U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Relatório Final de Estágio

Mestrado Integrado em Medicina Veterinária

SPATIAL ANALYSIS OF SMALL RUMINANTS BRUCELLOSIS OCCURRENCE IN TRÁS-OS-MONTES AND ALTO DOURO, 2010-2014

Carolina Perez da Costa de Albuquerque Duque

Orientador

Professor Doutor João José Rato Niza Ribeiro

Co-Orientadores

Professora Doutora Carla Maria Teixeira de Oliveira

Doutor Alfredo Jorge da Cruz Sobral

Porto 2016

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Relatório Final de Estágio

Mestrado Integrado em Medicina Veterinária

SPATIAL ANALYSIS OF SMALL RUMINANTS BRUCELLOSIS OCCURRENCE IN TRÁS-OS-MONTES AND ALTO DOURO, 2010-2014

Carolina Perez da Costa de Albuquerque Duque

Orientador

Professor Doutor João José Rato Niza Ribeiro

Co-Orientadores

Professora Doutora Carla Maria Teixeira de Oliveira

Doutor Alfredo Jorge da Cruz Sobral

Porto 2016

Foreword

This study was carried as part of the professional training period that composes the final year of the Integrated Veterinary Medicine Master of Instituto de Ciências Biomédicas Abel Salazar in University of Porto, Portugal. The choice of this subject came as an opportunity to continue a previous project that embraces the official veterinary services with the academic and scientific community.

Brucellosis is a public health issue worldwide and in Portugal this disease is still endemic in small ruminant population in some regions, being Trás-os-Montes and Alto Douro the most problematic.

The opportunity to work in an investigation team allowed me to explore the fields of veterinary epidemiology and public health in a wider perspective and to gather novel competencies. The work developed raised interesting questions regarding scientific approaches to practical field issues and the methodology used was always chosen and adjusted to the continuous discover.

Overviewing the work that it was done, I am more convinced that an One Health approach to animal health is the key to better understand the dynamics enrolled in the ecology of diseases. By integrating geography and mathematics among natural sciences in the investigations, it may be possible to ultimately help policy makers and other professionals in official authorities to find the most efficiency strategies to eradicate infectious animal diseases.

Summary

Small ruminant brucellosis is a widespread zoonosis caused by *Brucella melitensis* with important effects on both animal production and public health. At the present time the disease persists in limited regions in Portugal where special vaccination programs are being applied. This study aims to evaluate the advantages that can be gained by using a geographic information system in the study of the epidemiology of sheep and goat brucellosis in Trás-os-Montes and Alto Douro, a region in North-eastern Portugal between 2010 and 2014 by means of spatial analysis techniques.

Disease mapping was the first step in this spatial analysis, so maps representing prevalence and incidence by village were constructed. The next step was the detection of spatial dependence by means of Global and Local Moran Index. The spatial analysis showed that proximity of villages might play an important role in the prevalence of brucellosis across Trás-os-Montes and Alto Douro. However, the results of incidence risk are more curious since there was not found a significant association between spatial proximity and new cases of disease, with the exception of 2013. This previous result raises the question whether the direct contact between flocks or the sharing of pastures play together a determinant role in dissemination of the disease in the region. Furthermore, there were found no significant differences between clusters of high risk of prevalence and incident villages. The risk of infection was different between municipalities and farmer's organizations and higher with the increase in herd size and lower with time.

This study provides a suitable background for assessing the spatial patterns of distribution of animal diseases in Portugal and shows that geographic information systems can be used as a sentinel system that collects information on the animal population, evaluates disease indicators as well as the spatial and temporal trends.

Keywords: Brucellosis; spatial analysis; clusters; geographic information systems

Sumário

A brucelose dos pequenos ruminantes é uma zoonose com impacto na produção animal e saúde pública cujo agente bacteriano é *Brucella melitensis*. Atualmente, a doença persiste em determinadas regiões em Portugal onde programas de vacinação especiais estão implementados. Este estudo tem como objetivo avaliar as vantagens que podem ser obtidas por meio de um sistema de informação geográfica no estudo da epidemiologia da brucelose ovina e caprina em Trás-os-Montes e Alto Douro a partir de técnicas de análise espacial. Avaliaram-se dados da ocorrência de doença ao nível da exploração fornecidos pela DSAVRN para o período entre 2010 e 2014.

O mapeamento da doença foi o primeiro passo da análise espacial, pelo que foram construídos mapas representativos da prevalência e incidência da região. De seguida, foi avaliada a dependência espacial por meio do índice global e local de Moran. A partir dos resultados concluiu-se que a proximidade entre as freguesias pode desempenhar um papel importante para a prevalência da brucelose em Trás-os-Montes e Alto Douro. No entanto, no que respeita à incidência não foi encontrada uma associação significativa entre a proximidade espacial e novos casos da doença, com exceção do ano 2013. Esta última evidência levanta a questão: será que o contacto direto entre rebanhos ou a partilha de pastagens desempenham em conjunto um papel determinante na disseminação da doença na região. Além disso, não foram encontradas diferenças significativas entre freguesias de elevado risco de prevalência e freguesias incidentes. O risco de infecção foi diferente entre concelhos e organizações de agricultores e maior para o número de animais por rebanho e menor com o tempo (anos).

Este estudo constitui um modelo-base de avaliação dos padrões espaciais de distribuição de doenças animais em Portugal e mostra que os sistemas de informação geográfica podem ser usados como um sistema sentinela que recolhe informações sobre a população animal, avalia indicadores de doença e tendências espaciais e temporais.

Palavras-chave: brucelose, análise especial, clusters, sistemas de informação geográfica

Acknowledgments

To Professor João Niza Ribeiro, Professor Carla Oliveira and Doctor Cristiana Mendes for the opportunity to work in this project and for the orientation that was determinant to this study.

I wish to acknowledge the assistance of all members in the Department Geoepi in INEB/I3S who received me in their team right from the start, integrating and helping me along the internship.

To my family, my parents and my sister, for all the support and encouragement and to my grandmother Cesaltina.

To all my friends.

To my cats.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

% Percent

B2 non-brucellosis free herd

B2.1 infected herd

B3 brucellosis free herd

B4 officially brucellosis free herd

B3S/B4S herds suspected of brucellosis

B. melitensis *Brucella melitensis*

CFT Complement Fixation test

CIR Cumulative Incidence Ratio

DGAV Directorate General of Food and Veterinary Services

DL Portuguese government law decree

DSAVR Directorate Regional of Food and Veterinary Services

DSAVRN Directorate Regional of Food and Veterinary Services of North Region

ESDA Exploratory Spatial Data Analysis

GIS Geographic Information System

km^2 Square kilometers

LISA Local Indicators of Spatial Association

OIE Office International des Epizooties

OPP Livestock Producers Organizations

PISA.Net Informatic Database for the Ruminants Eradication Programs

RBT Rose Bengal Plate test

SIR Standardized Incidence Ratio

SNIRA National Identification and Registration System

SPR Standardized Prevalence Ratio

SRB Small Ruminants Brucellosis

WHO World Health Organisation

Part A: Introduction

1 Small Ruminants Brucellosis (SRB): a brief review

1.1 Agent

Brucellosis is an infectious disease caused by bacteria from genus *Brucella*. These gram-negative bacteria are cocobacilli and facultative intracellular parasites that belong to the α-2 Proteobacteria Philum. *Brucella* species have different host preferences and thereby they are capable of causing disease in innumerous species. There are six classical species namely *B. melitensis* biovars (bvs) 1-3 isolated from sheep and goats, *B. abortus* biovars 1-6 and 9 isolated from bovines and other *Bovidae* species, *B. suis* bvs 1-3 isolated from swine, bv 4 from elk and bv 5 from small rodents, *B. canis* isolated from dogs, *B. ovis* isolated from sheep and *B. neotomae* from desert rats[(1).] Recently four other species were identified: *B. pinnipedialis* isolated from pinnipeds[(2),] *B. microti* from voles, fox and soil[(3),] *B. ceti* from cetaceans[(2)] and *B. inopinatta* from a woman' breast implant[(4).] The majority of the reported cases in humans is by *B. melitensis*, *B. abortus* and *B. suis* in descendent order of occurrence and rarely *B. canis*[(5).]

Sheep and goats brucellosis (excluding the infection by *Brucella ovis*) is mainly caused by one of the three biovars of *B. melitensis spp*. and in rare occasions infections by *B. abortus* or *B. suis* can occur[(6)].

1.2 Sources of infection

Some of the most common sources for this bacterium are placentae, aborted fetuses and fetal fluids. Fetal and vaginal fluids are extremely rich in large number of this agent and their excretion lasts at least 2 to 3 months in goats and 21 to 64 days in ewes after parturition[(7) (8).] These excretions enable the transmission of the organism to others within the herd and as a consequence, the surroundings of the places where animals gave birth get easily contaminated specially during lambing season. The great resistance of *B. melitensis* to environmental conditions contributes to the contamination of pastures, husbandries and transport vehicles[(9) (10).] The persistency of infection of the udder and supremamary lymph nodes leads to the continuously excretion of *Brucella* in milk throughout lactation and after for periods up to six months in goats enabling the spread of the hazard for other animals and humans who consume unpasteurized milk and other dairy product[(11)]. Goat milk is a major source of infection particularly to humans and young animals. In opposite to goats, in sheep the excretion of the organism through milk is shorter and limited to approximately two months in the lactation following the infection[(12)]. The excretion of *Brucella* in urine and rarely in feaces was mentioned to be important during lambing season and in young animals feed with milk from infected animals. Males can shed the infection through semen although it is less common. The introduction of infected animals is one of the most important sources of infection in flocks or herds previously free from disease, and the animal movement can occur several months before the first signs of abortion are noticed.

1.3 Routes of transmission

The transmission of *Brucella* occurs via direct contact with contaminated animal products or following exposure to mechanical disseminators of the agent such as carnivores and scavengers previously in contact with contaminated carcasses or other detritus[13]. The common routes of infection in small ruminants are the oral and nasopharyngeal routes followed by contact with mucosas and serosas from digestive or respiratory system and in less extend via conjunctival mucosa or penetration of the skin[14]. Transmission by infected aerosols may also occur[15]. Transmission requires close contact between infected and susceptible animals or infected tissues of placenta, aborted fetus and vaginal discharges or the ingestion of contaminated food and water. Likewise, venereal transmission of *Brucella melitensis* can happen but in a lesser extent as evidences show[15]. Dogs, rodents and other animal species in contact with infected sheep and goats or infected materials are susceptible to become infected as well and convey and disseminate the agent. In addition, wild ruminants in direct or indirect contact with infected goats and sheep may as well become infected and maintain the infection in the natural habitat. The spread of disease within a country and between herds usually follows the movement or gathering of infected animals[15].

1.4 Risk factors

The susceptibility to infection increases with age, so SRB is predominantly a disease of sexually mature animals. Pregnant females are the most susceptible, in part due to the production of high quantities of the hormone erithritol in placenta. Although young animals are generally more resistance to infection, they can carry and spread the disease throughout their lives. In goats the susceptibility to disease varies between breeds and sheep milking breeds appear to be more susceptible than those kept for meat production[16]. The features of the production systems influence the occurrence of brucellosis in flocks. Generally, SRB is associated to extensive management systems where practices of mixing of flocks and sharing pastures or enclosures at night[9] [11] are most common. The main risk factors identified in outbreaks investigations in Trás-os-Montes and Alto Douro in 2013 by the official veterinary services were "direct contact with other holdings", "introduction of animals" and "pasture sharing"[17]. Transhumance and the mixture of species in markets and fairs contribute also to the dissemination of the disease. The lack of hygiene is a crucial risk factor for SRB so the disinfection of stables, removal of strum and aborted fetuses and infected tissues after parturition are determinant measures frequently deficient in infected herds that would help prevent further transmission to other animals. Mainar-Jaime & Vázquez-Boland[18] assessed the contribution of veterinary services and farmer characteristics to brucellosis prevalence and they concluded that veterinary activity and the availability of those services are protective factors, as well as farmer characteristics such as “youth”, “education” and “being a member of a milk cooperative”.

1.5 Pathogeny, pathology and clinical signs

An important characteristic of *Brucella* infection is its capability to persist and replicate in phagocytic cells of the reticuloendothelial system as well as in non-phagocytic cells (epithelial cells lines and trophoblasts). The invasion of phagocytic cells is determinant for the pathology of brucellosis since these bacteria can persist in the interior of these cells for a long time, replicate and cause a persistent or recurrent infection. The pathogenic potential is dependent on its ability to cross mucosal barriers, survive and replicate within the host cells[19].

The susceptibility to brucellosis increases after sexually maturity and as a result the main clinical signs are abortion, reduced milk yield and orchitis. Although abortion is generally the first clinical sign of brucellosis, in goats this rarely happens more than once and the infection progresses into a latent and chronic infection so they often remain shedders at subsequent parturitions. In spite of the equal susceptibility to infection in sexually mature animals, the sign that best describes the acute infection is the disturb of reproductive function with abortion in last trimester or birth of weak and infected offspring[20]. In males, the effects in the reproductive system are orchitis or epididymitis[21] that generally lead to hypertrophy and thickening of the testis, chronic infection and ending in temporary or permanent infertility. Up to date, there is no evidence that there are significant differences in *B. melitensis* infection between the three biovars[21].

1.6 Diagnoses

The diagnosis of SRB is based on laboratory tests and bacterial isolation on appropriate culture media stands as the gold standard test. A wide range of serological screening tests are available for use but the most frequently applied and recommended by European Community are the buffered Brucella antigen (BBAT), particularly Rose Bengal (RBT), and complement fixation (CFT) tests. The indirect enzyme-linked immunosorbent assays (I-ELISA), also recommended by European Community, the fluorescence polarisation assay (FPA), polymerase chain reaction assays (PCR) and brucellin skin test are alternative diagnostic techniques[15].

In Portugal, for eradication purpose, a combination of the RBT and CFT tests is applied. RBT is commonly used for the screening of brucellosis in areas free from disease and a combination with CFT is applied as a confirmatory test. In herds free from disease RBT and CFT results are interpreted in series. In order to increase the likelihood of detecting infected individual animals in areas non-free from brucellosis, the two previous tests are interpreted in parallel. Moreover, the results of these tests should always be interpreted bearing in mind the health and vaccination status of the herd. The isolation of *B. melitensis* remains the chosen method for the accurate diagnosis[22].

1.7 Geographical distribution

Sheep and goats brucellosis remains a major worldwide source of disease in animals and humans. Successful measures of eradication have limited the geographical distribution of the disease but even within the same country the distribution patterns vary widely. Despite these achievements, it remains a foremost problem in some Mediterranean countries, Central Asia and Middle Eastern countries, Africa and in South and Central America (Mexico, Peru and Northern Argentina)(6). In Iberian Peninsula this disease has decreased progressively in the last years. Particularly in Portugal, the measures taken have not been successful in eradicating the disease in all its territory, especially in Trás-os-Montes and Alto Douro where it remains a burden.

1.8 Impacts on animal production and profitability

The laboratory biosafety manual of the World Health Organization (WHO) classifies *Brucella* and especifically *B. melitensis* as an hazard in Risk group III(6). In veterinary public health, brucellosis always deserved a special attention for its impact in animal health and international commerce. A new focus of infection can interfere with the international commerce of animals and animal food products, which leads to high socioeconomical losses and damages in public health. The global market and the intracommunity exchanges are built in a high and uniform level of confidence for the most part due to the establishment and communication of the health status of the herds in the Community, so the persistency of disease is a barrier to the free movement of animals(23). The structure of the production systems that focus in the surveillance at flock level, the fast exchange of animals and the traffic of animals and animal products through long distances, or the unsatisfactory security in the chain of production contribute to the increase of the impact of this disease(5).

The costs of SRB include those associated with the treatment of human causalities, the losses in farmers' income (variable abortion rates, neonatal losses, weight losses in premature animals, reduced fertility, decrease in milk production) and bigger expenses for the country with the implementation of eradication and control programs, distribution of vaccines, veterinarians and assistant workers' salaries and payment of indemnities(9). In addiction, the ban of animal movements causes serious consequences to local economy and global trade. While in industrialized countries, the animal losses decrease the potential of milk and meat supply which can lead to the increase in the costs of the consumer; in developing countries the losses are more obvious since the supply of milk and meat is affected by the abortions and neonatal death of the animals(11). In Portugal and particularly in the region Trás-os-Montes and Alto Douro the production and commerce of small ruminants represents a major strength for the local economy and an impulse against the rural exodus.

1.9 Zoonosis

Humans become infected with *B. melitensis* mainly through consumption of contaminated milk and unpasteurized dairy products, via direct contact with infected animals or infected tissues or products and inhalation of infected aerosols. The clinical signs are unspecific and can be misleading. Some include recurrent febrile episodes from unknown origin, also described as "undulant fever", headaches, anorexia, abdominal pain and arthralgia(24).

The human cases of brucellosis in Portugal, predominantly in rural areas, are related to the consumption of unpasteurized milk and fresh cheese from infected animals and there is an occupational risk to farmers, veterinarians and laboratory or abattoir workers that handle closely infected animals and tissues(9).

1.10 Small Ruminant Brucellosis Control and Eradication Program in Trás-os-Montes and Alto Douro, Portugal

Brucellosis is a problematic disease in Portugal and its eradication has been a most important goal. The reduction of infection in small ruminants is particularly refractory to methods strictly of control. In addiction to that, the distinctive epidemiological features of this disease such as higher contagiousness, less satisfactory detection of individual infection (when comparing to bovine brucellosis(25)) and specific risk factors in different geographical regions across the country contribute to its endemicity. Under these circumstances, the persistence of the disease in some areas led to the establishment of special control and eradication programs. The region of Trás-os-Montes and Alto Douro, in Northeast Portugal, was the area with the highest prevalence of brucellosis for sheep and goats in 1999. The Special Control Program on brucellosis in sheep and goats in this region was initiated in 2001 by means of prophylactic, sanitary and medical measures with a special focus on a compulsory vaccination program with Rev1 of young animals over 3 months and adults. Rev. 1 vaccine contains a live attenuated strain of *B. melitensis* derived from a virulent isolate of this specie which development is dependent upon streptomycin(26) (25). Its application aims to reduce transmission and disease burden at herd level. Although it is the most effective available SRB vaccine, the biggest disadvantages of its use rise particularly in adult animals. These problems are the induction of a positive serological response for a variable time period that interferes with the diagnosis of field-strain infections(27), abortions in pregnant ewes, excretion of the vaccinal organism and the possibility that animals can carried the organism for the rest of their lives(16). These difficulties can be over past by the administration of Rev1 by conjunctival route(28), the use of reduced doses of the vaccine or restrict its administration to young animals between 3 and 6 months. This measure has been extremely advantageous for the efficiency of the general program and as result herd prevalence in the region decreased from 34.95% in 2001 to 18.08% in 2004. In 2005 some measures and policies were reviewed and some of them include the immunization of only young animals, the identification of vaccinated animals, serological control tests in all animals performed (depending on the herd sanitary status) followed by slaughter of positive animals, stamping out in particular cases, replacement of flocks with only vaccinated animals, restrictions and control on animal movements. Animal slaughtering is decided based on the interpretation of RBT and CFT results. Since the start of the program, herd prevalence in the region decreased to 8% in 2013.

The Animal Protection Department from (DSPA) the Directorate General of Food and Veterinary Services (DGAV) of the Portuguese Ministry of Agriculture and Sea formulates all control and eradication programs. At regional level, five Regional Food and Veterinary Directories (DSAVR) represent DGAV. The national authorities work with other entities in the design of such programs and in filed activities such as the OPP (Livestock Producers Organizations). These are farmer's organizations in charge of field activities included in the SRB eradication program such as animal identification, collection of blood samples for serological control and vaccination of animals with Rev1. In herds that are not associated with these organizations (ex.OPP), the same procedures are executed by the official services. The national animal health laboratories, private and public, perform the diagnoses tests and give technical support.

1.11 Herd registration and animal identification

Animal identification is essential for controlling animal diseases and for safeguard the traceability of animals and animal food products. The DSAVR is responsible for registration and attribution of the mandatory identification code of all small ruminants' herds in each region. The code is a unique combination of a number of characters that begins with the code of the country (PT in case of Portugal) followed by a letter representing the group of animals. The next two letters designate the region and the municipality where the herd is located and then it follows a group of three numbers that distinguish the herd in that municipality. Individual identification of sheep and goats includes one or two ear-tags and electronic identification with a microchip. All animals must be identified within six months after birth and all times before leaving the original holding. Exceptionally, animals in extensive management systems or free-range farming can be identified until nine months old. For movement purposes young animals may be identified with a temporary ear-tag. Producers according to DL 142/2006, from July 27th[29], execute the annual declaration of small ruminants directly in the National Animal Identification and Registration System (SNIRA) through the "idigital" platform. In addition, the informatics database for the ruminants eradication programs (PISA.Net) should be kept updated with information concerning the identification of herds, animals under control, the controls to herds and animals and the results of the diagnoses tests, the health and vaccination status of the herds and the slaughters. Animal movements are controlled and registered in "idigital" plataform and restrictions on movements are applied according to the health and vaccination status of herds.

1.12 Brucellosis health status

As regards the rules governing brucellosis status of sheep and goats herds reported in annex I of DL 244/2000 three main classifications are identified: herds non-free from brucellosis (B2), herds free from brucellosis (B3) and herds officially free from brucellosis (B4). There are also complementary classifications to specific circumstances: “infected herd” (B2.1) if it was officially confirmed the presence of *B. melitensis* through bacterial isolation; “herd suspected of brucellosis” (B3S and B4S) when the brucellosis status is suspended in both free and officially free from disease herds. These classifications are assigned based on the serological search for *B. melitensis* by RBT and CFT in individual animals according to Council Decision 90/242/CEE.

To further clarify the terminology used in the current document three brucellosis states of a herd must be accounted. Positives are herds with at least one animal positive to serological test (B2 and some B3S, B4S herds); infected are the herds with at least one animal where it has been isolated the bacterium, meaning B2.1 herds; suspected if there has been reported abortions or other clinical signs that suggest SRB, epidemiological surveys about human or animal cases that indicate the possible presence of infected animals, possible contact with infected or suspected flocks, and lastly, herds subject to serological control and waiting for conclusive laboratory results, that can be either B3S or B4S.

1.13 Epidemiology of SRB in Trás-os-Montes and Alto Douro, Portugal

The epidemiology of this disease is complex and multiple factors are involved. The identification of risk factors associated with seroprevalence of brucellosis in small ruminants has been already explored in several epidemiological studies[(30) (31) (18).] In Portugal, particularly in Trás-os-Montes and Alto Douro, there are a limited number of studies relating small ruminant brucellosis and risk factors; nevertheless the general results are consistent with previous discoveries. In 1997, Maria Vaz [(32)] identified the extensive production system, the transhumance, the mixing of animals at risk with infected ones, the long duration of parturition season and replacement animals as some risk factors in Serra da Estrela region. In this section, these results are most relevant in terms of similarity of production and socioeconomical conditions with Trás-os-Montes. Some risks factors identified by Coelho *et al.* [(33)] are higher number of animals, predominant species involved, type of production and introduction of animals from herds non-free or with unknown brucellosis sanitary status. Fonseca [(34)] referenced as the main risk factors the “open” flocks (entrance and exit of animals), the use of common pastures, the contact with high-risk flocks and the contamination of pastures with abortion tissues. The transmission of infection between neighbouring herds had been previously mentioned to be one of the main routes of dissemination of the disease since animals frequently underpass the fences, when those exist, or they get in contact with other flocks while sharing pastures and roads. The contact with dogs among other animals that might transport infected aborted foetus or placentas was also stated. In this scenario it is clear the importance of identifying the source of new infection cases and the routes of dissemination between herds and regions. The movement of animals between herds plays also a major influence in the dissemination of the disease, particularly in this region.

2 Geographic Information Systems

Geographers first used computers as a tool to display and analyse data in automatized cartography[35]. The foundation of Geographic Information Systems (GIS) goes back to the seventies in the last century and was born as a need to explain the environmental problems and social concerns of the population at that time. In the following decade there was a great spread of the use of GIS through many disciplinary fields, but it was only in the eighties that it became more accessible. The possible applications of these systems with roots in mathematical, analytical and digital cartography were fast discovered and it did not take long until they were used in managing of databases and geographic analysis worldwide. GIS can be applied to all problems where the location and the characteristics, or attributes in the GIS literature, of these geographic sets are relevant. One of the most known studies in spatial epidemiology and also one of the firsts is the map of cholera epidemic in London in 1855 by John Snow[36]. In the study, the addresses of the victims were represented in a map and the careful observation of the distribution of those addresses near a source of putative pollution raise the hypothesis that cholera could be transmitted by contaminated water supply. By the 1980s, the use of GIS in veterinary sciences began to take the first steps. Epidemiology and public health are two of the main fields of application of GIS in veterinary sciences as expected according to their strong relations to the characteristics of the place and space in the study of disease occurrence[37]. Geographical epidemiology is a sub discipline that describes the geography of disease at different scales and evaluates the factors associated with the spatial distribution of incidence, prevalence and other measures of disease. Yet, environmental epidemiology focuses on modeling relative risk for a certain area while controlling for the effects of environmental risk factors[38]. Furthermore, the possibilities and potentialities of GIS to address health related problems in several levels (local, regional, national and global) comes in part from its capacity to spatially analyse data through new and efficient methods.

Spatial data have three main components: features, supports and attributes. Features are units (points, lines, areas and volumes) with a spatial location and particular properties that together create a class. Each feature has three specific properties that form the support: size, shape and orientation. Lastly, the attributes are observations or values linked to features and frequently used in the statistical analysis of the data. Each feature can have more than one attribute and each analysis can have more than one attributes associated[39].

2.1 Spatial Analysis

The First Law of Geography[40] states that nearby attributes in space tend to have values more similar than those further apart. This relation can be used as the basic premise to solve problems of spatial dependence in health sciences that can be also quantified through the use of spatial statistics. According to Keith C. Clark[41], spatial analysis should be used in all data with an association with a certain geographical location and when there is a plausible role for its spatial distribution in the analysis. There are three main types of spatial data analysis: visualization, exploratory spatial data analysis (ESDA) and modeling construction[42]. The first focuses in mapping the distribution of data and describe spatial patterns; the second looks for significance in the data set and identifies non-common spatial patterns and formulates hypothesis that lead to future research; and the last one uses mathematical modeling to study how predictors influence or explain a response variable.

Maps are powerful means to communicate information and they can be design according to their target audience so they are easier to interpret. This tool to display animal health information can also be used to conduct studies that take advantage of the spatial attributes of data in GIS and try to explain spatial patterns of disease while ultimately help answer certain questions on disease occurrence. There are several methods to analyze patterns of disease, but not all of them are adequate to study specifically infectious diseases. Brucellosis, being an infectious and contagious disease, is characterized by the occurrence of cases of disease somehow related for the reason that those are not entirely independent and time and space variations are an important part of the disease pattern. Subsequently, it is wised to use methods of spatial autocorrelation to address the exploratory analysis of infectious disease patterns. Upsurge in the use of GIS in the spatial analysis of infectious diseases was also noticed in the increase of studies of brucellosis[43] [44].

2.1.1 Exploratory spatial data analysis

ESDA consist of a conjunction of procedures and techniques for computing, analysing and interpreting data with an explicit focus on its spatial attributes. Accordingly, these techniques can be used to describe spatial distribution, find patterns of association, discriminate patterns of instability and find atypical observations[45]. Spatial autocorrelation measures the level of association between data units within a specific spatial context and it can be computed by means of Moran's Index among other statistics. It allows risk assessment of the occurrence of health events very useful in geographical disease clustering tests. This method points out the presence or absence of a stable pattern of spatial dependence for the whole data set, specially useful in small data sets, but with poorly significance in large data sets. To overcome this limitation, local indicators of spatial association (LISA) can be implemented by way of Getis-Ord local statistic and local Moran's Index statistic[46]. The combination of ESDA techniques and GIS helps describing spatial interdependencies between general and local units.

Part B: Spatial Analysis of Small Ruminants Brucellosis Occurrence in Trás-os-Montes and Alto Douro, Portugal

1 Objectives

The present study aims to evaluate the use of GIS in small ruminants brucellosis eradication program by supplying the animal health officers with appropriate indicators and knowledge of the epidemiology of this disease in Trás-os-Montes and Alto Douro, Portugal. To carry out this investigation, the hypothesis that the distribution of *Brucella melitensis* infection in small ruminants' herds in Trás-os-Montes and Alto Douro could be explained in part by the spatial proximity of villages using an explicit geographical approach was tested. This is of interest to answer if distance between villages could be one key factor that explains the risk of infection in that region.

The five main objectives to be achieved in this study are: (i) to describe the geographical patterns of small ruminants brucellosis in Trás-os-Montes and Alto Douro, a region in Northeast of Portugal; (ii) to establish if new cases of infection occur randomly across the study area or if there can be identified spatial patterns of proximity; (iii) to evaluate the utility of exploratory spatial analysis techniques in the implementation of new strategies in the SRB eradication and control programme; (iv) to identify regions where interventions can be improved and where more epidemiological investigations could be necessary; finally, (v) to investigate the association between brucellosis and risk factors that characterize the herds.

2 Material and Methods

2.1 Study area

Trás-os-Montes and Alto Douro is a region in Northeast of Portugal with a total area of 12669.17 km^2 and geographical location between 42º0'13.3" N to 40º48'40.6"N and 6°11'6.9"W to 8°7'53.3"W limited in the North and East by Spanish border, in South by the region of Beira Interior and in the West by the regions of Minho and Douro Litoral. The region includes the districts of Bragança and Vila Real with a total of twelve and fourteen municipalities, respectively, and eight of the twenty-four municipalities of the district Viseu and one of Guarda district (Vila Nova de Foz Coa). The total number of villages in the region is 714 and in this study were analysed 706. SRB is known to be endemic in this region and its prevalence is one of the highest in the whole country.

2.2 Population

The epidemiological unit considered was the herd and for the purposes of the study all herds included are registered in the national database PISA.net and are enrolled in the special SRB eradication program in the regional administrative food and animal health services of Bragança, Chaves-Mirandela and Vila Real-Douro Sul. Thereby, all herds involved in the study have a known brucellosis status. The herds from the municipality of Mondim de Basto in Vila Real district were exceptionally excluded from the analysis due to lack of accessible information as it belongs to the regional administrative food and animal health services of Braga. When studying the spatial distribution of the disease the epidemiological unit chosen was the smallest administrative area, the village.

2.3 Study design

This study was conducted as a retrospective analysis of the data concerning the SRB eradication and control program from 2010 to 2014. The follow-up data of the sanitary status of the herds and other attributes were used to measure disease occurrence and the results were displayed in maps to highlight the health status at village and municipality level. Afterwards, it was performed an exploratory spatial data analysis and applied spatial statistics methods to investigate general clustering and identify spatial local clusters and outliers and the results were analysed and consolidated for the report of high risk areas. Finally, three different statistical models were executed in order to study the possible association between disease indicators and some risk factors.

2.4 Data source

Data of small ruminants' herds, including herd identification, number of animals, brucellosis sanitary status and location were collected for the period between 2010 and 2014 from the national Animal Health Information System database PISA.net provided by the Directorate Regional of Food and Veterinary Services of North Region (DRSAVRN). This database supports information on health activities related to the implementation, control and monitoring of official plans for the eradication of brucellosis in sheep and goats, among others diseases.

Administrative boundary maps of the region at village, municipality and district level were obtained from the database of Portuguese cartography available in the online portal of the Portuguese General Direction of Territory from the Ministry of Environment[47].

2.5 Data editing

Data were organized into databases using Microsoft® Excel 2010 and Microsoft® Access 2010 and all disease indicators were calculated for each year database. The editing involved check for duplicates, correction of undefined villages names and herds identification and cross tables to determine and select herds with suspended sanitary status as a consequence of the suspected or confirmed presence of *Brucella melitensis* from those with sanitary status suspended for other reasons. This last task was accomplished by crossing information regarding the results of serological tests with herd brucellosis status.

2.6 Measures of disease occurrence

In order to diagnose the health status of the population, a group of epidemiological indicators was tested. This class of indicators also named impact indicators measures the level and trends of occurrence due to a particular disease. Reductions in values of the measures are expected following health interventions but the interpretation of trends depends on the quality of the data used and the accuracy and plausibility of the results should be evaluated.

As mentioned previously, three health states were considered to classify herds in the following calculations: suspected, positive and infected. Depending on the criteria used, a combination of positive and infected can be considered in the calculation. A herd is considered prevalent in a certain moment if the disease is present (coded as "1"), otherwise is considered absent (coded as "0") and it is incident in a time interval if there was isolated the agent (coded as "1"), otherwise is not incident (coded as "0").

Estimates on small ruminants brucellosis prevalence and incidence at village level were performed according with the data available of the sanitary status of each herd from the population in study at the same date, first of January, in each year. Prevalence can be defined as the amount of disease (the presence of infection or antibodies) in a known population at a certain time[48]. In this study the prevalence of B2 is a percentage that represents the probability of a village having positive (B2) or infected (B2.1) herds. The population comprehends all herds (B2, B2.1, B3, B3S, B4, B4S) in each village at that point in time. The calculation is based on:

$$\boldsymbol{Prevalence\ B2_{it}} = \frac{\sum_{j=1}^{n_i} Herds\ B2.1, B2}{\sum_{j=1}^{n_i} All\ herds\ (B2.1, B2, B3, B3S, B4, B4S)} \times 100$$

Where *i* is each village, *t* the year and n_i the number of herds. For example, in 2010 there were five herds B2 and two herds B2.1 in the village Carregosa and the total number of herds in that village was seven at that time, the prevalence of B2 is: $((5 + 2) \div 7) \times 100 = 100\%$.

On the other hand, incidence is the number of new cases that occur in a known population over a specified period of time[48]. As measure of risk, it can be used to quantify the proportion or percentage of herds in each village that are initially free of disease that become infected over a specific period of time. In this study it was calculated the percentage of non-infected herds (B2, B3, B3S, B4S, B4) at the beginning of a period, one year, that become infected (B2.1) during that period for each village. Since the data available for the five-years were the sanitary status of the herds at the same day in each year, the annual incidence was determined by the population at risk at the beginning of that year and the number of new infected herds according to the records of the following year. This measure is given by:

$$\textbf{Incidence risk B2.1}_{it} = \frac{\sum_{j=1}^{n_i} New\ herds\ B2.1\ \ at\ t+1}{\sum_{j=1}^{n_i} Herds\ at\ risk\ at\ t\ (B2, B3, B3S, B4, B4S)} \times 100$$

Thus, in first of January 2011 in the village Vilar Chão one herd had become infected and there were twelve herds at risk in the beginning of the previous year, 2010, then the incidence of B2.1 for that village in 2010 is: $(1 \div 12) \times 100 = 8\%$.

Two other measures (prevalence of positive herds and incidence of B2) were estimated. The prevalence of positive herds was similar to the previous calculation with the only difference in the numerator that included herds B2, B2.1 and herds with suspended sanitary status (B3S and B4S) (supplementary formula 1). This measure might overestimate the overall presence of the disease, however when a sanitary status is suspended a positive result on at least one serological test was recorded justifying the inclusion in the infected group. The incidence of B2 was calculated as the proportion of herds at risk (in this case, B3, B3S, B4 and B4S) that become B2 or B2.1 (supplementary formula 2). Although B2 herds are not clearly infected, they are still in the process to become free from disease (B3).

The cumulative incidence rate is a measure of disease that accounts the velocity with which new cases of disease develop for a specific time period[49]. The measure is then given by the equation:

$$\textbf{CIR of B2.1}_i = \frac{\sum_{t=1}^{5} New\ herds\ B2.1\ \ in\ village\ i}{\sum_{t=1}^{5} Herds\text{-}time\ at\ risk\ in\ village\ i}$$

The cumulative incidence rate can be the chosen method applied in small data sets since it accounts more numerically stability than other age- or time-specific rates. According to the longitudinal design of this study which includes follow-up data, an estimate on the cumulative incidence rate by village for the period of study can be obtained, assuming a dynamic population, dividing the time into t intervals $(t = 1, \ldots, 5)$ having lengths of one year each.

For instances, in Milhão, a village of the county Bragança, there were no incident herds in 2010, two incident herds in 2011, one in 2012 and zero incident herds in 2013 and 2014. The number of herds at risk was eleven herds in 2010, nine in 2011 and 2012 and ten in 2013 and 2014. The CIR is approximately: (0+2+1+0+0)/(11+9+9+10+10)=0.33. The denominator in the equation above accounts an approximation to the total “herd-time at risk” during the study-period and they summarize all the periods of observation for each herd during the time they are free from disease, so they stop contribute to this component as soon as they get infected and become B2.1. It is also implicit that the same herd can become infected more than one time, so it can enter the calculation at different times. In sum, it was calculated an approximation of the cumulative incidence rate of B2.1 herds in each village during the years 2010-14.

In order to overcome the differences in the number of herds per village and thereby allow the comparison between the values of prevalence and incidence ratios along different villages, it was performed an indirect method of standardization to compare subpopulation ratios using a proportion of observed to expected counts[50]. The standardized prevalence ratio (SPR) and standardized incidence ratio (SIR) can be used as indirect standardization methods for summarizing the brucellosis prevalence and incidence ratios, respectively, across groups of data. This is a measure of risk and if it takes the value 1 indicates identical risk for that particular village when compared with all Trás-os-Montes area, whether values greater than 1 indicate a higher risk, and values less than 1 indicate a lower risk. The SPR is given by the sum of all prevalent counts in each village occurring each year, meaning the observed cases, and divide this total by the expected cases given by the product of the general population proportion (the sum of prevalent herds of that year divided by the sum of all herds) and the sum of all herds by village (supplementary formula 3). A SPR for brucellosis of 1,62 in Pombal would mean that there are sixty two percent more prevalent herds in that village than in the reference population (in this case the population of Trás-os-Montes and Alto Douro). Additionally, the SIR is given by the sum of all incident counts (meaning, B2.1 herds) by village occurring each year, representing the observed cases, and divide this value by the number of expected cases given by the product of the general population incidence ratio (the sum of incident herds of that same year divided by the sum of all herds at risk) and the sum of all herds at risk by village (supplementary formula 4). These measures of risk may give a better understanding on the overall prevalence and incidence of brucellosis in the region.

2.7 Exploratory spatial data analysis

2.7.1 Global spatial statistics

The Global Moran Index[46] is a statistic that measures the structure dependence in spatial patterns and it is based on the degree of covariance of all observations within a complete region under study and is given by the equation in supplementary formula 5. As an inferential statistic, it is grounded in probability theory and it has to be interpreted in the context of the null hypothesis and the computed z-score and p-value. In this study the null hypothesis is that the spatial pattern of SRB prevalence and incidence in Trás-os-Montes and Alto Douro are the result of random distribution, so they are spatial independent.

2.7.2 Local indicators of spatial association

All LISA have in common two fundamental principles: the LISA value for each observation is a reflection of the spatial clustering of similar values in its neighborhood and the summation of the total LISA values is proportional to a global indicator of spatial association. In order to decompose the global indicator was used the LISA local Moran's Index statistic given by the following equation:

$$I_i = \frac{z_i}{S_i^2} \sum_{j=1 \wedge j \neq i}^{n} w_{ij}\, z_j \ , \qquad S_i^2 = \frac{\sum_{j=1 \wedge j \neq i}^{n} z_i^2}{n-1} - \bar{x}^2$$

Where z_i is the deviation of an attribute x_i for feature i from its mean($\bar{x}$): $(x_i - \bar{x}\,)$; w_{ij} is the spatial weight between feature i ad j and n is the total number of feature.

These indicators have two major purposes: identify significant local spatial clusters around individual locations and access the significant outliers, so they may give an overview of the influence of individual locations on the magnitude of global statistics[46]. Local Moran's Index provides the diagnostic for local instability, hence spatial objects with a strong impact on global Moran's I, in other words, locations with values very different from the mean can be identify. A positive value for local Moran's Index indicates spatial clustering of similar values (high-high or low-low) also named clusters and a negative value indicates association of different values (high-low or low-high), also entitled outliers. In this study there will be identified not only villages of high prevalence surrounded by other villages of high prevalence (high-high clusters) but also villages of low prevalence surrounded by villages of low prevalence (low-low clusters) and villages of high prevalence surrounded by villages of low prevalence (high-low outliers) and vice versa (low-high outliers).

2.7.3 Spatial weights matrix

In order to represent the spatial structure of the observations a file containing the row-standardized spatial weights that defines spatial relationships among features (villages) within the region was generated. These matrices consist of rows and columns representing the observations and the non-zero elements for row-column pair represent contiguous locations[51] [52]. For the conceptualization of spatial relations was chosen the parameter contiguity corners and edges based on a binary contiguity scheme (combining common border and vertex contiguities) so that it would best fit the non-uniform shape of the polygons that represents the different villages and so only neighboring polygon features that shared a boundary or overlap would influence computations for the target polygon feature. The number of nearest polygons, in this case the neighbors, that will be associated with the cluster analysis and specifically with each cluster of polygons was specified to 353 so it would match approximately half of the total number of villages in that region. The row-standardization form was chosen to reflect the weighted average of observations at neighboring locations and each element of the matrix is divided by the row sum, and the summation of all row elements is 1.

2.7.4 Maps design

Since georeferenced data of the herds was not available, all herds were aggregated in the smallest administrative area (village) and a six-digit code entitled "Dicofre" in both herd and map databases was used to georeference all attributes to maps. All maps and spatial analysis were produced and performed, respectively, in GIS software ArcGIS® (v 10.2.1)[53].

2.8 Statistical analysis

According to the structure of the data collected, two binary logistic regression models were chosen to study the predictors of the likelihood that the risk of prevalence and incidence would not be different among municipalities. The dependent variables for each model are prevalence and incidence by herd (categorical measures). The predictors in both models include municipalities (categorical), herd size (continuous measure) and year (continuous measure). The significant level was set to $\alpha=0.05$.

The hypothesis that incidence was not related with the presence of prevalence clusters, both village-specific, was assessed using the Fisher's Exact Test. The hypothesis test was the same for each year and had as null hypothesis the absence of association between villages that are clusters of prevalence and villages that had incidence of brucellosis and the alternative hypothesis was that the villages that are clusters of prevalence were not independent from the incident ones. Clusters of prevalence were categorized in two classes, one that includes clusters of high risk (high-high), and another that includes areas of low risk (villages that are low-low clusters, outliers, both high-low and low-high, and villages that are any of these) and incidence was categorized also in two classes: 0 (incidence equals zero) and 1 (incidence different than zero). The significant level considered was $\alpha=0.05$.

The final statistical model performed was a binary logistic model to study the risk of prevalence in sixteen OPP that work with herds in that region. The dependent variable was the herd prevalence (coded 0= no, 1= yes) and the predictors were OPP, herd size (continuous measure) and year (continuous measure). The significant level was also set to $\alpha=0.05$.

The statistical analysis were performed by SPSS® Statistics 22 software[54] and statistical program R®[55].

3 Results

3.1 Descriptive epidemiology

According to the data analyzed, the total number of herds by year ranged between 4960 and 5229 and the number of animals between 282576 and 300594 (supplementary figure 1). The number of herds had increased between 2010 and 2011 and then declined until 2013 when it was at its lowest. By 2014 it had increased to a higher level than the one reported in 2012. The number of small ruminants had a similar behaviour until 2013 when instead of decreasing it increased despite the lowest number of herds, and by the same time in the following year it was lower despite the significant increase in the number of herds. The average number of animals per herd was approximately 57, with a maximum of 762 and a minimum of 1 animal. In this study, while prevalence of the disease in the region decreased during the five years, incidence did not have the same behaviour. From 2010 to 2011 it had increased reaching the highest value during the study interval and then decreased in 2012. Incidence again increased in 2013 and decreased in 2014 (supplementary figure 2).

Among the herds at risk (all except the B2.1) in 2010, 43 (2,12%) became infected in the district Bragança, 26 (1,49%) in Vila Real, 13 (1,75%) in Viseu and 1 (1,85%) in Guarda. By 2014, only 15 (0,73%) had become infected in Bragança, 12 (0,68%) in Vila Real and no one in Viseu or Guarda.

3.2 Spatial analysis

Supplementary figure 3 shows the spatial distribution of the average number of small ruminants by village across the region during five years. The highest category represented by the darkest shade of brown is predominantly located in villages in the northern and north-eastern part of the region and a clear low number of animals are located south-western.

The spatial pattern of prevalence of B2 from 2010 to 2014 is presented in figure 1. The colours indicating the levels of prevalence of each village have changed in the maps over the years, with a steady decrease of high prevalence villages represented in the darker shade of brown and a more slight decrease in medium levels of prevalence. The spatial distribution maps of small ruminant brucellosis incidence of B2.1 (figure 2) showed that new cases of brucellosis seem to occur all around Trás-os-Montes and Alto Douro between 2010 and 2014. Despite the slight decrease in the incidence level over the years, incidence appears to be geographically randomly distributed across villages. The number of incident villages has decreased in the study period with the exception of 2013.

The cumulative incidence rate (figure 3) was categorized into five classes for a better analysis. Only eleven villages had a rate greater than 0.18 and twelve had a rate between 0.09-0.17 from 2010 to 2014. The maximum rate (0.5) belongs to village Sampaio in Bragança district, where 2 herds became newly infected over the 5 years. On the other hand, the village Alvadia had 6 newly infected herds during the same period with an incidence rate of 0.11.

The supplementary figures 5 and 6 show the maps of the standardized prevalence and incidence ratios. The number of villages with a ratio close to one, meaning identical risk among the observed and general population, decreased over the years while the number of villages with a ratio close to zero tended to increase, which means that the risk across the villages is likely to decrease, indicating that a higher number of villages had fewer risk to be or become infected when comparing to the expected. In opposite, the number of villages with higher risk of prevalence did not change over the years with the exception of 2012 when it was at its lowest, whereas the SIR decreased over the years. When comparing these to the maps of the ratios of crude prevalence and incidence the patterns of the overall seem to be very similar year by year.

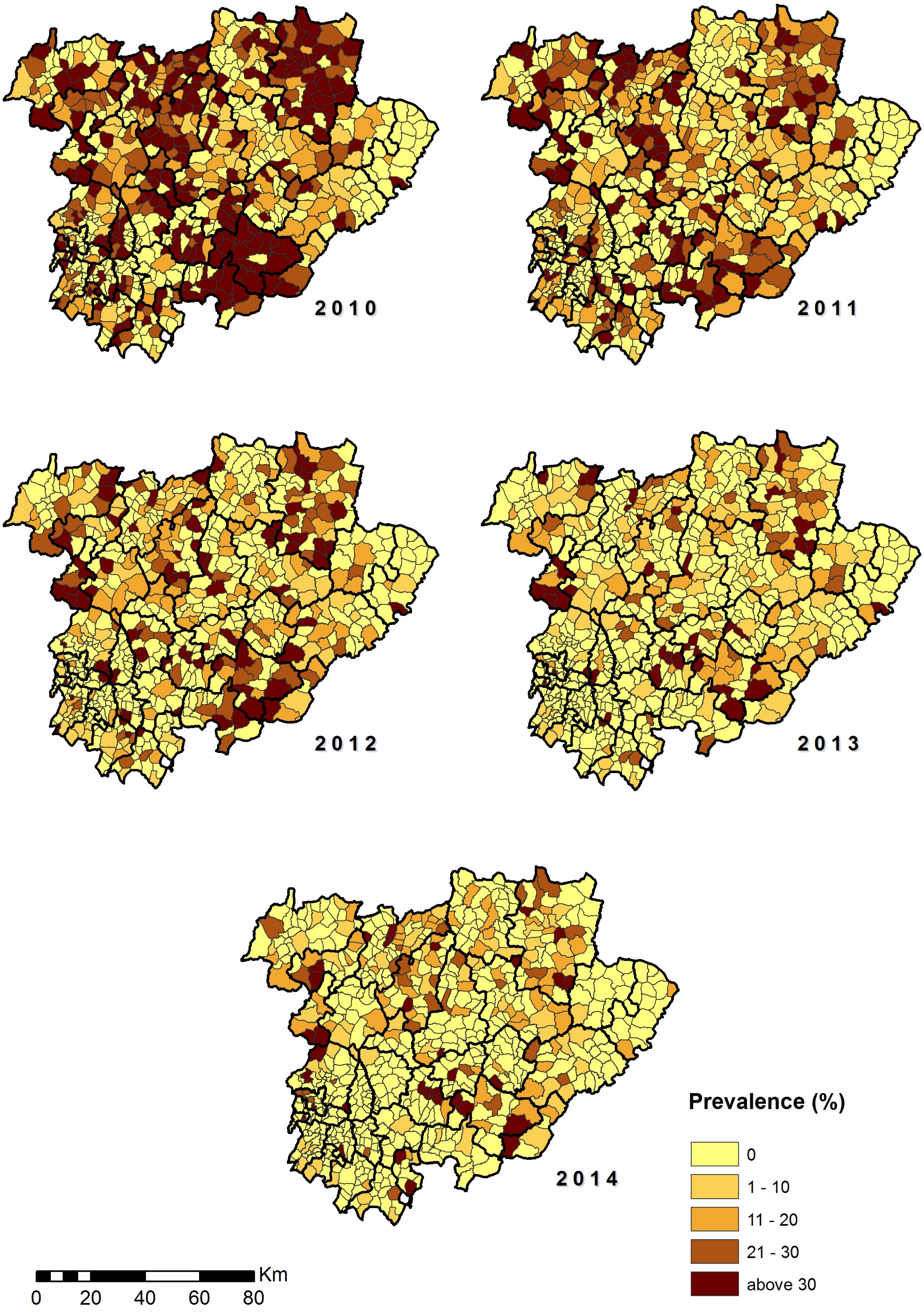


Figure 1 Choropleth maps showing spatially SRB prevalence by village (per year) in Trás-os-Montes and Alto Douro, 2010-2014.

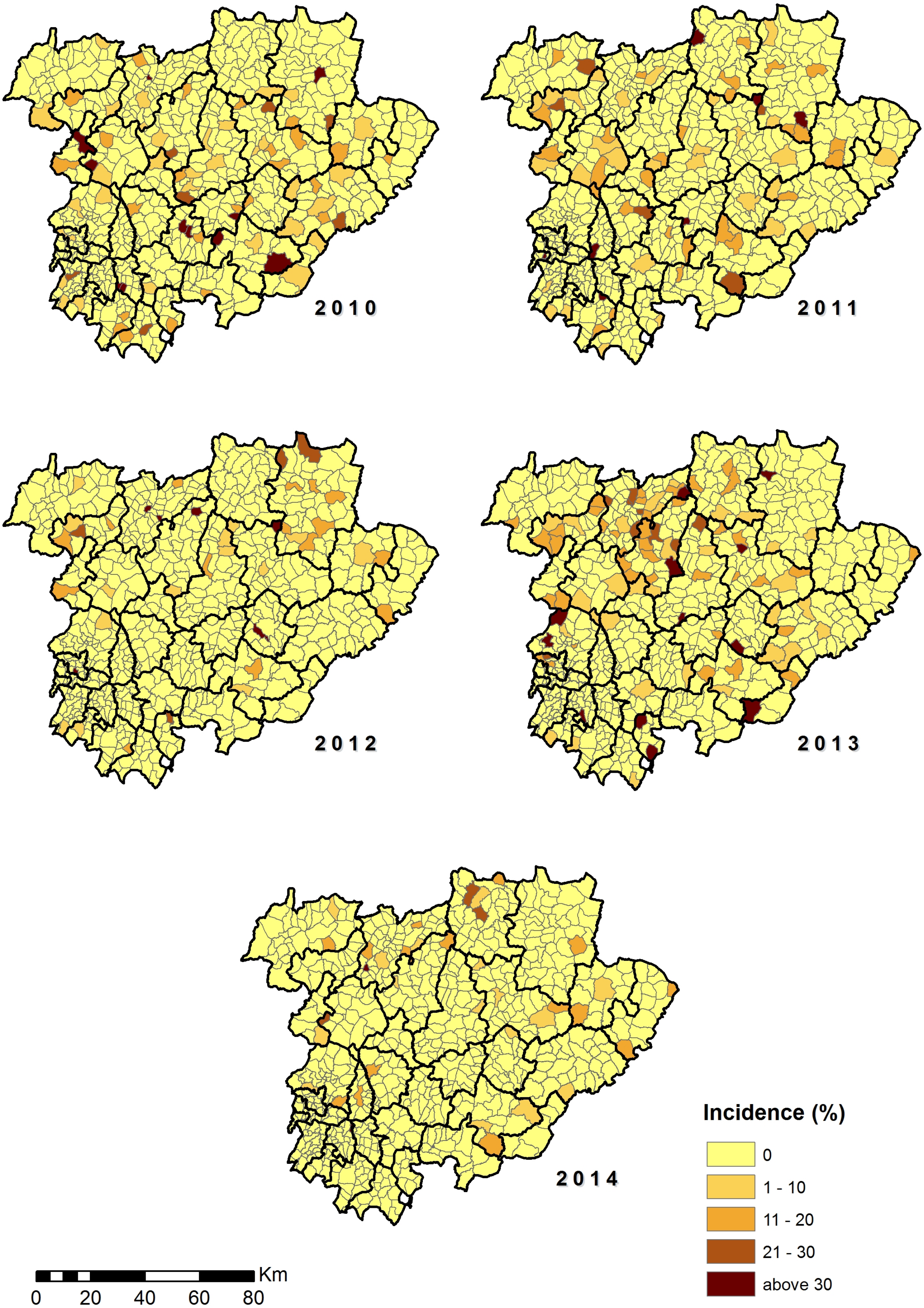


Figure 2 Choropleth maps showing spatially SRB incidence by village (per year) in Trás-os-Montes and Alto Douro, 2010-2014.

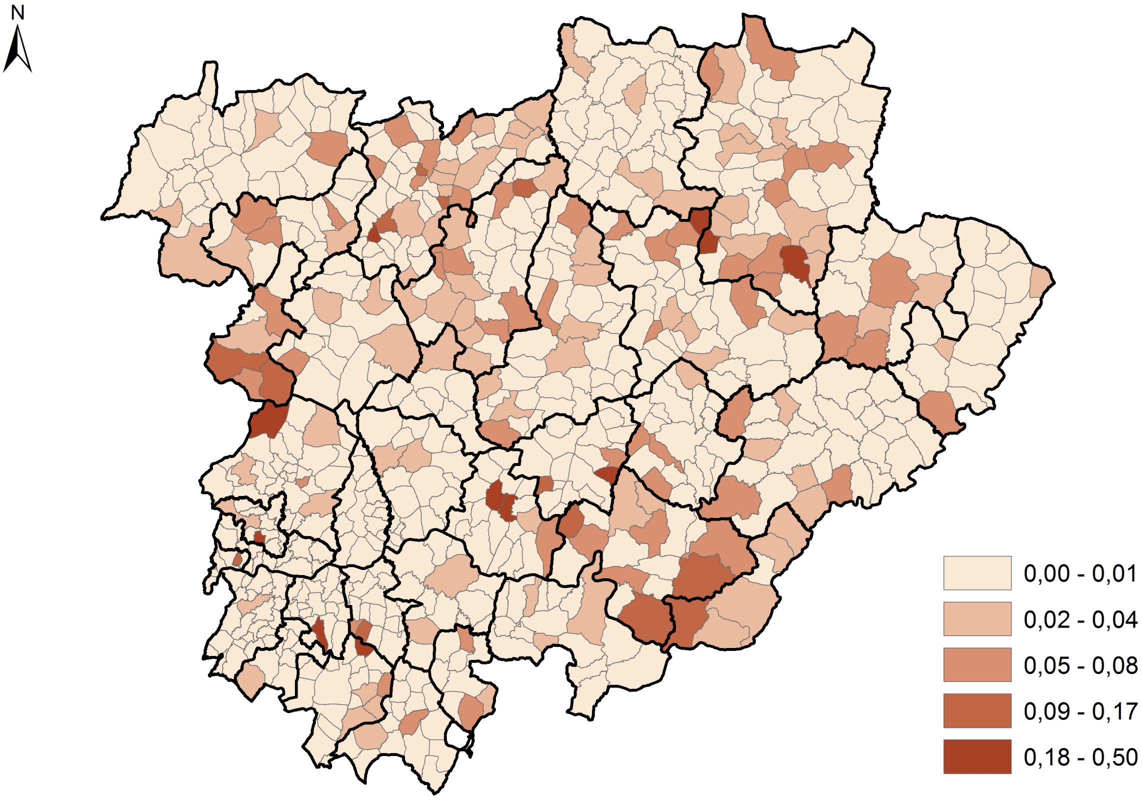


Figure 3 Cumulative incidence rate of small ruminant brucellosis by village in Trás-os-Montes and Alto Douro, 2010-2014.

3.2.1 Global spatial statistics

The results of Global Moran's statistic for prevalence of B2 are presented in table 1. The outcomes confirm the global spatial association between brucellosis prevalence and the spatial location of villages. In spite of the low values of Moran's Index in all the five years there is less than 1% likelihood that the clustered pattern of brucellosis prevalence in Trás-os-Montes and Alto Douro could be the result of random chance. The same statistical method was applied to incidence of B2.1 and it was found only in 2013 a statistically significant spatial autocorrelation that translates into the clustered pattern of the incidence distribution map of 2013.

The Global Moran's Index of cumulative incidence rate by village was 0.090, and given the z-score of 4.138, there is less than 1% likelihood that its clustered pattern could be the result of random chance.

Table 1 Results from the global Moran I's statistic for brucellosis prevalence of B2 in Trás-os-Montes and Alto Douro, 2010-2014.

Global Moran's Index						
Year	**Moran's Index**	**Expected Index**	**Variance**	***z-score***	***p-value***	**Pattern**
2010	0.184847	-0.001418	0.000517	8.195056	0.000000	Clustered
2011	0.161276	-0.001418	0.000514	7.174307	0.000000	Clustered
2012	0.125429	-0.001418	0.000512	5.606355	0.000000	Clustered
2013	0.126206	-0.001418	0.000504	5.683737	0.000000	Clustered
2014	0.096984	-0.001418	0.000501	4.396159	0.000011	Clustered

3.2.2 LISA analysis

The LISA cluster analysis identified significantly high-risk clustered areas of small ruminants brucellosis prevalence at the village level during each year of the study period (figure 4). The number of high-risk villages, which are those included in clustered areas of high risk, decreased over the years with a maximum of 44 villages in 2010 and 2011 and a minimum of 19 in 2014. In 2010 high-high clusters were found predominantly in the east side while in the next four years they were more evenly distributed around the study region. A total of 44 high-high clusters were identified in 2010. The number of villages belonging to high-low outliers decreased until 2013 reaching only 2 villages that turned to 7 villages in the following year. Over the study period there was not found low-low clusters. Only one village was part of a cluster during all period, Guiães in Vila Real, in the first four years as part of a high-high cluster and in 2014 in a high-low outlier; while 569 villages were never part of clusters or outliers during the same period (table 2). In the map of 2010 two main clusters can be identified, one in the municipality of Bragança and the other in Torre de Moncorvo, Vila Nova de Foz Coa, Vila Flor and Freixo de Espada à Cinta municipalities. The municipality of Bragança had clusters in all the five years with a combination of different villages. The same happened to Torre de Moncorvo. Two and then three villages in Ribeira de Pena were persistently high-risk clusters since 2010.

Table 2 Cluster and outlier analysis for brucellosis prevalence of B2 in Trás-os-Montes and Alto Douro, 2010-2014.

Cluster-outlier type				
Year	**Number of villages**			
	High-high	**High-low**	**Low-high**	**Low-low**
2010	44	12	5	0
2011	44	8	5	0
2012	36	8	6	0
2013	23	2	4	0
2014	19	7	1	0

In the maps of supplementary figure 7 two attributes of the region are represented so it could be compare the percentage of incidence by village with the presence of prevalence clusters in the same year. When looking closer by year it might seem that the incident villages do not match in most part the prevalence cluster ones.

The maps of LISA cumulative incidence rate are showed in figure 5. For the study period, 23 villages were high-risk areas, which means that they were part of high-high clusters. From these 23, 8 are from district Vila Real, 12 from Bragança and 3 from Viseu. In Vila Real 4 out of the 8 high-risk villages belong to Chaves. The outliers found include 8 villages, 3 low-high and 5 high-low. Additionally, any low risk areas were identified. Supplementary figure 8 shows the LISA clusters and outliers of incidence risk in 2013, the same year where a global cluster pattern was identified in the region.

3.3 Statistical analysis

With regard to the risk analysis, supplementary figure 10 shows the map of the regression coefficient exponential of prevalence by municipalities. The municipalities of Miranda do Douro, Vimioso and Macedo de Cavaleiros had a significantly lower risk of prevalence when compared to Vinhais considered in this analysis as the reference (odds ratio=1). The five municipalities with higher risk were Ribeira de Pena, Valpaços, Sabrosa, Carrazeda de Ansiães and Torre de Moncorvo. Herd size was identified as statistically significant (OR_{adj} 1.006 and 95% CI 1.005-1.007) and the year showed to be also statistically significantly (OR_{adj} 0.793 and 95% CI 0.729-0.863).

The statistical analysis of prevalence clusters and incidence by village during 2010-2014 revealed that the p-values of the Fisher's Exact Test were all lower than the significance value (supplementary table 2), which means that the variables are not significantly associated and the null hypothesis is not rejected. In conclusion, villages that are clusters of prevalence are not associated with incident villages in the same year.

In respect of the final analysis, from the sixteen OPP analysed, one was consider the reference (OPP 208) and other two were excluded from the results due to lack of sample size (OPP 213 and OPP 251). The OPP were categorized in 3 classes of risk according to low or high statistical significance of odds ratio. Four OPP (113, 201, 206, 214) were associated to a higher risk of prevalence (95% confidence interval, CI, 1.97-6.00) and other four (OPP 207, 210, 212, 321) were linked to lower risk (95% CI 0.02-0.74). The other five OPP (121, 203, 205, 209, and 211) are not statistically significant (95% CI 0.63-1.75). The results of the variables herd size (OR_{adj} 1.007 and 95% CI 1.006-1.008) and year (OR_{adj} 0.655 and 95% CI 0.629-0.681) were similar to those recorded in the first analysis. All the results are in supplementary table 3.

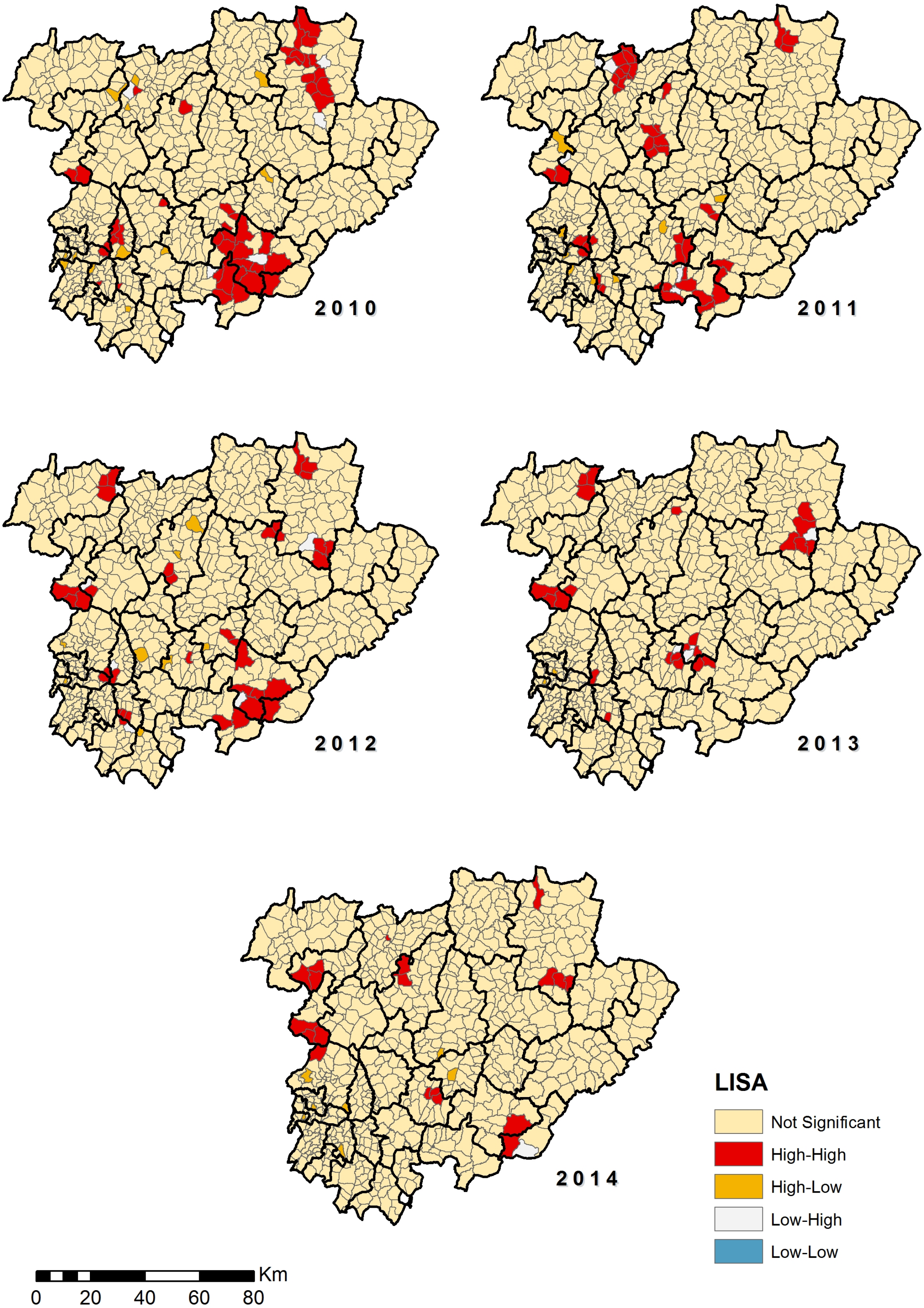


Figure 4 Cluster maps of Local Indicators of Spatial Association (LISA) of small ruminant brucellosis prevalence in Trás-os-Montes and Alto Douro between 2010 and 2014.

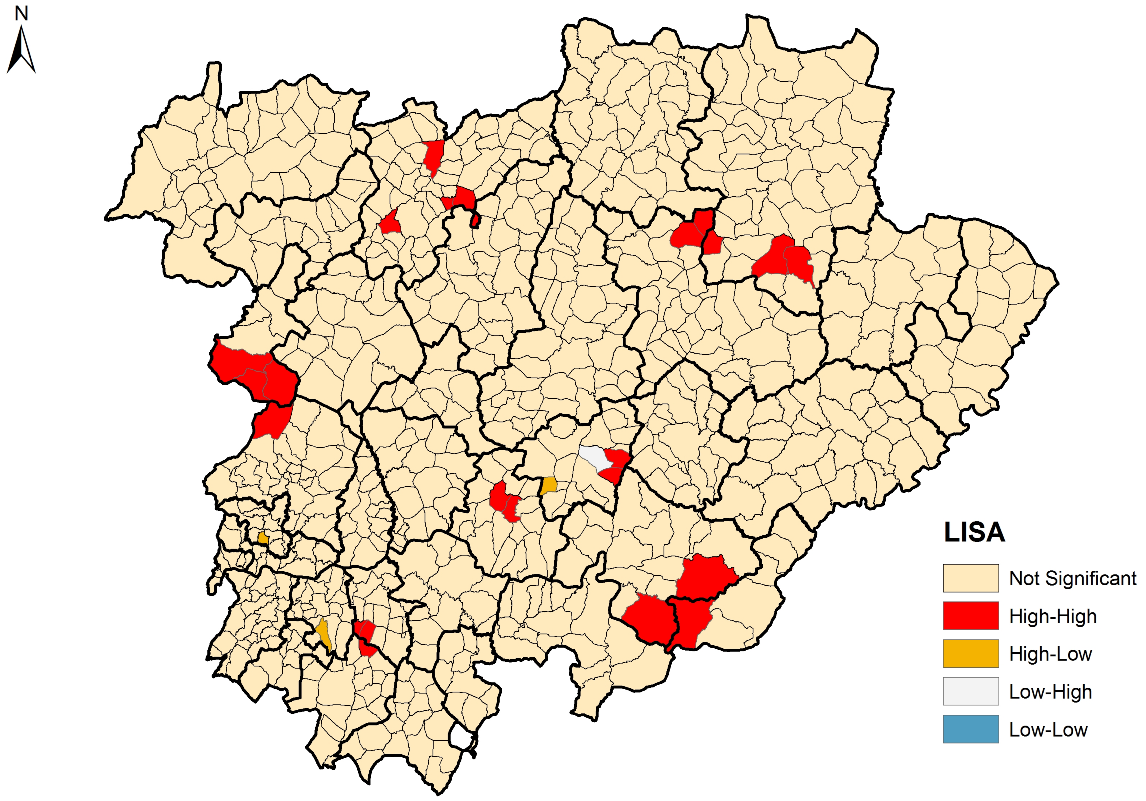


Figure 5 Cluster map of Local Indicators of Spatial Association (LISA) of cumulative incidence rate of brucellosis in Trás-os-Montes and Alto Douro, 2010-2014.

4 Discussion

As it was expected, the results from the summarized prevalence and incidence of SRB in Trás-os-Montes and Alto Douro showed a noticeable decrease in both measures. Even though prevalence decreased drastically in the first years, the same was not detected in the last two years and there might be a starting tendency in prevalence to slowly decrease and stabilize in lower levels. A marked drop of incidence was noted, but the unexpectedly increase in 2013 caught the attention. Although prevalence and incidence levels have decreased over the years, the changes in incidence are not as tarnished as in the prevalence, suggesting that (a) the resources applied and measures taken might have not been as efficient in decreasing the occurrence of new cases of disease as to controlling the presence of the disease, or (b) they have not been applied efficiently. The spatial patterns of brucellosis prevalence showed also a decreased equally in higher prevalence villages and in the number of villages with the disease, evidences consistent with the previous results. The effectiveness of the SRB eradication program is then notable predominantly in the first years when prevalence was very high, but when prevalence reached lower levels the effects become less notorious. The idea of spatial association of SRB prevalence that follows from the visual inspection of the maps is confirmed by the positive and significant value of the Moran's Index and the identification of local clusters. Moreover, several villages maintained the disease during several years and few are clusters during more than one year. The key to understand the maintenance of brucellosis in the same area is to better scrutinize the risk factors associated with this disease in that specific location so that the viable predominant routes of transmission of the agent, for instance, could be addressed with special measures. The longer duration of lambing season, hygiene of parturition, general management of the herd (hygiene, new-born, replacement animals, separation of serologically positive animals from pregnant ewes and their lambs), herd size and brucellosis campaign measures (frequency of testing, vaccination, duration of the delay in removal from the herds of positive animals)(9) are some of the factors herd-specific that may help explain this pattern in certain villages. Based on a previous study carried out in this same area to identify potential risk factors, the sanitary management of the herd, hygiene and displacement of animals without control play significant roles in brucellosis epidemiology in Trás-os-Montes and Alto Douro, where the major risk detected concerned the introduction of animals from non-free brucellosis or non-classified herds(33).

The findings on the standardized ratios for both prevalence and incidence are similar to those found in the maps of the same crude disease indicators with small differences. Some of those changes are in specific villages that although having the disease, when compared to the expected population they were found to have lower risk. The advantage of the use of a standardization method as to a metric in this study is the ability to perform more detailed comparisons within a risk region, especially when in presence of an aggregation level, in this case at village level. As a result, the effect of the number of herds in each village did not distort comparisons between different areas.

Animals are grouped in flocks and since this is a contagious disease transmitted by direct and indirect contact with infected animals or their contaminated secretions and objects, it would be expected that geographic proximity would play a major role in the transmission of the disease among different herds and flocks previously free from disease. This hypothesis could also be extrapolated to villages, and so those with higher incidence would be geographically associated in incidence clusters across the region. Consequently, the expected outcome of the spatial analysis of the incidence maps was the presence of statistically significant spatial association and existence of incidence clusters. The results from both analyses showed that incidence was independent from the location of villages with the exception of the distribution map of 2013, in which local clusters of incidence where identified. Considering that most herds in that area are under extensive manage conditions(22) and the practice of communal grazing is frequent, the contact between flocks from different herds and villages is a plausible risk factor. The results presented above suggest that incidence by village in each year is not spatial autocorrelated, so incidence in one village is not primarily affected by the percentage of incidence in neighbouring villages. This raises the following question: if proximity between villages is not a major risk factor for incidence of infection, what other risk factors play an important role in the appearance of new cases of disease in Trás-os-Montes and Alto Douro? The introduction of animals from infected herds or herds with unknown status was presented as one of the main risk factors for the introduction of the disease in brucellosis free herds[56]. As a consequence, further investigations regarding animal movement are important to understand both herds with a major role in sheep and goats commerce but also both in and out-going chains of animals in the region. The sources of purchased animals –dealers, markets, and other farmers- should then be more carefully evaluated particularly in this region.

The cumulative incidence rate reflects the fluctuations of the population of herds at risk during the time period in each village. The fact that spatial association was found in this analysis and local clusters were identified, contrasting to the analysis of the individual maps of incidence called our attention. Perhaps, due to the fact that this measure incorporates the oscillations of incidence in the course of the years, this disease indicator had more strength in reproducing what happened during that period in each village.

The percentage of vaccination coverage was not reviewed in this study; however, vaccination is referred as one of the major methods for controlling the infection and elimination of the disease[57] and its direct impact, especially on the incidence, must be taken in account. The increase in the number of immune animals, the reduction in the number of abortions and mostly the excretion of the agent emphasize the necessity to reinforce its application in herds located in higher risk villages and areas. The conventional vaccination of young animals under extensive management conditions (common in this region) is proved to be not effective in eliminating all sources of infection as the adults continue to be unprotected and the infection can freely disseminate. A strategy including whole-flock vaccination every two years in herds under those conditions could be the only feasible option for Trás-os-Montes primarily in villages or counties with a higher prevalence of *B. melitensis* infection[58]. In case of moderate to low village prevalence the combination of immunization of young replacement animals and test and slaughter in adults is recommended[59]. The presence of risk factors that cannot be controlled justifies the application of Rev1 vaccine also in herds with low prevalence.

Test and slaughter of positive animals in big flocks with high prevalence is proved to be ineffective and unreliable since some of the infected animals might not react to the diagnostic tests, especially if in the latent phase of the disease and the elimination of the organism from the herd is a no-end mission. On the other hand, this method is most effective when applied to all flock in infected herds and particularly in newly infected herds in healthy areas. Although expensive and difficult to justified to producers, it consists in a fast and reliable method to eradicate permanently brucellosis and more economical in a long-term perspective. When this policy cannot be applied an alienation of vaccination of a wider spectrum of the animal population is necessary.

Successful schemes will depend on incorporating new affordable techniques of epidemiological planning that identify problem or higher risk herds, or areas such as villages that are identified as clusters of prevalence in more than one year, or even use this analysis as a preliminary prioritizing scale for further investigations in herds belonging to those high risk clusters. Intervention target-specific can address the particular risk factors and limitations and hopefully lead to an efficient eradication program. Moreover, the maintenance of brucellosis-free herds and villages may be the first step to gradually eliminate the sources of infection and simultaneously targeting higher risk villages and municipalities. Implementing more regulated measures in the control of animal movements can also help to prevent the dissemination of the infection.

Under the multivariable analysis, were detected several risk counties. The odds ratio of prevalence was more than 3 times higher in herds from the municipalities of Ribeira de Pena, Valpaços, Sabrosa, Carrazeda de Ansiães and Torre de Moncorvo comparing to Vinhais. Interestingly to us, the three with the lowest risk of prevalence are geographically contiguous and share limits with Bragança, a high-risk municipality. Furthermore, the odds ratio of incidence was 7 times higher in Ribeira de Pena and Carrazeda de Ansiães municipalities also when compared to Vinhais. Carrazeda de Anseães and Miranda do Douro are a high and low risk municipalities, respectively, in both analysis. In relation to herd size, our results agree with other researches, which have also noted an increase in risk of infection with an increase in the number of animals. The year had also a significant influence on the risk of infection but in the opposite direction as expected due to the general trend of decrease of SRB.

The evidence of lack of association between prevalence clusters and incident villages suggests an independent process between the occurrence of new cases and the coexistence of the disease again in accordance with the results of the spatial analysis. The OPP associated (113, 201, 206, 214) to a higher risk of prevalence interestingly work mainly in counties from Vila Real district with the exception of OPP 201 that works in Bragança and Guarda districts.

It was not possible to establish if time-space clusters could be identified, since it was only measured spatial association by year, so further work in this area can been beneficial. The three biggest limitations of this study are: (a) the exclusion in the analysis of the municipality of Mondim de Basto for reasons already clarified; (b) the population of herds included in the study is possibly a subpopulation of the actual small ruminants' herds across the region since some might not be register in the SRB eradication program or the sanitary status might have not been established so far; finally, (c) regarding the sanitary status, the use of data by year might have underestimated the real evolution and surveillance of health status in herds across the region. This latter fact is however questionable once we evaluate the speed with which herds can update their sanitary status. For instances, in order to a B2.1 herd change to the B3 status it would take more than one year, and during that time it would had been through at least four controls of diagnostic tests to all animals.

One of the strongest points of this study is the use of core group of indicators of health status for quantify disease occurrence and prioritize disease interventions. The used of the sanitary status as an indicator of presence or absence of disease is a new method that can be useful for planners from animal health officers for managing village and municipality level activities and select high priority herds to be monitored.

The control of small ruminant brucellosis requires the same basic key elements common to other animal diseases: surveillance to identify animals and herds; control of transmission of infection to herds previously free from disease and eradicate the reservoirs in order to eliminate the source of infection to susceptible animals and herds. This study shows that with GIS and spatial analysis it was possible to visualize regions with high incidence and prevalence and identify spatial patterns of disease, which have led to the identification of higher risk villages and target regions. This tool can be readily use by the veterinary services to implement a systematic surveillance and evaluate the disease at village and municipality levels so it is possible to design strategic intervention plans in problem areas and herds and thereby allocate the available resources according to a priority risk scale. The success of eradication program is influenced by several factors such as the implementation of an efficient surveillance system with an adequate laboratory support and understanding and sharing of eradication goals between producers, veterinary services and other relevant intervenient(1). The eradication of brucellosis is strongly related to the degree of control of the sources of infection, which is consequently associated with the cooperation between authorities and producers. The latter play a determinant role in this campaign since being aware of the dangers and economical losses related to this disease emphasizes the importance of self-responsibility.

Conclusions

This study shows that brucellosis prevalence in Trás-os-Montes and Alto Douro is clustered in certain villages while incidence seems to be randomly distributed across the region, except in 2013, suggesting that proximity between villages might not be the main route of transmission of the disease from infected to non-infected villages. The association between OPP, municipalities, herd size and year with disease could interest investigators to understand the underlying risk factors associated with SRB in that region.

A core group of indicators resembling the ones used in this study could be useful for animal health authorities to integrate a scheme of prioritization of animal health problems at village, county or district level. Moreover, a spatial decision support system could be design to incorporate these novel techniques and contribute in the future to identify new infection focus, evaluate and classify the risk of neighboring regions, determine the risk of infection and prevent herds at risk from being exposed. The use of GIS requires available and quality data, which implies a continuous updated database. Further work in this area should be directed towards collecting more information flock or herd-specific and acquiring georeferenced data from herds, working place and activity profiles would bring more advantages in the investigation of brucellosis and other animal diseases.

Finally, this study draws the attention of the animal health services to the benefits of mapping disease occurrence so that when planning future interventions, resources may be better allocate to high priority areas.

Bibliography

1. FAO-OIE-WHO (2006) **Brucellosis in humans and animals.** WHO Library Catalogue Data, 1–88.
2. Foster G, Osterman BS, Godfroid J, Jacques I, Cloeckert A (2007) *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. **Internatioonal Journal of Systematic and Evolutionary Microbiology**. 57(11), 2688–2699.
3. Scholz HC, Hubalek Z, Sedlácek I, Vergnaud G, Tomaso H, Al Dahouk S, *et al.* (2008) *Brucella microti* sp. nov., isolated from the common vole *Microtus arvalis*. **International Journal of Systematic and Evolutionary Microbiology,** 58(2), 375–82.
4. Scholz HC, Nockler K, Llner CG, Bahn P, Vergnaud G, Tomaso H, *et al.* (2010) *Brucella inopinata* sp. nov., isolated from a breast implant infection. **International Journal of Systematic and Evolutionary Microbiology**. 60(4):801–8.
5. Liu D (2014) Manual of Security Sensitive Microbes and Toxins. In: **Manual of Security Sensitive Microbes and Toxins,** 313–318.
6. OIE (2012) Caprine and ovine brucellosis (excluding *Brucella ovis*). **OIE Terrestrial Manual 2012.** 968–977.
7. Blasco JM, Molina-Flores B. (2011) Control and eradication of *Brucella melitensis* infection in sheep and goats. **Veterinary Clinics of North America Food Animal Practice** ; 27(1), 95–104.
8. Entessar F, Ardalan A, Ebadi A, Jones LM. (1967) Effect of living Rev.1 vaccine in producing long-term immunity against *Brucella melitensis* infection in sheep in Iran. **Journal of Comparative Pathology.** 77(4), 367–376.
9. Verger JM, Plommet M, editors. *Brucella melitensis* [Internet]. Vol. 32, Current topics in veterinary medicine and animal science. Martinus Nijhoff Publishers; 1984.
10. Thimm BM (1982) Brucellosis: Distribution in Man, Domestic and Wild Animals. **Sitzungsberichte der Heidelberger Akademie der Wissenschaften, Mathematisch-Naturwissenschaftliche. Springer-Verlag Berlin Heidelberg**
11. Beran GW Handbook of Zoonoses. Second Edi. 1994.
12. Louza A (1993) Brucelose- modelo de zoonose de impacto sócio-económico, Parte II. **Medicina Veterinária.** 44:61–7.
13. Ficht T (2011) *Brucella* taxonomy and evolution. **Future Microbiology**. 5(6):859–866.
14. Crespo F (1994) Influencia de los elementos y factores geográficos en la epidemiología de la brucelosis del ganado ovino y caprino. **Papeles de Geografia**. 20(6),189–209.
15. European Commission (2001) Brucellosis in Sheep and Goats. **Scientific Committe for Animal Health and Animal Welfare.** 20.
16. Alavi-Shoushtari SM, Zeinali A (1995) Responses of female lambs to Rev-1 (brucellosis) vaccination. **Preventive Veterinary Medicine**. 21(4):289–297.
17. European Commission (2014) Report of the meeting of the “Bovine, Sheep and Goat Brucellosis” sub-group of the Task Force for monitoring disease eradication. Vila Real, Portugal.
18. Mainar-Jaime RC, Vázquez-Boland JA (1990) Associations of veterinary services and farmer characteristics with the prevalences of brucellosis and border disease in small ruminants in Spain. **Preventive Veterinary Medicine**. 40(3-4):193–205.
19. Aleixo MJ, Ferreira ML, Antunes F (1999) Brucellosis. **Acta Médica Portuguesa** 12, 323–30.

20. Xavier MN, Paixão TA, den Hartigh AB, Tsolis RM, Santos RL (2010) Pathogenesis of *Brucella* spp . **Open Veterinary Science Journal.** 4, 109–118.
21. Garin-Bastuji B, Blasco JM, Grayon M, Verger JM (1998) *Brucella melitensis* infection in sheep - present and future [Review]. **Veterinary Research.** 29(3-4), 255–74.
22. DGAV Programa de erradicação da brucelose dos pequenos ruminantes. 2014;(1407167984934).
23. Assembleia da República (2000) Decreto-lei n^{o} 244/200 de 27 de Setembro. **Diário da República - I Série A.** 5207.
24. OIE (2006) Brucellosis in humans and animals. 1–102.
25. Fensterbank R (1986) Brucellosis in cattle, sheep and goats: diagnosis, control and vaccination. **Revue Scientifique et Techique** 5 (May), 605–618.
26. Elberg SS, Henderson DW, Herzberg M, Peacock S (1995) Immunization against Brucella infection. IV. Response of monkeys to injection of a streptomycin-dependent strain of Brucella melitensis. **Journal of Bacteriology.** 69, 643–648.
27. Schurig GG, Sriranganathan N, Corbel MJ (2002) Brucellosis vaccines: past, present and future. **Veterinary Microbilogy** 90, 479–496.
28. Fensterbank R, Pardon P, Marly J (1982) Comparison between subcutaneous and conjunctival route of vaccination with Rev. 1 strain against *Brucella melitensis* infection in ewes. **Annales des Rechearches Veterinaires.** 13(4), 295–301.
29. Assembleia da República (2006) Decreto-Lei n^{o} 142/2006 de 27 de Julho. **Diário da República, 1^{a} série.** 5367–5368.
30. Reviriego FJ, Moreno MA, Domínguez L (2000) Risk factors for brucellosis seroprevalence of sheep and goat flocks in Spain. **Preventive Veterinary Medicine.** 44(3-4), 167–173.
31. Lithg-Pereira PL, Mainar-Jaime RC, Alvarez-Sánchez MA, Rojo-Vázquez FA (2001) Evaluation of official eradication-campaigns data for investigating small-ruminant brucellosis in the province of León, Spain. **Preventive Veterinary Medicine.** 51(3-4), 215–225.
32. Maria Vaz Y (1996) Analysis of policies for the eradication of brucellosis from sheep and goats in Portugal. **University of Reading, England.**
33. Coelho AM, Coelho AC, Roboredo M, Rodrigues J (2007) A case-control study of risk factors for brucellosis seropositivity in Portuguese small ruminants herds. **Preventive Veterinary Medicine**. 82(3-4), 291–301.
34. Fonseca AP (2011) Situação e perspectivas do controlo da brucelose dos ovinos e caprinos em Portugal. In: **V Congresso Sociedade Portuguesa de Ciências Veterinárias: Livro de Resumos**.
35. Tobler WR (2011) Automation and Cartography. In: **The Map Reader: Theories of Mapping Practice and Cartographic Representation**. 137–140.
36. Snow J (1854) On the mode of communication of cholera. Delta. 1–97.
37. Kazmi SJH, Usery EL (2001) Application of remote sensing and gis for the monitoring of diseases: A unique research agenda for geographers. **Remote Sensing Reviews** 20(1), 45–70.
38. Haining R (2003) Spatial Data Analysis.
39. Waller L, Gotway C (2004) Applied Spatial Statistics for Public Health Data. JohnWiley & Sons, Inc., Hoboken, NJ.

40. Tobler WR (1970) A computer movie simulating urban growth in the Detroit region. **Economic Geography**. 234–240.
41. Clarke KC, McLafferty SL, Tempalski BJ (1996) On epidemiology and geographic information systems: a review and discussion of future directions. **Emerging Infectious Diseases** 2(2), 85–92.
42. Bailey TC, Gtroll AC. (1995) Interactive Spatial Data Analysis. p 413.
43. D'Orazi A, Mignemi M, Geraci F, Vullo A, Di Gesaro M, Vullo S, *et al.* (2007) Spatial distribution of brucellosis in sheep and goats in Sicily from 2001 to 2005. **Veterinari Italiana.** 43(3):541–548.
44. Mainar-Jaime RC, Ligh-Pereira PL, Epp T, Waldner C (2005) The application of spatial analysis tools in Small-Ruminant Brucellosis Eradication Programs in Northern Spain. **International Journal of Applied Research in Veterinary Medicine.** 3(3), 179–188.
45. Anselin L (1996) The Moran scatterplot as an ESDA tool to assess local instability in spatial association. In: **Spatial Analytical perspectives on GIS.** p. 111–125.
46. Anselin L (1995) Local indicators of spatial association — LISA. **Geography Analysis.** 27(2), 93–115.
47. Portuguese General Direction of Territory (2015) Portuguese cartography [Internet]. Available from: http://www.dgterritorio.pt/cartografia_e_geodesia/cartografia/carta_administrativa_oficial_de_portugal__caop_/caop__download_/
48. Thrusfield M, Blackwell V, Scientific UK, Tiller R, Gee JE, Frace MA, *et al.* (2010) Veterinary Epidemiology. Vol. 76 SRC p. 5837-5845
49. Breslow NE, Day NE, International Agency for Research on Cancer. **Statistical methods in cancer research Volume I- The Analysis of Case-Control Studies**. Stat Methods Cancer Res. 1980;1, 346.
50. Little TD, editor (2013) The Oxford Handbook of Quantitative Methods. Vol. 1: Foundat, Oxford Library of Psychology. Oxford University Press
51. Anselin L, Hudak S (1992) Spatial econometrics in practice. A review of software options. **Regional Science and Urban Economics.** 22(3), 509–536.
52. Getis A, Aldstadt J (2004) Constructing the Spatial Weights Matrix Using a Local Statistic. **Geographycal Analysis** 36(2), 90–104.
53. ESRI. ArcGIS for Desktop [Internet] Available from: http://www.esri.com
54. IBM Analytics. IBM SPSS Statistics Software [Internet]. Available from: http://www.ibm.com/analytics/us/en/technology/spss/
55. Bell Laboratories R software [Internet]. Available from: https://www.r-project.org
56. Coelho AM, Coelho AC, Góis J, Pinto M de L, Rodrigues J (2008) Multifactorial correspondence analysis of risk factors for sheep and goat brucellosis seroprevalence. **Small Ruminant Research**. 78(1-3), 181–185.
57. B G-B, Benkirane A (1995) Use of REV 1 Vaccine in Small Ruminants and Cattle. **FAO/WHO/OIE Round Table on the use of Rev. 1 Vaccine in Small Ruminants and Cattle.**
58. Robinson A (2003) Guidelines for coordinated human and animals brucellosis surveillance. **FAO Animal Production Health Paper.**
59. European Commission (2013) Report on the meeting of the task force for monitoring animal disease eradication in the member states.

Annexes

Supplementary formula 1 Prevalence of positive herds

$$\boldsymbol{Prevalence\ of\ positive\ herds_{it}} = \frac{\sum_{j=1}^{n_i} Herds\ B2.1, B2, B3S, B4S}{\sum_{j=1}^{n_i} All\ herds\ (B2.1, B2, B3, B3S, B4, B4S)}$$

Supplementary formula 2 Incidence risk of B2

$$\boldsymbol{Incidence\ risk\ B2_{it}} = \frac{\sum_{j=1}^{n_i} New\ herds\ B2.1, B2\ at\ t+1}{\sum_{j=1}^{n_i} Herds\ at\ risk\ at\ t\ (B3, B3S, B4, B4S)}$$

Supplementary formula 3 Standardized prevalence ratio

$$\boldsymbol{SPR_{it}} = \frac{observed_i}{expected\ _i} = \frac{\sum_{j=1}^{n_i} Herds\ B2.1, B2}{\frac{\sum_{i=1}^{708}\sum_{j=1}^{n_i} Herds\ B2.1, B2}{\sum_{i=1}^{708}\sum_{j=1}^{n_i} All\ herds} \times \sum_{j=1}^{n_i} All\ herds}$$

Supplementary formula 4 Standardized incidence ratio

$$\boldsymbol{SIR_{it}} = \frac{observed_i}{expected\ _i} = \frac{\sum_{j=1}^{n_i} New\ herds\ B2.1}{\frac{\sum_{i=1}^{708}\sum_{j=1}^{n_i} New\ herds\ B2.1}{\sum_{i=1}^{708}\sum_{j=1}^{n_i} Herds\ at\ risk\ (B2, B3, B3S, B4, B4S)} \times \sum_{j=1}^{n_i} Herds\ at\ risk}$$

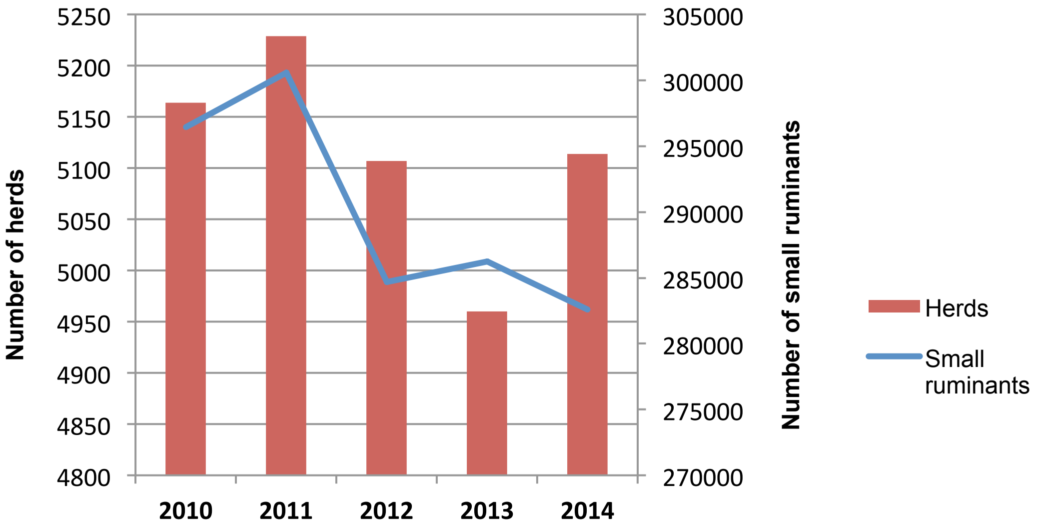
Supplementary formula 5 Global Moran Index

$$\boldsymbol{I = \frac{n}{S_0} \frac{\sum_{i=1}^{n} \sum_{j=1}^{n} w_{i,j}\ z_i\ z_j}{\sum_{i=1}^{n} z_i^2}}$$

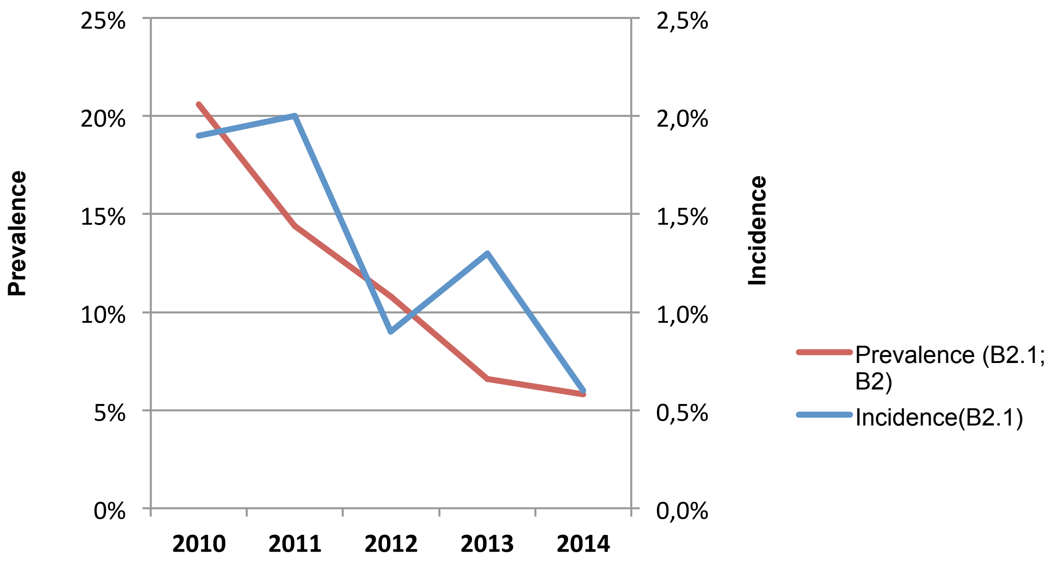
Where z_i^2 is the deviation of an attribute for feature i from its mean$(x_j - \bar{X}\ )$, $w_{i,j}$ is the spatial weight between feature i ad j and S_0 is the aggregate of all the spatial weights.

Supplementary Table 1 Results from the Global Moran's I statistic for small ruminant brucellosis incidence of B2.1 in Trás-os-Montes and Alto Douro, Portugal between 2010-2014.

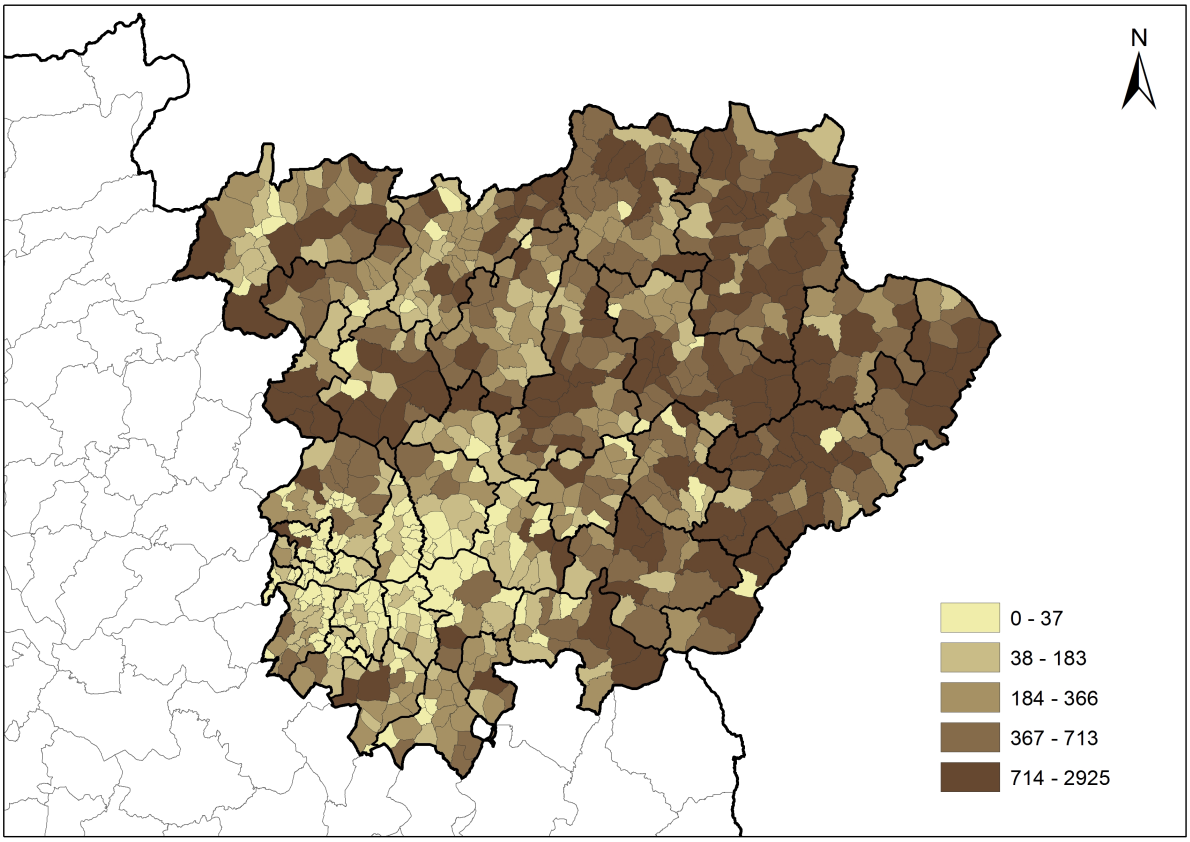
Global Moran's Index						
Year	**Moran's Index**	**Expected Index**	**Variance**	***z-score***	***p-value***	**Pattern**
2010	0.022320	-0.001418	0.000474	1.089780	0.275810	Random
2011	0.021240	-0.001418	0.000495	1.018653	0.308368	Random
2012	-0.002872	-0.001418	0.000424	-0.070611	0.943708	Random
2013	0.042462	-0.001418	0.000479	2.004089	0.045061	Clustered
2014	-0.004262	-0.001418	0.000362	-0.149445	0.881203	Random



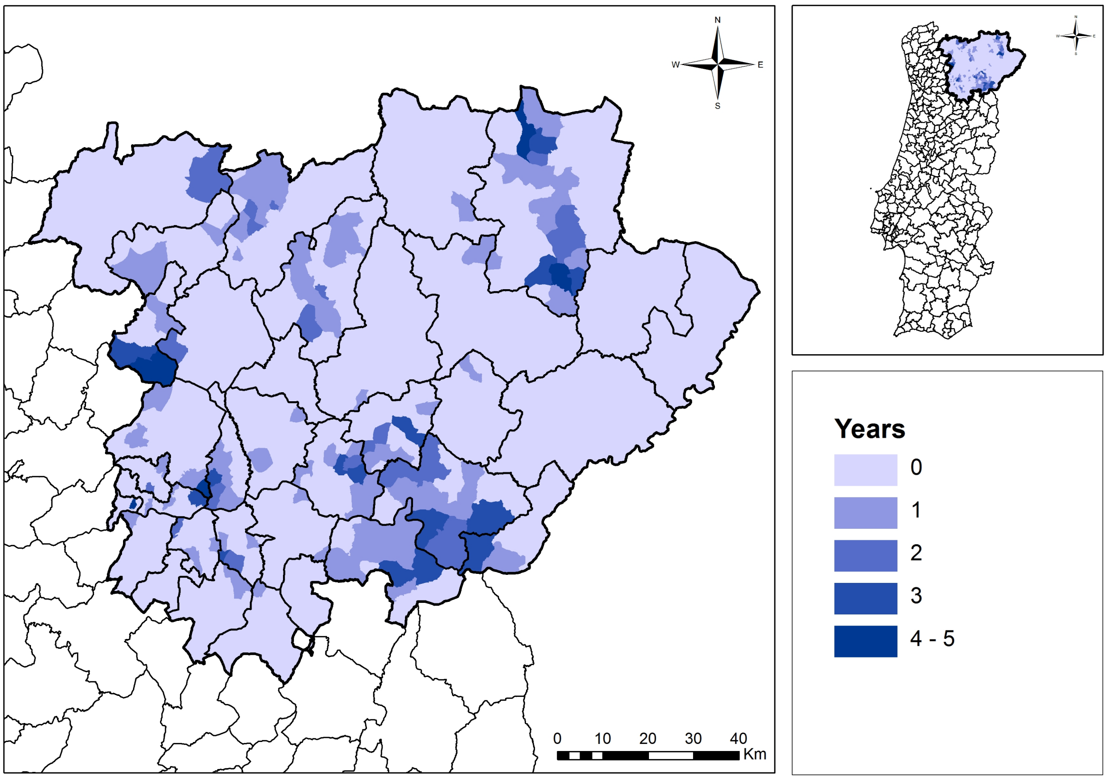
Supplementary figure 1 Annual distribution of herds and small ruminants in Trás-os-Montes and Alto Douro, 2010-2014.



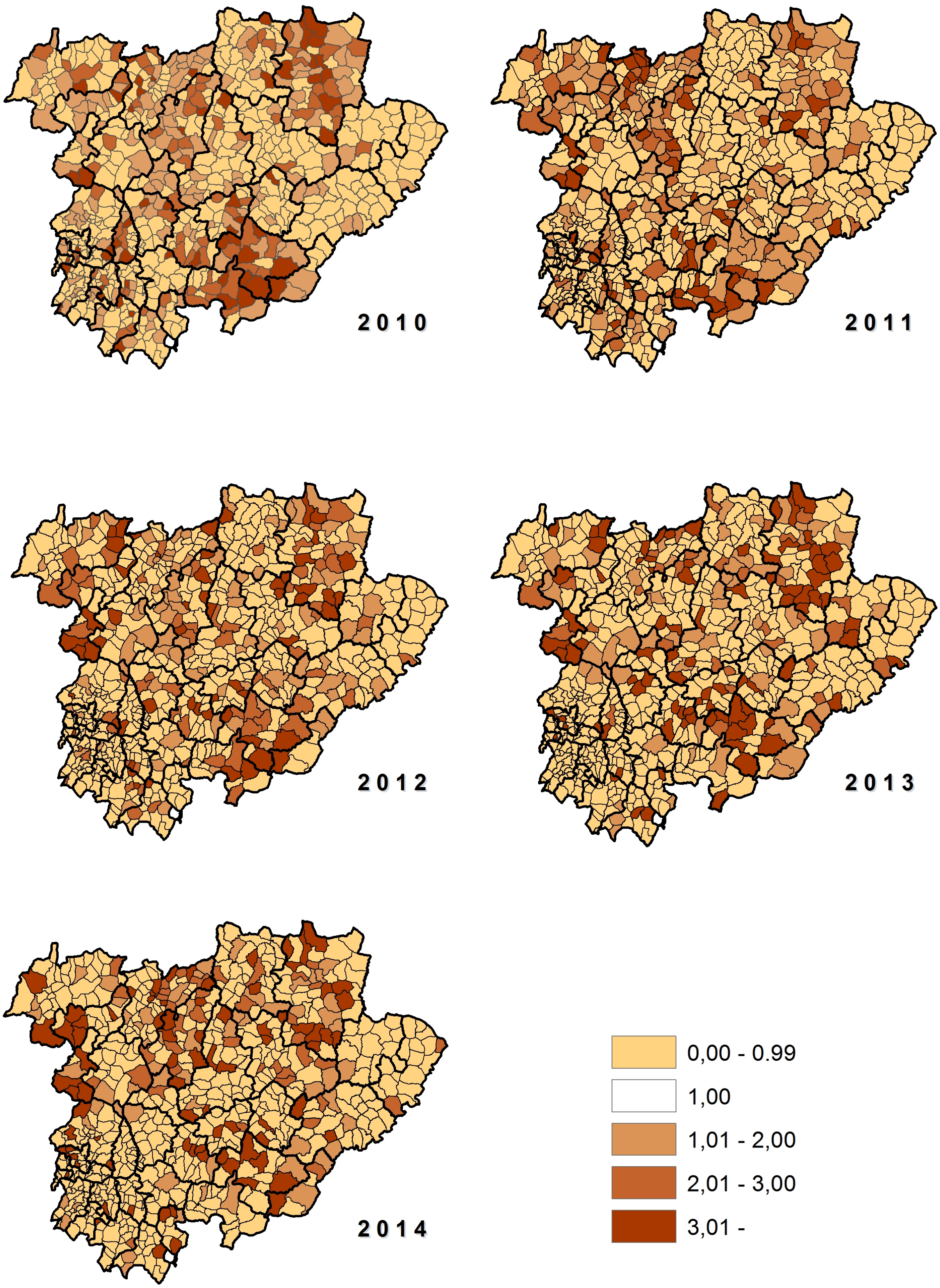
Supplementary figure 2 Annual prevalence and incidence in Trás-os-Montes and Alto Douro, 2010-2014.



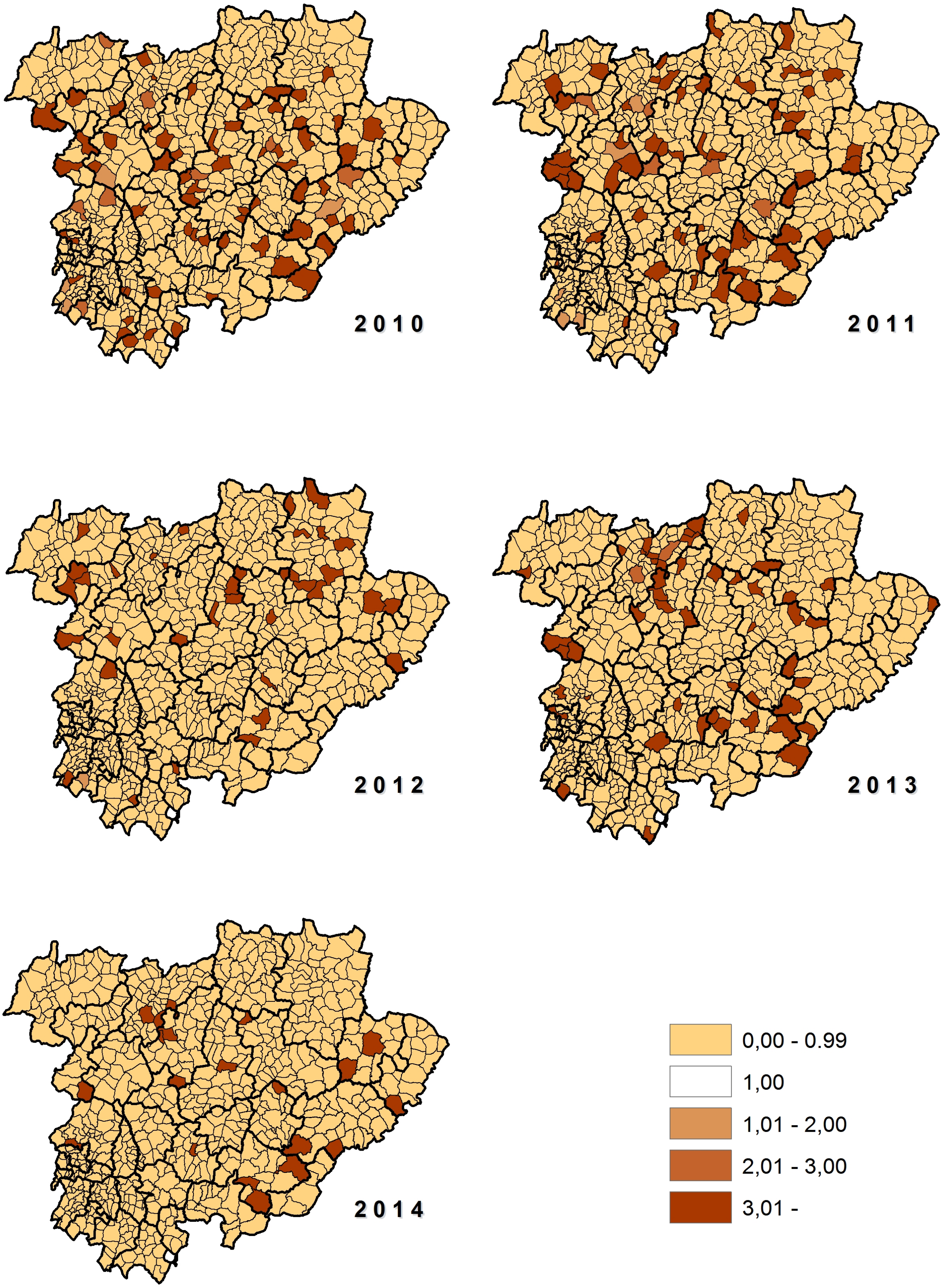
Supplementary figure 3 Map with the average number of small ruminants across Trás-os-Montes and Alto Douro from 2010 to 2014



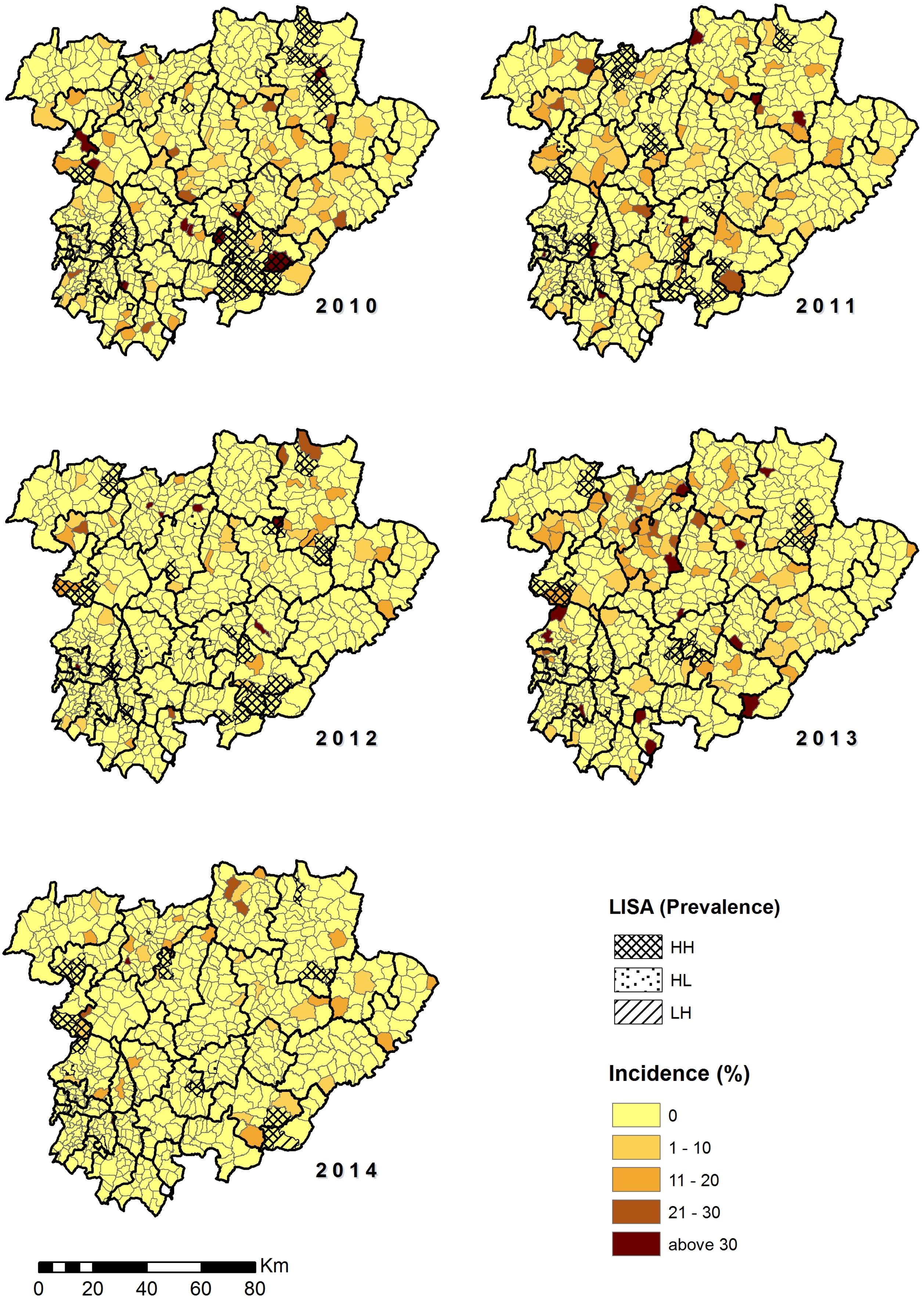
Supplementary figure 4 Number of years being a cluster/outliers of prevalence of B2 between 2010-2014 across Trás-os-Montes and Alto Douro



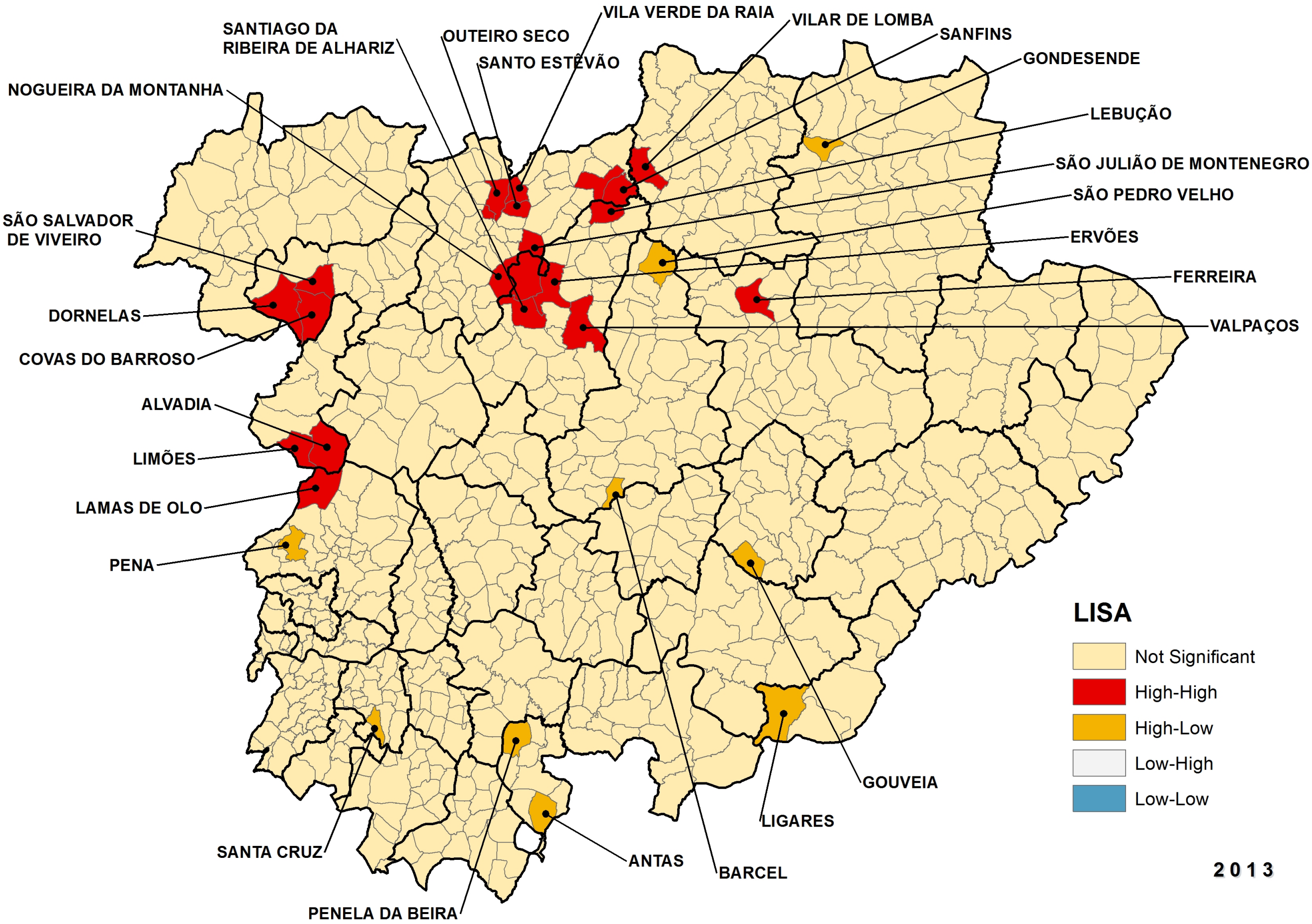
Supplementary figure 5 Distribution of small ruminant brucellosis: standardized prevalence ratio in Trás-os-Montes and Alto Douro between 2010 and 2014.



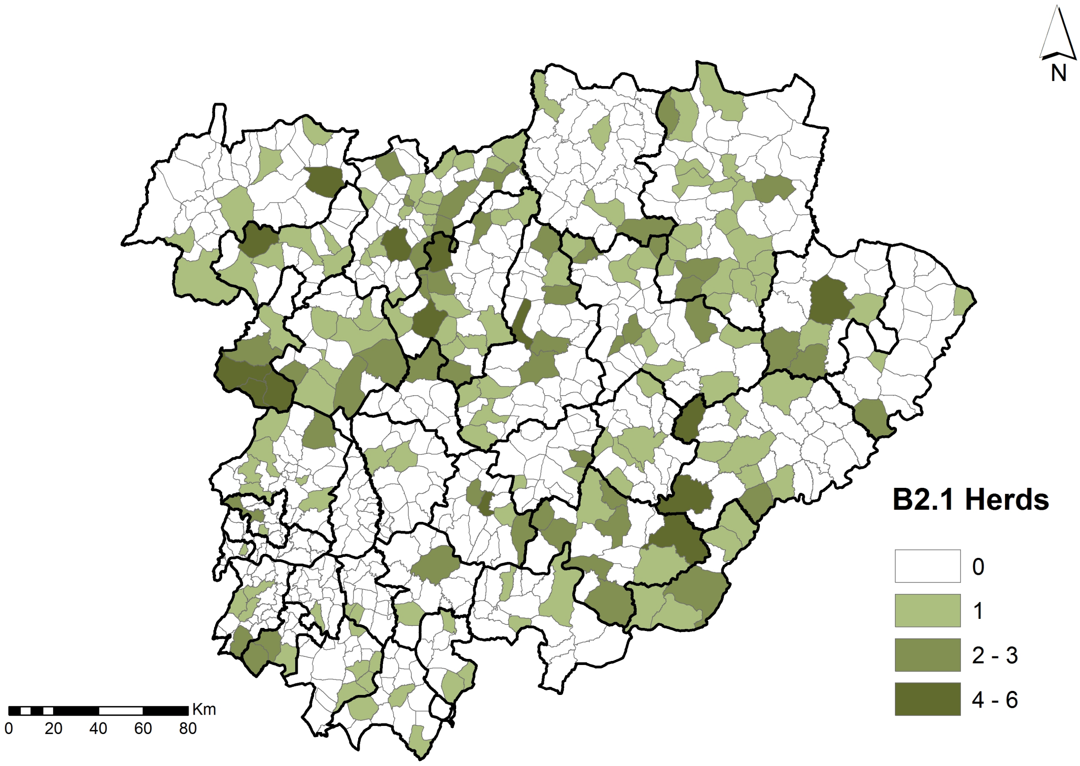
Supplementary figure 6 Distribution of small ruminant brucellosis: standardized incidence ratio in Trás-os-Montes and Alto Douro between 2010 and 2014.



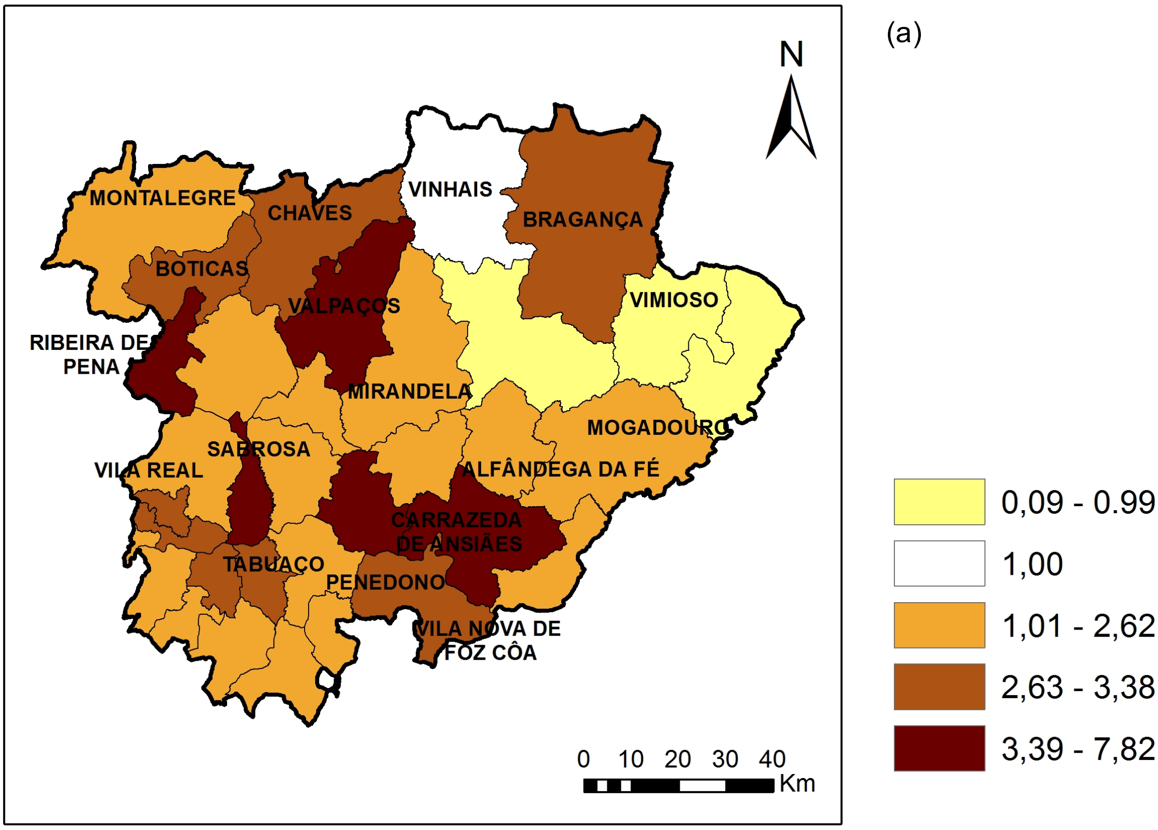
Supplementary figure 7 Maps of incidence of B2.1 and clusters and outliers of prevalence of B2 in Trás-os-Montes and Alto Douro during 2010-2014.

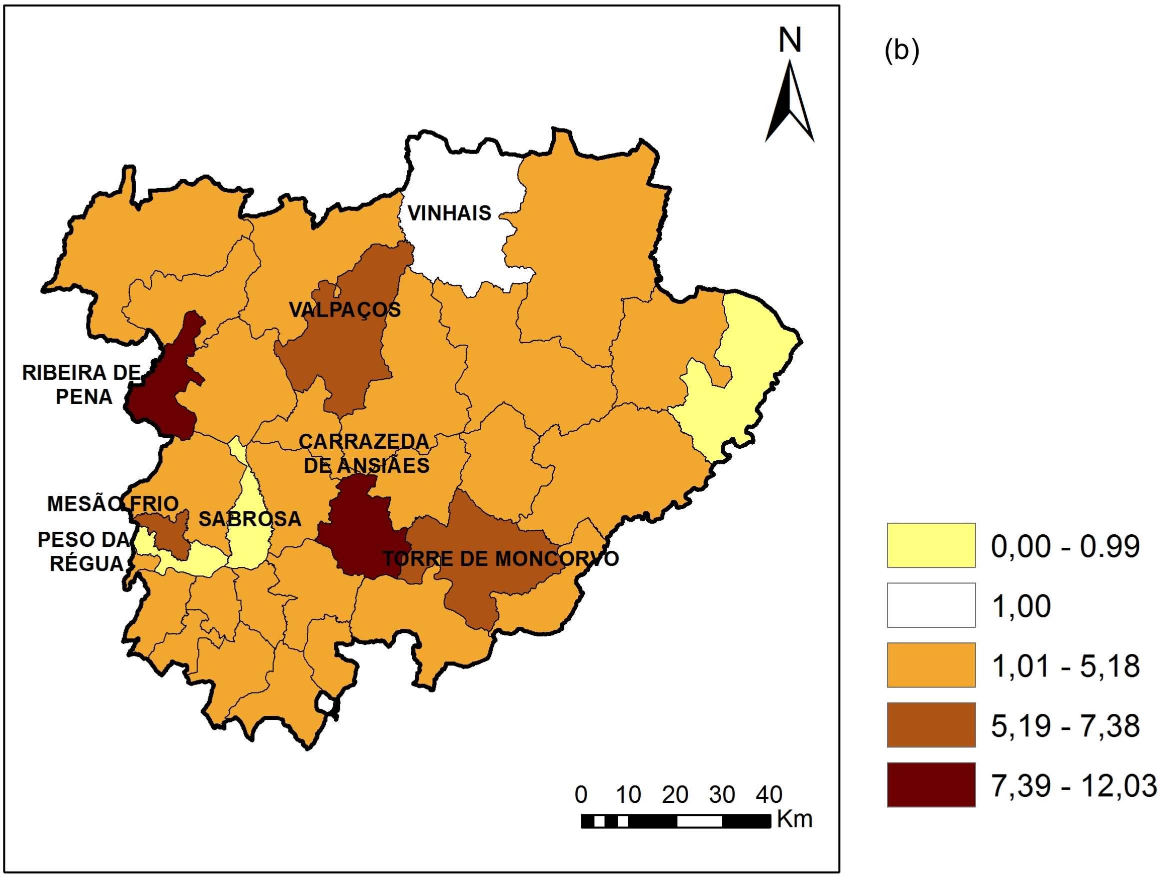


Supplementary figure 8 Cluster and outliers map of LISA of incidence of B2.1 in 2013 in Trás-os-Montes and Alto Douro with the legend of villages.



Supplementary figure 9 Distribution of reported incident B2.1 herds in Trás-os-Montes and Alto Douro, 2010-2014.





Supplementary figure 10 Maps with the odds ratio resultant of multivariable analysis of prevalence (a) and incidence (b) across the municipalities of the region Trás-os-Montes and Alto Douro, 2010-2014.

Supplementary Table 2 Results from the Fisher's Exact Test for prevalence clusters (Clusters HH) and incidence (IncB2.1) of small ruminant brucellosis in Trás-os-Montes and Alto Douro, Portugal between 2010-2014.

			2010		**2011**		**2012**		**2013**		**2014**	
			IncB2.1		IncB2.1		IncB2.1		IncB2.1		IncB2.1	
			0	1	0	1	0	1	0	1	0	1
Clusters HH	0	Observed	593	69	593	69	634	36	594	89	652	35
		Expected	592.6	69.4	596.4	65.6	633	37	595	88	652	35
	1	Observed	39	5	43	1	33	3	21	2	18	1
		Expected	39.4	4.6	39.6	4.4	34	2	20	3	18	1
Fisher's Exact Test			0.800		0.113		0.442		0.756		1.000	

Supplementary Table 3 Study of the risk of prevalence in different OPP in Trás-os-Montes and Alto Douro, Portugal between 2010-2014 (results from the binary logistic model).

	OR	**95% CI**	
OPP321	0.102	0.017	0.324
OPP210	0.217	0.151	0.307
OPP212	0.551	0.416	0.727
OPP207	0.557	0.416	0.742
OPP121	0.890	0.634	1.233
OPP205	0.908	0.716	1.153
OPP203	1.116	0.909	1.377
OPP209	1.117	0.856	1.453
OPP211	1.255	0.892	1.745
OPP201	2.495	1.970	3.166
OPP214	2.575	2.067	3.220
OPP113	2.891	1.988	4.161
OPP206	4.436	3.278	5.995
Year	0.655	0.629	0.681
Herd size	1.007	1.006	1.008