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New Assessment of Bovine Tuberculosis Risk Factors in Belgium Based on Nationwide Molecular Epidemiology^{∇†}

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This assessment aimed to elaborate a statistical nationwide model for analyzing the space-time dynamics of bovine tuberculosis in search of potential risk factors that could be used to better target surveillance measures. A database comprising *Mycobacterium bovis* molecular profiles from all isolates obtained from Belgian outbreaks during the 1995-to-2006 period ($n = 415$) allowed the identification of a predominant spoligotype (SB0162). Various databases compiling 49 parameters to be tested were queried using a multiple stepwise logistic regression to assess bovine tuberculosis risk factors. Two isolate datasets were analyzed: the first included all *Mycobacterium bovis* isolates, while the second included only data related to the SB0162 type strain. When all *Mycobacterium bovis* isolates were included in the model, several risk factors were identified: history of bovine tuberculosis in the herd ($P < 0.001$), proximity of an outbreak ($P < 0.001$), cattle density ($P < 0.001$), and annual amplitude of mean middle-infrared temperature ($P < 0.001$). The approach restricted to the predominant SB0162 type strain additionally highlighted the proportion of movements from an infected area during the current year as a main risk factor ($P = 0.009$). This study identified several risk factors for bovine tuberculosis in cattle, highlighted the usefulness of molecular typing in the study of bovine tuberculosis epidemiology, and suggests a difference of behavior for the predominant type strain. It also emphasizes the role of animals' movements in the transmission of the disease and supports the importance of controlling trade movements.

Despite significant historical efforts and the implementation of eradication plans, bovine tuberculosis (bTB) remains a pre-occupant issue in the European Union, with some member states recently facing a reemergence of the disease (10). Some countries succeeded in biologically eradicating bTB after implementing control measures, while others, declared officially tuberculosis free (OTF), still report outbreaks every year, despite ongoing eradication and control programs (10). Belgium was declared OTF in 2003, yet 5 to 10 outbreaks are reported every year (12). In 2008, an increase in the number of reported outbreaks was noticed (12), as shown in Fig. 1.

Numerous risk factors for bTB have been identified in cattle around the world. These risk factors include a variety of parameters in relation to wildlife, cattle contacts, movements, density of animals, etc. (reviewed in reference 20), but a num-

ber of studies lack standardization. Furthermore, bTB transmission cycles underlying the failure to eradicate *Mycobacterium bovis* in cattle in some areas remain poorly understood, and several transmission hypotheses have been formulated: inadequate control measures, agro-environmental factors, latency, wildlife reservoirs, and movements of infected animals (15). Partly because bTB control programs are an economical burden, national animal health authorities are considering downscaling current control measures, e.g., cancelling testing at purchase and reducing herd testing. Nevertheless, animal movements were shown to be a risk factor in other countries, such as the United Kingdom (15, 16). Before these reductive measures are applied, it therefore seems appropriate to investigate the true risk represented by animal movements in the country.

A database including all *M. bovis* isolates grown from outbreaks reported between 1995 and 2006 in Belgium was compiled. This database was instrumental in analyzing bTB dynamics in Belgium during the 1995-to-2006 period. A full literature review for bTB risk factors allowed the identification of several potential risk factors to be tested in Belgium (20). A statistical model initially developed on the basis of data collected in the United Kingdom (15) was then adapted to the Belgian data set in order to test these potential risk factors.

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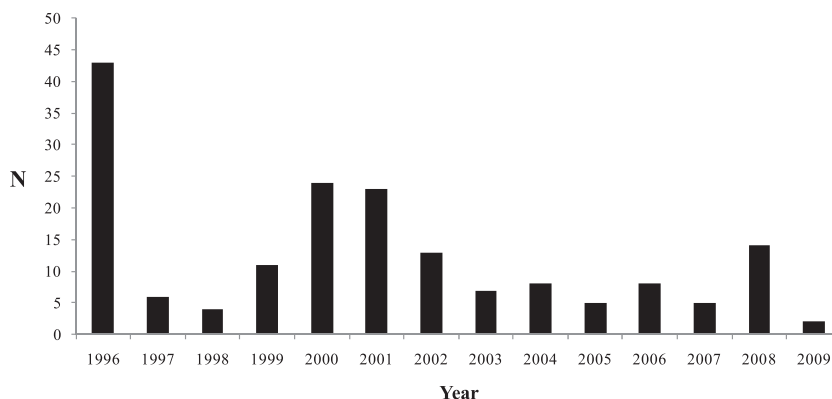


FIG. 1. Trends for numbers of bTB outbreaks (N) in Belgium between 1996 and 2009, as reported to the World Animal Health Organization. The situation worsened in 2008 as the number of outbreaks almost tripled compared to that for the year 2007. (Based on data from reference 12.)

In addition, recent studies focusing on *M. bovis* strains isolated in cattle and badgers from the United Kingdom confirmed the limited number of strains circulating in the United Kingdom, even though the bTB herd prevalence is elevated (14, 38). On the other hand, the situation in Belgium is totally opposite: there is a wide diversity of cocirculating strains, with one predominating, and the herd prevalence is under 0.1% in the cattle population (12). It was thus decided that two approaches would be followed, one including all strains isolated in the country during the period of interest (1995 to 2006) and one focusing on the predominant strain type, in order to possibly highlight a difference in behavior.

This molecular epidemiology approach, never carried out so far in Belgium, is valuable for health authorities in reassessing and adapting current control measures applicable for surveillance of bTB and in challenging possible reductions in herd and individual testing.

MATERIALS AND METHODS

Database compilation. A literature review of bTB risk factors allowed the identification of several parameters to be tested as possible risk factors. These parameters were called predictors. All predictors used were derived from the databases described below. These predictors were all compiled into a unique database. The analysis of bTB dynamics was bidimensional. The temporal reference point was the year, and the spatial unit was defined as follows: the territory was divided into 5-km by 5-km cells, identified thanks to their *x* and *y* Lambert coordinates; these cells were named pixels (<http://users.skynet.be/belgique/belgica.zip>).

A database of all *M. bovis* isolates grown from sampled organs suspected of bTB infection at the Belgian national reference laboratory for bTB between 1 January 1995 and 31 December 2006 was the starting point of the analysis. Sampling was performed at the slaughterhouse when suspect lesions of bTB were observed, according to the European legislation (11). Once the presence of *M. bovis* was confirmed, molecular typing tools allowed for individual genotyping of each strain. Three techniques were used in parallel to identify the strain type: spoligotyping, restriction fragment length polymorphism-IS6110 (IS6110-RFLP), and mycobacterial interspersed repetitive unit (MIRU)-variable-number tandem repeat (VNTR). These techniques have been widely used for the identification of *M. bovis* strains (9, 31) and were combined in the study area (1), where IS6110-RFLP proves to improve the discriminatory power of MIRU-VNTR, given that 48% of the area isolates display 8 or more copies of IS6110 (1). Isolates found in Belgium between 1995 and 2006 have previously been classified into 12 lineages, according to their combined RFLP/VNTR/spoligotype molecular profiles, which allowed for the identification of a specific strain type characterized by its unique SB0162 spoligotype as predominant. SB0162 was identified in 27% ($n = 112$) of all isolates ($n = 415$) (in accordance with the international nomenclature given at <http://www.Mbovis.org/>) (35).

Other databases were released by the Federal Agency for the Safety of the Food Chain (FASFC); a complete list of all registered cattle herds of the country as well as the annual census of all herds (the number of animals per herd, as defined on 31 December of each year, with data available from 2000 and after) were made available. Cattle movement data that took place between 1995 and 2006 were extracted from the National Cattle Tracing System (SANITEL).

The Nature and Forest Division (NFD) provided data for several wildlife species. Annual estimated populations of red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*), fallow deer (*Dama dama*), and mouflons (*Ovis orientalis*) were included in the model.

Land cover data were also part of the model. Different types of land cover were considered: pasture, crops, forest, humid areas, urban areas, and other vegetation. The length of forest-pasture edge was also available for analysis and was defined as a specific number of meters per pixel: the more important the length of forest-pasture edge, the greater the risk of potential contact between wildlife and cattle in pasture was assumed to be.

Remotely sensed data for several bioclimatic indicators were used as bioclimatic data. The collection of these data has previously been described (19). Altitude was included in the model as well.

Risk predictors. Predictors were converted for each pixel, per year, to include biological, demographic, climatic, and topographic variables, such as distance to the center of a bTB outbreak pixel, densities of wildlife populations, eco-climatic data, land cover, and movements and density of cattle, all compiled and re-sampled at the 5-km resolution.

The predictor named "disease persistence" (persistence of bTB [PBTB]; antecedent of bTB) was included in the model as follows. For each pixel, and for each year of the period, it was specified if bTB was present or not. A note of 0 was allotted if no bTB outbreak had been registered for the pixel in the previous year, while a note of 1 meant the presence of bTB in the pixel.

In case of the presence of bTB, the *M. bovis* strain was specified. Cattle populations were converted in order to include "density of cattle" per pixel in the model. Data concerning wild animal populations were originally available at the district level; they were further transformed and converted for availability at the pixel level under the name "density of wild species." Regarding land cover predictors, data included in the model were the percentages of occupation within the pixel for the different types of vegetation.

Raw data extracted from the National Cattle Tracing System (SANITEL) were preliminarily transformed before inclusion in the model, as follows. Each movement was referenced with two data points: the first data point was the pixel of the location from which the animal moved (pixel off) and the other one for the pixel of the location to which the animal moved (pixel on). Both data points had to be paired and were coupled to three variables: the total number of cattle head movements into a pixel, the total number of movements from an outbreak pixel, and the resulting proportion of movements that originated from outbreak pixels. These three variables were added to the model. Movement data were then analyzed in two ways. First, we investigated the impact of movements that were registered the year before the eventual occurrence of bTB in a given pixel. Second, we assessed the impact of movements taking place during the year of occurrence of bTB outbreaks in a given pixel. A total of 49 predictors were considered, as summarized in Appendix S1 in the supplemental material.

TABLE 1. Summary of statistics for the predictors presenting a significant relationship with the occurrence of bTB in all isolates, as determined by multivariate analysis using the backward-selection approach^a

Predictor	Estimation	SE	Z	P	Significance of effect
PBTB	2.0708656	0.2032527	10.189	<2e-16	***
Logtb5km	-2.2286674	0.2306585	-9.662	<2e-16	***
BOV03	0.0002091	0.0000361	5.791	7.01e-09	***
Red deer	-3.985e-02	1.467e-02	-2.716	0.006617	**
Roe deer	-7.058e-03	2.314e-03	-3.050	0.00229	**
FORkm ²	-9.887e-02	2.613e-02	-3.784	0.000154	***
CH0107A1	4.202e-02	9.909e-03	4.241	2.23e-05	***
CH0107A2	-2.166e-02	1.001e-02	-2.164	0.030494	*
CH0114P1	-5.942e-03	1.700e-03	-3.496	0.000472	***
DTM	-2.204e-03	7.034e-04	-3.133	0.001732	**

^a Z, the Z-statistic testing the logistic coefficient; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; PBTB, presence of bTB the previous year; Logtb5km, logarithm of the distance to the center of the infected pixel (km); BOV03, density of cattle in 2003; roe deer, density per pixel; red deer, density per pixel; FORkm², surface of the pixel occupied by forests (km²); CH0107A1, land surface temperature annual amplitude (°C); CH0107A2, land surface temperature biannual amplitude (°C); CH0114P1, normalized difference vegetation index phase of annual cycle; DTM, altitude (m).

Statistical analyses. A stepwise multiple logistic regression analysis was applied to data in order to investigate the potential association between bTB occurrence and the predictors. This model was originally created by Gilbert and collaborators to assess the importance of animal movements in the transmission of bTB in Great Britain (15); this model was further adapted to the peculiar situation of the study area and for inclusion of the molecular biology aspect. A unique multiannual database, including all the information regarding the 49 predictors per pixel and per year, was built. For each year and each pixel, the absence or presence of bTB was specified. First, the following predictors were entered in the model: PBTB (status of bTB in the previous year) and short-distance spread (number of infected pixels in the previous year in a doughnut-shaped window 5 km in radius). These two predictors were shown to have a significant impact on the presence of bTB in the pixel. Then, other variables were added to the model by use of a standard-entry stepwise procedure. The model was restricted to predictors with the highest predictive power, and only those presenting more than 1% log likelihood change after removal were retained. In other words, these best predictors were systematically tested with the other families of predictors. Finally, all the predictors showing a significant effect were tested together. The 49 predictors could not be entered all together in the model at the same time, because some of them were correlated (altitude, densities of wild species, and forest surfaces), and any significant effect could thus have been masked. A predictor was considered a significant risk factor when presenting a positive or a negative relationship with the presence of bTB ($P < 0.05$).

The model was applied in two stages. The first stage included all *M. bovis* strains identified in Belgium during the period of concern. The second stage focused on the predominating type strains (SB0162), isolated in the country between 1995 and 2006 (35).

The whole statistical process was carried out with R software (29).

RESULTS

All *Mycobacterium bovis* strains. The predictors presenting a significant relationship as determined by the analysis of all bTB isolates ($n = 415$) are summarized in Table 1. Two predictors were positively and significantly related to the presence of bTB in a pixel: persistence of bTB (PBTB) and cattle density (BOV03). The distance from the center of an infected pixel (Logtb5km) showed a negative relationship with bTB, which means the closer a herd was from the center of an infected pixel, the greater the risk of being infected was. These three predictors exhibiting the highest level of significance were tested with each of the other families of predictors (movements, wildlife, bio-climatic predictors, and land cover) in a backward-selection approach. At each step, the variable with the lowest Wald statistics value was discarded. Finally, all predictors presenting a significant relationship with the presence of bTB were kept. Red deer and roe deer densities presented

significantly negative relationships with the presence of bTB, as did forest density per pixel, which would suggest that, to date, no wildlife reservoir of bTB exists in Belgium.

The backward-selection approach involving bio-climatic predictors as well as altitude revealed that annual amplitude of mean middle-infrared (MIR) temperature presented a positive relationship with the presence of bTB. On the other hand, biannual amplitude of mean MIR temperature, normalized difference vegetation index (NDVI) phase of annual cycle, and altitude showed a negative relationship with the risk of bTB.

Once all the families of predictors had been tested separately with the three best predictors, the variables presenting a significant effect were all tested together, as illustrated in Table 2. Some predictors then lost their significant effect, mostly because of colinearity (red deer and roe deer densities, percentage of forest cover per pixel, and altitude).

Predominant *Mycobacterium bovis* spoligotype (SB0162). Only *M. bovis* isolates characterized as SB0162 strain types were included in the second step of the model ($n = 112$). All results of this approach are presented in Table 3. Persistence of bTB (PBTB) presented a positive significant relationship, while cattle density showed no relationship with the presence of bTB. The distance to the center of an infected pixel (Logtb5km) showed a negative relationship with bTB. Only these two variables were thus tested with the other families of predictors (backward-selection method). The proportion of movements originating from infected pixels during the current year presented a significant relationship with the presence of bTB, as did crop surface. Regarding bio-climatic variables, the annual amplitude of the mean MIR temperature presented a significant effect on the risk of bTB occurrence. As when all *M. bovis* strains were included in the model, the biannual amplitude of the mean MIR temperature and the NDVI phase of the annual cycle showed significant negative relationships with the risk of bTB (Table 3).

After all the families of predictors were tested separately, a model including all variables significantly related to the presence of bTB was tested. As for the all-*M. bovis*-strains approach, several predictors lost their significant effect. The only predictors showing a significant relationship then were the proportion of movements originating from infected pixels dur-

TABLE 2. Summary of statistics for the predictors presenting a significant relationship with the occurrence of bTB in all isolates, as determined by multivariate analysis for significant predictors tested together^a

Predictor	Estimation	SE	Z	P	Significance of effect
PBTB	1.978e+00	2.050e-01	9,650	<2e-16	***
Logtb5km	-1.801e+00	2.497e-01	-7.213	5.48e-13	***
BOV03	2.284e-04	3.855e-05	5.926	3.11e-09	***
Red deer	2.205e-03	5.507e-03	00.400	0.688828	
Roe deer	-1.044e-02	1.988e-02	-0.525	0.599500	
FORkm ²	-6.515e-02	7.395e-02	-0.881	0.378362	
CH0107A1	3.630e-02	1.093e-02	3.322	0.000892	***
CH0107A2	-2.135e-02	1.036e-02	-2.061	0.039292	*
CH0114P1	-5.094e-03	1.867e-03	-2.729	0.006352	**
DTM	-1.282e-03	8.819e-04	-1.454	0.145924	

^a Z, the Z-statistic testing the logistic coefficient; *, P < 0.05; **, P < 0.01; ***, P < 0.001; PBTB, presence of bTB the previous year; Logtb5km, logarithm of the distance to the center of the infected pixel (km); BOV03, density of cattle in 2003; roe deer, density per pixel; red deer, density per pixel; FORkm², surface of the pixel occupied by forests (km²); CH0107A1, land surface temperature annual amplitude (°C); CH0107A2, land surface temperature biannual amplitude (°C); CH0114P1, normalized difference vegetation index phase of annual cycle; DTM, altitude (m).

ing the current year (positive relationship) and the biannual amplitude of the mean MIR temperature (Table 4) (negative relationship).

DISCUSSION

The model allowed for highlighting of several factors correlated with the presence of bTB nationwide (e.g., Belgium) and is the first study of that kind. A first approach considered all the strains isolated between 1995 and 2006, while a second approach focused on the predominant strain type characterized by its SB0162 spoligotype, most frequently isolated in Belgium over the past 13 years (35).

A history of bTB in a given pixel was shown to represent a significant risk factor for the presence of bTB, both in the all-inclusive and in the predominant-strain-restricted approaches. This observation confirms the results of previous studies carried out in other countries. Indeed, British groups demonstrated that bTB outbreaks occur in a repeated way in the same areas (36). It is likely that the source of infection has not been cleared and/or that permanent factors would make these areas particularly prone to the reemergence of bTB.

The proximity of an infected pixel turned out to be a significant risk factor for bTB as well. The greater the distance to the center of an infected pixel is, the lower the risk of infection is.

This was previously observed in the Republic of Ireland (18). In their study, Griffin and collaborators demonstrated that, in a short period of time, bTB outbreaks most frequently affect several herds at the same time rather than a sole herd, because the contiguity with other herds under restriction was a risk factor. Another study carried out in the same country and including 215 dairy herds showed that neighboring with an infected herd was associated with an outbreak in a particular herd. Nevertheless, a bTB infection confirmed for adjacent herds could point to a common source of infection (8). North American scientists highlighted the importance of contacts between animals over fences as a particular risk factor for the transmission of *M. bovis* between infected and healthy animals (22, 25). Thus, the results observed in a low-prevalence situation seem to confirm what was observed in areas where bTB prevalence is high.

Density of cattle is a significant risk factor for bTB in Belgium. In our study, this predictor was identified as a significant risk factor in the first approach, including all *M. bovis* isolates, but not when the statistical model was applied to SB0162 only. Intensive farming is a risk by itself because of the closer proximity of animals to each other and thus the increased contacts and interactions between them. Airborne transmission is indeed the principle route of infection in cattle (13). The higher the density of the animal population, the higher the probability

TABLE 3. Summary of statistics for the predictors presenting a significant relationship with the occurrence of bTB in SB0162-type strains, as determined by multivariate analysis using the backward-selection approach^a

Predictor	Estimation	SE	Z	P	Significance of effect
PBTB	2.0134	0.3923	5.132	2.86e-07	***
Logtb5km	-3.2453	0.3537	-9.176	<2e-16	***
qNB	1,7932	0,6607	2,714	0,00665	**
Roe deer	-0,010778	0,004339	-2,484	0,013	*
CROPkm ²	0,08612	0,02782	3,096	0,001961	**
CH0107A1	0,053690	0,018834	2,851	0,00436	**
CH0107A2	-0,059615	0,022151	-2,691	0,00712	**
CH0114P1	-0,009562	0,003118	-3,067	0,00216	**

^a Z, the Z-statistic testing the logistic coefficient; *, P < 0.05; **, P < 0.01; ***, P < 0.001; PBTB, presence of bTB during the previous year; Logtb5kmNoI, logarithm of the distance to the center of the infected pixel (km); qNB, proportion of movements from infected pixels that took place during the current year; roe deer, density per pixel; CROPkm², surface of the pixel occupied by crops (km²); CH0107A1, land surface temperature annual amplitude (°C); CH0107A2, land surface temperature biannual amplitude (°C); CH0114P1, normalized difference vegetation index phase of annual cycle.

TABLE 4. Summary of statistics for the predictors presenting a significant relationship with the occurrence of bTB in SB0162 type strains, as determined by multivariate analysis for significant predictors tested together^a

Predictor	Estimation	SE	Z	P	Significance of effect
PBTB	1.665550	0.420790	3.958	7.55e-05	***
Logtb5km	-2.729520	0.381493	-7.155	8.38e-13	***
qNB	1.856831	0.705736	2.631	0.00851	**
Roe deer	-0.006700	0.005178	-1.294	0.19569	
CROPkm ²	0.029300	0.024411	1.200	0.23003	
CH0107A1	0.038892	0.020954	1.856	0.06345	
CH0107A2	-0.059532	0.021977	-2.709	0.00675	**
CH0114P1	-0.005458	0.003630	-1.504	0.13269	

^a Z, the Z-statistic testing the logistic coefficient; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; PBTB, presence of bTB during the previous year; Logtb5km, logarithm of the distance to the center of the infected pixel (km); qNB, proportion of movements from infected pixels that took place during the current year; roe deer, density per pixel; CROPkm², surface of the pixel occupied by crops (km²); CH0107A1, land surface temperature annual amplitude (°C); CH0107A2, land surface temperature bi-annual amplitude (°C); CH0114P1, normalized difference vegetation index phase of annual cycle.

of close contacts between them. The highest incidence of bTB is generally observed in areas where intensive farming is practiced (5). The trends in dairy cattle are going toward intensification in industrialized countries, which means fewer, much bigger herds and, as a result, increased contacts between animals and an increasing risk of bTB transmission (34). Under intensive conditions, aerogenic transmission of *M. bovis* prevails (23).

Contrary to what has been observed elsewhere, animal movements from an outbreak to another herd were not shown to be a significant risk factor when all *M. bovis* strains were included in the statistical model. The low rate of outbreaks observed every year did not permit the highlighting of this risk factor in the country, contrary to the studies carried out by Gilbert and collaborators in Great Britain (15). On the other hand, it is difficult to determine whether the differences between the two countries can be explained by differences in the control of cattle movements or by the level of prevalence. A study focusing on the analysis of cattle movements between 1985 and 2003 in the United Kingdom relied on molecular typing to identify most outbreaks reported to occur in the North-East of England between 2002 and 2004 (16). Animal movements had a major impact if animals were moved from a zone where bTB was endemic to a bTB-free area. The second approach, which included predominating strain type SB0162, identified the proportion of movements from infected pixels during the current year as a significant risk factor.

Several wild species play an important role in the transmission of *M. bovis* to cattle. This is the case for badgers in the United Kingdom and in the Republic of Ireland (4, 8, 17) and for brush-tail possums in New Zealand (24). Deer infected with *M. bovis* were discovered in North America (22), in the United Kingdom (6, 7), in the Republic of Ireland (28), in Spain (2), and in France (40). *M. bovis* has frequently been isolated in wild boar in Western Europe, especially in France, Spain, and Italy (26, 32, 40). Even when *M. bovis* is not yet isolated from wildlife, this risk must not be dismissed. The influence of wildlife densities on the emergence of bTB outbreaks in Belgium was thus tested. In our study, nevertheless, no relationship could be observed between the densities of the main wild species tested (roe deer, red deer, wild boar, and incidentally fallow deer and mouflons, mainly present in Belgium parks and domestic herds) and the presence of bTB,

suggesting once more that they do not represent a risk for cattle contamination, as *M. bovis* is probably not circulating in wildlife species to date. On the other hand, the presence of bTB presented a negative relationship with wildlife population densities, and the same effect was observed for land covered by forests. It is most likely that both observations are linked, as these two variables correlate. One should recall that data on wildlife species were available only for relatively large administrative units, hence the lack of an apparent statistically significant relationship could be caused by the lack of high-resolution data, and those results should be interpreted cautiously.

The analysis of the SB0162 type strain data identified the proportion of a pixel occupied by crops as a significant risk factor. A hypothesis that may explain this observation is that farms are concentrated around culture areas for the supply in fodder.

Several bio-climatic factors happened to appear as significant risk factors for the emergence of bTB. The annual amplitude of the temperature on the earth's surface would be a risk factor, as shown for both approaches (that using all strains versus that using the predominating strain). Climate indeed influences the survival of *M. bovis* in the environment (27). The environmental survival of *M. bovis* would be inversely proportional to mean daily temperatures, as suggested previously for New Zealand (21). Temperatures just above 0°C coupled with a strong hygrometry are in favor of *M. bovis* survival (3). Tanner and Michel also observed a longer survival time for *M. bovis* in feces in the winter and under moist conditions in the Kruger National Park, South Africa (33). Nevertheless, scientific opinions diverge regarding the importance the environment plays in the survival of *M. bovis* as well as all the survival times suggested by the different studies that focused on this aspect. Some authors suggest that the survival times for infective doses of *M. bovis* on fomites are relatively short under natural conditions (24). Older studies described longer survival times: *M. bovis* mixed artificially with cow feces and exposed on pasture land survived at least 35 weeks in the winter, 28 weeks in autumn, and up to 14 weeks in the summer in southern England (36a). In northern Europe, *M. bovis* mixed with organic matter survived for 22 to 47 weeks when exposed to sunlight at 24 to 34°C but up to 104 weeks if buried 5 cm below the surface of shaded soil (14a). More recently, Young and collaborators suggested that *M. bovis* BCG remains viable in

soil for more than 15 months (39). Many studies focusing on survival times of *M. bovis* in the environment reached their conclusions under experimental conditions. In 1997, scientists who worked on data collected in England and Wales suggested that bTB occurrence was linked to seasonality and to climatic changes from one year to another (37). It is constantly reported that temperatures just above 0°C and a strong hygrometry are in favor of *M. bovis* survival, and these conditions are frequent in North-West Europe in the wintertime. The potential impact of climate change on *M. bovis* survival and on the occurrence of bTB outbreaks should gain further attention, as scientific opinions still diverge to date. Scientists from the United Kingdom recently evoked the potential role of free-living protozoa as an environmental reservoir of *M. bovis*, which could contribute to the environmental persistence of the mycobacteria (30). This possibility should not be neglected either.

The risk of bTB seems to decrease as altitude increases. The highest areas of the country are located in the south and east of Belgium, regions where few or even no outbreaks were reported to occur to date. This trend could be put in relation with forests, mostly located in the same regions.

This is the first nationwide study analyzing bTB risk factors combining three typing techniques. The statistical analysis of relationships between bTB outbreaks and *M. bovis* strain types allowed the identification of some risk factors: antecedents of bTB in a herd or in a small area, proximity of an outbreak, and cattle density. These observations should pave the way for increased vigilance in matters of epidemiological investigations and eradication of ongoing outbreaks. Animal movements from infected areas were shown to be at risk for the predominant *M. bovis* SB0162 strain type circulating in Belgium; it is thus essential for vigilance in the control of movements and skin testing at purchase not to be slackened. Wildlife does not seem to represent a risk in Belgium to date, but epidemiological surveillance is crucial in light of the situation in neighboring countries, such as France or the United Kingdom. Studies focusing more specifically on the role of environment and climate in the persistence of *M. bovis* should be undertaken as well. In addition, the results of this study also suggest a difference of behavior for the SB0162 type strains, underlying the importance of molecular epidemiology for investigation of potential differences of virulence according to the strain.

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