Research in Veterinary Science 159 (2023) 72–80

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Once bitten twice shy: Risk factors associated with bovine tuberculosis recurrence in Castilla y Leon, Spain

Pilar Pozo [a,b,*], Javier Bezos [a,c], Beatriz Romero [a,c], Anna Grau [d], Jesus Nacar [d], Jose Luis Saez [e], Olga Minguez [f], Julio Alvarez [a,c]

[a] *VISAVET Health Surveillance Centre, Universidad Complutense de Madrid, Madrid, Spain*
[b] *SaBio, Instituto de Investigación en Recursos Cinegéticos IREC-CSIC-UCLM-JCCM, Ciudad Real, Spain*
[c] *Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid. Spain*
[d] *Dirección General de Producción Agropecuaria e Infraestructuras Agrarias, Consejería de Agricultura y Ganadería de la Junta de Castilla y León, Valladolid, Spain*
[e] *Subdirección General de Sanidad e Higiene Animal y Trazabilidad, Dirección General de Sanidad de la Producción Agraria, Ministerio de Agricultura, Pesca y Alimentación, Madrid, Spain*
[f] *Consejería de Sanidad de la Junta de Castilla y León, Valladolid, Spain*

A R T I C L E I N F O

Keywords:
Bovine tuberculosis
Recurrence
Persistence
Cattle
Survival analysis
Case-control study

A B S T R A C T

Persistence of bovine tuberculosis (bTB) in cattle herd remains a major challenge in disease elimination due to the ineffectual removal of all infected animals in a bTB breakdown. Characterization of herds with a higher probability of experiencing further bTB breakdowns can help to implement specific risk-based policies for disease control and eradication. Here, our aim was to identify herd- and breakdown-level risk factors in bTB infected herds in Castilla y Leon, Spain, associated with a decreased time to recurrence and an increased risk of recurrence using a mixed effects Cox proportional hazards model and a multivariable logistic regression model, respectively.

Results revealed that location (province), herd size and number of incoming animals/contacts were good predictors of a decreased time to bTB recurrence and an increased risk of becoming a recurrent herd. Additionally, the duration of the previous outbreak and the number of IFN-γ herd-tests applied in it were associated with increased odds of (an early) recurrence. Risk factors identified here can be used for early identification of herds in which bTB eradication may be more challenging and that should thus be subjected to increased control efforts. The characterization of high-risk herds may help to minimize the risk of reinfection and emphasize early detection and removal of bTB positive animals in the herd.

1. Introduction

Successful eradication of bovine tuberculosis (bTB) in cattle remains a major challenge in several regions worldwide. The chronic nature of the disease, together with the limitations in the sensitivity of available diagnostic tests and certain animal- and herd-level risk factors, hamper disease control due to the inability to effectively clear the infection from a herd (Alvarez et al., 2014; de la Rua-Domenech et al., 2006; Pollock and Neill, 2002). The role of between-herd movements of infected (but undetected) cattle has been widely evaluated, with its contribution demonstrated in several regions (Palisson et al., 2016; Picasso et al., 2017; Pozo et al., 2019). Wildlife reservoirs may also act as source of bTB when infected wild animals are in close contact with cattle, both in extensively and intensively managed herds (Doyle et al., 2022; LaHue et al., 2016). Additionally, local risk factors such as bTB prevalence of the area, the use of shared pastures, and the presence of infected neighboring farms (i.e., fence-to-fence contact) may increase the likelihood of infection, and for these reasons, increased awareness of farm biosecurity is of utmost importance (Biek et al., 2012).

Current control and eradication strategies in Spain are based on a combination of regular test and slaughter policies, consisting on the routine application of the single intradermal test (SIT), the interferon-gamma (IFN-γ) assay used as an ancillary test in bTB infected herds, and post-mortem inspection at the abattoir (Anon, 1964, 2022c). When positive animals are identified in the ante-mortem tests, these are slaughtered within 15 days and if the infection is confirmed post-mortem the herd is subjected to movement restrictions until undergoing two consecutive negative herd tests separated by at least 60 days.

* Corresponding author at: VISAVET Health Surveillance Centre, Universidad Complutense de Madrid, Madrid. Spain.
E-mail address: ppozo@ucm.es (P. Pozo).

https://doi.org/10.1016/j.rvsc.2023.04.011
Received 18 January 2023; Received in revised form 4 April 2023; Accepted 15 April 2023
Available online 19 April 2023

Several studies have demonstrated the impact that herds experiencing recurrent infections have on the progress of bTB eradication programs. Up to 59% of the bTB positive herds identified in New Zealand during 2005–2011 had experienced a previous bTB episode (Dawson et al., 2014). Similarly, around 23% of the bTB breakdowns in Great Britain during 2003–2005 recurred within a year and up to 38% within two years (Karolemeas et al., 2011). Additionally, up to one third of derestricted herds in Ireland in 2013 disclosed reactors in follow-up tests within 5 years, and a similar proportion of breakdowns initiated by routine slaughter surveillance had experienced a previous breakdown in the preceding 3 years (Byrne et al., 2020). Despite the surveillance and control efforts in place and the progressive decline in the bTB prevalence in Spain [2.2% in 2002 and 1.5% in 2020, (Anon, 2022b, 2022c)], a study conducted between 2009 and 2011 identified residual infection as the most frequent cause of new bTB breakdowns (Guta et al., 2014). Given the substantial economic loses and the detrimental effect in public health and productivity linked to bTB (Caminiti et al., 2016; Tschopp et al., 2022), and the limited information regarding the farm- and disease-level predictors that might predispose to bTB recurrence in Spain, we analyzed bTB breakdown information of positive herds in Castilla y Leon, a high-prevalence Autonomous region of Spain. Here, our aim was to identify risk factors in bTB infected herds in the region during the 2010–2020 period associated with i) a shorter time to experience a subsequent bTB breakdown (time to recurrence), and ii) an increased risk of experiencing a new bTB outbreak after being considered free of the disease (risk of recurrence). Information obtained here may help to predict which herds are at higher risk of experiencing additional bTB breakdowns, what could aid in applying targeted control efforts to prevent bTB recurrence in previously infected herds or, if the disease is already present, to act early on in a breakdown.

2. Materials and methods

2.1. Study definitions and data sources

The study population consisted of herds in which at least one animal was positive in a bTB herd test to either the skin test, the IFN-γ assay and/or bacteriology between 2010 and 2020. Here, we replicated the study definitions for bTB breakdowns used in previous research performed on the same study region (Pozo et al., 2020): briefly, all bTB-positive related tests separated by ≤18 months in a given herd were considered part of the same bTB breakdown; therefore, a bTB breakdown was considered resolved when no bTB positive results to any diagnostic test used in the herd were found in the 18 months following the last bTB positive herd test. The date of the first bTB negative herd test after that last bTB positive herd test is named from hereon the derestriction date (Byrne et al., 2021).

Demographic data available for each herd/breakdown was extracted from the SITRAN Information System (Anon, 2021) and included the herd identification, herd production type (beef/fattening, bullfighting, and dairy/mixed), location, and number of animals present in the herd. Information on the number of movements and number of incoming animals to/from each herd was also accessible for the period 2007–2020. Data on bTB tests including date, number of tested animals, and number of reactors in ante-mortem (SIT and/or IFN-γ assay) and post-mortem tests (bacteriology in animals sampled at the abattoir) applied in the framework of the Spanish bTB eradication program during the study period was also collected.

Available explanatory variables for each herd depending on the model used (see below) included herd production type, location (province), number of incoming connections (i.e., herds from which animals were received, in-degree) and number of incoming animals in the three years previous to the start of the bTB breakdown. Furthermore, explanatory variables derived from the previous bTB breakdown experienced in a herd included its duration [measured in number of days elapsed between the first bTB positive test and the derestriction date, see (Pozo et al., 2020)], the median herd size when it started (based on number of animals >6 weeks, i.e., tested using the skin test), the number of confirmed animals (based on bacteriology) throughout the previous bTB breakdown and specifically in the last test before it was considered resolved, the number of times (herd-tests) the IFN-γ assay was applied, and the number of bTB positive herd-tests (to any diagnostic technique) during the whole previous bTB breakdown. Given that herds are subjected to movement restrictions until they recover the officially tuberculosis free (OTF) status, in-degree and number of animals entering the farm in the three years prior to the start of the previous bTB breakdown were divided by the number of months the farm was OTF during those three years (i.e., was not subjected to movement restrictions, "weighted in-degree and weighted number of incoming animals"). According to the Spanish bTB eradication program [10], the ancillary IFN-γ test is applied for the detection of the maximum number of infected animals in bTB positive herds where there is conclusive evidence of the presence of bTB through bacteriological culture. The variable number of IFN-γ herd-tests applied refers to the number of herd-tests in which the IFN-γ was used as an ancillary test in animals >6 months.

Previous bTB breakdown duration and herd size at the start of the previous bTB breakdown were categorized into four categories based on quartiles, whereas number of confirmed animals, number of positive routine/follow-up herd tests and number of IFN-γ tests applied during the previous bTB breakdown, weighted in-degree and number of incoming animals in the three years prior to the start of the previous bTB breakdown were categorized using terciles. The effect of these variables (e.g., in-degree and number of incoming animals) on the outcome was also evaluated on log-transformed distributions. The variable number of animals confirmed through bacteriology in the last test of the previous bTB breakdown was a dichotomized into "none" or "one or more" according to non-normal distribution of observations (median = 0, IQR = 0–0, max = 33, Table 1).

2.2. Time to recurrence (survival analysis)

A database formed by all resolved breakdowns registered in herds located in Castilla y Leon between 2010 and 2020 was used for this analysis. Since only resolved breakdowns were considered (i.e., in which no new positive herd tests were observed for at least 18 months) the last possible date of a positive herd-test was July 1st 2019. The outcome variable was the period between bTB breakdowns (or time to recurrence), that is, the number of days between the bTB breakdown derestriction date and the start of a subsequent bTB breakdown (the first bTB-positive herd test following the derestriction date). When herds experienced more than one bTB breakdown during the study period these were kept in the database as different observations although lack of independence between breakdowns from the same herd was accounted for in the analysis (see below). The censoring point for observations (breakdowns) that were not followed by a new breakdown was the end of the study period (December 31st, 2020).

The association between each of the available explanatory variable and the outcome variable "time to recurrence" was explored using a mixed effects Cox proportional hazards model, in which the herd was included as a random effect to account for the lack of independence between observations originating from the same herd. The survival distributions for the categories of each of the explanatory variables considered were plotted using Kaplan-Meier survival estimates and compared using the log-rank test. Univariable mixed Cox regression models were fitted to compare the hazard ratios (HR) of experiencing a further bTB breakdown considering the effects of the $X_1 \ldots X_n$ explanatory variables considered, so that:

$$\lambda(t) = \lambda_0(t)e^{X_i\beta + Z_i b},$$

where $\lambda_0(t)$ is an unspecified baseline hazard function, X_i and Z_i are the design matrices for the fixed and random effects, respectively, β is the

Table 1
Results of the univariable and multivariable logistic regression models using the recurrent status (case/control) for 2638 herds experiencing at least one bTB breakdown in Castilla y Leon during 2010–2020 as the outcome variable.

Variable (number of herds with information)	Level	Controls		Cases			Univariable			Multivariable		
		Number	%	Number	%	Total	OR (95% CI)	P-value[a]	P-value[b]	OR (95% CI)	P-value[a]	P-value[b]
Herd type (n = 2648)	Beef/ Fattening	1744	85.6	533	87.4	2277	1 (NA)	–	0.502			
	Bullfighting	38	1.9	9	1.5	47	0.77	0.495				
	Dairy/ Mixed	256	12.5	68	11.1	324	0.87	0.334				
Province (n = 2648)	Avila	345	16.9	110	18.0	455	1 (NA)	–	0.002	1 (NA)	–	
	Burgos	192	9.4	40	6.6	232	0.65 (0.4–1)	0.038		0.78 (0.5–1.2)	0.257	0.002
	Leon	209	10.3	54	8.9	263	0.81 (0.6–1.2)	0.263		0.89 (0.6–1.3)	0.546	
	Palencia	73	3.6	21	3.4	94	0.90 (0.5–1.5)	0.704		0.79 (0.4–1.3)	0.424	
	Salamanca	774	38.0	258	42.3	1032	1.05 (0.8–1.4)	0.734		1.09 (0.8–1.4)	0.559	
	Segovia	197	9.7	41	6.7	238	0.65 (0.4–1.0)	0.036		0.80 (0.5–1.2)	0.295	
	Soria	63	3.1	38	6.2	101	1.89 (1.2–2.9)	0.006		2.58 (1.6–4.2)	<0.001	
	Valladolid	29	1.4	8	1.3	37	0.86 (0.4–1.9)	0.727		0.84 (0.3–1.9)	0.695	
	Zamora	156	7.6	40	6.6	196	0.80 (0.5–1.2)	0.295		0.91 (0.6–1.4)	0.673	
Previous bTB breakdown duration (days, n = 2648)	1–84	578	28.4	130	21.3	708	1 (NA)	–	0.005	1 (NA)	–	0.014
	85–158	473	23.2	148	24.3	621	1.39 (1.0–1.8)	0.014		1.42 (1.1–1.9)	0.014	
	159–481	495	24.3	164	26.9	659	1.47 (1.1–1.9)	0.003		1.41 (1.1–1.9)	0.015	
	>481	492	24.1	168	27.5	660	1.52 (1.2–2.0)	0.002		1.20 (0.9–1.7)	0.208	
Herd size at the start of the previous bTB breakdown (n = 2638)	1–44	567	27.9	100	16.4	667	1 (NA)	–	<0.001	1 (NA)	–	<0.001
	45–79	540	26.6	137	22.5	677	1.44 (1.1–1.9)	0.011		1.22 (0.9–1.6)	0.188	
	80–134	479	23.6	157	25.7	636	1.86 (1.4–2.4)	<0.001		1.43 (1.1–1.9)	0.017	
	>134	442	21.8	216	35.4	658	2.77 (2.1–3.6)	<0.001		1.96 (1.5–2.6)	<0.001	
Number of bTB positive herd tests in the previous bTB breakdown (n = 2648)	1–2	1504	73.8	413	67.7	1917	1 (NA)	–	0.006			
	3	328	16.1	87	14.3	293	1.54 (1.2–2.0)	0.002				
	>3	206	10	110	18.0	438	1.22 (0.9–1.6)	0.105				
Confirmed animals in the previous bTB breakdown (n = 2648)	0	1311	64.3	372	61.0	1683	1 (NA)	–	0.259			
	1	342	16.8	106	17.4	448	1.09 (0.9–1.4)	0.483				
	>1	385	18.9	132	21.6	517	1.21 (0.9–1.5)	0.105				
Number of confirmed animals in the last test of the previous bTB breakdown (n = 2648)	0	1833	89.9	546	89.5	2379	1 (NA)	–	0.757			
	>0	205	10.1	64	10.5	269	1.05 (0.8–1.4)	0.756				
Number of IFN-γ tests in the previous bTB breakdown (n = 2648)	0	1213	59.5	329	53.9	1542	1 (NA)	–	0.034			
	1–2	433	21.2	139	22.8	572	1.18 (0.9–1.5)	0.145				
	>2	392	19.2	142	23.3	534	1.34 (1.1–1.7)	0.013				
Weighted in-degree in the previous 3 years to the start of the previous bTB breakdown (n = 2648)	0–0.02	731	35.9	144	23.6	875	1 (NA)	–	<0.001	1 (NA)		<0.001
	0.02–0.06	761	37.3	112	18.4	873	0.75 (0.6–1.0)	0.032		0.68 (0.5–0.9)	0.006	
	>0.06	546	26.8	354	58.0	900	3.29 (2.6–4.1)	<0.001		2.88 (2.3–3.6)	<0.001	
Weighted number of incoming animals in the previous 3 years to the start of the previous bTB breakdown (n = 2648)	0–0.03	722	35.4	152	24.9	874	1 (NA)	–	<0.001			
	0.03–0.30	684	33.6	190	31.1	874	1.32 (1.0–1.7)	0.022				
	>0.30	632	31.0	268	43.9	900	2.01 (1.6–2.5)	<0.001				

bTB, bovine tuberculosis; OR, odds ratio.
[a] Walds test.
[b] Chi-square test.

vector of fixed-effects coefficients and *b* is the vector of random effects coefficients (Therneau, 2020). The proportional hazards assumption was evaluated for each covariable by computing Schoenfeld residuals.

Variables with $p \leq 0.2$ in the univariable analyses were selected for their potential inclusion in a multivariable Cox proportional hazards mixed model. First, multicollinearity between potential covariables was assessed using the variance inflation factor (VIF) to ensure a mean VIF of <5 among the variables before being offered to the multivariable model (Belsley et al., 1980). For correlated variables, AIC was used to perform variable selection so that the variable yielding the lower AIC in the univariable models was included in the multivariable analysis (Dohoo et al., 2003). The final multivariable model was selected based on the lowest AIC among all possible combination of variables using a backward selection procedure.

In order to assess the potential effect of including breakdowns in which the infection could not be confirmed using bacteriology in the variables included in the final model, the same analysis was conducted considering alternatively only observations starting from a previous breakdown in which the infection was confirmed or only those in which it was not confirmed.

2.3. Case-control study of predictors for bTB recurrence

Factors associated with an increased risk of experiencing a new breakdown (recurrence) in herds that had previously resolved another breakdown were evaluated through a case-control study. Here, the analyses were performed at the herd level, and cases were defined as those herds that experienced more than one bTB breakdown during the 2010–2020 study period (recurrent herds). A control was any herd that i) had experienced only one bTB breakdown during the 11-year span, and ii) in which the minimum period elapsed between the derestriction date and the end of the study period (31st December 2020) was at least 1420 days (~47 months). This threshold was selected to avoid including as controls herds that could eventually become cases since it was the 75% percentile of the distribution of between-breakdown durations (see Results). Mann-Whitney test was first used to detect significant differences in bTB breakdown duration and herd size between recurrent and not recurrent herds.

The potential risk factors were then tested in a univariable logistic regression model using the recurrent status (case/control) as the outcome variable. Covariables used in the analysis were the same ones considered in the survival analysis, but regarding outbreak-associated variables, in the case of herds experiencing more than one outbreak (i.e., case herds) variables referred always to the first bTB breakdown registered during the study period. Explanatory variables that were significant in the univariable model at a liberal $p < 0.2$ were considered for inclusion in a multivariable model. Multicollinearity between potential risk factors was evaluated applying again the variance inflation factor (VIF) to ensure a mean VIF of <5 among the variables (Belsley et al., 1980) before being offered to the multivariable model. In the event of correlation between variables, the variable yielding the lower AIC value was in the univariable models was selected (Dohoo et al., 2003). The final multivariable model included the selected risk factors along with significant two-way biologically plausible interactions and was built using a backward selection procedure based on a likelihood ratio test with a threshold of significance of $p \leq 0.05$. Results in the model were expressed as odds ratios (ORs) and 95% confidence intervals (CIs). The Hosmer–Lemeshow statistic was used to test the goodness of fit of the final model (Hosmer and Lemeshow, 2000).

In order to assess the potential impact of the threshold (1420 days) used to select controls (namely, the risk of including as controls herds that could still become cases), analyses were replicated using only cases with a duration between breakdowns <1420 days so that no overlap between the durations of the case and control herds existed.

Statistical analyses were performed using R version 4.1.1 (R Core Team, 2021). Survival analyses and Cox proportional hazards models were fitted using the survival (Therneau et al., 2021) and survminer (Kassambara et al., 2021) packages. Multiple comparisons, network analyses, graphics and model fitting were conducted using PMCMRplus (Pohlert, 2020), igraph (Csardi and Nepusz, 2006), ggplot2 (Wickham, 2009), ggspatial (Dunnington, 2018), dplyr (Wickham et al., 2019), lme4 (Bates et al., 2015), glmmTMB (Brooks et al., 2017), and DHARMa (Hartig, 2020) packages.

3. Results

3.1. Description of the study population

During 2010–2020, there were 19,068 tested cattle herds in Castilla y Leon, of which 4946 (25.9%) yielded at least one positive result in a bTB-diagnostic test at some point during the study period. Out of these, 2377 (48.1%) tested bTB positive in only one herd test, 883 (17.9%) in two and 1686 (34.1%) in three or more herd tests. Considering the breakdown definition applied in this study, over the 11-year time span there were 6130 bTB breakdowns in the region. Three hundred and twenty-nine (5.4%) bTB breakdowns were excluded from the database as these were either not resolved before the end of the study period or the herd was not tested again after the positive test that initiated the bTB breakdown. Twenty bTB breakdowns were disclosed in units classified in categories other than the ones considered here (e.g., pastures, $n = 7$, and breeding heifers, $n = 2$) and, given the differences in their management (pastures often correspond to units harboring animals from different herds for seasonal grazing) and the low number of observations, were excluded from the database for simplicity. Finally, 1185 (20.5%) bTB breakdowns were omitted from further analyses as these were resolved after July 1st 2019 (i.e., <18 months before the end of the study period). The remaining 4596 bTB breakdowns used in the analysis were disclosed in 3936 herds, of which 15.5% ($n = 610/3936$) herds experienced more than one bTB breakdown.

Most (42.0%) breakdown herds were located in Salamanca ($n = 1655$), followed by Avila (17.0%, $n = 670$) and Leon (9.6%, $n = 379$). Salamanca was also the province with a higher proportion (42.3%, $n = 258/610$) of recurrent herds, followed again by Avila (18.0%, $n = 110$) and Leon (8.9%, $n = 54$, Fig. 1). Out of the total number of recurrent herds, 8.2% ($n = 50$) had three bTB breakdowns, and were mainly located in Salamanca (50%, n = 25/50), followed by Soria (16%, 8/50) and Avila (14% n = 7/50, Fig. 1). The majority of the breakdown herds were beef/fattening (86.8%, $n = 3416/3936$) followed by dairy/mixed (11.7%, $n = 461$), and bullfighting (1.5%, $n = 59$) herds (Table 2). In terms of bTB breakdowns, again the majority (86.9%, $n = 3993$) were disclosed in beef/fattening herds, followed by dairy/mixed (11.6%, $n = 533$), and bullfighting (1.5%, $n = 70$) herds (Table 2).

Overall, the median bTB breakdown duration in Castilla y Leon was 126.5 days [Interquartile range (IQR) = 79–406]. The median duration of the interval between bTB breakdowns in recurrent herds was 918 days (IQR = 644–1420).

3.2. Time to recurrence (survival analysis)

A total of 4596 observations (periods starting after a resolved bTB breakdown) were considered in this analysis. Up to 85.6% (3936/4596) bTB breakdowns were not followed by a subsequent bTB breakdown during the study period and were thus considered censored observations in the analysis. The remaining 660 bTB breakdowns that followed a previously resolved bTB breakdowns were disclosed in 610 herds.

Kaplan–Meier curves for the 10 predictor variables are shown in Supplementary material A-C, and results of the univariable analyses are shown in Supplementary material D. No information on herd size at the start of the previous bTB breakdown was available for 15 bTB breakdowns from 15 herds, as these took place in fattening units ($n = 14$) in which no routine tests were performed prior to the disclosing test and a bullfighting herd with no prior whole-herd tests. All variables except

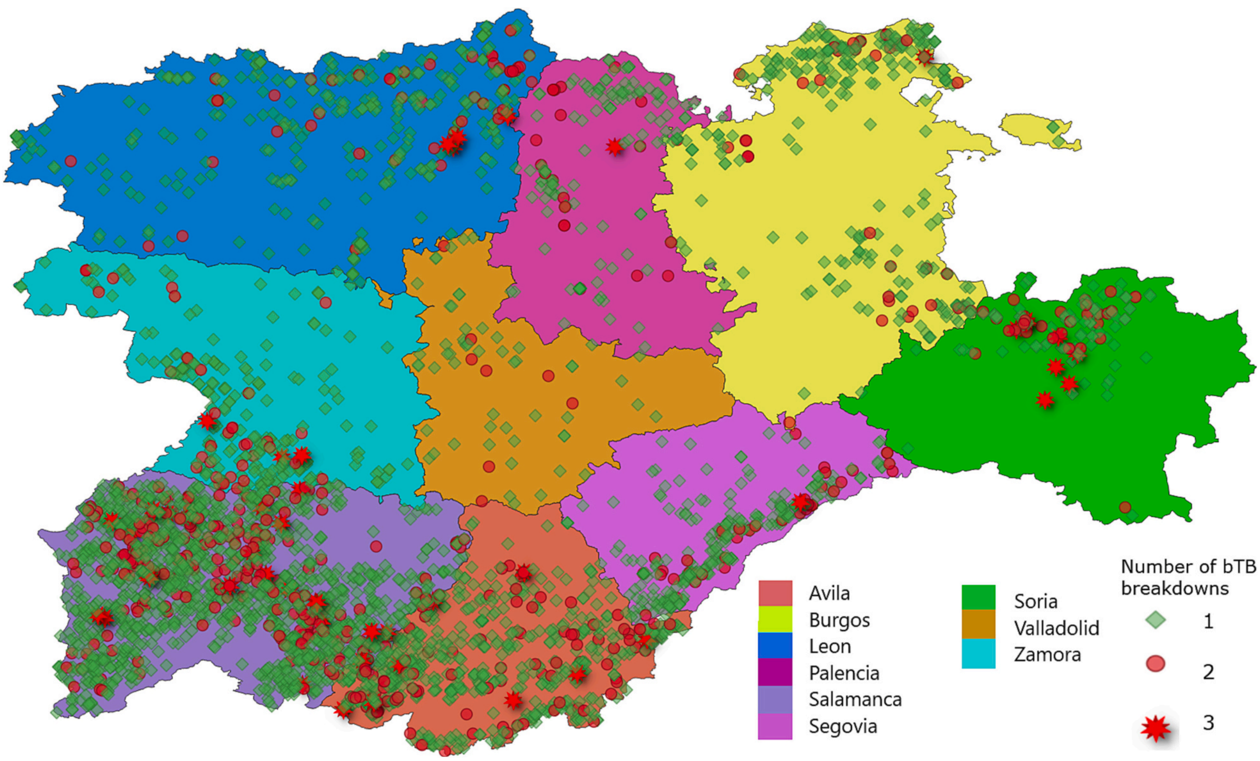

Fig. 1. Spatial distribution of recurrent (red) and not recurrent (green) herds in Castilla y Leon included in the study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Number of herds and number of bTB breakdowns included in the study per production type/province.

		Number of bTB breakdowns		Number of herds					
		Total	%	Total	%	Recurrent	%	Not recurrent	%
Production type	Beef/fattening	3993	86.9	3416	86.8	533	87.4	2883	86.7
	Bullfighting	70	1.5	59	1.5	9	1.5	50	1.5
	Dairy/mixed	533	11.6	461	11.7	68	11.1	393	11.8
Province	Avila	787	17.1	670	17.0	110	18.0	560	16.8
	Burgos	335	7.3	294	7.5	40	6.6	254	7.6
	Leon	437	9.5	379	9.6	54	8.9	325	9.8
	Palencia	169	3.7	147	3.7	21	3.4	126	3.8
	Salamanca	1938	42.2	1655	42.0	258	42.3	1397	42.0
	Segovia	348	7.6	306	7.8	41	6.7	265	8.0
	Soria	201	4.4	155	3.9	38	6.2	117	3.5
	Valladolid	74	1.6	66	1.7	8	1.3	58	1.7
	Zamora	307	6.7	264	6.7	40	6.6	224	6.7
Total		4596		3936		610		3326	

herd type and number of confirmed animals through bacteriology in the last test of the previous bTB breakdown were potentially associated ($p < 0.2$) with the time to recurrence and were thus considered for the multivariable analysis (Supplementary material D). A mean VIF value of <5 among the variables was observed.

According to the final multivariable Cox regression model (based on lower AIC) the variables province of origin, herd size at the start of the previous breakdown, number of IFN-γ tests in the previous bTB breakdown and weighted number of incoming animals in the 3 years prior to the start of the previous bTB breakdown significantly ($p < 0.001$) influenced the time to recurrence in bTB breakdowns Castilla y Leon during 2010–2020 (Fig. 2). The variable weighted number of incoming animals was retained in the models over the weighted in-degree based on lower AIC. Time to recurrence was significantly shorter in bTB breakdowns disclosed in herds located in Soria ($p < 0.001$, HR = 1.92, 95% CI 1.4–2.7) compared to those in Avila (reference category) (Fig. 2 and Supplementary material D). An increasing trend in the risk of recurrence with increasing herd size at the start of the previous bTB breakdown was also identified, with large herds (>141 animals) being at a significantly higher risk of having a subsequent bTB breakdowns earlier than small herds (1–46 animals). An increasing number of IFN-γ tests in the previous bTB breakdown was also significantly associated with a decreased time to recurrence. Time to recurrence was shorter with increasing weighted number of incoming animals in the three years prior to the previous bTB breakdown (Fig. 2 and Supplementary material D).

A limited variability in the herd-level random effects estimated in models assessing time to recurrence effects was observed (standard deviation between herds <0.01). When the log- transformed instead of the categorized variables were offered to the multivariable model, none of them were selected.

When the same model was fitted using the subset of periods between bTB breakdowns in which bTB was not confirmed through bacteriology in the preceding bTB breakdown (67.6%, n = 3108 out of the total

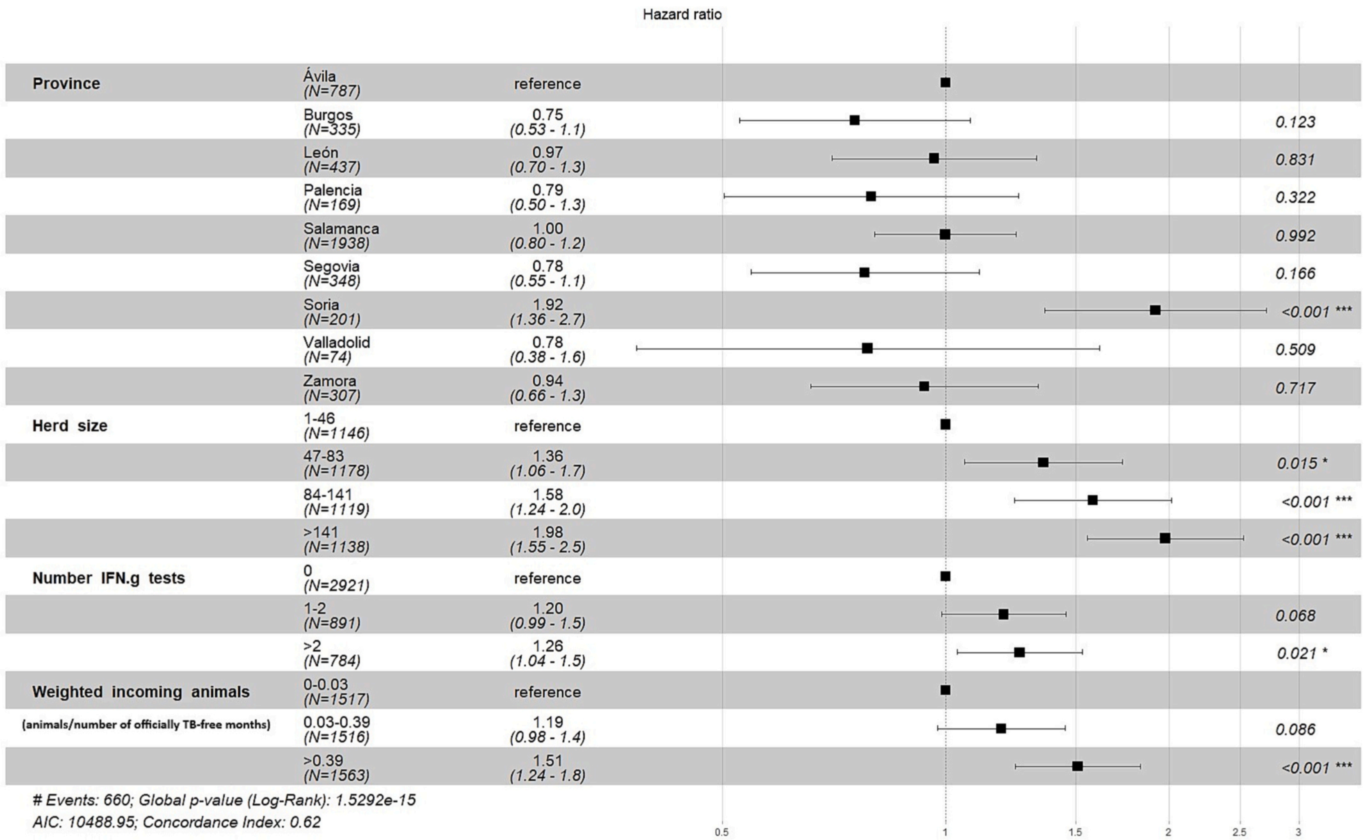

Fig. 2. Forest plot of the overall multivariable survival analysis. Hazard ratios are shown with 95% confidence intervals (CI). No information on herd size was available for 15 bTB breakdowns. Multivariable analysis was performed on 4581 periods between bTB breakdowns, of which 660 ended up with a subsequent bTB breakdown.

number of periods between bTB breakdowns), no substantial (≤20%) variability in the hazard ratios was observed (Supplementary material E). However, when only observations in which bTB was confirmed in the previous bTB breakdown (32.4% of the total periods between bTB breakdowns) were considered, herd size and weighted number of incoming animals in the previous three years were still significantly associated with time to recurrence along with previous bTB breakdown duration, whereas province and number of IFN-γ tests was not (Supplementary material F).

3.3. Case control study of recurrence

Over the study period, there were 610 case herds that experienced at least two bTB breakdowns (recurrent herds or cases) and 2038 control herds that did not suffer a subsequent bTB breakdown at least 1420 days after the previous one was resolved (non-recurrent herds or controls) which were subsequently used in this analysis.

Recurrent herds experienced significantly longer bTB breakdowns (190.0 days, IQR = 91.0–532.0) and had larger herd size (101.0 animals, IQR = 57.0–163.0) than non-recurrent herds (143.0 days, IQR = 83.0–457.0 and 73.5 animals, IQR = 41.0–124.8, respectively, $p <$ 0.001, Mann-Whitney tests). Most bTB breakdowns in both recurrent (67.7%, n = 413) and non-recurrent (73.8%, n = 1504) herds were characterized by only 1–2 bTB positive herd tests (Table 1). Overall, bTB was confirmed through bacteriology in at least one animal in 965 herds, of which 24.7% (n = 238) were case herds and represented 39.0% out of the total number of recurrent herds included in the analysis, compared with 35.7% (n = 727) of confirmed breakdowns in non-recurrent herds. A low proportion of both recurrent and non-recurrent herds were bTB confirmed in the last test of the previous bTB breakdown (<11%, Table 1). The IFN-γ assay was used in the previous bTB breakdown at least once in 46.1% (n = 281/610) recurrent herds and 40.5% (n = 825/2038) non-recurrent herds.

No information on herd size at the start of the previous bTB breakdown was available for 10 herds. The univariable analyses revealed that 7 out of the 10 predictor variables were potentially ($p < 0.2$) associated with the risk of bTB recurrence (Table 1). As movement network-related variables (weighted in-degree and number of incoming animals) were highly correlated (rho = 0.87, $p < 0.001$), weighted in-degree was included on the final multivariable model based on lower AIC. According to the final model performed on 2638 herds with complete information on all predictors, the variables province, previous bTB breakdown duration, herd size at the start of the previous bTB breakdown and weighted in-degree were all significantly associated with being a recurrent herd (Table 1). bTB infected herds with a higher risk of relapse were mainly located in Soria (OR = 2.58, 95% CI = 1.6–4.2), larger (> 134 animals, OR = 1.96 compared to ≤44 animals, 95% CI = 1.5–2.6), had a previous bTB breakdown that lasted for 159–481 days (OR = 1.41 compared to <85 days, 95% CI = 1.1–1.9), and were in contact with a higher (weighted in-degree >0.06) number of herds (OR = 2.88 compared with weighted in-degree <0.02, 95% CI = 2.3–3.6). No significant interaction terms were found. The Hosmer-Lemeshow test showed no significant lack of fit (Chi-square = 3.08, p = 0.929). When the same analysis was performed excluding case herds with recurrence times >1420 days (upper 25% of the distribution of duration in cases) similar results were obtained, with low-to moderate (<39%) variation in certain coefficients of the model but no overall difference in the significance of the variables and the directionality of the effects.

4. Discussion

Despite the continuing decreasing trend in bTB prevalence in endemic regions, limitations in sensitivity of currently available diagnostic tests can limit their efficacy, as infected animals may remain undetected in the herd acting as sources of infection for other susceptible animals. Persistence of bTB-causing bacteria in infected herds has been recognized as a major contributing factor impairing disease eradication in several studies (Karolemeas et al., 2011; More and Good, 2015). Inaccurate identification and delayed removal of bTB infected animals may increase the risk of recurrence of bTB breakdowns, which is associated with severe socio-economic consequences to farmers, stakeholders, and governments worldwide. We aimed to identify herd and breakdown related factors associated with i) decreased time to recurrence and ii) increased risk for bTB infected herds during 2010–2020 in Castilla y Leon, Spain to become recurrent.

Epidemiologically related bTB positive herd tests were considered part of the same bTB breakdown following the definition previously framed for herds in the same region (Pozo et al., 2020). Two approaches were used in this study to characterize the risk of bTB relapse in herds already known to be infected. On the one hand, a breakdown-level assessment of the time to recurrence was undertaken in an attempt to identify factors associated with decreased time to recurrence. On the other hand, the risk of becoming a bTB recurrent herd was evaluated in a group of bTB positive herds to identify factors that could point at failures in eradicating bTB at the herd-level using a logistic regression model. Results of both univariable survival and logistic regression analyses revealed that both herd factors (province, herd size, number of incoming connections/animals) and factors associated with the previous bTB outbreak (duration, number of bTB positive tests and IFN-γ herd-tests) were potentially associated with a shorter time to bTB recurrence and a higher risk of being a recurrent herd. In addition, the number of confirmed animals through bacteriology in the previous bTB breakdown was also associated with a decreased time to recurrence.

The multivariable analyses revealed that province, herd size and number of incoming animals/contacts were good predictors of decreased time of bTB recurrence and increased risk of becoming a recurrent herd (Supplementary material D and Table 1). The province effect was mostly explained by the increased odds of recurrence of herds located in Soria, even though the herd-level prevalence in this province is comparatively lower compared with others such as Salamanca and Avila. Additionally, the largest proportion of recurrent herds were located in these two provinces, where in fact a higher herd prevalence values have been found in the last years (e.g., herd prevalence in Soria in 2021 = 0.34% Vs. Salamanca = 2.53% and Avila = 1.66% (Anon, 2022)). Nevertheless, the proportion of bTB-infected herds in Soria that became recurrent was in fact the highest (37.6% compared with e.g. 25.0% in Salamanca, Table 1) suggesting in fact that in spite of the low herd prevalence in this province the epidemiology of the infection is different compared with other provinces in the region, as also previously suggested in an analysis of chronically infected herds (Pozo et al., 2020) and of detection of bTB-related lesions in the abattoir (Pozo et al., 2021). Results obtained here and in the abovementioned studies suggest that early- and/or late-stage infections in cattle may be missed in Soria, with unresolved bTB infections leading to further bTB breakdowns after herds are being (wrongly) considered cleared. Interestingly, when the survival analyses were performed only on the subset of bTB breakdowns confirmed through bacteriology, province was no longer a significant predictor, although sample size was limited since in fact Soria was the province in which less bTB breakdowns/bTB-infected herds were confirmed through bacteriology (17.4% of breakdowns and 26.7% of herds compared with e.g., 33.8% and 39.1% of Salamanca, data not shown). Further research is being conducted, including the analysis of the genetic diversity between spoligotypes recovered from different farms (and wildlife reservoirs), to disentangle the factors associated with the different pattern experienced by bTB-positive herds in this province.

Increased herd size at the start of the previous breakdown was also associated with a shortened time to recurrence and higher probability of becoming a recurrent herd (Fig. 2, Supplementary material D, and Table 1). This could be due to an increased probability of finding reactors (both true and false positives) in larger herds as repeatedly demonstrated in several studies (Byrne et al., 2021; Olea-Popelka et al., 2004). Alternatively, this result could suggest the effect of other management measures more common in larger herds not explicitly considered through the available covariables. One of such management measures could be increased number of incoming movements/animals, but in fact this risk factor was explicitly considered in the model (and was found to be significantly associated with both reduced time to recurrence and increased risk of becoming a recurrent herd). This suggests that, despite the routine use of pre-movement tests in animals being transported in Spain [with the exceptions contemplated in the Spanish Eradication Program such as herds located in source provinces with no prior MTBC confirmation or herds tested in the prior 6 months, among others (Anon, 2022c)], cattle movements could contribute to an increased risk of experiencing bTB breakdowns as suggested previously in the region (Pozo et al., 2020; Pozo et al., 2019). Along the same lines, studies conducted in the Republic of Ireland and Northern Ireland, demonstrated that ~7% of the confirmed bTB breakdowns were directly attributed to the introduction of infected animals, and up to 28.8% of bTB reactors or a lesion at routine slaughter could be attributable to purchased bTB infected animals (Clegg et al., 2008; Doyle et al., 2017). As herd bTB recurrence may be influenced by residual infection and local sources of (re)infection (More and Good, 2015), the use of molecular characterization techniques such as Multi-Locus VNTR Analysis [MLVA, (Milne et al., 2019)] and whole genome sequencing (van Tonder et al., 2021) could help to assess if the observed association between recurrence and connectivity is due to the entry of new strains (in which case it would be a case of reinfection due to new introductions rather than recurrence). Furthermore, these high throughput approaches have been applied to quantify the role of factors such as the presence of wildlife reservoirs or contaminated environments in bTB persistence in cattle (Crispell et al., 2019) as opposed to new disease introduction through the entry of infected animals through cattle movements (Pozo et al., 2019). A limitation of our study though was the lack of information on potential exposure to infected wildlife reservoirs, which are known to play a key role in bTB maintenance and spread in certain parts of Spain (Gortazar et al., 2017; Guta et al., 2014). In this sense, integration of molecular characterization data on the strains recovered from domestic animals and wildlife from the same geographical region may provide better insights on the relative role of wildlife interactions in disease persistence (Aranaz et al., 2004; Romero et al., 2008). Additionally, with no information on the *M. bovis* genomes responsible for the different bTB breakdowns that occurred in each herd, the proportion of bTB breakdowns derived from residual infection vs. reinfection with a new strain could not be calculated here as it was outside the scope of our study.

The variables number of IFN-γ herd-tests applied in the previous bTB breakdown and previous bTB breakdown duration were associated with time to recurrence and with the risk of becoming a recurrent herd respectively. Both predictors were highly correlated (rho = 0.79, $p < 0.001$) as the longer the bTB breakdown lasts (often associated with confirmation of the outbreak through bacteriology), the higher probability the IFN-γ assay may be used to increase diagnostic sensitivity. In any case, both variables indicate that longer breakdowns (which are typically also more severe) were associated with increased odds of (an early) recurrence. Breakdown length was also a significant predictor of bTB future risk in a study conducted in Ireland, with bTB breakdowns >230 days having significantly higher risk of future bTB breakdown relative to short (<130 days), whereas the period of time a herd was under movement restrictions in the prior 5 years was an important predictor of recurrence in Northern Ireland (Byrne et al., 2021; Doyle et al., 2016).

In the survival analysis, we extracted the information of all bTB breakdowns for herds with more than one bTB breakdown to construct the Cox proportional hazards models using herd as a random effect.

However, in the case-control study, information on the first bTB breakdown for herds with two or more bTB breakdowns was used in the model. To evaluate the potential effect of this selection, we performed a sensitivity analysis by comparing the results of three separate models using information only from the first bTB breakdown (default), only from the second bTB breakdown or from one of the first or second bTB breakdown selected at random in each herd, and found little (<20%) variation in all coefficients (data not shown), suggesting our results were robust.

Considering our bTB breakdown definition, out of the 3936 herds that experienced at least one bTB breakdown included in this study, only 12.9% ($n = 507$) experienced a new bTB breakdown within the next 3-year time span (data not shown), which is considerably lower than the 30.2% of herds that were derestricted in 2012 and subsequently re-restricted in the following three years in Ireland (Houtsma et al., 2018), and the 33% of derestricted herds in 2013 that failed a follow-up test within 5 years (Byrne et al., 2021). Differences in the proportion of recurrent herds within 3 years in Spain and Ireland may be related to disparities in the bTB breakdown definition, as bTB breakdowns here were considered resolved 18 months after the last bTB positive herd test while in Ireland a herd was considered derestricted after two consecutive clear full-herd SICTT tests conducted at least two months apart. In this sense, we consider our definition may avoid overestimation of recurrence due to ineffective clearance of the disease in just two consecutive clear full-herd SICTT tests.

Previous analyses performed on chronically infected herds in the region demonstrated that the herd history of bTB (number of SIT reactors and bacteriology-positive animals), herd size, inward contacts in the previous three years, local bTB prevalence, and province were significantly associated with increased bTB breakdown duration (Pozo et al., 2020). Our results here further demonstrate the impact of some of these variables and others in an increased risk of recurrence of bTB infected herds, pointing out at possible characteristics that could be used to develop a systematic risk-based scheme where herds may be identified and ranked based on their risk of experiencing future and more problematic bTB breakdowns. Control efforts, such as increased testing efforts and shorter testing intervals between follow-up tests after the disclosure of reactors, should be applied on herds with the characteristics identified here to minimize the impact of residual infection and bTB persistence at the herd level, and to predict the probability of relapse. This may help to develop and implement specific measures targeted at minimizing the risk of reinfection and reinforce early detection and removal of bTB positive animals in the herd.

5. Conclusions

Understanding how bTB is able to persist in cattle herds, especially in endemic regions, is crucial for disease management. Here, we characterized herd- and bTB breakdown-level risk factors associated with bTB persistence and recurrence in positive herds located in a high bTB prevalence region of Spain. Our results suggested that beforehand identification of infected herds located in certain areas, with increasing herd size, and high number of incoming contacts/animals could help to develop optimized strategies to minimize the risk of bTB recurrence. Moreover, the severity of bTB breakdowns, in terms of longer bTB breakdown duration and number of IFN-γ herd-tests performed on the previous breakdown, increased the probability of bTB recurrence. These potential features could be used to develop a systematic risk-based scheme in which herds may be identified and classified based on their risk of experiencing future and more problematic bTB breakdowns.

Funding

This research is a contribution to the project Integrated Strategies for Tuberculosis Control and Eradication in Spain (ERATUB; RT2018–096010-B-C22, Ministerio de Ciencia, Innovación y Universidades). The Ministerio de Agricultura, Pesca y Alimentación supported this publication. PP is a recipient of a Juan de la Cierva Formación grant (FJC2021–046805-I) funded by the Ministerio de Ciencia e Innovación and Agencia Estatal de Investigación (MCIN/AEI/10.13039/501100011033) and by European Union NextGenerationEU/PRTR. JA was the recipient of a Ramon y Cajal contract from the MINECO (RYC-2016-20422).

Declaration of Competing Interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper. Ethical review and approval were not required for the animal study because all data was collected as part of authorized regulatory surveillance in the framework of the Spanish National Eradication Program for Bovine Tuberculosis. Written informed consent for participation was not obtained from the owners because approval from premises owners was not required for this study.

Acknowledgements

We thank Guillermo Rodriguez Diez and Moises Gonzalez Sanchez for help in data collection. Authors are greatful to Ministerio de Ciencia, Innovación, Agencia Estatal de Investigación and Ministerio de Agricultura, Pesca y Alimentación for his support and funding.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2023.04.011.

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