

Evaluation of PCR for Jaagsiekte Sheep Retrovirus Detection from Iranian Sheep Flocks

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Abstract: Ovine pulmonary adenomatosis (OPA), also known as Jaagsiekte, is a contagious progressive pulmonary neoplasia of sheep and rarely goats. Using a representative sample of Iranian sheep comprising 125 flocks, the sensitivity and specificity of PCR for Jaagsiekte sheep retrovirus (JSRV) was estimated. By combining and adapting existing methods, the characteristics of the diagnostic test were estimated (in the absence of a gold standard reference) using repeated laboratory replicates. As the results of replicates within the same animal cannot be considered to be independent, the performance of the PCR was calculated at individual replicate level. The median diagnostic specificity of the PCR when applied to individual animals drawn from the Iranian flock was estimated to be 0.997 (95% confidence interval [CI] 0.996-0.999), whereas the median sensitivity was 0.107 (95% CI 0.077-0.152). Considering the diagnostic test as three replicates where a positive result on any one or more replicates results in a positive test, the median sensitivity increased to 0.279. Reasons for the low observed sensitivity were explored by comparing the performance of the test as a function of the concentration of target DNA using spiked positive controls with known concentrations of target DNA. The median sensitivity of the test when used with positive samples with a mean concentration of 1.0 target DNA sequence per 25 μ L was estimated to be 0.160, which suggests that the PCR had a high true (analytical) sensitivity and that the low observed (diagnostic) sensitivity in individual samples was due to low concentrations of target DNA in the blood of clinically healthy animals.

Key words: Jaagsiekte sheep retrovirus • Diagnostic test validation • PCR • Modelling

INTRODUCTION

Jaagsiekte sheep retrovirus (JSRV) is the aetiological agent of ovine pulmonary adenocarcinoma (OPA), an infectious lung tumour of sheep occurring in almost all countries but absent from Australia, New Zealand and Iceland. Currently, there is no treatment or vaccination for JSRV infection and clinical OPA is inevitably fatal. OPA can cause substantial losses in affected flocks and, in order to prevent spread of JSRV infection, a reliable diagnostic test for detection of infected sheep is needed.

No cost effective serological assays are available for JSRV, since the virus does not induce a specific antibody response in infected animals [1, 2]. Current JSRV diagnostic tests are based on virus detection, e.g. from blood or bronchoalveolar lavage samples, observation of clinical signs of OPA in advanced clinical cases and identification of OPA lesions at post mortem examination. However, no routine assays for pre-clinical diagnosis of JSRV infection are available.

PCR for JSRV is not used routinely since there are reservations regarding its suitability for diagnosis of JSRV infection outside the research [3, 4]. Viable implementation of any assay into routine diagnostics is dependent upon the accuracy of the diagnostic test. Hence, thorough validation of the test against the target population is essential.

In this study, we used blood samples from a national survey commissioned by the Iranian Government for validation of a JSRV PCR assay. The diagnostic test used is similar to the hemi-nested PCR described by De las Heras [3]. Previous work suggested that the diagnostic accuracy of this test is highly dependent upon [5] the specimen tested and [6] the stage of disease in the animal being sampled. De las Heras noted that the sensitivity of the test when based on blood samples from infected but clinically healthy animals was too low to provide a reliable result at the individual animal level and these authors recommended flock level testing. This conclusion was based on sampling from six animals infected with JSRV, but with no clinical evidence of disease [3].

Voigt suggested that the sensitivity of a similar JSRV PCR used with blood samples may be as low as 10% at the individual animal level; this estimate was based on a study population of 47 Grey Heath sheep with histologically confirmed OPA lesions [4]. These experimental studies used small sample sizes with repeated sampling of individual animals and confirmatory tests in live and dead animals [4].

Although certain findings from these two experimental studies may not be applicable for diagnosis of JSRV infection under field conditions, the observed association between disease status and diagnostic accuracy is of relevance because in prevalence surveys it is expected that the majority of animals tested will be clinically healthy, i.e. the likelihood of detecting an individual infected sheep will be low. Diagnostic sensitivity and specificity are population parameters that describe the test performance for a given reference population [7]. So it is important to question the accuracy of the JSRV PCR assay under given circumstances, in our case when applied to the Iranian sheep flock. The answer to this question has implications for future disease monitoring and control in the target population.

Hughes and Totten(2003) proposed that the sensitivity of PCR assays should be specified as a function of the number of target DNA molecules present [8]. However, in field samples the concentration of target DNA is unknown and estimates of sensitivity and specificity of the test used are not functions of concentration but rather averages over the range of possible concentrations which occur biologically within the subjects being sampled.

An extensive body of literature exists on methods of validation for diagnostic tests in the absence of a gold standard reference test. Hui and Walter[9] defined the necessary conditions for test sensitivities and specificities to be estimated using maximum likelihood methods [9]. Later additions include Bayesian approaches [10] and allowance for covariance between tests [11]. The use of non-gold standard methods, particularly Bayesian methods, in diagnostic testing features heavily in modern veterinary epidemiology [12].

In this study, the primary objective was to estimate the accuracy of the JSRV PCR when applied to the Iranian sheep flock. A secondary objective was to present a novel statistical approach for estimating sensitivity and specificity of a diagnostic test in the absence of a gold standard reference test, using laboratory replicates to increase the amount of data available for analysis.

MATERIALS AND METHODS

Data: Data were collected from a representative random sample of 125 Iranian sheep flocks. Only flocks with at least 50 breeding ewes were eligible to take part in the study. In each flock, blood was collected from a random sample of animals, typically 27 sheep and each blood sample was subsequently tested for the presence of JSRV proviral DNA using a hemi-nested PCR [13], except that 800 ng DNA were used per replicate and the second round was a Taqman PCR using the carboxyfluorescein (FAM) labelled probe 5'-AGCAAACATCCGAGCCTTAAGAGCTTTC-3' using an Applied Biosystems SDS7000.

Samples from each flock were tested separately and comprised three replicate aliquots from each blood sample, along with a set of three positive controls of varying JSRV DNA concentrations (each with one aliquot) and typically four negative control samples (each with three replicate aliquots). The negative controls were from differing sources, namely, cow blood, Icelandic sheep blood, distilled water and a buffer solution. A total of 499 negative control samples were available, each with three replicate aliquots; samples from one flock were tested with three rather than four negative controls.

Table 1 summarises the test results of the negative controls and field samples. We ignored the source of the negative control samples, as there was no evidence to suggest any differences associated with source in the mean proportion of replicates falsely testing positive. A total of 121 positive control samples was included in the analysis. Table 2 provides a summary of the positive control data.

Table 1: Observed test results from field and negative control samples

Number of positive replicates	Number of field samples	Number of negative control samples
1	221	456
0	107	12
3	15	1
4	8	0
Total	3684	495

Each observation is the number of replicates out of a total of three which tested positive for JSRV.

Table 2: Observed test results from positive control samples

Mean number of DNA plasmids	Number of samples	Number of samples positive for JSRV
8	122	97
13.5	123	108
24	122	110

Statistical Method: In the analysis of field samples three issues were relevant to statistical estimation of the sensitivity and specificity of the JSRV PCR. Firstly, test results from individual blood samples were not validated against a gold standard reference test to determine the true status of each sample. Secondly, replicate aliquots were available from each blood sample, which increased the amount of data available; however, these results could not be assumed to be independent and therefore an appropriate adjustment was needed to correct for correlations among replicates. Finally, the probability of a flock being free from the infectious agent (i.e. the within flock prevalence can equal zero with non-zero probability) needed to be accounted for. To accommodate each of these complications, we used a Bayesian non-gold standard latent variable model [12], with conditional dependence between replicates from the same sample [11], where the latent variable denoting within flock JSRV prevalence has a mixture distribution [5].

This study was based on a single diagnostic test with conditionally dependent replicates and was considered to be a special case based on the approach of Dendukuri and Joseph [11], with the sensitivity and specificity being the same in each test. The observed data within a single flock were modelled using a multinomial distribution, which defines the probability of observing animals with zero, one, two or three positive replicates, given a fixed total number of animals sampled.

The statistical model allowed the prevalence of JSRV to vary between flocks estimated sensitivity and specificity across all flocks. The likelihood function for a single flock is multinomial and the likelihood function for all flocks in study is the product of the likelihood functions for individual flocks, where allowed the prevalence of JSRV in each flock to vary independently. In this investigation a Bayesian model with uninformative priors for all parameters and fitted the model using JAGS, an open source software package for running Markov chain Monte Carlo analyses similar to WinBUGS.

In the analysis of control samples we estimated the sensitivity and specificity of the test when applied to the JSRV positive and JSRV negative control samples. These control samples were primarily used as quality assurance checks during the laboratory testing process; however, they also provided potential bounds on the accuracy of the test when applied to samples of unknown status. Analysis of the negative control samples followed the same method as for the field samples, since they were distinct samples with three replicates each, with the knowledge that the true status of the sample was negative.

The positive control samples required a different approach, since we had no replicates but rather a single sample at three different dilutions. We adopted the parametric approach of Hughes and Totten [8], which discriminated between ‘observed’ sensitivity and ‘true’ sensitivity. The former includes test error due to the aliquot under study contains no copies of the target DNA sequence; [5] or although target DNA is present, the PCR fails to amplify the DNA. It is argued that ‘true’ sensitivity only includes the error associated with [6] and that sensitivity should be a function of the number of target DNA molecules. The observed sensitivity may be estimated using standard methods, such as logistic regression with dilution as a covariate. In contrast, estimating true sensitivity requires certain probabilistic assumptions, e.g. the number of DNA molecules follows a Poisson distribution.

RESULTS

Field Samples: Fitting our statistical model to the field data, we estimated that sensitivity (S) of the PCR had a posterior median of 0.107 and a 95% CI of 0.077-0.152. In contrast, The observed results found that the test was highly specific, with a posterior median for specificity (C) of 0.997 (95% CI 0.996-0.999). Estimates of the posterior densities for S and C shown estimated covariance within sample replicates was low, with a median of 2.59×10^{-3} when JSRV was present (cov_s) and a median of 3.63×10^{-6} when JSRV was absent (cov_c).

Control Samples: Fitting the statistical model to the negative control samples (Table 1), the estimated 95% CI was 0.982-0.993 for S and 1.04×10^{-6} to 1.41×10^{-4} for cov_c . Using the method of Hughes and Totten for estimating the true S of the test on the positive control samples, median S estimates for mean concentrations of 1, 6, 12.5 and 25 target DNA molecules per 25 μ L were 0.160, 0.648, 0.886 and 0.987, respectively [8]. In contrast, the raw observed S of the test using the data in Table 2 were 0.793, 0.884 and 0.901 for mean concentrations of 6, 12.5 and 25 target DNA molecules per 25 μ L, respectively. The method also allows for explicit estimation of C ; however, given that results had median estimates for C from both the field samples and negative control samples in excess of 0.99, it assumed that the probability of observing a false positive is zero.

Estimates of the posterior density for S at the three observed concentrations, plus extrapolation when the mean concentration is one copy of target DNA sequence in 25 μ L. A key parameter in the mechanistic model used

Table 3: Posterior estimates of test accuracy applied to field samples.

Parameter	Median	95% Confidence interval
P (0; I*) ^a	9.92×10^{-1}	$1.92 \times 10^{-1}; 3.14 \times 10^{-1}$
P (0; I)	7.21×10^{-1}	$9.87 \times 10^{-1}; 9.96 \times 10^{-1}$
P (1; I*)	7.61×10^{-3}	$6.24 \times 10^{-1}; 7.92 \times 10^{-1}$
P (1; I)	2.48×10^{-1}	$1.14 \times 10^{-2}; 4.67 \times 10^{-2}$
P (2; I*)	2.29×10^{-2}	$3.82 \times 10^{-3}; 1.25 \times 10^{-2}$
P (2; I)	8.16×10^{-3}	$3.81 \times 10^{-7}; 3.61 \times 10^{-5}$
P (3; I*)	8.59×10^{-6}	$3.54 \times 10^{-7}; 3.58 \times 10^{-5}$
P (3; I)	8.39×10^{-6}	$2.61 \times 10^{-3}; 2.13 \times 10^{-2}$

^a Number of positive replicates; Infection status. I* and I denote infection positive and infection negative for JSRV, respectively.

by Hughes and Totten(2003) is the probability that each of the target molecules in the sample being tested fails to escape amplification by PCR, where when estimated to be 0.160. As assumed that the specificity is 1.0, then this is equivalent to the S when the mean concentration is 1.0 target DNA sequence per 25 μ L [8].

Summary Probabilities: Table 3 contains summary statistics for each of the eight independent conditional probabilities estimated from this model. These summarise the probabilities of observing zero or more positive replicates conditional on whether JSRV infection is truly present. The probability of observing one or more positive replicates is considerably larger when JSRV is truly present, as should be expected.

DISCUSSION

This study estimated that the median sensitivity of the JSRV PCR was 0.107 per individual replicate, where this accounted for covariance between replicates from the same sample. It was found that the covariance between replicates was low, which was unsurprising, given the observed data: out of a total 3,361 sets of triple replicates, only 106 had one positive replicate, 14 had two positive replicates and four had three positive replicates. Hence, there is little obvious covariance between positive replicates, even from positive animals.

Considering the performance of the PCR when applied to control samples it was interesting to note that estimates of C using the field samples were slightly higher than those derived from the negative controls. This could in part be explained by the fact that S and C in the field data were negatively correlated. This is to be expected, since $S + C$ must be greater than 1 to have a 'legitimate' (better than random guessing) diagnostic test. In our modelling, we assumed uninformative independent priors for S and C . An alternative would be to explicitly model this correlation using a joint prior, as for Chu studing [6].

For the positive control samples, we found that the estimates of observed S were lower than estimates of true S ; this was to be expected, since the former also includes the error due to individual samples not containing any target DNA. Table 3 showed an alternative and potentially more informative assessment of test accuracy. The probability of observing no positive test replicates, given that JSRV is truly present within the blood sample, is in excess of 0.70. If define a positive test result as where any one of the available three replicates is positive, then we can approximate the sensitivity of the test across all three replicates by summing the individual probabilities in rows 3, 5 and 7 in Table 3, giving a median observed sensitivity of 0.279. This result is in line with observations in previous studies, where PCR results based on blood samples were compared with other diagnostic procedures [3, 4, 14]

The primary objective of this investigation modelling was to estimate the diagnostic accuracy of the JSRV PCR when applied to the Iranian sheep flock. A necessary and interdependent part of this process is estimation of the prevalence of JSRV within each flock sampled. the observed explicitly allowed the prevalence of JSRV to vary independently within each of the study flocks. If, instead, the primary goal of our study was estimation of prevalence and, in particular, at regional or national flock level, then a natural extension to our model would be to incorporate it into the hierarchical framework of Branscum designing [5].

In previous work, the study population consisted largely of animals which were likely to be in early and late stages of OPA; these studies concluded that the 'observed' sensitivity in preclinical animals is considerably lower than in animals with clinical OPA [3, 4]. Samples for the current study were taken from a random selection of Iranian sheep; the vast majority of these animals were clinically healthy. The test characteristics assessed in this validation based on a representative field sample would therefore be applicable for prevalence studies or diagnostic screening of clinically healthy sheep.

the 'true' estimated sensitivity of the JSRV PCR as a function of the number of target DNA molecules present using the associated spiked positive control samples to assess the detection limit of the PCR. There were two key findings from this analysis. Firstly, estimated a median sensitivity of 0.160 when the mean number of target DNA molecules per 25 μ L is 1.0; this observation is indicative of a high technical performance of the PCR assay and a good 'true' sensitivity. Secondly, comparing the estimate of 0.160 with the median sensitivity from field samples of

0.107 strongly suggests that the concentrations of target DNA in the latter are generally very low. The fact that only a few samples from infected animals tested positive in more than one replicate leads to the same conclusion and hence potentially explains the low 'observed' sensitivity.

The used a non-gold standard method to validate the diagnostic test. The use of non-gold standard methods in practice requires considerable care, e.g. requiring sufficient observations to enable robust estimation [14]. In the work presented, we required data from at least four flocks, each with different levels of prevalence, in order to calculate test characteristics.

The JSRV PCR assay was assessed to have a generally low 'observed' sensitivity when used with blood samples from clinically healthy sheep. Therefore, improvements and adjustments would be necessary should the test become part of routine diagnostic investigations. Theoretically, the assay could be further enhanced, but, given that various technical refinements have been implemented in the past, the PCR seems to have reached what is currently possible with state of the art technology. On an individual animal level, the 'observed' sensitivity could be improved by testing specimens which have a higher concentration of JSRV proviral DNA in infected animals, e.g. bronchoalveolar lavage samples. An obvious next step is estimation of the flock level sensitivity of the test when applied to the Iranian sheep flock. This is, however, a considerably more complex task, since flock level sensitivity depends jointly on the accuracy of the JSRV PCR assay at individual animal/replicate level, the number of animals sampled from within each flock and, crucially, the distribution of within flock prevalence of JSRV in the population under study. Using non-gold standard methods, which make use of laboratory replicates to maximise available data, the JSRV PCR was assessed to have a high 'true' sensitivity and low 'observed' sensitivity, where the latter can be explained by the low concentration of JSRV proviral DNA in the blood of infected sheep. The analytical method presented is generic and applicable to diagnostic test validation when repeated measurements are available.

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