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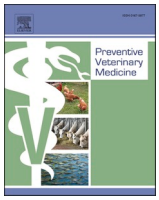

Estimation of the specificity of an antibody ELISA for paratuberculosis generated from a sector of the UK cattle population using results from a paratuberculosis control programme

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ABSTRACT

In the United Kingdom (UK) a voluntary programme to control paratuberculosis in cattle based on herd management and serological screening has been operating since 1998. The programme assigns a risk level to each participating herd according to the within herd seroprevalence and the confirmation of the presence of infection with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) by faecal culture or polymerase chain reaction (PCR). From the outset a general concern over the specificity of the paratuberculosis antibody enzyme-linked immunosorbent assay (ELISA) resulted in the use of a faecal screen for the causal organism to negate or confirm infection in individual seropositive animals. Progress in improving the diagnostic tests has been gradual throughout the life of the programme and the under-pinning approach to using tests to determine the risk of paratuberculosis for a herd required to be re-examined. This study used a large data set of more than 143,000 test results over five years from the lowest paratuberculosis risk level category of herds to estimate the specificity of a commercially available paratuberculosis antibody ELISA for cattle. In each year of the study the estimated specificity reached or exceeded 0.998. We also examined the apparent impact that annual or more frequent application of the single intradermal comparative cervical tuberculin (SICCT) test for tuberculosis (TB), using purified protein derivatives of *Mycobacterium bovis and Mycobacterium avium* subspecies *avium,* had on specificity of the antibody ELISA for paratuberculosis. We found a statistically significant difference in three of the five years with herds that were officially tuberculosis free and not subject to frequent SICCT testing. This difference was small and considered to be of little practical importance for the paratuberculosis assurance programme. We concluded that, in the UK the mandatory TB surveillance programme of cattle herds is not a limiting factor in the use of serological testing to support herd-level assurance schemes for paratuberculosis. Furthermore, in paratuberculosis, where shedding of MAP is intermittent and the sensitivity of the commercially available PCR tests for detection MAP is highly variable, faecal screening of seropositive animals is an unreliable method for negating infection in seropositive cattle.

1. Introduction

Infection with the bacterium *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*) and the consequent disease, paratuberculosis is of socioeconomic importance through its impact on the health, welfare and productivity of ruminants (Anon, 2022a). Considerable effort is made in many countries to control the disease in cattle and to a lesser extent in sheep and goats (Whittington et al., 2019). The identification and elimination of infected animals has been one element of these control programmes. Effective tests to detect infection are a prerequisite for this approach, however paratuberculosis is a complex, slowly progressive disease, with the greatest susceptibility to infection occurring in young animals, but the disease only manifesting several years later (Chiodini et al., 1984). During the prolonged period of subclinical infection the animal may shed *MAP* intermittently in the faeces, while the production of specific antibodies can occur relatively late in the disease (Stabel,

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2000), preceding, by a relatively short period, both the shedding of large numbers of *MAP* (Nielsen and Toft, 2006), and the development of clinical disease. Consequently, while the diagnostic tests may be accurate in the detection of antibody when it is present in milk or serum, and in the detection of *MAP* when it is shed in faeces, they have limited sensitivity for much of the life of the infected animal (Nielsen and Toft, 2008), and such animals may not test positive for both antibody and the causative organism at the same time. This can lead to concerns not only that the sensitivity of tests to detect *MAP* in faeces may be too low for the purpose of herd level assurance programmes, but conversely that the seropositive animals with negative results for faecal shedding may be false positives and the specificity of the antibody ELISA may also be limiting.

To use diagnostic tests effectively in paratuberculosis control programmes stakeholders should share an understanding of the strengths and limitations of the tests. However, the difficulty in evaluating diagnostic tests for paratuberculosis in relation to the complex pathogenesis of this disease has resulted in inconsistent methodologies and consequently a difficulty in comparing studies to arrive at firm estimates for test sensitivity and specificity (Nielsen and Toft, 2008). Test sensitivity estimates for the enzyme-linked immunosorbent assay (ELISA) from 30 % to 47 % have been generated from studies conducted in cattle from four different regions of the world, excluding the United Kingdom (UK), and underline the difficulties in establishing clarity, which to some degree is to be expected given the way the disease develops. Nevertheless, the specificity reported in these studies was uniformly greater than 99 %, although the number of cattle in the test populations was relatively limited and did not exceed 970 (Collins et al., 1991; Sweeney et al., 1995; Reichel et al., 1999; Kalis et al., 2002; Fry et al., 2008; Kohler et al., 2008).

In the UK there is concern that the frequent use of the single intradermal comparative cervical tuberculin (SICCT) test for tuberculosis (TB) screening of the cattle population may adversely affect the specificity of the antibody ELISA for paratuberculosis. The SICCT test entails the intradermal injection of purified protein derivatives (PPD) of tuberculins extracted from *Mycobacterium bovis* and *Mycobacterium avium* subspecies *avium* (*MAA*) (Anon, 2022b). The close genetic relationship between *MAA* and *MAP* (Turenne and Alexander, 2020) raises the question as to whether the SICCT test can stimulate the production of antibodies that cross-react in the paratuberculosis ELISA thereby reducing the specificity of the paratuberculosis antibody ELISA. Experience in the UK and Ireland has been that carrying out serological testing for paratuberculosis in the period shortly after the SICCT test for TB will result in a higher-than-expected proportion of cattle that test positive for antibody in the paratuberculosis ELISA (Kennedy et al., 2014). It is unclear, however, whether this observed increase in the expected number of paratuberculosis antibody positive animals occurs similarly in herds that are considered to be free of paratuberculosis as well as in infected herds. Therefore It is important to examine this factor further to develop a fuller understanding of the limitations of the antibody ELISA in UK paratuberculosis control programmes.

The herd level paratuberculosis assurance programme in the UK is governed by the Cattle Health Certification Standards (CHeCS) (CHeCS, 2021) and delivered by veterinary laboratories licensed by CHeCS and supported by the private veterinary surgeons of the participating farmers and in 2020 there were 3391 herds taking part in the programme. The participating cattle farmers must adhere to defined biosecurity standards and their adult cattle are subjected to annual herd tests. The herd test results are used to classify each participating herd according to its paratuberculosis risk level. Those herds that make up the lowest risk level for paratuberculosis (risk level 1), where no evidence of the disease was found after a defined qualifying period, offer a population of animals in which the specificity of the paratuberculosis ELISA can be estimated for UK conditions. The majority of herds in the CHeCS assurance programmes are pedigree beef herds, as a declaration of the CHeCS paratuberculosis status of each herd is a requirement at all the major pedigree beef breeds sales of breeding stock (NBA, 2022). The primary objective of this study was therefore to use the antibody test results from CHeCS paratuberculosis risk level one herds to estimate the specificity of the antibody ELISA in a large and relevant population of animals. The second objective was to investigate the impact frequent tuberculosis SICCT testing had on the specificity of the paratuberculosis antibody ELISA by comparing the specificity estimates from risk level 1 herds that were exposed to annual or more frequent tuberculosis SICCT testing with those where the frequency of SICCT testing was no more than once in every four years. Achieving these objectives would provide information to address two of the main concerns of stakeholders in the CHeCS paratuberculosis risk level programme.

2. Materials and methods

The CHeCS paratuberculosis programme defines risk level 1 herds as those that have been subjected to at least three consecutive annual ELISA tests of all animals of two years of age and older without *MAP* infection being confirmed by serial faecal screening of ELISA positive animals. Animals that test positive for antibody to paratuberculosis by ELISA may be serially tested using faecal culture or polymerase chain reaction (PCR) to screen for the presence of *MAP*. Any animal that is then faeces screen negative is categorised as test negative for the purposes of the scheme. Any animal that is positive by faecal screen is categorised as infected and the herd test is positive with the loss of risk level 1 status. Any animal that tests positive for antibody and is not subject to further testing is categorised as infected with the loss of herd risk level 1 status (CHeCS, 2021). The population of adult animals within herds that maintain risk level 1 status from one year to the next will include some animals that are positive for antibody to paratuberculosis and negative by faecal screen, but which under the scheme

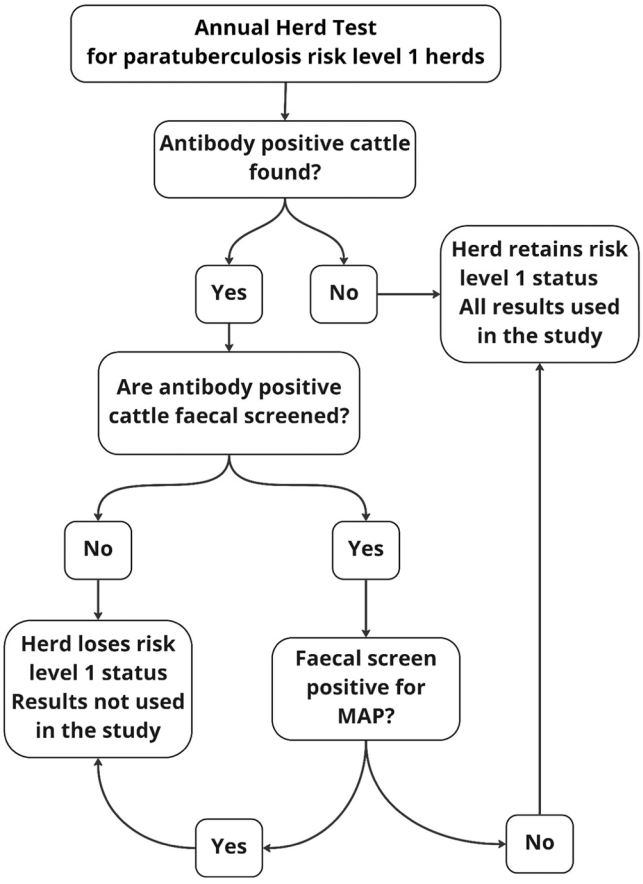

Fig. 1. Annual herd test outcomes for paratuberculosis risk level 1 herds.

rules are categorised as negative and by inference considered to be false positive antibody ELISA test results (Fig. 1). Therefore the set of results from risk level 1 herds that maintain their status provides the opportunity to estimate the specificity of the paratuberculosis antibody ELISA for this population of animals. The farmers' veterinary surgeons carry out all blood and faeces sampling that is required for the scheme.

We examined the herd test results from one CHeCS licensed programme provider, Scotland's Rural College Veterinary Services Premium Cattle Health Scheme (PCHS, 2022). The terms and conditions of membership of the Premium Cattle Health Scheme allow for test results to be analysed for the purpose of research providing no personal data are used in any analysis or publication. All personal data were excluded from the data retrieval for this study. We selected the results from all herds that maintained risk level 1 within the year and examined the test result data over a period of five consecutive years. Each testing year began on the 1st of August and ended on the 31st of July and the period of study commenced on the 1st of August 2015 and ended on the 31st of July 2020. These herds were located throughout the UK. As animals were traded between herds and were frequently tested before and after movement and may also have been included in two different herd tests within the one testing year we only included the first paratuberculosis antibody ELISA test carried out within one year for each animal. It is understood that paratuberculosis infection may be present in some of the risk level 1 herds and some of the animals categorised as false positives in the ELISA, may be infected with *MAP* and therefore be true positives. However, for the purposes of these analyses the possibility that animals were true positives was discounted. The specificity was, therefore, calculated using the total number of animals tested in the antibody ELISA minus the animals that tested positive as the numerator; and the total animals that were tested as the denominator. This was calculated from the antibody ELISA results of all herds that met the above selection criteria and is therefore a relative specificity related to the CHeCS risk level 1 standard. Additionally two subsets of the total were examined and compared; namely those herds from the counties of UK that were subjected to six- or 12-month TB herd routine surveillance testing described as the "high risk area" and "edge area" counties, and defined in this study as high frequency SICCT testing, and those herds from Scotland, which is officially TB free and were defined as "low frequency SICCT testing". The default testing interval for cattle herds in Scotland is 48 months, although herds may be exempt from this requirement if they meet certain additional criteria (APHA, 2021).

Ninety-five per cent confidence intervals for the specificity estimates were calculated (Newcombe and Altman, 2000) and the "N-1" Chi-squared test (Campbell, 2007) was used to compare the differences in specificity seen between years and between the areas of high and low frequency herd bTB testing. The relative risk of a sample testing positive in the antibody ELISA from animals in herds exposed to high frequency tuberculosis SICCT testing was calculated with 95 % confidence intervals. These analyses were completed using Medcalc Software, version 20.023 (Software, 2022; Medcalc, 2022). Over-dispersion of the proportion of animals testing antibody positive within herds was assessed on a logistic model in which the number of the animals that tested positive out of the number of animals tested in a herd was the dependent variable and the only independent variable was the year of the test (treated as a non-quantitative factor). The model was compared, using a chi-squared test, with an equivalent quasi-binomial model using generalised linear model function *glm()* within the statistical software R (R Core Team, 2022).

All laboratory procedures were carried out to ISO 17025 standard and were subject to independent audit and certified each year by the UK Accreditation Service (UKAS, 2022). The serological test used for the entire period of the study was an indirect antibody ELISA test for paratuberculosis produced by Innovative Diagnostics (Innovative Diagnostics, 2022a). The standard kit interpretation was used. Samples that gave a result of 70 % or more (sample optical density divided by the optical density of the positive control multiplied by 100) were classified as positive. Samples that gave a result of less than 70 % positivity were classified as not positive. During the five years covered by the study three different faecal *MAP* tests were used to serially test all antibody positive animals to allow low seroprevalence herds to maintain risk level 1 status. These were either liquid culture, using Trek para-JEM (ThermoFisher, 2022a) where the culture fluid was tested for the presence of *MAP* by PCR using the Thermofisher MagMAX Total Nucleic Acid isolation kit and Thermofisher VetMAX *MAP* screening kit (PCR method 1), or the direct faecal PCR using the Thermofisher MagMAX Total Nucleic Acid isolation kit and the Thermofisher VetMAX *MAP* screening kit (PCR method 1) until one month after the start of testing year five (ThermoFisher, 2022b). Thereafter the ID Gene EZPrep & MagFAST kit was used for *MAP* DNA extraction and ID Gene Paratuberculosis Duplex real-time PCR (PCR method 2) for detection (Innovative Diagnostics, 2022b). The interpretations for the threshold cycle (CT) values used in the two PCR methods during the study are provided in Table 1. Animals were only categorised as *MAP* faecal screen negative once this screening process had been completed with negative results.

3. Results

In the course of the five years of the study 1187 animals tested positive for antibody at a herd test for risk level 1 herds and were screened by faecal culture or PCR for *MAP*. Of these 148 animals were found to be positive (0.125) for *MAP*. They were from 25, 19, 22, 30 and 27 herds across years one to five, giving a total of 123 herds that consequently lost risk level 1 status. The antibody ELISA results from these herds in the year that infection was confirmed were excluded from the analysis. Of the 1521 herds that were tested and maintained risk level 1 status there were 367 herds that were tested and included in only one year; 367 in two years; 269 in three years; 242 in four years and 276 in five years of the study. Similarly of the 79,252 animals that were tested and included in the study 41,835 were tested in one year only; 20,064 were tested in two years; 9844 were tested in three year; 5236 in four years and 2273 animals were tested in each of the five years. Therefore while animals were tested in multiple years, in year 5 when 33,438 animals were tested, the overlap with animals tested in year one accounted for 0.068 of the animals tested.

Table 1
MAP PCR interpretations used in the study.

Method 1, 1st August 2015–30 th September 2017		
CT value	Interpretation	Outcome for Individual
<37	Positive	Infection confirmed
>=37	Inconclusive	To be resampled
No amplification by 40 cycles	Negative	Infection excluded
Method 1, 30th September 2017–30th September 2019		
CT value	Interpretation	Outcome for Individual
<37	Positive	Infection confirmed
>=37	Inconclusive	Further aliquot tested. If any amplification before 40 cycles sample is categorised as positive and infection confirmed. If no amplification sample is categorised as inconclusive and animal must be resampled.
No amplification by 40 cycles	Negative	Infection excluded
Method 2, 1st October 2019 until end of study (30th August 2020)		
CT Value	Interpretation	Outcome for Individual
<33	Positive	Infection confirmed
>=33	Inconclusive	Further aliquot tested. If any amplification before 40 cycles sample categorised as positive and infection confirmed. If no amplification sample is categorised as inconclusive and animal must be resampled.
No amplification by 40 cycles	Negative	Infection excluded

In year one 22,367 animals were tested from 753 herds and there was an increase in the number of herds and animals tested across the five years to reach 904 herds from which 33,348 animal were tested in the final year as more herds progressed to risk level 1 status. Specificity of the antibody ELISA consistently exceeded 0.999 in years 1, 2 and 3 and exceeded 0.998 in years 4 and 5 (Table 2) and in the final year the specificity was calculated as 0.9986 with a 95 % confidence interval of 0.9981–0.9989. Although the differences seen in specificity estimates between years were small, only exceeding 0.001 in the comparison between years 1 and 4, there was a statistically significant difference in the specificity estimates between years 1 and 4, 1 and 5, 2 and 4, 2 and 5, 3 and 4 and 3 and 5 (Table 3). The overall specificity for the five years of testing, which included 143,804 antibody ELISA results was 0.9989 with a 95 % confidence interval of 0.9987–0.9991.

In the course of the five years of testing there were samples that tested positive in the antibody ELISA in 118 annual herd tests across 103 herds. In 91 of the herd tests there was a single positive; 17 had two positive; six had three positive; three had four and a single herd had five positives (Table 4). Ninety of the herds had one or more test positive in only one year, but 11 had one or more test positives in two years and two had one or more test positive in three years. Only three of the herds with positive antibody results lost their risk level 1 status in subsequent years of the study following the detection of further antibody positives and confirmation of *MAP* in the faecal screen. These were herds with a single positive in year 4. These results indicate overdispersion of positives ($p < 1 \times 10^{-20}$) which signifies that there were more herds with multiple positives than would be expected if test positive animals were randomly distributed amongst herds.

The number of eligible herds that were identified within the high frequency TB SICCT testing area was 84 in year 1, rising to 112 in year 4, before dropping to 103 herds in year 5, during which time the number of eligible tests rose from 2194 in year 1–2762 in year 5 (Table 5). In the low frequency TB SICCT testing area there were 299 herds in year 1 rising to 351 in year 5, during which time there were 9807 samples rising to 15,142 (Table 5). The specificity calculated from the high frequency bTB SICCT subset exceeded 0.995 in all years and ranged from 0.9955 (95 % CI of 0.9917–0.998) in year 2–0.9982 (95 % CI of 0.9955–0.9995) in year 3 (Table 5). In the low frequency TB SICCT testing subset the calculated specificity exceeded 0.999 in each of the five years. While the difference in calculated specificity between these subsets did not exceed 0.005 in any year, a significant difference was seen between the subsets in the year 1 (difference of 0.0033: 95 % CI 0.0014–0.0069), year 2 (difference of 0.0040; 95 % CI 0.0019–0.0078) and year 5 (difference of 0.0020; 95 % CI 0.0004–0.0048). There was no statistical difference between the calculated specificity of these two subsets in years 3 or 4 (Table 6). Similarly, the 95 % confidence intervals for the relative risk for a positive antibody ELISA result associated with exposure to high frequency SICCT testing exceeded 1 in years 1, 2 and 5, demonstrating an increased risk of a positive antibody result in the high frequency SICCT testing areas for these years (Table 7).

Table 3
The difference in specificity between years for the tests from herds that retained risk level 1 status in that year.

Years	Difference in specificity	95 % Confidence Interval		Chi-square value	degrees freedom	P value
		Lower	Upper			
Year 1-Year 2	0.00015	0.00035	0.00062	0.395	1	0.530
Year 1-Year 3	0.00019	0.00032	0.00067	0.612	1	0.434
Year 1-Year 4	0.00106	0.00048	0.00164	11.767	1	0.001
Year 1-Year 5	0.00081	0.00026	0.00134	7.892	1	0.005
Year 2-Year 3	0.00004	0.00044	0.00053	0.028	1	0.866
Year 2-Year 4	0.00091	0.00035	0.00149	9.828	1	0.002
Year 2-Year 5	0.00066	0.00013	0.00120	5.888	1	0.015
Year 3-Year 4	0.00087	0.00030	0.00146	8.807	1	0.003
Year 3-Year 5	0.00062	0.00008	0.00116	5.090	1	0.024
Year 4-Year 5	0.00025	0.00036	0.00088	0.650	1	0.420

4. Discussion

Understanding the characteristics of the tests used to support an animal disease control programme is essential if effective control is to be achieved and the expectations of the participating cattle owners and their veterinary surgeons are to be managed. The moderate sensitivity of the antibody ELISA for paratuberculosis has been recognised as a limiting factor and that may undermine the confidence in the use of this test in a control programme. In contrast, the published work indicates that the specificity of most paratuberculosis antibody ELISAs is equal to

Table 2
Test specificity for years 1–5 for all test results from herds that retained risk level 1 status in that year.

	Number of herd tests	Number of animals			Specificity	95 % Confidence Interval	
		Total tested	Positive	Not positive		Lower	Upper
Year 1	753	22,367	14	22,353	0.9994	0.9990	0.9997
Year 2	844	28,383	22	28,361	0.9992	0.9988	0.9995
Year 3	863	28,186	23	28,163	0.9992	0.9988	0.9995
Year 4	892	31,430	53	31,377	0.9983	0.9978	0.9987
Year 5	904	33,438	48	33,390	0.9986	0.9981	0.9989
Total		143,804	160	143,644	0.9989	0.9987	0.9991

Table 4
The frequency of test positives in herd tests where risk level 1 status was retained.

		Number of positives in herd test						
	Total herd tests	0	1	2	3	4	5	Total herd tests with positive results
Year 1	753	742	8	3				11
Year 2	844	830	9	2	3			14
Year 3	863	845	16	0	1	1		18
Year 4	892	850	33	8	0	1		42
Year 5	904	871	25	4	2	1	1	33
Total Herd Tests	4256	4138	91	17	6	3	1	118

90 herds were positive in only one year; 11 were positive in two years and two were positive in three years.

Table 5
Test specificity from herds in high and low frequency SICCT testing regions by year.

	Number of Animals Tested				95 % Confidence Interval	
High Frequency SICCT Testing Region	Total Tested	Positive	Not Positive	Specificity	Lower	Upper
Year 1	2194	8	2186	0.9964	0.9928	0.9984
Year 2	2211	10	2201	0.9955	0.9917	0.9978
Year 3	2257	4	2253	0.9982	0.9955	0.9995
Year 4	2713	6	2707	0.9978	0.9952	0.9992
Year 5	2762	8	2754	0.9971	0.9943	0.9987
Low Frequency SICCT Testing Region						
Year 1	9807	3	9804	0.9997	0.9991	0.9999
Year 2	12,452	6	12,446	0.9995	0.9990	0.9998
Year 3	13,152	11	13,141	0.9992	0.9985	0.9996
Year 4	14,436	14	14,422	0.9990	0.9984	0.9995
Year 5	15,142	14	15,128	0.9991	0.9984	0.9995

or greater than 0.99 (Collins et al., 1991; Sweeney et al., 1995; Reichel et al., 1999; Kalis et al., 2002; Fry et al., 2008; Kohler et al., 2008), although some ELISAs have been shown to have lower specificity when tested in the same populations where other ELISAs demonstrated specificity of greater than 0.99 (Collins et al., 2005). Estimating the specificity of the different antibody ELISAs for paratuberculosis has been done in test populations of animals where *MAP* infection was known, or considered to be absent. Within such populations specificity can be affected by the presence of cross-reacting antibody to shared antigens of other endemic infections, which is why large numbers of animals are desirable within a control cohort to potentially include a diverse and therefore more relevant measure of non-specific reactivity. Prior exposure to related vaccines can interfere with later diagnostic tests for natural infection, as in the case of experimental vaccination of cattle with Bacillus Calmette–Guérin (BCG) (Vordermeier et al., 2016). Maternal antibodies in calves could potentially induce a false-positive serology test result, while the presence of rheumatoid factor (anti--immunoglobulin G) in individual animals triggers false positive results at a low frequency for most serological tests. It is for these reasons that specificity should be estimated in the larger population from which the animals or herds originate (Greiner and Gardner, 2000). While published reports of the performance of a test carried out elsewhere in the world provide evidence of the expected performance of the test, validating the test within the population in which it is to be used provides additional confidence in the actual performance. Therefore, specificity estimates generated from populations of cattle in other parts of the world only offer an indication of how serological tests for paratuberculosis might perform when used in the UK, and vice versa. By using a large population of animals from herds in the UK that were either free from paratuberculosis or where it was present at very low prevalence within the herd we had the opportunity to generate a specificity estimate for the indirect antibody ELISA for paratuberculosis within the population of cattle in which the test is used and to examine this over multiple years. The results were consistent across years where specificity exceeded 0.998 in all years and taken across the five-year period the specificity was 0.9989 with a 95% confidence interval of 0.9987–0.9991. There were minor differences in specificity between the years examined that were of statistical significance, but they were marginal. The largest difference between years was less than 0.001 and therefore it was considered unlikely to be of practical significance for the control programme. That there is over-dispersion of positives within the herd tests points to herd specific factors that may impact on the herd level specificity of the ELISA. The primary herd specific factor could be that the observed clustering may be due to unconfirmed paratuberculosis in the herd, in which case the specificity in this study has been under-estimated because the positives are true positives. Alternatively, the positives may truly be false positives due to some other herd-level factor(s) unrelated to paratuberculosis. Further investigation of such anomalous herds is required to advance understanding of this phenomenon.

These results are consistent with the previously reported specificity estimates, but achieve greater precision, because of the very much larger test population and the demonstration of consistent findings across the five years of the study. The agreement with the specificity estimated for the paratuberculosis antibody ELISA in studies from other parts of the

Table 7
The relative risk of exposure to high frequency SICCT testing for positive paratuberculosis antibody ELISA results by year.

		95 % confidence interval			
	Relative Risk	Lower	Upper	Z statistic	P value
Year 1	11.920	3.1648	44.8946	3.663	0.0002
Year 2	9.386	3.4148	25.8004	4.341	0.0001
Year 3	2.119	0.6753	6.6489	1.287	0.1981
Year 4	2.280	0.8771	5.9291	1.691	0.0908
Year 5	3.133	1.3154	7.4606	2.579	0.0099
Years 1–5	4.016	2.608	6.1841	6.312	<0.0001

Table 6
Difference in specificity between high and low frequency SICCT testing regions by year.

		95 % confidence interval				
Year	Difference	Lower	Upper	Chi-square	Degrees Freedom	P value
Year 1	0.003340	0.001447	0.006878	21.839	1	<0.0001
Year 2	0.004041	0.001900	0.007833	28.124	1	<0.0001
Year 3	0.000936	-0.000332	0.003736	1.735	1	0.1877
Year 4	0.001242	-0.000124	0.003876	3.023	1	0.0821
Year 5	0.001972	0.000412	0.004806	7.398	1	0.0065

world indicates that in the UK the impact of endemic bovine tuberculosis and high frequency herd SICCT testing on the specificity of the paratuberculosis ELISA may be of limited importance. The finding that the estimate of test specificity in the high frequency SICCT testing areas exceeded 0.995 in all years further supports this. Nevertheless, the high frequency SICCT testing population would appear to be different in that there was a statistically significant difference between the ELISA specificity estimates for this population and those derived from the low frequency SICCT testing areas in three of the five years (years 1, 2 and 5). This is further supported by the demonstration of a significant relative risk of exposure to high frequency SICCT testing in these years. As with the differences observed between years, any difference between the high and low frequency testing areas was minimal, not exceeding 0.005 and likewise is considered to be of limited practical significance. Other mycobacteria share antigens with *MAP* (Bannantine and Kapur, 2020) potentially resulting in cross-reacting antibodies that can be detected in serological tests for *MAP*. The two populations examined, high frequency and low frequency SICCT areas, exist in widely separated parts of the UK with the possibility of different prevalence of other mycobacteria in the environment. This along with the different bTB status might offer possible explanations for the minimal differences observed in specificity between high and low frequency SICCT testing areas. Experience has been that carrying out serological testing for paratuberculosis in the period shortly after a SICCT test for TB will result in higher-than-expected number of animals that test positive in the paratuberculosis ELISA (Varges et al., 2009), but this can be minimised by delaying the blood sampling to beyond 70 days after SICCT testing (Kennedy et al., 2014) and a period of three months from SICCT testing to carrying out the annual paratuberculosis herd test is advised in the CHeCS programme (CHeCS, 2021). However, the few studies that have investigated the effect of the SICCT test on diagnostic antibody tests for paratuberculosis have been in *MAP*-infected herds. Therefore, it is not clear whether the increase in seropositive results might actually reflect an enhanced sensitivity of the antibody ELISAs for paratuberculosis following tuberculin skin testing of cattle, as has been suggested previously (Picasso-Risso et al., 2019), or that the increase is due to stimulation of cross reacting antibody that creates false positive results.

Previously it has been shown in a TB-infected herd that the sensitivity of two *M. bovis* antibody ELISAs was enhanced when samples were collected at day 15 following administration of the SICCT test compared to the results on samples collected prior to the SICCT test (Casal et al., 2014). Further in the experimental situation not only was this increased sensitivity observed, but the control animals that had not been infected with *M. bovis* did not produce antibody to *M. bovis* following the intradermal injection of tuberculin (Waters et al., 2015). It was concluded that the injection of bovine PPD transiently elevated existing antibody titres through the stimulation of specific B cells that were already present as a result of *M. bovis* infection, a phenomenon known as anamnestic boost (Casal et al., 2014). This has been observed in other species and the increased sensitivity of serological testing stimulated by the SICCT test has been shown to have practical value for the diagnosis of TB in New World camelids (Bezos et al., 2013). It may be that the same reaction occurs in cattle that have been exposed to *MAP* where the SICCT leads to a transient increase in specific antibodies that results in enhanced sensitivity of the paratuberculosis ELISA.

As with studies involving naturally occurring populations that are subjected to intervention there will be sources of bias that can impact on the certainty of these estimates (Nielsen, 2020). Firstly, a proportion of these herds will have progressed from a CHeCS paratuberculosis risk level where infection was recognised in the herd to achieve the risk level 1 status (lowest level of risk of being infected) by removing animals that tested positive by the antibody ELISA, irrespective of whether serial faecal testing was positive. In this way any animal that was uninfected, but with a tendency to produce false positive antibody results would be removed prior to the herds making up the population that was under examination in this study. This could be expected to elevate the specificity estimate as false positives were removed from the population (Nielsen and Toft, 2008). As a consequence of the structure of the extracted data we were not able to examine the previous history of these herds to assess this factor, either to determine the herds that had never removed a test positive animal, or the proportion of animals that had been removed. Neither could we examine the number of antibody positive animals that were faecal screen negative and retained in the herd historically. Therefore it was not possible to examine for this source of bias. While this bias can be expected to have some impact on the precision of the specificity estimates, these estimates are nevertheless relevant to the population of all CHeCS risk level 1 herds in the UK, where some herds will inevitably have undergone removal of paratuberculosis ELISA positives. It should also be recognised that in each year the population contained animals that were tested in previous years, but by the final year only 0.068 of the animals that were tested had been present in the first year of testing. This turnover in animals is affected firstly by the expected annual replacement rate of 16% in beef breeding herds (AHDB, 2023) and secondly that new herds achieved risk level 1 status in each of the years in the study and some lost status or left the programme. While the degree of overlap in herds and animals tested across the years prevents the specificity estimates for each year being independent, the close similarity in the specificity estimates across the years should give scheme participants the confidence that the specificity can be expected to be consistent from year to year.

The third probable bias affecting the specificity estimate for the antibody ELISA is that it is relative to the sensitivity of the CHeCS risk level 1 testing protocol and is strongly affected by the performance of the faecal screen used to test antibody positive animals. An inadequate sensitivity to detect *MAP* shedding in the antibody positive animals that were truly infected would allow these to be misclassified as false positives by the antibody ELISA. Were this to be the case more infected herds would be included in the low-risk population and the specificity of the ELISA would be under-estimated. The test for faecal screening was not consistent across the period of examination. Faecal culture was used progressively less frequently from the start of the period of the study, because of the prolonged incubation time required to resolve the herd status with a consequent impact on trading for the affected herd. A different extraction method and PCR test was used to test faecal samples in the final year. No difference was seen in the ELISA specificity between years one to three and again between years four and five, but there was a statistically significant difference between each of the first three years and the last two years. This raises the possibility that the change in the confirmatory faecal test method, firstly from liquid culture with PCR confirmation; to one extraction and PCR kit to a second extraction and PCR kit in the final year, had some impact on the estimated specificity of the antibody ELISA, marginally reducing it. Finally, the possibility that there was also some change in the performance of the commercial antibody ELISA kit that was used over the entire period of the study cannot be excluded. Indeed, there may well be other unrecognised factors that could have acted differently on this population.

While the focus of this study has been on estimating the specificity of the antibody ELISA, the practice of using a serial testing approach to the confirmation of infection is a problem for the test programme too. Faecal culture has long been the gold standard test for paratuberculosis, however, there are limitations to faecal screening that call this practice into question. *MAP* is not distributed homogenously in faeces and therefore small numbers of organisms below the limit of detection may prevent detection in one subsample, while another subsample may have higher numbers of the organism and test positive. The harsh decontamination method used to prevent overgrowth of competing bacteria and fungi during culture may prevent some strains of the organism from growing at all, especially where the number of *MAP* organisms in the sample is low (Whittington, 2010). This second constraint is not a consideration for the PCR as the sample is not subject to decontamination and the organism does not need to be viable to be detected. However, the small volume of faeces used for this test in relation to the patchy distribution

of the organism in faeces has the potential to impact negatively on test sensitivity and repeatability. Cattle faeces is also a challenging matrix for PCR due to the presence of PCR inhibitors and the high volume of other bacteria that dilute *MAP* DNA (Fock-Chow-Tho et al., 2017). The DNA of the organism must be extracted from the sample before the PCR test itself is run and the *MAP* outer membrane is particularly tough and resistant to disruption (Sting et al., 2014). There are therefore a number of variables that directly affect PCR performance: test matrix, sample volume, extraction system and PCR test. The impact that this has on the variation between test performance can be important and lead to much greater variation than occurs in the performance of serological tests. In one study that evaluated six commercially available extraction systems in conjunction with a single PCR kit, the detection of known positive samples ranged from 18 % to 94 % (Leite et al., 2013). In a follow-up study examining three extraction kits the proportion of culture positive samples identified from animals that were also antibody positive ranged from 23 % to 89 % (Fock-Chow-Tho et al., 2017). The relevance of the number of organisms present in the faecal samples used to estimate the sensitivity of PCR testing was shown in a comparison of three commercial kits using samples that were positive in the Trek culture system. The three kits examined detected as positive all 25 samples from high shedders, as defined in the Trek culture system, but for low shedders the detection rates were 48 %, 72 % and 88 % for the three kits examined (Prendergast et al., 2018). When using faecal tests in the field, the poor repeatability of positive results between samples from animals that are shedding small number of the organisms, might lead the owners of affected cattle to question the validity of a positive result that cannot be replicated.

Furthermore, infected animals can be expected to shed *MAP* intermittently in their faeces. For instance, in a five-month longitudinal study conducted in two low prevalence dairy herds, all but one of 13 cows that were detected as faecal positive at the beginning of the study yielded at least one positive faeces sample subsequently, but only 48 % of the samples collected at weekly intervals were positive for *MAP* by culture. The proportion of culture positive results in those animals defined as progressors (high numbers of *MAP* present) was 73 % (Beaver et al., 2017). In the same study, 68 % of the faeces samples were positive by PCR, suggesting superior sensitivity to the culture system used, but also demonstrating the relatively low repeatability of faecal screening in some infected individuals. In one experiment where 20 three-month-old calves were infected and monitored for the next five years only two animals were consistently faecal positive, despite infection being identified in seven animals when examined postmortem at the end of the study (Begg et al., 2018).

The variability in test performance, together with the intermittent shedding of *MAP* exhibited by infected animals, means that the predictive value of a negative faecal screening test result is low and raises questions in relation to how the examination of faeces for *MAP* should be used in programmes designed to offer assurance of a low risk of the presence of paratuberculosis in a herd. This is a particular challenge as despite the high specificity of the antibody ELISA it is to be anticipated that occasionally herds categorised as low risk will experience more antibody positives than expected and it will be necessary to investigate the herd further.

Despite these uncertainties, this study has shown that under UK conditions the specificity of the paratuberculosis antibody ELISA compares well with the estimates generated in other studies from around the world where TB is not endemic in cattle. As the CHeCS paratuberculosis risk level 1 herds came from all parts of the United Kingdom, including areas of high TB prevalence and where TB herd surveillance testing is carried out annually or every six months, it can be inferred that if the use of the TB SICCT test does increase the number of false positive antibody results in herds that are defined by the paratuberculosis control programme as being of the lowest risk category, this increase is unlikely to be of significance for the success of the programme.

5. Conclusion

Paratuberculosis herd level control and assurance programmes in the UK rely on serology as the primary screening test. A review of test performance in published studies indicates that the specificity of the tests is high when serum samples are used and will therefore not lead to any important degree of unnecessary culling or wastage. The examination of a large data set from CHeCS paratuberculosis risk level 1 herds where one commercial antibody ELISA was used and the confirmation test was either liquid culture or PCR, resulted in an apparent relative specificity for the antibody ELISA of at least 0.998. In the risk level 1 herds subjected to at least annual herd SICCT for tuberculosis the overall specificity did not fall below 0.995. However, previously published studies showed a variability in the sensitivity of PCR kits for faecal screening and in the performance of commercially available DNA extraction systems. Taken together this indicates that the weakness in the UK paratuberculosis programme lies not in the serological screening tests to demonstrate a seronegative herd, but in the faecal tests used to negate unexpected or contested antibody positive results. Ensuring the best methodology for faecal examination is adopted as standard for the programmes will not address the variability in *MAP* shedding among infected animals. Therefore alternative approaches to confirm MAP infection in a herd are required to support serology-based assurance programmes.

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Declaration of Competing Interest

The author was previously the manager of SRUC Veterinary Services and the SRUC Premium Cattle Health Scheme is a commercial enterprise.

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