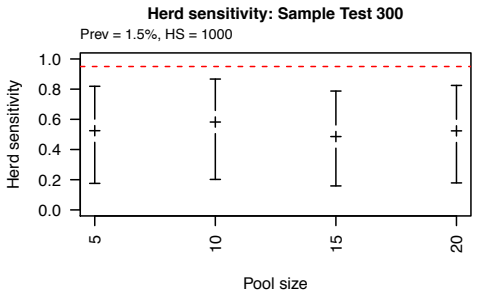

Herd sensitivity: Sample Test 300
Prev = 1%, HS = 2000
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

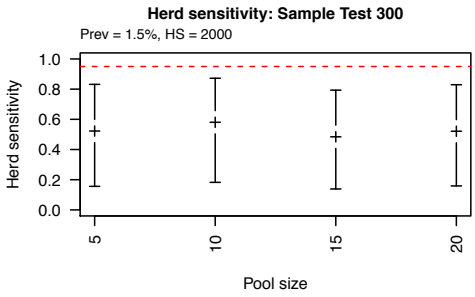

Herd sensitivity: Sample Test 300
Prev = 1.5%, HS = 50
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

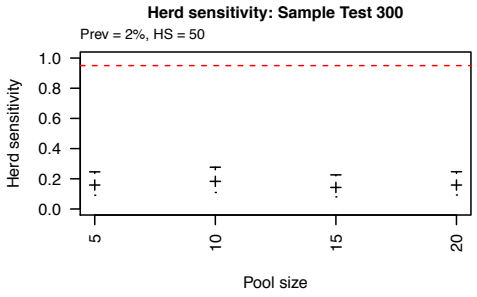

Herd sensitivity: Sample Test 300
Prev = 1.5%, HS = 100
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

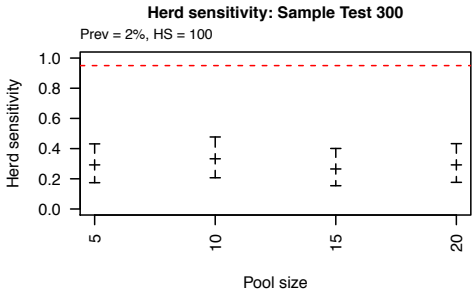

Herd sensitivity: Sample Test 300
Prev = 1.5%, HS = 200
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

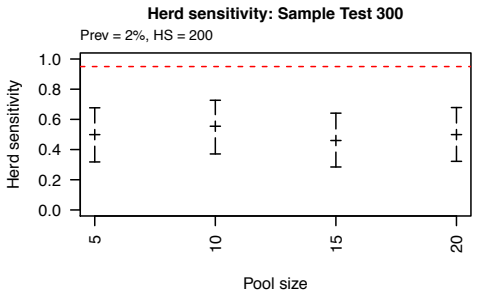

Herd sensitivity: Sample Test 300
Prev = 1.5%, HS = 500
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

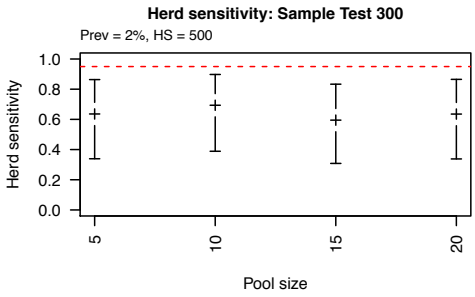

Herd sensitivity: Sample Test 300
Prev = 1.5%, HS = 1000
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

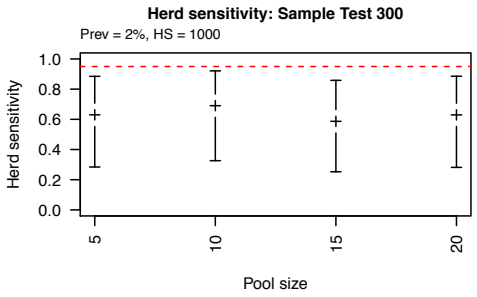

Herd sensitivity: Sample Test 300
Prev = 1.5%, HS = 2000
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size


Herd sensitivity: Sample Test 300
Prev = 2%, HS = 50
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size


Herd sensitivity: Sample Test 300
Prev = 2%, HS = 100
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size


Herd sensitivity: Sample Test 300
Prev = 2%, HS = 200
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size


Herd sensitivity: Sample Test 300
Prev = 2%, HS = 500
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size


Herd sensitivity: Sample Test 300
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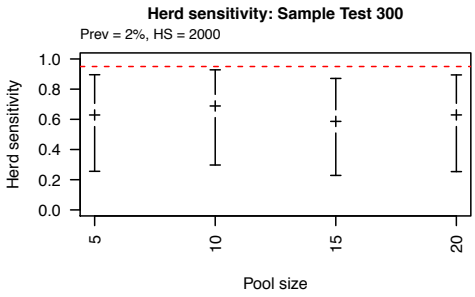
Herd sensitivity: Sample Test 300
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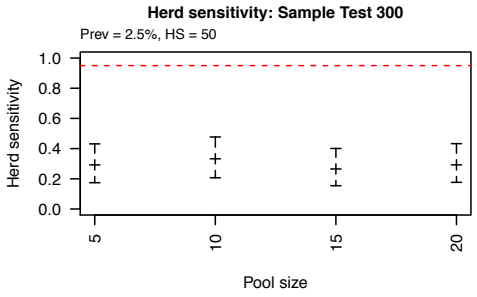
Pool size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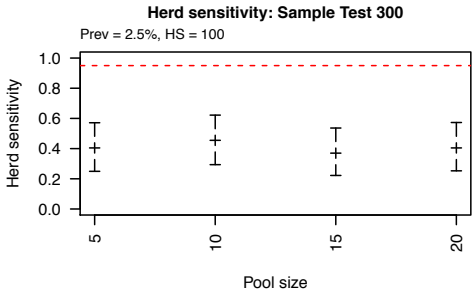
Pool size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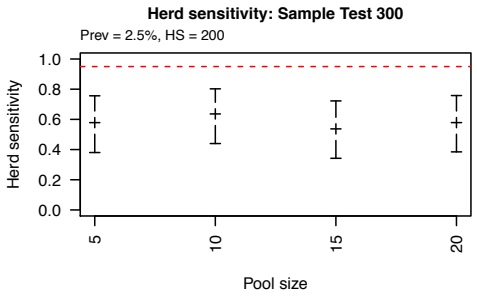
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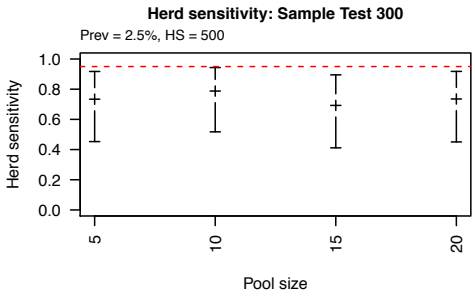
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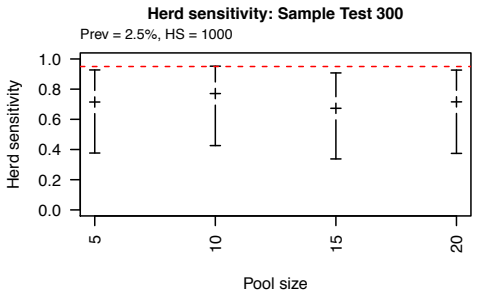
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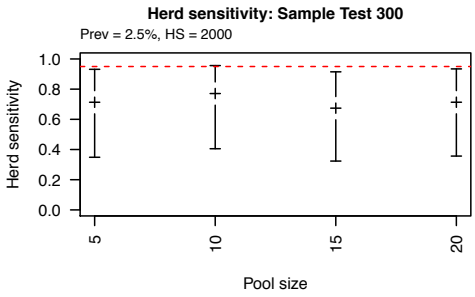
Herd sensitivity: Sample Test 300
Prev = 2.5%, HS = 2000
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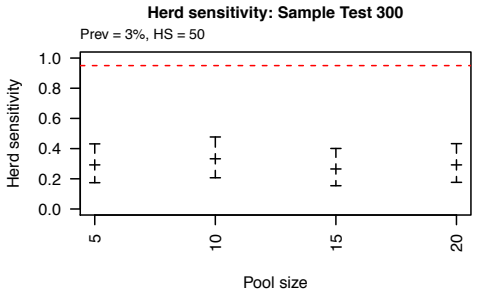
Herd sensitivity: Sample Test 300
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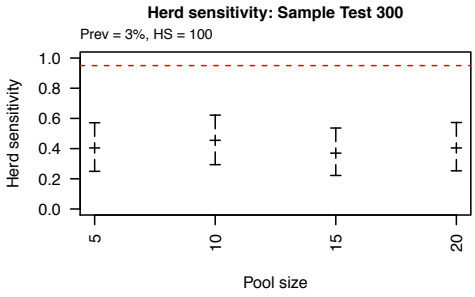
Pool size


Herd sensitivity: Sample Test 300
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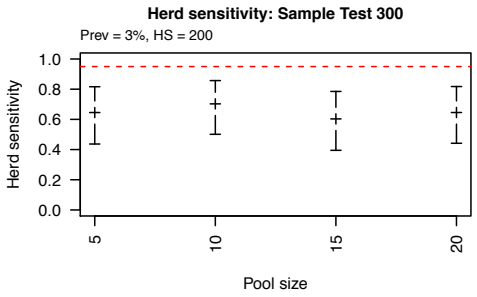
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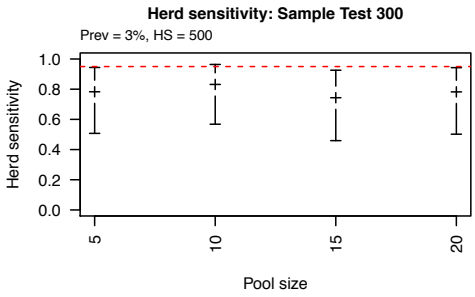
Pool size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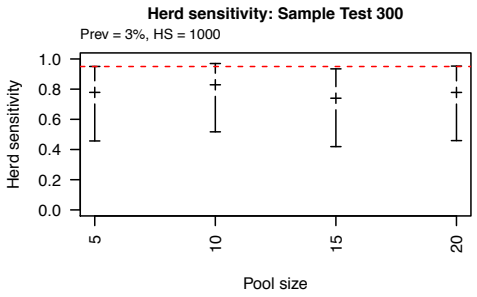
Pool size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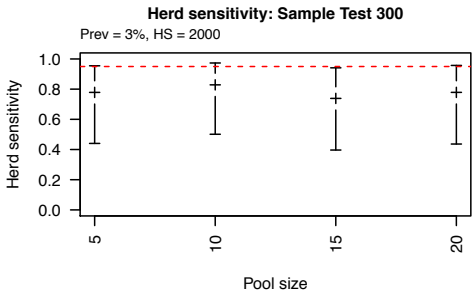
Pool size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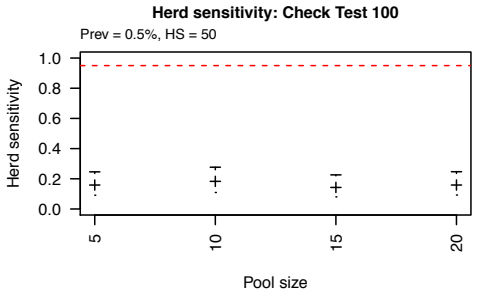
Pool size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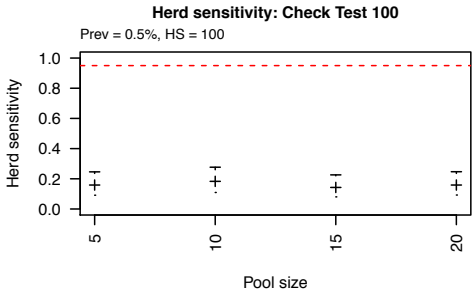
Pool size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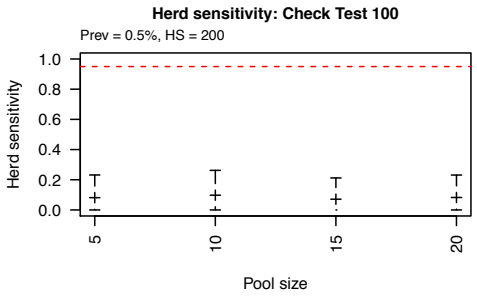
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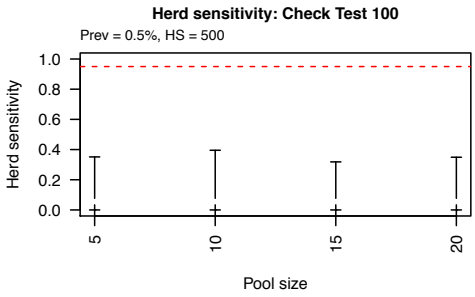
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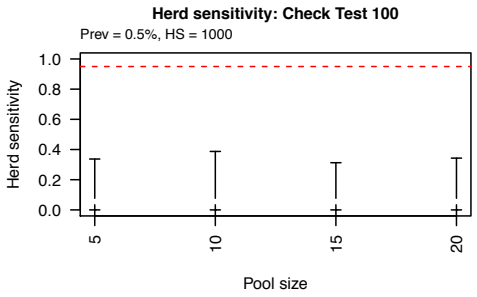
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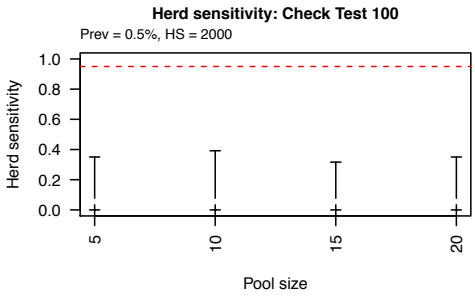
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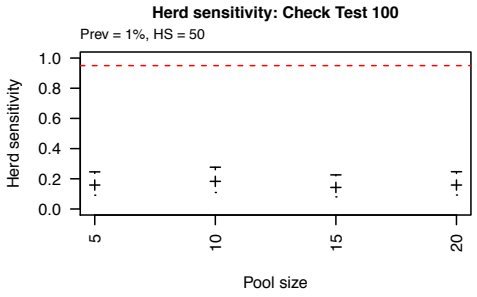
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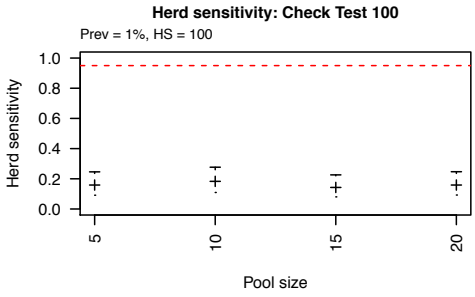
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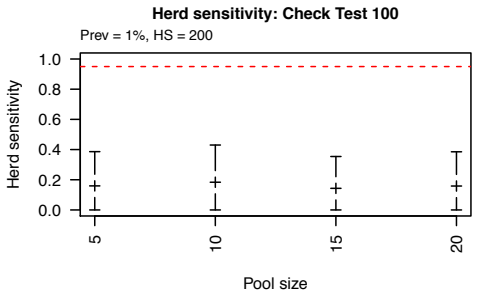
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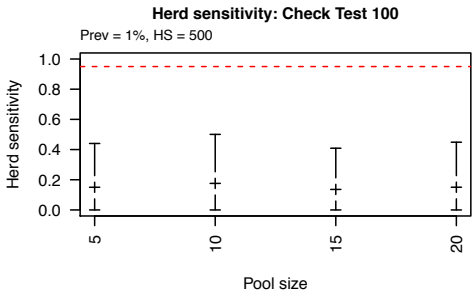
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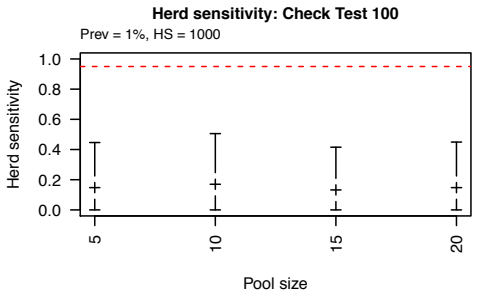
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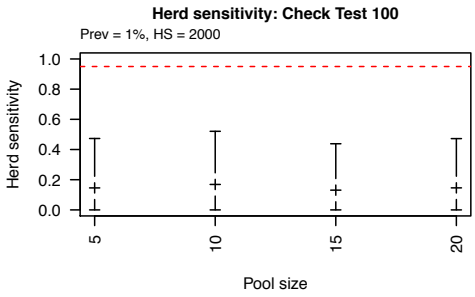
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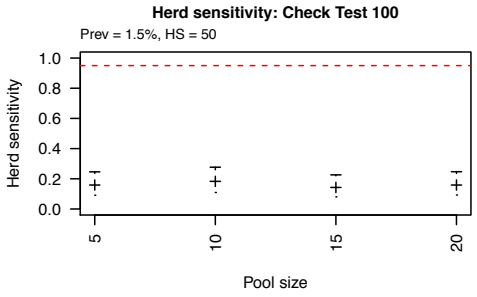
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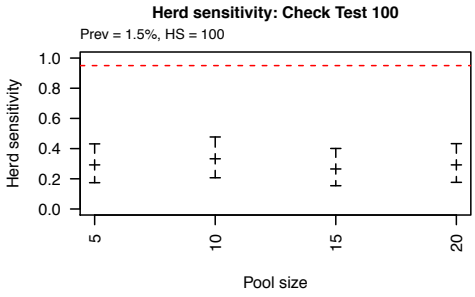
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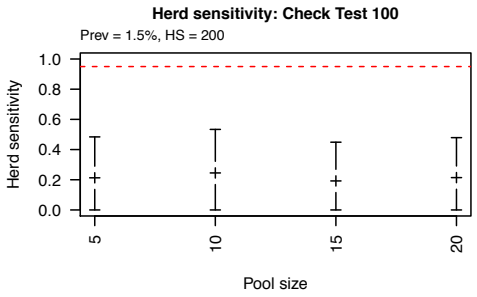
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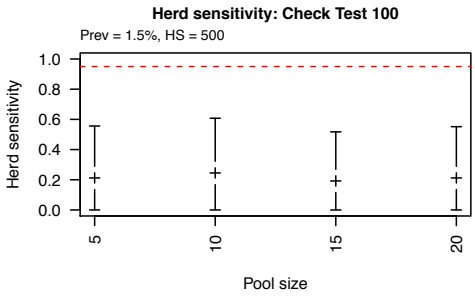
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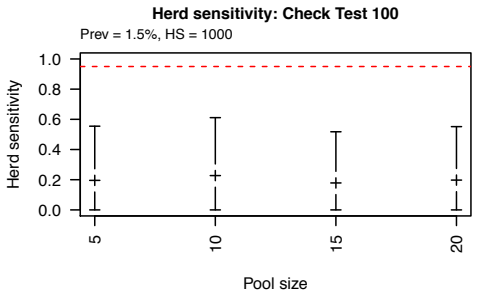
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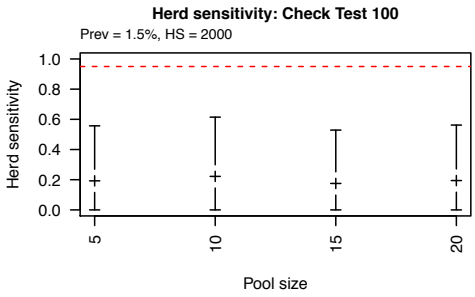
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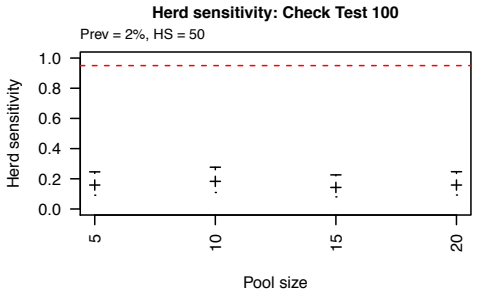
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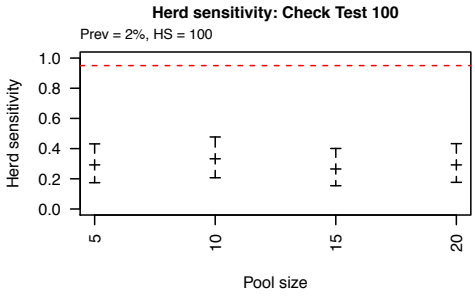
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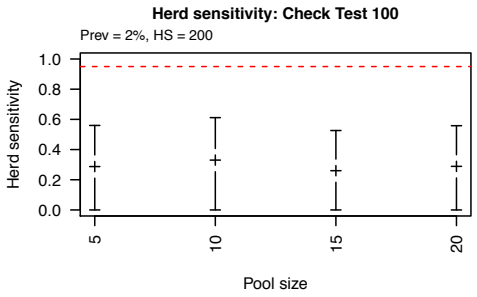
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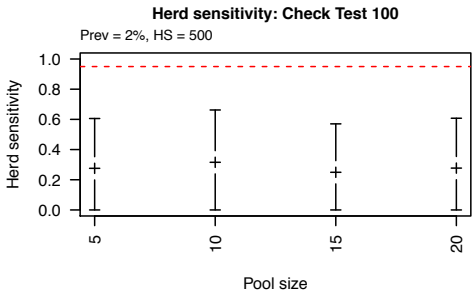
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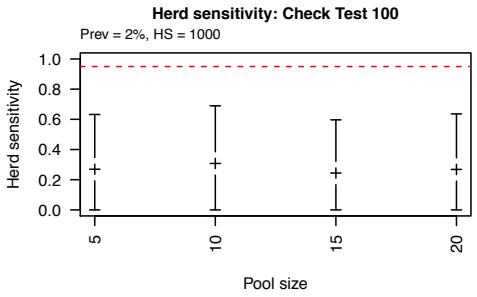
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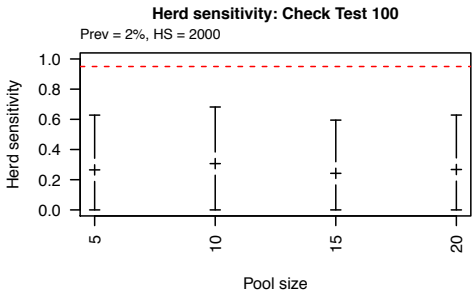
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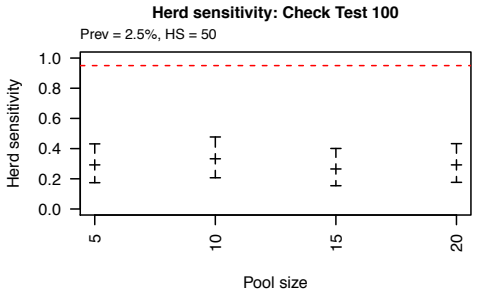
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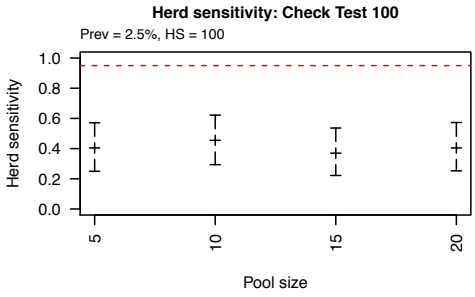
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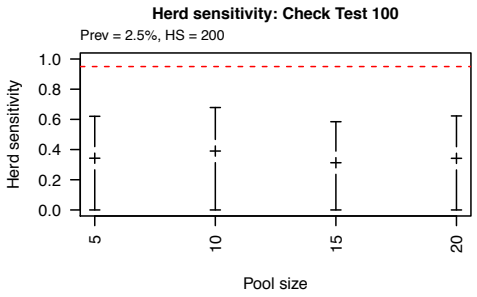
Pool size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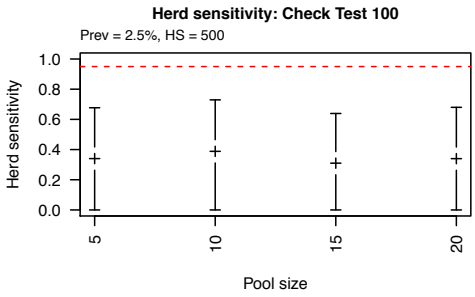
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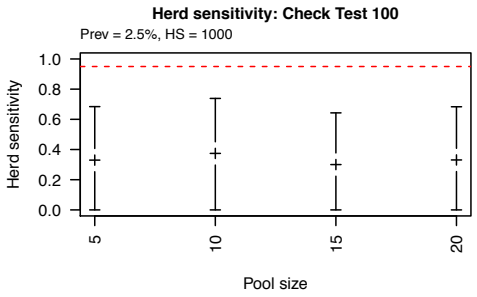
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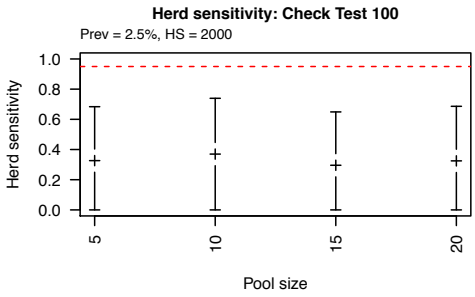
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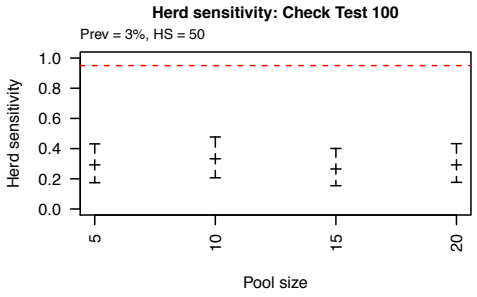
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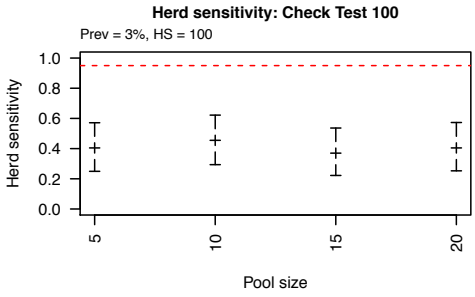
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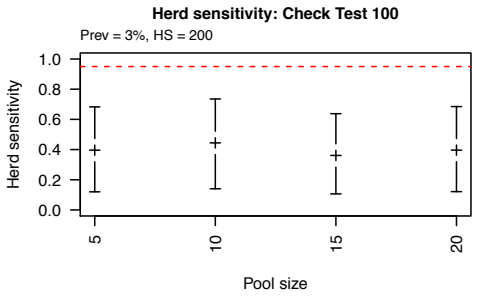
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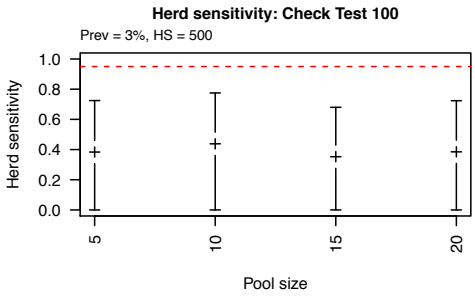
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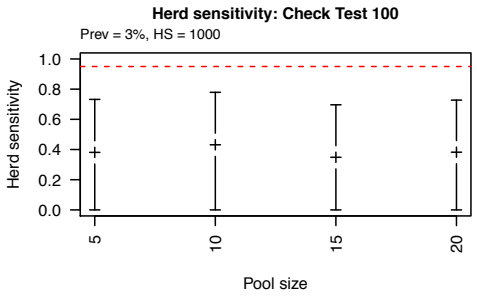
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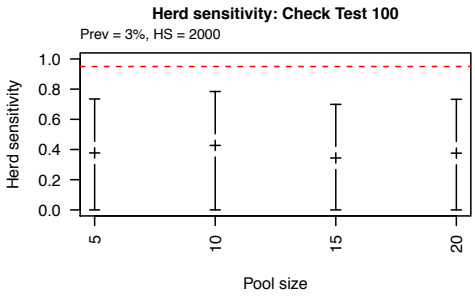
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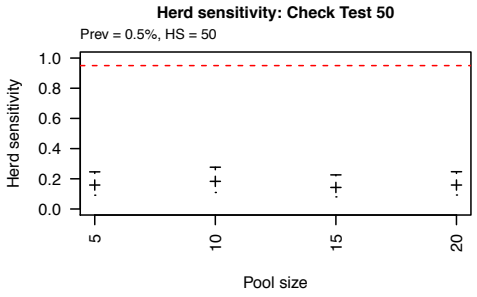
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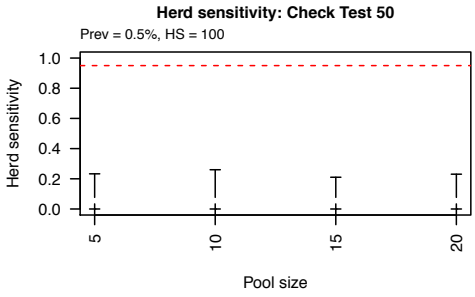
Pool size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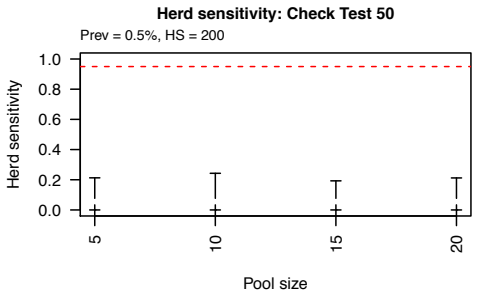
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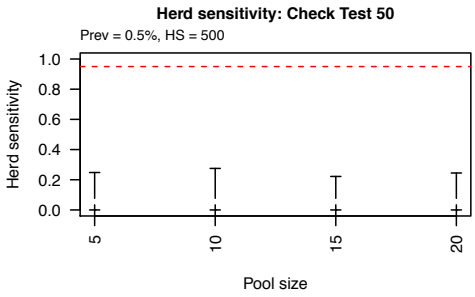
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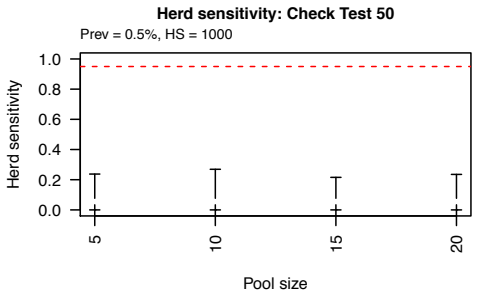
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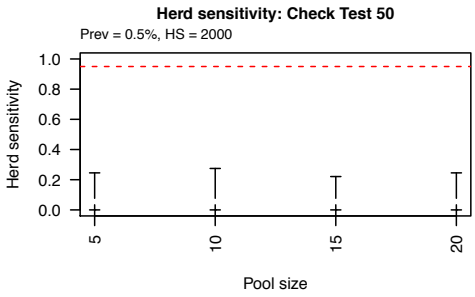
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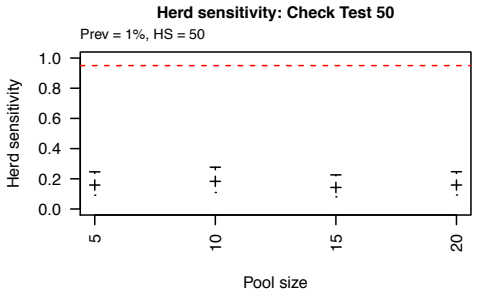
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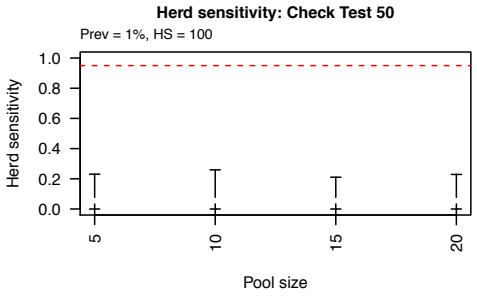
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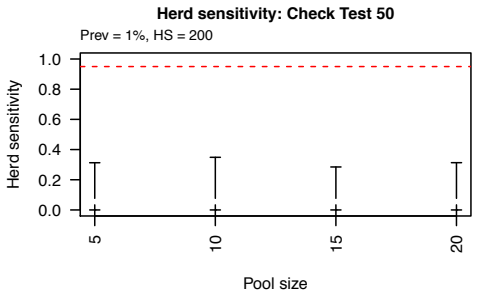
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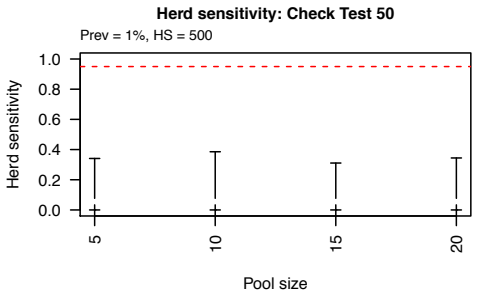
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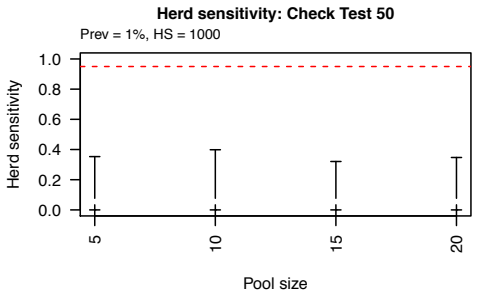
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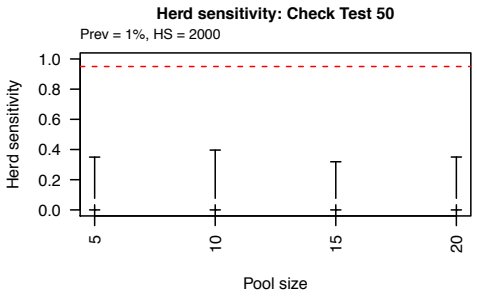
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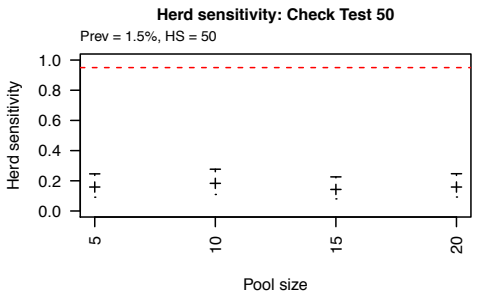
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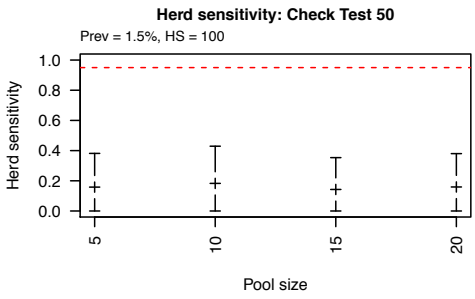
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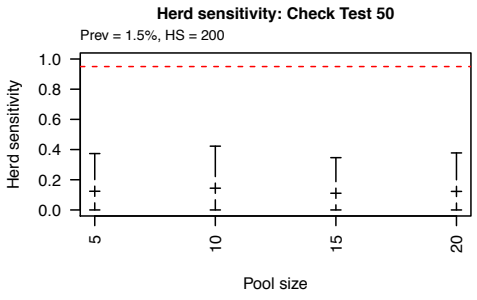
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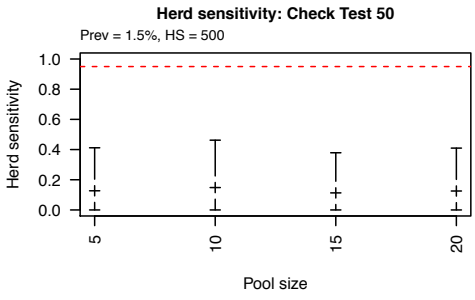
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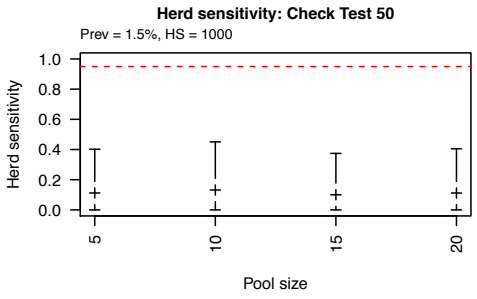
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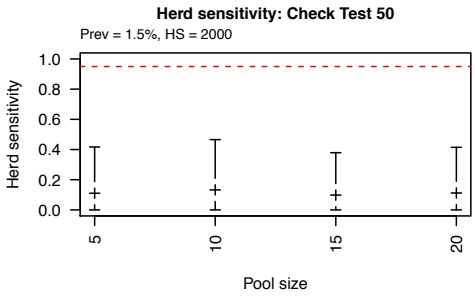
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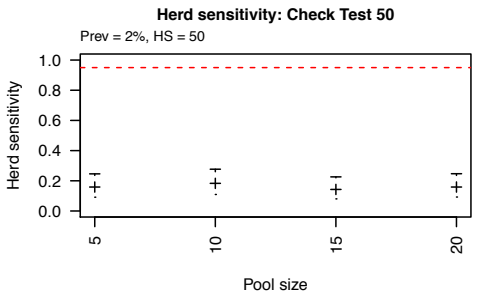
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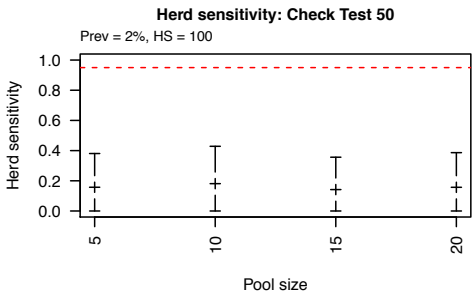
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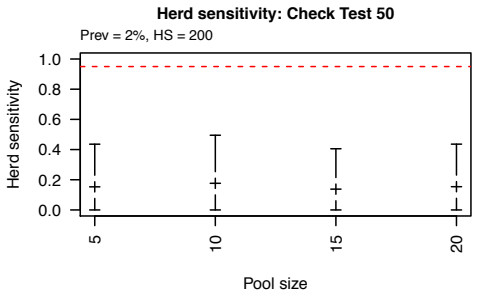
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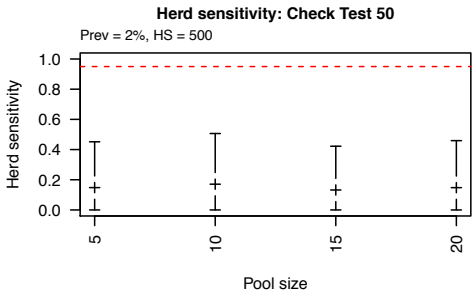
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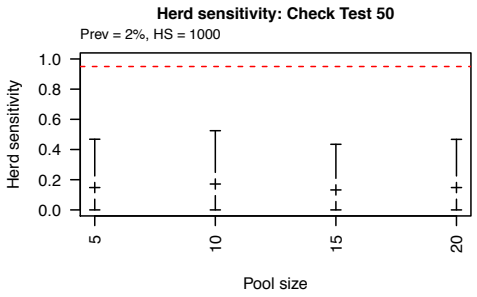
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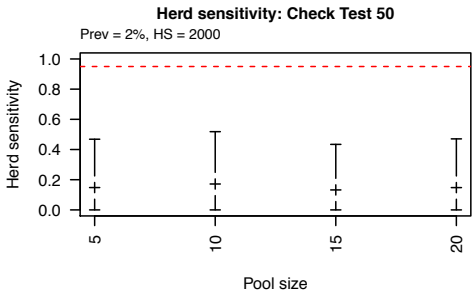
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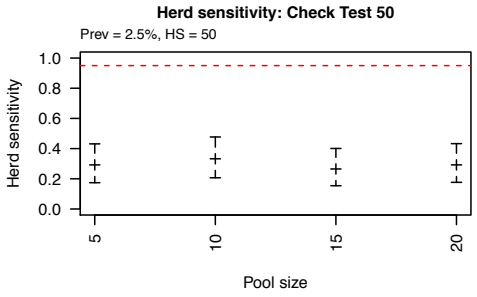
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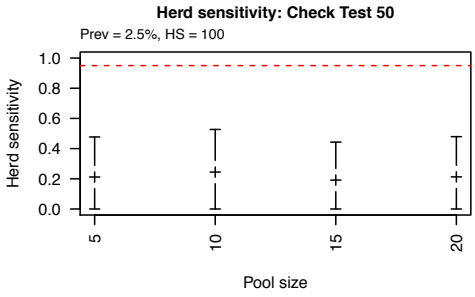
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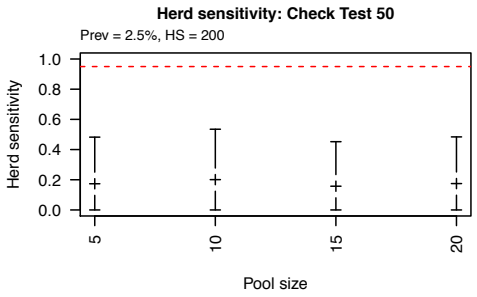
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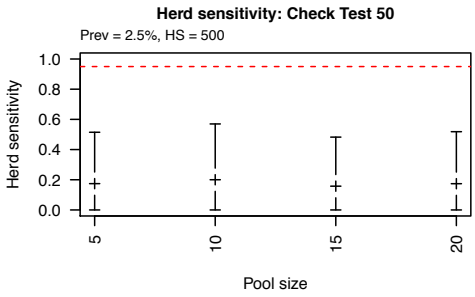
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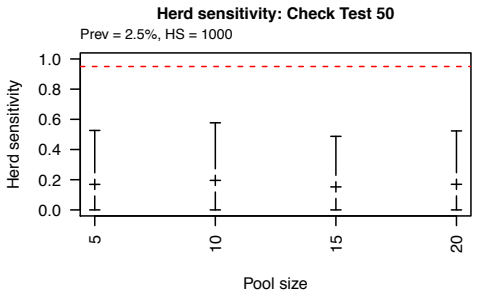
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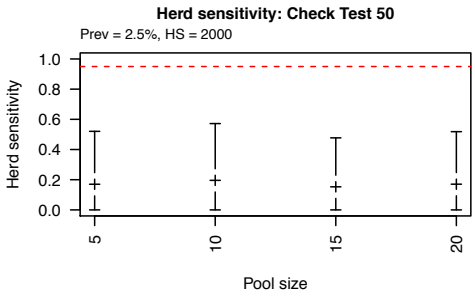
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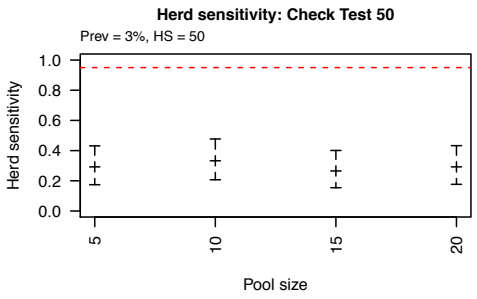
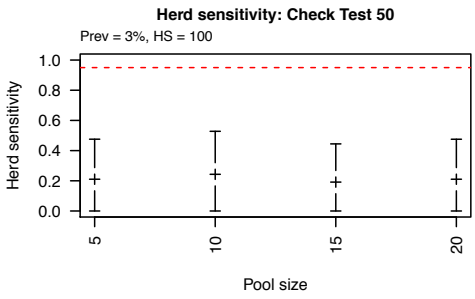
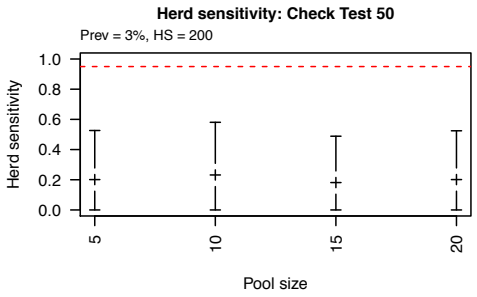
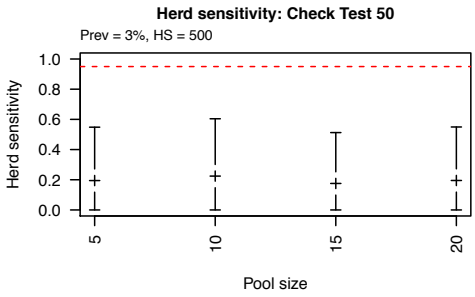
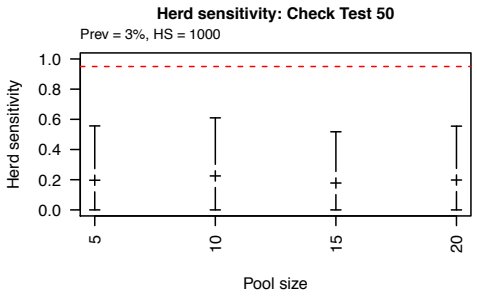
20
Pool size

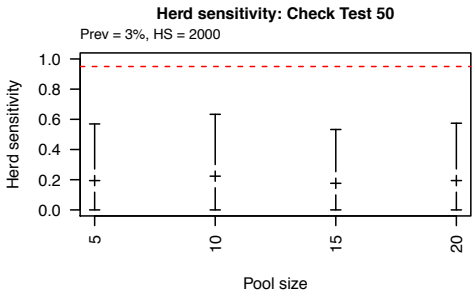

Herd sensitivity: Check Test 50
Prev = 3%, HS = 50
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size


Herd sensitivity: Check Test 50
Prev = 3%, HS = 100
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size


Herd sensitivity: Check Test 50
Prev = 3%, HS = 200
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

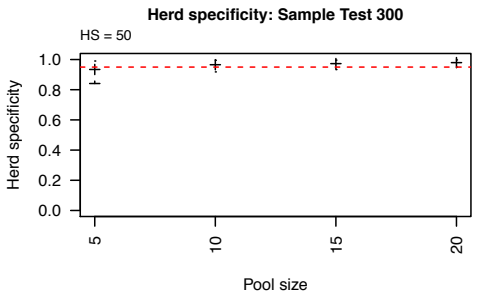

Herd sensitivity: Check Test 50
Prev = 3%, HS = 500
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

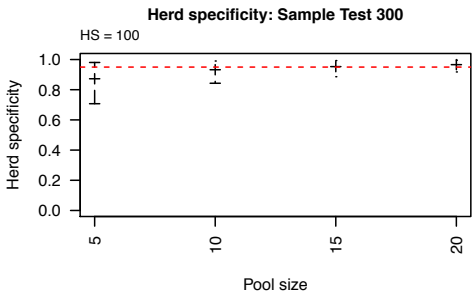

Herd sensitivity: Check Test 50
Prev = 3%, HS = 1000
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

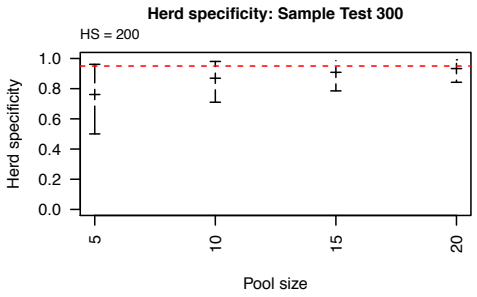

Herd sensitivity: Check Test 50
Prev = 3%, HS = 2000
Herd sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

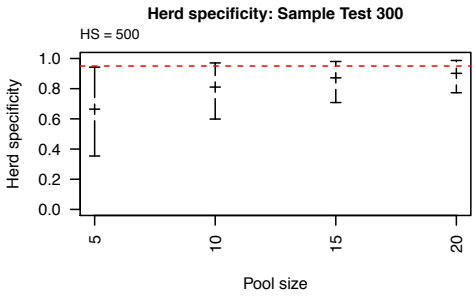
9.4 Appendix-4: Effect of pool size on herd-specificity

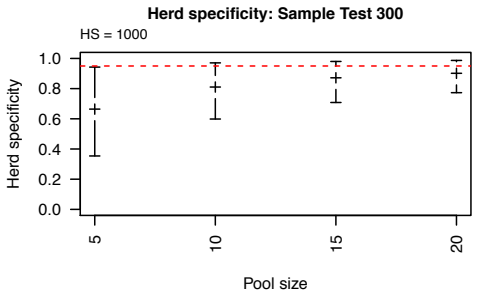
These figures are based on the sensitivity analysis conducted assuming imperfect specificity.

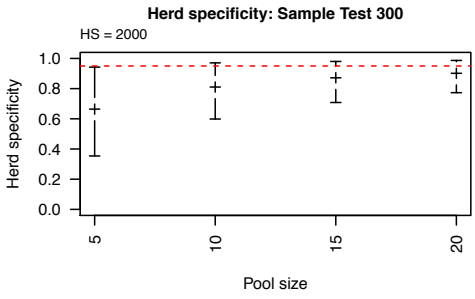


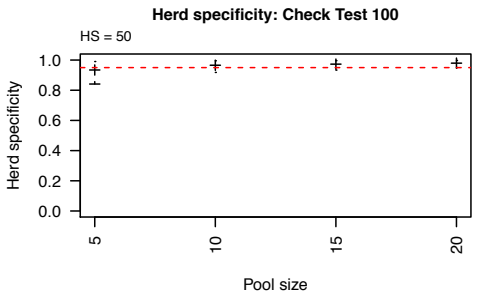


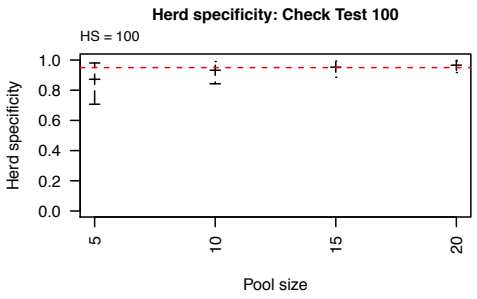


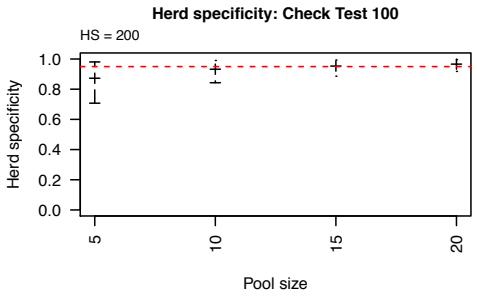


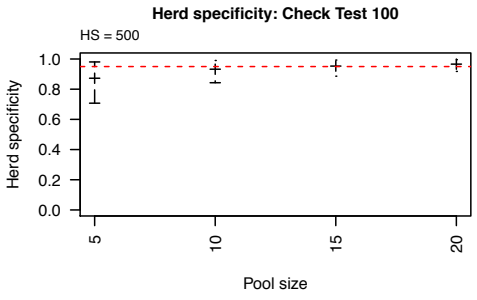


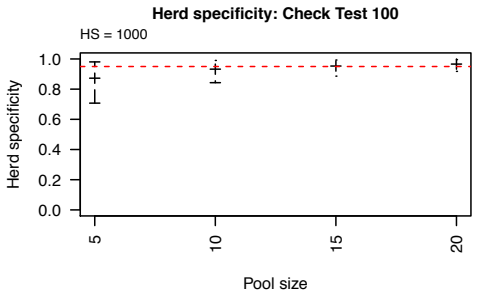


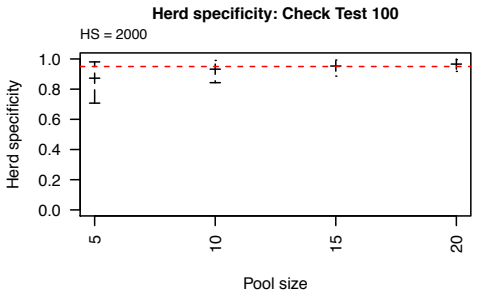

Herd specificity: Check Test 100
HS = 50
Herd specificity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

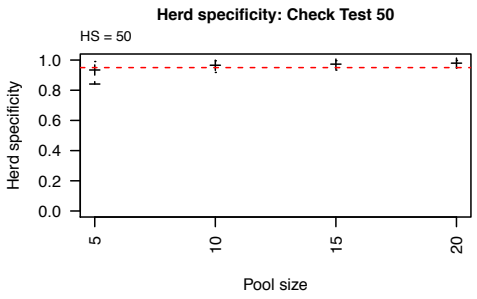

Herd specificity: Check Test 100
HS = 100
Herd specificity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

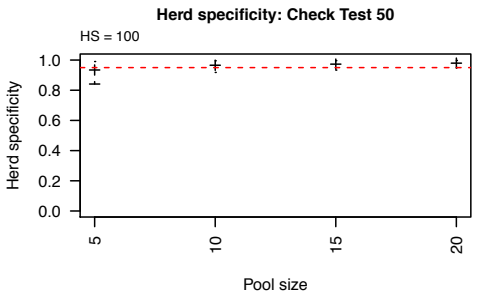

Herd specificity: Check Test 100
HS = 200
Herd specificity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

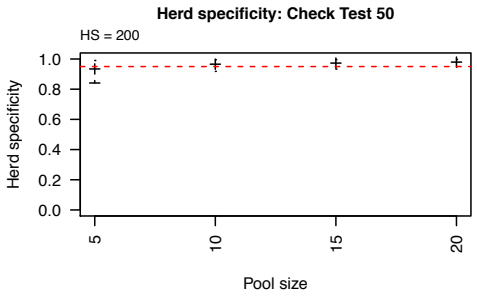

Herd specificity: Check Test 100
HS = 500
Herd specificity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

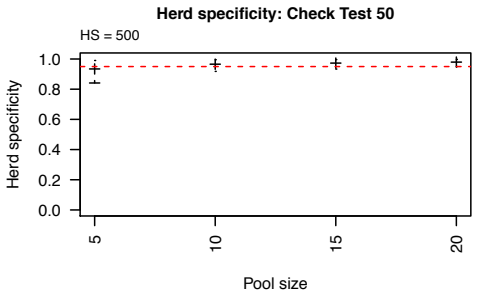

Herd specificity: Check Test 100
HS = 1000
Herd specificity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

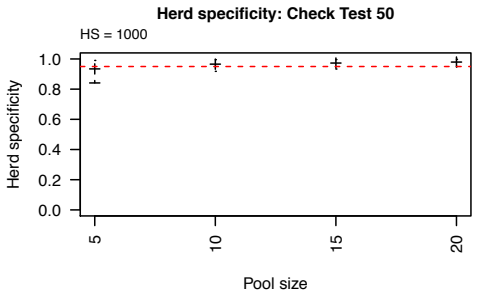

Herd specificity: Check Test 100
HS = 2000
Herd specificity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size

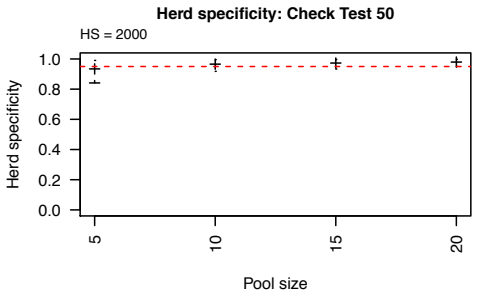

Herd specificity: Check Test 50
HS = 50
Herd specificity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size


Herd specificity: Check Test 50
HS = 100
Herd specificity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size


Herd specificity: Check Test 50
HS = 200
Herd specificity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size


Herd specificity: Check Test 50
HS = 500
Herd specificity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size


Herd specificity: Check Test 50
HS = 1000
Herd specificity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size


Herd specificity: Check Test 50
HS = 2000
Herd specificity
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Pool size