Analysis of the resistance behaviour of indicator bacteria to several antibiotics in bulk milk using spatial point pattern methodology

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Summary

Between 2002 and 2004, indicator bacteria (*Enterococcus*, *Escherichia coli*) isolated from 799 bulk milk samples were tested against thirty different antibiotics for resistance. Systematic sampling over space (every eighth Styrian cattle breeding farm on an unaligned grid) was conducted within the scope of the bovine virus diarrhoea (BVD) control programme. Results of resistance testing were assigned to their respective farms by means of LFBIS (information system for agricultural and forestry enterprises: *land- und forstwirtschaft-lichen Betriebsinformationssystem*) numbers. The distribution of resistance was evaluated using a geographic information system. To show the application of spatial point pattern analysis, results of resistance testing of *Enterococcus* to tetracycline and streptomycin is presented. Kernel density estimation and K functions were used to determine whether the distribution of the resistant samples was clustered, relative to isolates from samples that were sensitive to these antibiotics. Initially, spatial clustering of resistance (tetracycline and streptomycin separately) was investigated. The space-time scan statistic was subsequently used to search for space-time clusters of resistance for both antibiotics simultaneously.

Keywords

Austria, Bovine virus diarrhoea, *Escherichia coli*, Geographic information system, K function, Kernel density, Space-time scan statistics, Spatial point pattern.

Analisi, mediante la metodologia del modello del punto spaziale, del comportamento di resistenza ad alcuni antibiotici di batteri indicatori nel latte di massa

Riassunto

*Tra il 2002 e il 2004, alcuni batteri indicatori (*Enterococcus, Escherichia coli*) isolati da 799 campioni di latte di massa sono stati testati per la resistenza a trenta differenti antibiotici. E' stato condotto un campionamento sistematico nello spazio (ogni otto allevamenti di bovini di razza Siriana in una griglia non allineata) nell'ambito del programma di controllo del virus della diarrea virale bovina (BVD). I risultati dei test sulla resistenza sono stati assegnati alla loro rispettiva azienda tramite i numeri del LFBIS (sistema informativo per le imprese agricole e forestali:* land- und forstwirtschaft-lichen Betriebsinform-ationssystem*). La distribuzione della resistenza è stata valutata usando un sistema informativo geografico. Per illustrare l'applicazione dell'analisi mediante il modello di punto spaziale, sono stati presentati i risultati dei test sulla resistenza di* Enterococcus *alle tetracicline ed alla streptomicina. La stima della densità di Kernel e la funzione K*

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sono state usate per stabilire se erano presenti dei cluster nella distribuzione dei campioni, in relazione agli isolati che erano, al contrario, sensibili a questi antibiotici. Inizialmente è stata valutata l'aggregazione spaziale della resistenza alle tetracicline e alla streptomicina in maniera separata. La scansione statistica spazio-temporale è stata usata successivamente per riconoscere eventuali cluster spazio-temporali di resistenza simultanea ad entrambi gli antibiotici considerati.

Parole chiave
Austria, Densità di kernel, *Escherichia coli*, Funzione K, Modello del punto spaziale, Scansione statistica spazio-tempo, Sistema informativo geografico, Virus della diarrea virale bovina.

Introduction

The development of antibiotic-resistant strains of bacteria in livestock systems is not only of major concern in veterinary medicine, but might be a source of resistant pathogens in human populations. According to good veterinary practice (GVP), veterinarians are responsible for consumer health through diagnosis, identification of indications, drug selection, compliance with dosage regulations and documentation of drug use and monitoring of therapeutic success in animals treated with antibiotics.

The European Union Council Resolution on antibiotic resistance (*A strategy against the microbial threat*) calls on member states to develop multidisciplinary and cross-sector strategies to control antibiotic resistance (5). Antibiotic resistance of zoonotic pathogens in food, such as *Salmonella* spp., *Campylobacter* spp., *Listeria* spp. or pathogenic *Escherichia coli*, is also of importance. Indicator bacteria (commensals such as *Enterococcus* or *E. coli*) are considered a reservoir of resistant strains in the intestines of animals and are capable of transferring resistance genes (for example, via plasmids or transposons) to both pathogens of animal and human origin. The transmission of resistant bacteria from animals to humans may occur through food consumption or direct contact.

To explore possible spatial patterns of antibiotic resistance, data from an integrated control system for the improvement of food safety, established by the Department of Veterinary Administration of the Styrian Government, Austria (8) was analysed with spatial point pattern algorithms.

Materials and methods

Sample size

Between 2002 and 2004, indicator bacteria (*Enterococcus*, *E. coli*) isolated from 799 bulk milk samples were tested against thirty different antibiotics for resistance. Systematic sampling over space was conducted within the scope of the bovine virus diarrhoea (BVD) control programme. Therefore, Styria was overlaid with a squared grid (grid size 3 × 3 km) and, in each cell, farms were sampled systematically if one or more Styrian cattle breeding farms were present. If there were more than eight farms within one grid cell, every eighth farm was selected. This systematic sampling (on an unaligned grid) procedure was selected to produce a representative spatial sample distribution with respect to the spatial density of the farms.

Results of resistance testing were assigned to their respective farms by means of LFBIS (information system for agricultural and forestry enterprises: *land- und forstwirtschaftlichen Betriebsinformationssystem*) numbers. The distribution of resistance was evaluated using a geographic information system (VETGIS-Styria)

Detection methods

Tests and analyses were performed on bulk milk samples. For this purpose, 2 ml of milk was taken within the BVD control programme (bulk milk analyses). The samples were cooled and subsequently conditioned. Citrate azide tween carbonate (CATC) selective medium (Merck 1.10279) was used to isolate *Enterococcus* spp. Red *Enterococcus* colonies develop in the culture medium after an incubation period of 48 h at 37°C.

Resistance test – determination of minimum inhibition concentration values

Resistance testing and determination of minimum inhibition concentration (MIC) values were performed using the SensititreR system (MCS-Diagnostics, Veldpoort 28, NL-6071 JL Swalmen), a commercially available MIC technique using dehydrated antimicrobials in microtitre wells. The wells were inoculated according to National Committee for Clinical Laboratory Standards (NCCLS, now the Clinical and Laboratory Standards Institute®) guidelines (11) and incubated aerobically at 37°C for 18-22 h. The MIC was defined as the lowest concentration of antimicrobial with no visible microbial growth. An isolate was classified as resistant if the MIC exceeded the appropriate breakpoint (1). For example, the breakpoint for tetracycline in bulk milk is 16.

Statistical background

A spatial point pattern may be thought of as a set of locations (s1, s2, etc.) in a defined 'study region' R in which the 'events' of interest have been recorded. In this paper, events are of two types, namely:

- locations at which resistance was detected
- locations at which resistance was not detected.

Data were collected over a period of three years (t1, t2, t3) and, for each of the thirty antibiotics tested, a point patterns were available. Therefore, a multivariate spatiotemporal data set was available. To demonstrate the use of spatial point pattern analysis, *Enterococcus* resistance data for tetracycline and streptomycin was studied.

An observed spatial point pattern can be thought of as the realisation of a spatial stochastic process. The behaviour of general spatial stochastic processes may be characterised in terms of its first- and second-order moments. First-order moments are described in terms of the intensity $\lambda(s)$ of the process, which is the mean number of events per unit area at the point s (4). Several methods are available to estimate the first-order intensity (7). Perhaps the most popular is the kernel-density estimation. A window of fixed size is defined and is centred on a number of locations in turn, where these are arranged in a fine grid superimposed over R. Then estimates of the intensity at each grid point are obtained. Kernel estimation is a generalisation of this idea, where the window is replaced with a moving three-dimensional function (= kernel) which weights events within its sphere of influence according to their distance from the point at with the intensity is being estimated.

If a point process is stationary (and isotropic) there is a close mathematical relationship between the second-order intensity and an alternative characterisation of second-order properties known as the 'K function'. This is defined by the relationship $\lambda K(d) = E$ (#[events ≤distance d of an arbitrary event]), where $K(d)$ describes the extent of which there is spatial dependence in the arrangement of events. As far as the K function for a complete spatial random process is concerned, the important point is that the probability of the occurrence of an event at any point in R is independent of any other events that have occurred and is equally likely over the entire area of R. For a homogeneous process with no spatial dependence, the expected number of events with the distance d of a randomly chosen event is simply $\lambda\pi d^2$. In other words $K(d) = \pi d^2$, representing a circle with radius d. If there is clustering of point events one would expect to see an excess of events at short distances. For small values of d, the observed value of $K(d)$ will be greater than πd^2.

Given n1 events of primary concern (cases, i.e. resistant) and n2 events representing the 'natural' homogeneity (controls, i.e. sensitive), in the absence of clustering among the cases relative to the controls, if we pool the two sets of events we would expect the n1 case 'labels' to be attached at random to the combined set of events; this is called a random labelling of events (6). Under such random labelling it can be shown that the K functions for the cases $K1(d)$ and for the controls $K2(d)$ are identical.

To test the hypothesis of 'random labelling', which suggests that $K1(d) = K2(d)$, we might use a plot of the difference $D(d) = K1(d) - K2(d)$ against d to explore such a hypothesis. When

D(*d*) is plotted against *d*, peaks in this plot will show clustering over and above that environmental heterogeneity. A more formal assessment of the significance of peaks in this plot is the idea of simulation techniques. Under the assumption of complete spatial randomness, we may perform *m* independent simulations of *n* events in the study region. For each simulated point pattern we can estimate K(*d*) and the use the maximum and minimum of these functions for the simulated patterns to define upper and lower simulation envelopes. If the estimated K(*d*) lies above the upper envelope, we can speak of significant spatial clustering, if it lies below the lower limit, this is evidence of regularity in the arrangements of events (2). Such a method provides a useful complement to alternative approaches (3) which are essentially based on extensions to distribution function of inter-event distances.

Kernel density estimation and K functions (4) were used to determine whether the distribution of the resistant samples were clustered, relative to the distribution of locations where resistance was not detected. The calculations were performed with the help of the software package R (version 2.2.1).

Alternative tests for special clustering which do detect a range of clustering types are the space-scan statistics. The general statistical theory behind the spatial scan statistic is described in detail by Kulldorff (9).

The spatial scan statistic uses two different probabilistic models, based on the Bernoulli and Poisson distributions, respectively. With the Bernoulli model, there are cases and non-cases as a 0/1 variable. They may reflect cases (i.e. resistant) and controls (i.e. sensitive) from a larger population, or they may together constitute the population as a whole. With either model, the scan statistic adjusts for the uneven population density present in almost all populations, and the analysis is conditioned on the total number of cases observed. For each location and size of the scanning window, the alternative hypothesis is that there is an elevated rate within the window compared to outside.

The likelihood function of the chosen probability model is maximised over all windows, identifying the window that constitutes the most likely cluster. This is the cluster that is least likely to have occurred by chance. The likelihood ratio for this window is noted and constitutes the maximum likelihood ratio test statistic. Its distribution under the null hypothesis and its corresponding *p*-value is obtained by repeating the same analytic exercise on a large number random of replications of the data set generated under the null hypothesis, in a Monte Carlo simulation.

Initially, antibiotic resistance data were analysed separately for spatial clustering. The space-time scan statistic (10) was then used to search for space-time clusters.

Calculations for the period between 2002 and 2004 were performed using the software package SaTScan™ version 6.1 (10) with 999 Monte Carlo replications. For the specification of the spatial window, the maximum spatial window size was set to 50% of population at risk and the window shape was set to circular.

Results

Enterococcus strains were isolated from 799 samples and tested for resistance to thirty different antibiotics. A total of 120 (15%) isolates were multi-resistant, i.e. resistant against more than one antibiotic. The highest proportions of isolates were resistant to tetracycline (52%), streptomycin (21%), kanamycin (18%), flavomycin (15%) and chloramphenicol (13%).

To exemplify the use of spatial point pattern analysis, we analysed the resistance of *Enterococcus* strains to tetracycline and streptomycin. Figure 1 shows the sample point map for 2002 with resistant and sensitive samples for tetracycline and streptomycin. Figure 2, for example, shows the kernel density ratio for tetracycline resistance only, ignoring a possible temporal influence. To calculate this ratio, the kernel density for resistant samples was divided through the kernel density of all samples. The highest ratio can be seen in the east and some hot-spots in the north and west. That means that more than 90% of the samples

taken between 2002 and 2004 were resistant in these areas. Note that areas with ratios <60% were not plotted in Figure 2. To determine if these areas can be interpreted as spatial clusters, K functions K1(d) and K2(d) were estimated separately for resistant and sensitive samples and subtracted to obtain the difference function D(d)=K1(d)-K2 (d). To test for spatial clustering, upper and lower simulation envelopes were developed by performing 999 Monte Carlo replications. As D(d) lies between the upper and lower envelope (Fig. 3) no statistically significant ($p>0.05$) evidence for clustering of tetracycline can be seen. In addition, note that for streptomycin no statistically significant spatial clustering could be detected.

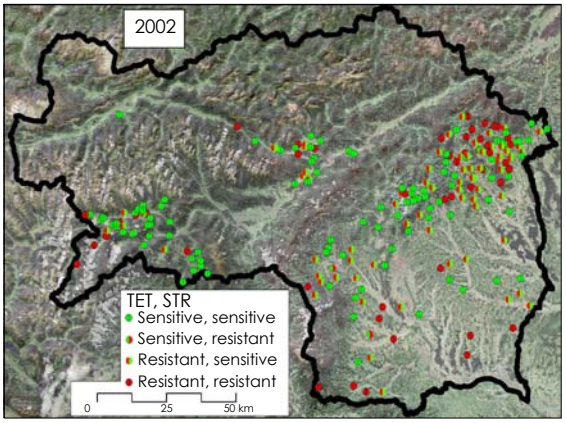

Figure 1
Point map of samples taken in 2002: resistant and sensitive samples, tetracycline (TET), streptomycin (STR)

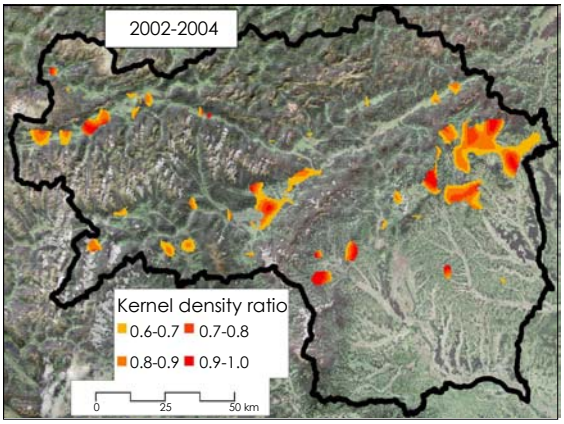

Figure 2
Kernel density ratio for resistance behaviour (tetracycline) in Styria (2002-2004)

To determine if simultaneous spatial clusters of resistant (tetracycline and streptomycin) samples existed, scan statistics using the Bernoulli model were calculated. Figure 4 shows the most likely spatial cluster for lower than expected resistant samples, ignoring the temporal component. This cluster shows no statistically significant results (p = 0.07).

When every year (2002, 2003, 2004) was analysed separately, significant simultaneous spatial clusters ($p<0.05$) for tetracycline and streptomycin were found in 2002 (Fig. 5). This spatial cluster was not seen in 2003 or 2004.

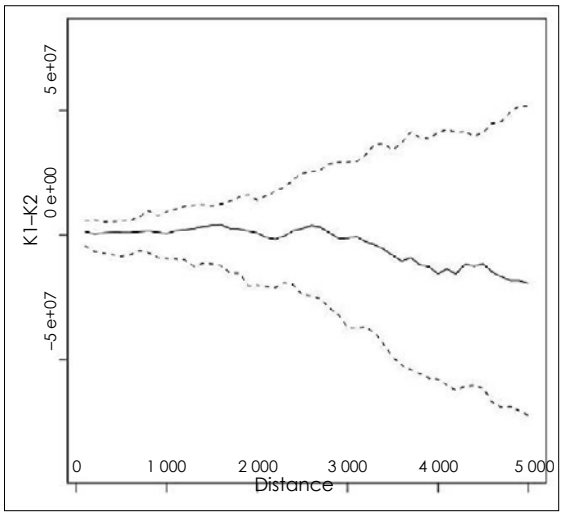

Figure 3
Difference between K functions (solid line) and simulated envelope to test for spatial clustering for tetracycline

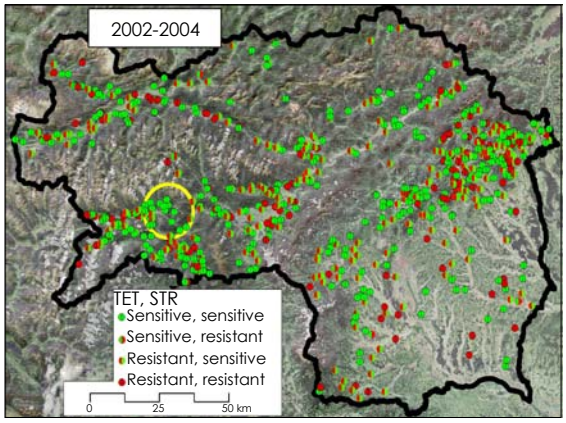

Figure 4
A simultaneous spatial cluster of lower than expected resistance, tetracycline (TET), streptomycin (STR) isolates of *Entercoccus* (2002-2004)

Discussion

Spatial point pattern analyses was used to simultaneously explore spatial and/or temporal clusters of the resistance behaviour of two antibiotics (tetracycline and streptomycin). Ignoring a possible temporal component, no clearly statistically significant spatial clusters could be found. The observed differences in the regional distribution of antibiotic resistance in the Styrian area may be caused by the agricultural structure of the area (small farms, farmers working in other jobs to obtain a supplementary income in the south), incorrect antibiotic dosage and insufficient accompanying measures during therapy. Another reason for higher resistance rates in the east of Styria may be the combination of cattle farming and intensive pig and poultry farming, if bacterial strains or resistant bacterial genes from swine or poultry can be transferred to cattle.

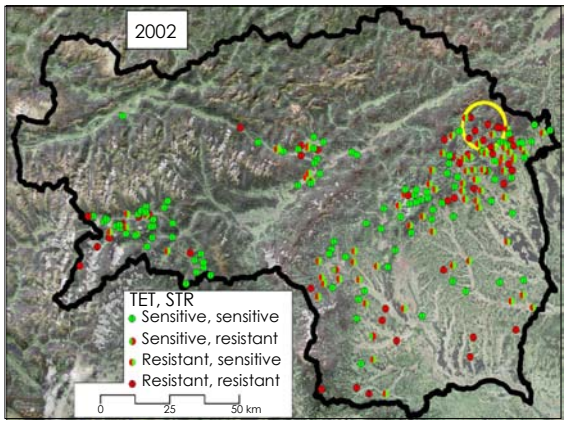

Figure 5
A simultaneous spatial cluster of higher than expected resistance (tetracycline, streptomycin) isolates of *Entercoccus* in 2002

Antibiotic resistance test results of thirty different antibiotics in Styria have been published annually since 2002 and are entered into a central database, which is linked to the VETGIS-Styria geographic information system. The simultaneous cluster of high antibiotic resistance for tetracycline and streptomycin detected in 2002 could not be found 2003 or 2004. This may be an indication this monitoring programme of residues of veterinary drugs had an effect. Furthermore, these data are combined with current analysis results from human medicine and with international animal production data, in order to assess the risk factors for transmission of resistance.

In summary, drug usage in livestock production must be based on the principles of GVP. Increased cooperation between human and veterinary medicine is required to investigate the problem of resistance transfer.

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