Predictors of Bluetongue development in Sardinia (Italy) identification, using multilevel logistic mixed model

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ABSTRACT

Objectives: The Bluetongue Virus is one of the most studied ruminant diseases, affecting particularly sheep and goats. This study aims to identify, for the first time, the specific risk factors influencing the disease development in Sardinia, using multilevel logistic regression model, in order to give a contribution to the sanitary programs and favour the early detection.

Methods: The data of the present retrospective study, collected from informatics systems of Istituto Zooprofilattico della Sardegna, are referred to all 15,780 Sardinian sheep farms observed for 3 years (2012-2014). The outcome of interest was dichotomous and defined the development of Bluetongue outbreak, after serological test or clinical signs. The effect of several region-specific prognostic factors on disease spread was investigated.

Results: The final model indicated that Bluetongue development was significantly associated with an increase in number of animals ($P < 0.0001$), number of cattle around farm ($P < 0.0001$), water surface area ($P = 0.002$), and amount of rainfall in the previous days ($P < 0.0001$). Furthermore, the altitude over 450 MASL ($P < 0.0001$), the vaccination prophylaxis ($P < 0.0001$) and the previous outbreak event ($P < 0.0001$) had a protective effect against the outcome.

Conclusion: The results of this study indicated that number of animals and the amount of rainfall were the most important risk factors that affected the Bluetongue development, while the vaccination prophylaxis was found to be an effective measure in decelerating the disease spread.

Key words: Multilevel logistic mixed model, Bluetongue disease, risk analysis, retrospective analysis, propensity score matching

INTRODUCTION

Bluetongue virus (BTV) is the most studied virus transmitted by *Culicoides* biting midges spp., belongs to the Orbivirus genus of the Reoviridae family. BTV determines a vector-borne disease affecting both wild and domestic animals [1; 2]; although all species of ruminants are susceptible, the disease clinical signs affect especially in sheep [3; 4; 5; 6]. The distribution of the Bluetongue (BT) disease is limited to those regions where competent vector species are present, characterized by particular ranges of rainfall, soil characteristics, photosynthetic activity, wind speed and temperature, and its transmission to those times of the year when climatic conditions are favourable for the cycle of transmission [7; 8; 9]. After its spread in southern Europe towards the end of the 1990s and continuous occurrence throughout the current decade [8], distribution of carrier and its climate adaptations make Bluetongue a disease considered endemic in many African countries and in large Mediterranean area, where occasional incursions of different BTV serotypes caused high morbidity and mortality rates [10; 11; 1]. The disease is now classified as notifiable by International Epizooties Office (OIE), due to its potential rapid transmission, large economic impact [12; 13] and serious consequence, mostly in naïve populations [14]. Twenty nine different BTV serotypes are currently known globally and the epidemiology is varied greatly during the years [15]. After most recent BT-epidemics and to progressive filling the gaps in Culicoides spp. knowledges [16], the Eropean Union (EU) began a large-scale entomological surveilliance program (EU regulation 1266/2007). Many epidemiological studies were performed during the years in order to identify the most important risk factors involved in Bluetongue development and spread within a particular region. Firstly Wright in 1993 [17] and Ward in 1994 and 1996 [18; 19] evaluated the role of temperature and precipitation in vector life dynamics. Subsequently, many studies evaluated the factors associated to Bluetongue disease or Culicoides distribution: land use [20], farm and animal density [7; 1; 21], altitude [22; 23], wind [24; 25]. Conte in 2007 [26] provided an epidemiological overview of Bluetongue in Europe, evaluating some of the major factors involved in disease spread. Santman-Berends in 2010 [27] and Turner in 2012 [28] performed risk factors analysis related to disease transmission by movements. In 2013 Faes [29] and Pascual-Linaza in 2014 [14], finally showed the results of a well-rounded studies, evaluating all the well-known risk factors, as the number of sheep and goats, type of farms, temperature, road, animal movements and vaccination. However, all those studies demonstrated the strictly association between BT development and region-specific risk factors, which can't be ignored in a good disease surveillance program. Furthermore, all studies published until this moment performed a macro risk evaluation, collecting all characteristics included as explicative variables by municipality level. In Sardinia since 2000, after the disease arrived for the first time with serotypes BTV-2 and BTV-4, several epidemic waves are followed, as shown in Figure 1.The aim of this study, focused primarily on the relationship between characteristic ecosystems suitable for bluetongue vectors and climate, is to punctually identify the specific risk factors that have a role by increasing or reducing the risk of developing BT in Sardinian farms, through a multilevel logistic mixed model, and consequently obtain the predictor risk value for each farm.

METHODS

Data

The present study was a retrospective review study conducted on the records relating to Sardinian sheep and goats farms, from 2012 to 2014. The study population consisted of all Sardinian ovine farms, which meet all the inclusion criteria: kind of sheep and goats farm, with start and end activities date present in National database, in business for the entire study period (start activities date minor/equal to January 1st, 2012; end activities date major/equal to December 31th, 2014), and animal census data recorded on National Database for all study year considered (from 2012 to 2014). The data consisted of anagraphical information as farm code, farm's owner name and surname, fiscal code, address, localization, municipality, province and Local Sanitary Area of reference, latitude and longitude, start date activities, end date activities, animal census data. The outcome considered is to the outbreak event for the years of reference. The case was defined as a BTV-infected sheep farm, where BTV was diagnosed by serological or virological test, as establish by OIE, 2009 [30], and for which was reported data suspect and data confirmation for on SIMAN, from 2012 to 2014. The date of outbreak occurrence was assumed to be the day of suspect reported by local veterinary doctor. Specific farm characteristics were recorded: the number of sheep and goats by annual census, the number of bovine in the same farm or around each farm, since has been demonstrated that cattle may act as a reservoir for infection [31; 32]. The information related to BTV vaccination was collected for each farm as dichotomous variable by study year. Inasmuch has been shown that the outbreak event generates a virus resistance in the surviving animals [33], variable regarding outbreak notification in the previous year was considered as self-immunization proxy, supposing to have similar effect to that of vaccination. The pH of territory was collected and included as categorical way (acid or basic), since as demonstrated previously the larval reproduction may be reduced in alkalized sites [26; 34]. Data about land use (rocks, garrigue, marshes, builders, meadows, woods, seeds, urban green areas, orchards/olive groves/vineyard, pasture, mediterranean vegetation), altitude (MASL), water surface as lakes or

rivers (m2), were collected. The amount of water that slide through the territory (i.e. rivers, lakes and large reservoirs), measured in square meters, was included and evaluated as proxy for the amount of mud since, as known by literature [35; 36], the C. *imicola's* ideal habitat is the mud, hot and humid environments. Altitude at which farms are located has been inserted as a proxy of climatic differences, assuming that the best C. *imicola* habitat is at low altitude. In order to study this associations the millimeters of rains related to two and three decades immediately previous data of event for all three years in study, two variables related to the amount of rain fallen in the previous monthly decades by the outbreak event were collected and included in the study. Specifically, for each outbreak farm we collected the data suspect of event by SIMAN and this date has been associated with the amount of rain fall in the previous two decades. In order to solve the problem of the association of the same variable to no-outbreaks farms (and therefore without data suspect on SIMAN), the Propensity Score Matching 1:5 method was used, matching five no-outbreaks with one outbreaks farm with similar characteristics at baseline: Provinces (1-8), Municipalities (1-377), altitude, latitude and longitude, type of land. This allowed linking the data suspect with the rainy monthly decades. All data were extracted by VETINFO (Informative Veterinary System of Health Minister), SIMAN (Animal Disease National Informative System) and BDN (National Database), or by collaboration with ARPAS (Methereological Sardinian Agency). All confidential information were encrypt before analysis process in order to guaranty the privacy.

Statistical analyses

Data accuracy and validity was evaluated, fields with registration errors, non-correspondance between database systems, other irregularities and irrelevancies like outliers were corrected or removed. Descriptive analyses were used to illustrate the characteristics of the sample and evaluate the baseline distributions. The nominal variables were described through frequencies and percentages, while the quantitative variables were expressed in terms of mean and standard deviation (sd). To check the main hypothesis of this study, a multivariable analysis of the risk factors contributing to Bluetongue occurrence in each of the three years in Sardinia (2012-2014) was conducted using a multilevel logistic model (Eq.(1)). The model included municipality and farms as random effects to account for the between-observation differences in the model and for the dependency or correlation of observations between municipalities.

Assuming $y_{it} \sim \text{Binomial}(nit, \pi it)$, considering the response y_{ij} as the number of successes from a series of r_{ij} Bernoulli trials (replications), and defining $\pi_{it} = P(\text{outbreak} = 1)$,

we have:

$$logit(\pi_{ijf}) = \beta_{0jf} + \sum_{k=1}^{n} \beta_k X_{kijf} + \mu_j + \delta_f \quad (1)$$

where πit is the expected probability of BTV outbreaks for j = 1, …, 377 municipalities, with i = 1, … n_j farms at municipality j; β_0 is the intercept; the summitry is a vector of n covariates (X) measured in each farm i and their respective coefficients β_k; μj represents the variation between municipalities and δ_f account for the variation between different measurement of the same farm. In addition three alternative statistical model formulations were explored using a combined model and considering different assumptions. First assumption: observations between years are independent. The most straightforward way to evaluate a combined model for the three years is to include this factor as fixed effect, which allows taking into account the similarities between farms within year with respect to environmental/climatic conditions, farmer's behavior and local time-dependent veterinary practices. The equation here will be the same than Eq. (1), including year as covariate, producing the Baseline Random Model (Baseline RM). Second assumption: observations between year are not independent. We also explored a multilevel logistic model for the study period, adding year as random effects to the "Model baseline", producing the Year Random Model (Year RM). The inclusion of year as random effect allows to control for the between-year differences in the model and to account for the correlation of observations between farms (Eq. (2)).

$$logit(\pi_{ijft}) = \beta_{0jft} + \sum_{k=1}^{n} \beta_k X_{kijft} + \mu_j + \delta_f + \theta_t \quad (2)$$

where θt represents the variation between years of observation, for each farm i in the municipality j.

The last assumption supposed that observations between years are not independent and there are time-dependent effects. To test this assumption the potential existence of time-dependent effects over the 3 years period was explored, controlling for between-year differences using a multilevel logistic model, including farm, municipality and year as random effects and including random slopes to evaluatethe possible interaction between each variable and year (Eq. (3)), producing the Slop Random Model (SRM).

$$logit(\pi_{ijft}) = \beta_{0jft} + \sum_{k=1}^{n} \beta_k X_{kijft} + \mu_j + \delta_f + \beta_{kt}\theta_t \quad (3)$$

The risk factors to be included in the model were selected by backward elimination process (p-value > 0.1), including random effect only after the best fit model selection, based on Akaike's and Schwarz's Bayesian information criteria (AIC and BIC) values, as suggested by Zuur et al., 2009 [37]. Since the primary goal of this work was to make a model that most accurately predicts the desired target value for new data, and in order to lead the phenomenon of over-fitting (where a model may fit the training data very well), internal and external model

validation were performed based on the data used to fit the model (2012-2014) and data about previous (2009-2011) and posterior (2015-2016) period for the external validation. The predicted performance of the model was evaluated by the area under the receiver operating characteristics curve (AUC), as explained by Taylor in 2008 [38], and the root mean square tests. The residual analysis was performed graphically using Pearson's residuals [39]. Residuals are constructed to closely follow a normal distribution, including both the fixed portion and the random portion of the model. Thus it's possible to check outliers by plotting residuals against observation numbers.

Software

All the tests were two-sided, with a level of significance of 0.05. The analyses were performed using the SAS 9.3 software (SAS Institute Inc., Cary, USA) for the propensity score matching, the STATA software (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP) for the model fitting, and R-language v. 3.4.0 using the libraries "glmmML" [40] and "rms.gof" [41] for the model validation.

RESULTS

All farms presents in Sardinia during study period were recruited, based on inclusion/exclusion criteria. Of them 2,610 farms were excluded because not in activities for the entire study period and 893 were excluded because animal census was missing. Data about a total of 12,277 farms were collected, with 36,832 total record analyzed. The case-events definition has been attributed to 5.152 farms since have developed outbreaks in one of the study years, the others 31,680 were defined as not cases. Descriptive baseline characteristics are reported in Table 1.

Multivariable analysis

The model's comparison criterion and the validation criterion reported in Table 2 highlight the superiority of the Years Random Model (Year RM), which includes the municipality, farm and year as random effects (Eq. 2), compared to the Baseline Random Model and the Slope Random Model. This model has been defined as the final model and the results, expressed as adjusted Odds ratio (ORadj) and Confidence Interval at 95% level (IC95%) for the fixed effect and as variance (σ^2) with Standard Deviation (SD) for the random effect, are shown in Table 3. The model included 8 fixed effects: number of animals; number of cattle around farm; altitude; amount of water (m2); vaccination (yes/no); outbreaks notification in the previous year (yes/no); amount of rainfall 20 days previous (millimeters); municipality. No one statistically significant interaction terms were found. As shown in Table 3, the results obtained by multivariable analyses, performed to explore the variation of the effect size for the considered factors on BTV occurrence, highlight that the presence of an increasing number of animals one by one inside livestock can be considered a significant risk factor favoring the outbreak event with an ORadj of 1.0012 at p-value <0.0001 [IC95% 1.0011–1.0013]. Likewise, cattle around farm (OR = 1.0004 [IC95% 1.0002–1.0005, p-value < 0.0001), as the water surface area increased of 1 m2 (OR = 1.00003 [IC95% 1.00001–1.00005], p-value = 0.002) and the growing amount of rainfalls during 20 days previous the outbreak occurrence (OR = 1.15 [IC95% 1.14–1.16], p-value < 0.0001) are factors that have a statistically significant effect on the BTV outbreak development. On the contrary the increase of altitude (OR = 0.997 [IC95% 0.996–0.997], p-value < 0.0001), vaccination prophylaxis on farm versus none vaccination (OR = 0.016 [IC95% 0.009–0.027], p-value < 0.0001) and the outbreak event happened in the prior year (OR = 0.16 [IC95% 0.13–0.20], p-value < 0.0001), have a protective effect against the illness event. According to the established validation criteria, the multi-level mixed model selected appears to be able to predict the number of outbreaks properly for the years in use (2012-2014) and for outbreaks occurred during the previous and posterior period (2009-2011; 2015-2016). The AUC values were 0.976 and 0.977 for the internal and external validation, respectively, suggesting a good predictive performance of the model. The root mean square tests were non-significant for internal model validations (Root MSE = 0.151, p-value = 0.98) and for external validation (Root MSE = 0.160, p-value = 1), indicating no evidence of failure. The mean of the residual's distribution very close to zero (3.42*10-3; sd = 2.67*10-3) also indicated a very goodness of fit of the model. The Pearson's residuals obtained by residual analysis are shown in Figure 2: in the graph the residual for outbreaks observation shows a potential outliers, suggesting a poor fit for those observations; however not specific outlier appears in no outbreak group.

DISCUSSION

The Sardinia Region is one of the main pastoral areas for farmed sheep in Italy and represents the most likely candidate region for the study of BTV distribution and prevalence characteristics. Given the need to provide specific information on the disease spread, since epidemiological studies based on Sardinian farms have never been performed before, this study conducted an investigation on the BTV transmission to define a punctual risk profile for all Sardinian farm, through a logistic multilevel mixed model, taking into account agro-meteorological aspects and farm characteristics. The inclusion in this study of all the farms present in the region and therefore the

TABLE 1. Baseline farms characteristics recorded at inclusion in the study, by event of outbreaks and no-outbreak and overall. The results are reported in terms of mean [standard deviation], median [I, III quartile], and n (%).

BASELINE FARM'S CHARACTERISTICS	OUTBREAKS (N = 5151)	NOT OUTBREAKS (N = 31680)	OVERALL (N = 36831)
Number of animals	329 (270); 264 [159 – 419]	214 (240); 159 [27 – 305]	230 (247); 177 [40 – 323]
Number of cattles	7 (2.4); 12 [5 – 30]	6 (1.8); 11 [4 – 28]	7 (2.4); 12 [5 – 30]
Whater (i.e. rivers, reservois, m2)	2152 (3273); 0 [0 – 3302]	1867 (3093); 0 [0 – 2483]	1907 (3120); 0 [0 – 2597]
Altitude (m)	242 (178); 200 [100 – 350]	351 (243); 300 [150 – 500]	356 (239); 300 [150 – 450]
Land type			
Rock, garrigue	222 (4.3%)	1907 (6%)	2129 (5.8%)
pond, marshe	149 (2.9%)	1021 (3.2%)	1170 (3.2%)
building	495 (9.6%)	2728 (8.6%)	3223 (8.7%)
meadow	598 (11.6%)	3691 (11.6%)	4289 (11.6%)
wood	644 (12.5%)	5344 (16.9%)	5988 (16.3%)
seeded, green area	1984 (38.5%)	9820 (31%)	11804 (32%)
olive-grove, vineyard	371 (7.2%)	2161 (6.8%)	2532 (6.9%)
pasture	438 (8.5%)	2813 (8.9%)	3251 (8.8%)
mediterranean vegetation	252 (4.9%)	2186 (6.9%)	2438 (6.6%)
pH territory			
Acid	4650 (90%)	28531 (90%)	33181 (90%)
Basic	501 (10%)	3149 (10%)	3650 (10%)
Wild ruminants			
Yes	4887 (95%)	29908 (94.5%)	34795 (95%)
Not	264 (5%)	1772 (5.5%)	2036 (5%)
Vaccination			
Yes	214 (4%)	8774 (28%)	8988 (24%)
Not	4937 (96%)	22906 (72%)	27843 (76%)
Outbreaks year previous			
Yes	123 (3%)	5021 (16%)	5144 (14%)
Not	5028 (97%)	26659 (84%)	31687 (86%)
Rain 20 days prev. (millimetres)	38.3 (15.2); 39 [29 – 46]	13.7 (9.9); 15 [5 – 19]	17.2 (13.8); 17 [7 – 22]

sample size so numerous are definitely the major strengths of this work, which is able to provide a picture of the regional situation as broad and varied as possible. Our results are in agreement with previous studies of BTV and indicate fundamental roles of all baseline factors included in the final logistic model [17; 18; 21; 22; 23; 28]. The variables considered in the model are able to accurately describe the main animals, business farm, territorial and climatic characteristics involved in the insect's life cycle and the development of the disease. We need to take into account that the effectiveness of the animals immunization may have been influenced by several factors, including the vaccine administration process on a farm, and we believe that the proper immunization of animals has been key to reduce the disease spread in the region and the implementation of a risk-based vaccination strategy will help

TABLE 2. Baseline Random Model – Baseline RM; Year Random Model - Year RM); Slope Random Model - Slope RM). The Akaike information criterion (AIC), Schwarz's Bayesian information criteria (BIC) and the area under the receiver operating characteristics curve (AUC) is also provided for each model.

	LR test (p-value)*	AIC	BIC	AUC
Baseline RM	83.93 (p < 0.0001)	13019.20	13098.32	0.964
Year RM	58.42 (p < 0.0001)	13012.41	13086.59	0.976
Slope RM	13.54 (p < 0.003)	13035.47	13101.41	0.958

** Likelihood-ratio test comparing the model to ordinary logistic regression, Eq(1) without μj and* δ_t

TABLE 3. Odds ratio (OR), 95% confidence intervals (IC 95%) and p-values obtained by the final multilevel logistic mixed models (Year Random Model - Year RM). Variance (σ^2) and Standard Deviation (SD) of the random effects of municipality, farm and year are also shown.

	OR Adjusted [IC 95%]	p-value
Altitude (m)	0.997 [0.996 – 0.997]	< 0.0001
Number of animals	1.0012 [1.0011 – 1.0013]	< 0.0001
Number of cattles	1.0004 [1.0002 – 1.0005]	< 0.0001
Whater (i.e. rivers, reservois, m2)	1.00003 [1.00001-1.00005]	0.002
Vaccination Yes Not	 0.016 [0.009 – 0.027 Ref.	 < 0.0001
Outbreaks year previous Yes Not	 0.16 [0.13 – 0.20] Ref.	 < 0.0001
Rain 20 days prev. (millimetres)	1.15 [1.14 – 1.16]	< 0.0001
Year 2012 2013 2014	 Ref. 6.0 [5.2 - 6.9] 0.81 [0.35 – 0.18]	 < 0.0001 < 0.0001
σ^2 (SD)	Farm: 0.12 (0.35) municipality: 3.06 (1.14) year: 7.48 (6.17)	

to speed the eradication of BTV in Sardinia. Furthermore, the identification of the major risk factors that contribute to the BTV event can be very useful also for cost effective projects and surveillance in other European regions with similar characteristics. Those results may be a practical support to assess and plan the diagnostic and preventive interventions. Most of the limitations are related to the retrospective type of this study (i.e. data traceability and accuracy), although the check practices should have limited, at least in part, the generation of possible selection bias. Another aspect to take into account is that some variables were used as proxy for factors which seems to be determinant influencing/limiting the C. imicola vital cycle, as know by literature. The proxy use could give a reflected measure of those environmental features that we can't directly measure, such as the amount of mud present in a territory approximated by observing the surfaces of lakes or reservoirs. Actually, the BT surveillance system in Sardinia provides the serological results after 20 days infection up to even 80 days after. Obviously all time would be reduced if the serological test was associated with virological control in those areas where, as the predictors indicate, it is estimated to be viral circulation. A future

FIGURE 1. Data about number of outbreaks for Bluetongue Virus in Sardinia (Italy), by epidemic season year.

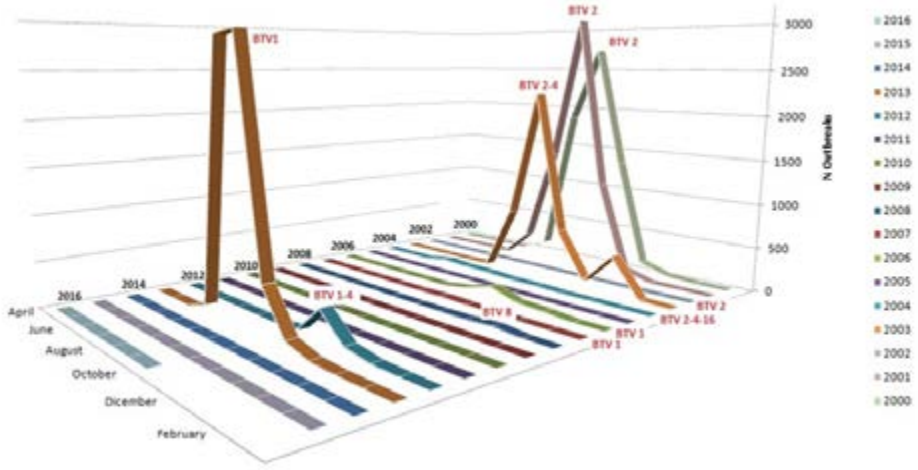
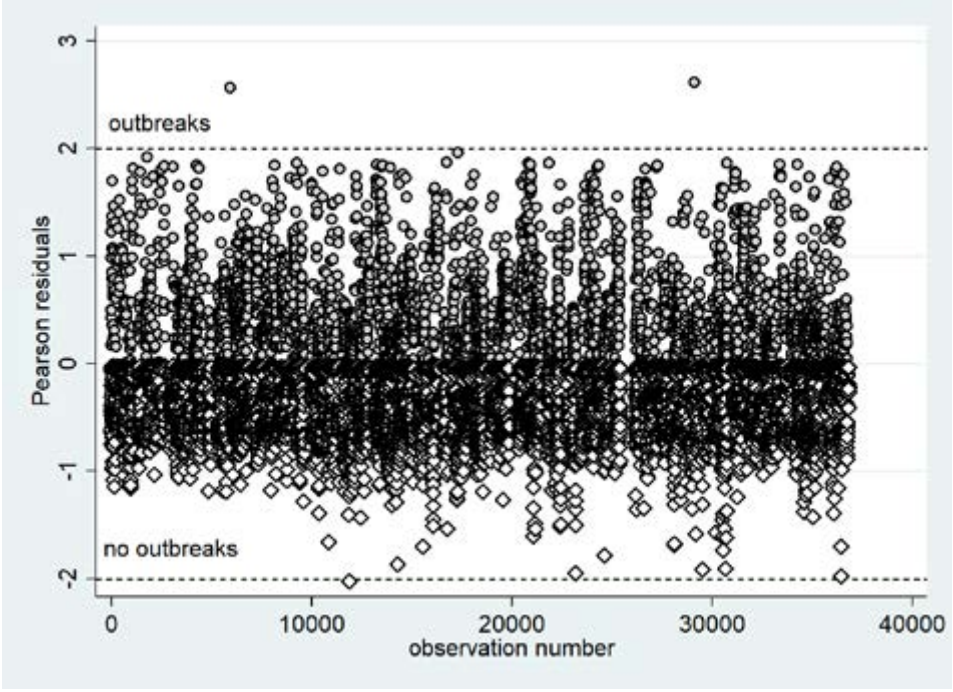


FIGURE 2. Graphical representation of Pearson's residuals plot versus observation numbers, by outbreaks cases (circles) and not cases (diamonds). Dash lines define the ±2 range, beyond which residuals are potential outliers.



research project aimed to contributing in the understanding of the phenomenon could be precisely focused on the study of the wild ruminants role in BTV occurrence as hypothesized by Cappai et al. In 2015 [42], the role played by the temperatures, and the wind on the insect vectors movement from company to company.

Conflict of interest

The authors declare no conflict of interest.

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