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# Spatial and phylogenetic analysis of vesicular stomatitis virus over-wintering in the United States

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ABSTRACT

From 2004 through 2006, 751 vesicular stomatitis (VS) outbreaks caused by vesicular stomatitis virus serotype New Jersey (VSNJV) were reported in nine states of the southwestern United States. The normal model of the spatial scan statistic and phylogenetic techniques were used to assess whether the spatial and genetic relations among VSNJV outbreaks were consistent with the hypothesis that VSNJV over-wintered in specific regions of the southwestern United States infected in 2004 and 2005, respectively. Use of the spatial scan statistic led to the identification of two clusters of outbreaks for which the Euclidean distance to the nearest outbreak reported in the previous or following year, whichever was shorter, was significantly ($P < 0.01$) shorter than the epidemic's (2004–2006) mean. Clusters were centered at Colorado and Wyoming and included 375 and 21 outbreaks, respectively. Results were supported by the phylogenetic analysis of 49 VSV samples collected from 2004 through 2006 in the United States and 10 VSV samples originated from Mexico. These findings, which were displayed using a publicly accessible web-based system referred to as the FMD BioPortal, were consistent with over-wintering of specific sub-lineages of VSNJV in a limited geographical region of the United States affected by a VS epidemic in 2005 and 2006.



## 1. Introduction

Vesicular stomatitis (VS) is a zoonotic disease of horses, cattle, swine, and certain wild life species caused by a rhabdovirus referred to as vesicular stomatitis virus (VSV). Two VSV serotypes, New Jersey (VSNJV) and Indiana, are responsible for the endemic presentation of the disease from northern South America through to southern Mexico (Rodriguez and Nichol, 1999).

VS is typically associated with low mortality and morbidity levels, but in susceptible populations the infection can spread rapidly over wide geographical areas (Rodriguez, 2002). VSV infection in humans is rare and results in mild influenza-like symptoms. In susceptible domestic animals, clinical manifestation of VSV infection is characterized by the presence of blister lesions that are primarily located in nostrils, lips, oral mucosa, and tongue. Blister lesions can also occur in teats and in the coronary band of the hooves. The pain caused by the rupture of

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vesicles often leads to the refusal of animals to eat, drink, and walk. Those signs may consequently cause weight loss and, in dairy cows, a severe drop in milk production. In addition to the potential zoonotic nature and direct economic impact of the disease, VS is important because its clinical signs in ruminants and pigs resemble those caused by foot-and-mouth disease (FMD) virus infection (Letchworth et al., 1999). Because FMD was eradicated from the United States more than 80 years ago, there is a reasonable concern that early signs of a potential FMD epidemic in the country may be erroneously confused with VS, leading to delays in diagnosis.

The southwestern United States has been incidentally affected by VSV epidemics during the last 100 years, with the last episodes being reported in 2004–2006 (Rainwater-Lovett et al., 2007). Previous studies suggest that VS epidemics in the southwestern United States are caused by genetically distinct VSV strains introduced from endemic areas of southern Mexico (Rodriguez, 2002; Rodriguez et al., 2000). VS epidemics typically follow a seasonal pattern in the southwestern United States and it is believed that the ability of the virus to over-winter explains the re-emergence of the disease frequently observed during the years that follow an initial introduction of the virus (Rodriguez, 2002). Mechanisms of VSV transmission are, however, not fully understood. Direct contact of susceptible animals with infected individuals or with fomites such as contaminated water, feed bunks, or milking machines seems to play a role in disease transmission. However, transmission between premises and over large geographical areas is believed to be mediated by certain species of insects, as suggested by the reduced risk for the disease observed in stabled animals and on farms that implement insect control programs (Hurd et al., 1999). Three insect species have been experimentally demonstrated to be biological vectors of VSNJV; *Simulium vittatum* (*Diptera: Simuliidae*) (Mead et al., 2004), *Lutzomyia trapidoi* (*Diptera: Psicodidae*) (Tesh et al., 1971) and *Culicoides sonorensis* (*Diptera: Ceratopogonidae*) (Perez de Leon and Tabachnick, 2006). In addition, VSNJV has been isolated from a variety of field-collected arthropods such as eye gnats (*Chloropidae spp.*), root-maggot flies (*Anthomyiidae spp.*), house flies (*Musca spp.*), biting midges (*Culicoides spp.*), black flies (*Simuliidae spp.*), sand flies (*Lutzomyia spp.*), and mosquitoes (*Aedes spp.*) (USAHA, 2008).

Insect-borne transmission may explain the seasonal pattern observed for VS epidemics in the southwestern United States. Trans-ovarian transmission, which has been suggested to occur in black flies (Mead et al., 2004) and sand flies (Tesh et al., 1972) could play a role in VS maintenance and spread in the region, specifically by potentially allowing over-wintering.

Spatial clustering and phylogenetic techniques were used in a web-based GIS framework to obtain evidence that the 2005 and 2006 VS outbreaks may have been associated with VSNJV over-wintering in certain regions of the United States affected by the disease in the previous year. Gaining knowledge on epidemiological aspects of VSV infection in the United States is important in order to develop effective prevention and control strategies for the disease. Moreover, such knowledge will also help to establish differences with the expected behavior of a potential FMD epidemic in the country.

## 2. Methods

### 2.1. Study population

From 2004 through 2006, 751 outbreaks caused by the VSNJV were reported in the United States' southwestern states of Arizona, Colorado, Idaho, Montana, Nebraska, New Mexico, Texas, Utah, and Wyoming (Table 1). All 751 outbreaks were confirmed by analysis of samples submitted to the national veterinary services laboratories (NVSL) at either Ames, IA, or Plum Island, NY.

Information on the geographic location (latitude, longitude) and susceptible species (bovine, equine, other) of the 751 VSNJV-infected premises was provided by the United States Department of Agriculture's Animal & Plant Health Inspection Service (APHIS). Infected premises were geo-located considering either the location of the front gate or the point where the private drive way intersects a public road. The system used to geo-locate the data was recorded for 460 (61.2%) outbreaks, from which 392 (85.2%), 42 (9.1%), and 26 (5.7%) were geo-located using global positioning system (GPS) receivers, by geo-coding the address, and by mapping or aerial photography interpolation, respectively. We have no reason to believe that missing information on the type of system used to geo-locate the outbreaks, or use of one system or another, was associated with any particular source of systematic error that may have introduced any kind of bias to the study. Moreover, most of the outbreaks (>85%) were geo-located using one single geo-location system, i.e., GPS receivers.

### 2.2. Identification of spatial clusters

The Euclidean distance (measured in km) to the nearest VSNJV outbreak reported in the previous or following year ($d$), whichever was shorter, was recorded for each of the 751 confirmed outbreaks. A variation of the spatial scan statistic, referred to as the normal model of the spatial scan statistic, was used to identify clusters

**Table 1**
Number of vesicular stomatitis outbreaks reported in the United States from 2004 through 2006, stratified by State and year of reporting.

| State | Year | | | Total |
|---|---|---|---|---|
| | 2004 | 2005 | 2006 | |
| Arizona | 0 [0] | 27 [1] | 0 [0] | 27 [1] |
| Colorado | 196 [18] | 102 [2] | 0 [0] | 298 [20] |
| Idaho | 0 [0] | 2 [0] | 0 [0] | 2 [0] |
| Montana | 0 [0] | 46 [1] | 0 [0] | 46 [1] |
| Nebraska | 0 [0] | 3 [2] | 0 [0] | 3 [2] |
| New Mexico | 80 [8] | 23 [2] | 0 [0] | 103 [10] |
| Texas | 16 [3] | 0 [0] | 0 [0] | 16 [3] |
| Utah | 0 [0] | 105 [2] | 0 [0] | 105 [2] |
| Wyoming | 0 [0] | 138 [4] | 13 [6] | 151 [10] |
| Total | 292 [29] | 446 [14] | 13 [6] | 751 [49] |

Numbers in brackets indicate the number of samples sequenced.

of outbreaks for which the value of $d$ was shorter than the background value of $d$ estimated for the whole region affected by the epidemic. The difference between the normal model of the spatial scan statistic and more traditional applications of the technique, such as the Bernoulli or Poisson models, is that the former is used to identify significant clusters of a continuous variable, rather than clusters of counts or rates. Because the parameter assessed here was measured as a continuous variable ($d$), use of the normal model of the spatial scan statistic was appropriate. A detailed description of this particular variation of the spatial scan statistic is available elsewhere (Kulldorff et al., 2009). As in any other application of the spatial scan statistic, circular windows of candidate clusters were sequentially placed over each infected premises with the size of window varying up to a maximum of 50% of the outbreaks (Kulldorff and Nagarwalla, 1995; Kulldorff et al., 1998). The mean value of $d$ was computed for premises located within each candidate cluster $c$ ($d_c$). The value of $d_c$ was compared with the mean value of $d$ estimated throughout the study region ($d_m$), so that the value of $d_c$ is included for the computation of $d_m$; thus, $d_m$ represents the null hypothesis of uniform distribution of $d$. Because this technique bases the detection of clusters on the computation of mean values for the variable of interest, results are sensitive to the presence of outliers in the dataset. No outliers were found in the dataset used here (Grubb's test, $P > 0.05$). A maximum cluster size of 50% of the outbreaks was selected because a larger cluster of outbreaks with a significant small value of $d_c$ would likely be the reflection of significant high values of $d$ outside the cluster rather than a true cluster of small values of $d_c$ within the cluster (Kulldorff et al., 1998). The values of $d$ observed at each location $i$ ($d_i$) and their corresponding geo-locations were randomly permuted to create 999 data sets that represent random distributions of the values. For each candidate cluster, a log likelihood ratio was computed for the set of observed values of $d$ ($L_d$) and for each of the 999 simulations ($L_s$). The value of $L_d$ was compared with the 999 values of $L_s$ to estimate the number of times ($N$) in which the condition $L_d > L_s$ was observed. The $P$-value of each candidate cluster c was subsequently computed as $N_c/(999 + 1)$. Clusters with a value of $d_c$ significantly ($P < 0.05$) shorter than $d_m$ were interpreted to represent geographical locations in which the probability of finding VSNJV outbreaks in consecutive years was significantly higher than expected under the null hypothesis of a homogenous probability distribution. Therefore, such clusters were considered geographical regions where VSNJV over-wintering was likely to occur. In the normal model of the spatial scan statistic it is possible to weight the values of the continuous variable to adjust for the uncertainty in true value of the parameter associated with each particular location. Because the variable of interest here ($d$) was computed from the data as a point estimate, no weighting factor was used in the analysis. The spatial scan statistic was run using SaTScan v. 8.0 (http://www.satscan.org) and the Grubb's test was computed using GraphPad Quickcalcs (http://www.graphpad.com/quickcalcs/index.cfm).

### 2.3. Genetic analysis

Fifty-nine VSNJV isolates were included in the phylogenetic analysis (Table 1). Although samples were collected from different States and at different periods of time, no formal random design was used for the sampling scheme. Ten of these isolates were isolated from Mexican outbreaks from 2000 through 2004, whereas the remaining 49 isolates were obtained from the southwestern United States (29 in 2004; 14 in 2005; 6 in 2006). Isolates collected from 2000 through 2005 ($n = 53$) have been described elsewhere (Rainwater-Lovett et al., 2007). The six VSNJV isolates collected in 2006 originated from Wyoming, the only state with confirmed infection in 2006, and were processed using previously described methods (Rainwater-Lovett et al., 2007). Briefly, viral RNA was extracted using the RNeasy Mini kit (Qiagen) and reverse transcribed with random hexamers and SuperScript II RNAse H reverse transcriptase (Invitrogen) as per the manufacturers' instructions. The hypervariable region of the phosphoprotein used in the phylogenetic analysis was amplified with Pfu DNA polymerase and the previously described primers NJP-102F and NJP-831R (Rodriguez et al., 1993). Single band products were confirmed visually on an agarose gel and purified using QIAquick PCR purification kits (Qiagen). PCR products were sequenced using a BigDye Terminator Sequencing kit on a 3730A automated sequencer (Applied Biosystems). Sequencher v4.1 software (GeneCodes) was used to analyze chromatograms and Clustal_X (Thompson et al., 1997) was used to create sequence alignments and a 450 nt region of the VSNJV phosphoprotein, commonly referred to as the hypervariable region, was used for phylogenetic analysis.

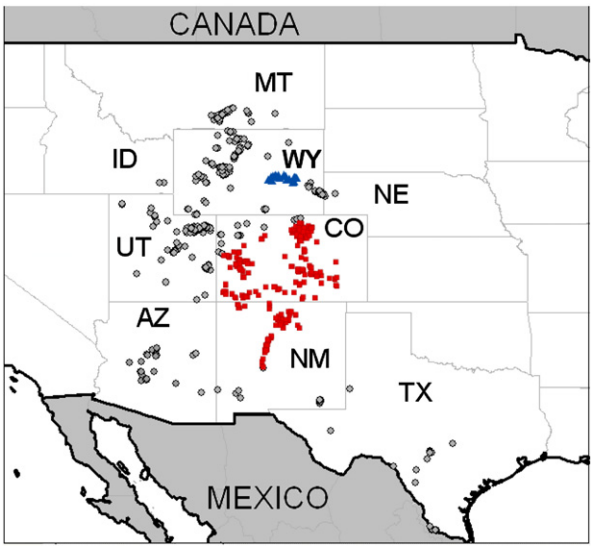


**Fig. 1.** Location of vesicular stomatitis virus outbreaks reported in the United States in 2004–2006. Triangles and squares indicate two clusters for which the distance to the nearest outbreak reported in the previous or following year, whichever was shorter, was significantly ($P < 0.01$) smaller than the epidemic's mean. Acronyms indicate the names of the States affected by the epidemic (AZ, Arizona; CO, Colorado; ID, Idaho; MT, Montana; NE, Nebraska; NM, New Mexico; TX, Texas; UT, Utah; WY, Wyoming).

Phylogenetic analysis was performed using the maximum-likelihood optimality criterion as implemented in PAUP* version β10 (Swofford, 1998). Parameters of nucleotide substitution were estimated using Modeltest, version 3.7 (Posada and Crandall, 1998). Sequences of the six isolates that were reported here for the first time were submitted to GenBank (Accession numbers: FJ595501–FJ595506). The associations between the spatial and phylogenetic clusters detected were assessed using Chi squared tests.

### 2.4. Visualization tools

A publicly accessible web-based system, referred to as the FMD BioPortal (https://fmdbioportal.ucdavis.edu/) was used to display the results of the spatial clustering and phylogenetic analyses. A description of the technical attributes and capabilities of the FMD BioPortal along with an illustration of its functionality has been presented elsewhere (Perez et al., 2009).

## 3. Results

Two clusters of VS outbreaks were identified with values of $d_c$ significantly ($P < 0.01$) smaller than the country's mean value ($d_m = 136$ km) (Fig. 1). The average value of $d_c$ was 2.47 times smaller than $d_m$ in a cluster of 375 outbreaks reported in Colorado and New Mexico ($d_c = 54.8$ km; $SD_{dc} = 50.6$; $n = 375$). A cluster centered in Wyoming included 21 outbreaks for which the value of $d_c$

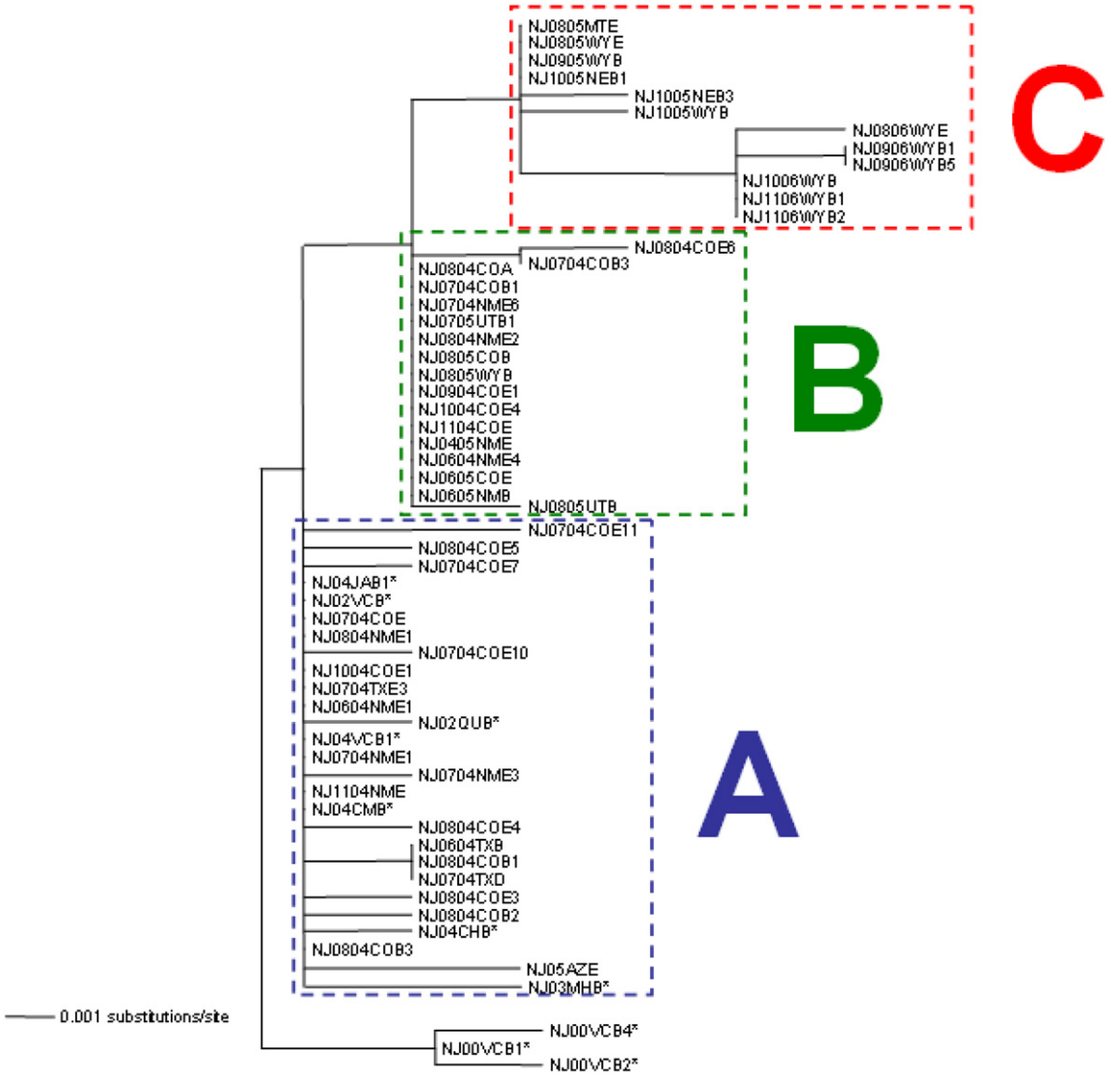


**Fig. 2.** Maximum-likelihood analysis of vesicular stomatitis viruses associated with outbreaks reported in the southwestern United States (2004–2006) and in Mexico (2000–2004). Sequence names indicate the serotype (NJ), the month (when available) and year of isolation, the United States (see Fig. 1 for reference) or Mexican (CH, Chihuahua; CM, Colima; JA, Jalisco; MH, Michoacan; QU, Queretaro; VC, Veracruz) state of origin, and the species affected (A, alpaca; B, bovine; D, donkey; E, equine). Mexican isolates are indicated by an (*). Groups indicate isolates from Mexico and from the United States in 2004 (A), and from the United States in 2004–2005 (B), and 2005–2006 (C).

was 11.3 times smaller than the value of $d_m$ ($d_c$ = 12.5 km; $SD_{dc}$ = 7.8; $n$ = 21).

Sequencing and phylogenetic analysis of 49 VSNJV United States isolates and 10 VSNJV Mexican isolates suggests the presence of at least three different groups of viruses. The first group comprised of 27 viruses, which included 19 and one United States viruses isolated in 2004 and 2005, respectively, and seven closely related viruses from Mexico isolated from 2002 through 2004. The sequences of four of the seven Mexican viruses were identical to those of isolates collected in Texas, Colorado, and New Mexico in 2004 (Fig. 2A). The second group included 10 isolates from 2004 and seven isolates from 2005 (Fig. 2B); 14 of those 17 isolates (82.4%) were part of the spatial cluster centered in Colorado and New Mexico (Fig. 1). The third group encompassed six isolates from 2005 and the six isolates from 2006 (Fig. 2C), from which nine (69.2%) were obtained from outbreaks located within the spatial cluster centered in Wyoming (Fig. 1). The second and third groups did not include viruses from Mexico. Associations between the cluster centered in Colorado and New Mexico and isolates from the first or second phylogenetic groups, and between the cluster centered in Wyoming and isolates from the second or third phylogenetic groups were significant (Fisher exact test, $P < 0.01$).

A video demonstration of these findings using the FMD BioPortal and including the simultaneous visualization of the geographical, temporal, and genetic relation of isolates is publicly accessible at (http://fmdbioportal.ucdavis.edu/vsv/demo.avi).

## 4. Discussion

Over-wintering has been proposed as a possible biological mechanism associated with the re-emergence of VSV during consecutive spring and summer seasons in the southwestern United States following initial introduction of the virus from endemic areas (Rainwater-Lovett et al., 2007). The study here presents results of the combined application of phylogenetic analysis and techniques for identification of spatial clustering. The results are consistent with the hypothesis that VSV-over-wintering was likely to occur in 2005 and in 2006 in two discrete geographical regions of the southwestern United States affected by the epidemic.

Two spatial clusters of outbreaks could be identified within which the average distance to previous or following year's outbreaks was significantly smaller than outside these clusters ($P < 0.01$) (Fig. 1). Spatial clusters of outbreaks that took place in consecutive years suggest the presence of ecological, epidemiological, or demographic factors or forces in the region that facilitate or promote disease infection or spread. One would expect that if over-wintering occurs, premises close to the place where the virus over-winters will be more likely to be infected during consecutive years than far-away located herds. Thus, the spatial clusters detected here may represent geographical locations where over-wintering is likely to occur. Note, however, that the estimates of spatial clustering detected here alone do not provide sufficient evidence that over-wintering has occurred in the region. Spatial clustering may also have resulted from a number of factors promoting the re-introduction of the virus into the region from endemic or infected zones, including, for example, the consistent movement of animals or introduction of contaminated products during consecutive years.

Phylogenetic analysis of the isolates, however, provides additional support for the hypothesis of over-wintering. Two of the three genetic lineages or groups of VSV identified during the epidemic (Fig. 2B and C) mostly overlap with the geographical location of the two spatial clusters identified. Isolates from the spatial cluster of herds located in Colorado and New Mexico correspond to a specific group of genetically related VSV identified in 2004 and 2005 (Fig. 2B). This group seems to be phylogenetically closely related to the group of VSV detected early in 2004, which was similar and in some cases identical to viruses originating in Mexico. Viruses from the spatial cluster of herds located in Wyoming were isolated in 2005 and 2006 and corresponded to a lineage phylogenetically distant from the Mexican and early 2004 United States isolates. Previously, it was shown that two distinct genotypes existed in spatially separated areas of Wyoming in 2005 (Rainwater-Lovett et al., 2007) with an isolate from western Wyoming belonging to Group B (Fig. 2) and those isolated in eastern Wyoming belonging to Group C. It is likely that viruses from the Group C genotype, found in eastern but not in western Wyoming, gave rise to the 2006 viruses that were isolated only in eastern Wyoming, and not in any other part of the southwestern United States.

There are a number of factors that may have biased the results of the spatial and phylogenetic clustering analyses. Spatial heterogeneity of the susceptible population may have resulted in significantly smaller values of $d$ in densely populated regions, which may have biased the results of the spatial analyses towards the detection of clusters in most densely populated areas. On the other hand, the phylogenetic analysis may have been affected by a selection bias associated with the absence of a formal random design to select samples for sequencing. Incomplete or absence of information on the distribution of the susceptible population and absence of a random sampling design are common drawbacks in the investigation of outbreaks and epidemics. For those reasons, arguable, the use of either a phylogenetic or spatial approach, as typically occurs in most scientific contributions dealing with analogous problems, would not have resulted in sufficient evidence to test the hypothesis of over-wintering. Here, we conducted both phylogenetic and spatial analytical approaches and, because sources of potential bias are likely independent for each of the methods, consistency in the results was interpreted as evidence that conclusions were unlikely influenced by potential sources of bias. Consequently, combined interpretation of the phylogenetic and spatial clustering analyses provided more evidence than if either of the methods would have been conducted alone and suggest that VSV may have over-wintered in 2004–2005 in certain regions of New Mexico and Colorado and in 2005–2006 in a limited area of eastern Wyoming. Moreover, virus over-wintering likely resulted in a distinct genetic lineage that evolved from that

**Table 2**
Association between phylogenetic groups of vesicular stomatitis virus (VSV) and spatial clusters of VSV outbreaks for which the distance to the nearest outbreak reported in the previous or following year, whichever was shorter, was significantly ($P < 0.01$) smaller than the epidemic's mean estimated for VSV outbreaks reported in the United States in 2004–2006.

| Cluster | Phylogenetic groups | | *P*-value* |
|---|---|---|---|
| Colorado and New Mexico | A + B | C | <0.001 |
| Inside | 30 | 0 | |
| Outside | 7 | 12 | |
| Wyoming | A | B + C | 0.003 |
| Inside | 0 | 10 | |
| Outside | 20 | 19 | |

* Fisher's exact test.

detected in the previous year within each of the specific clusters, as suggested by the associations estimated between lineages A and B and the cluster centered in Colorado and New Mexico, and between lineages B and C and the cluster centered in Wyoming (Table 2). Thus, when results of the phylogenetic and spatial analyses are interpreted together, they suggest that certain regions of Colorado, Wyoming, and New Mexico may offer ecological, epidemiological, or demographic conditions that facilitate over-wintering, adaptation and evolution of the VSNJV.

Unfortunately, no VSV isolates collected after 2004 from Mexico were available to us for comparison with the strains collected in the United States in 2005 and 2006. However, the phylogenetic analysis presented here shows that 2005 and 2006 outbreaks in the United States were caused by monophyletic viral lineages whose closest ancestors are viruses isolated in the previous year and in the same geographical region. The geographical region affected by the epidemic in 2006 was also affected in 2005, it is far-away located from the Mexican border, and no VSV outbreak was detected in any other region of the southern United States in 2006. For those reasons, interpretation of the results of the phylogenetic and spatial analyses conducted here indicates that it is most likely that the new lineages of VSV identified in the United States in 2005 and 2006 were the result of local evolution of the virus following a period of over-wintering, rather than the consequence of new virus introductions from Mexico.

Specific factors associated with conditions that favor over-wintering are still to be assessed. One may hypothesize, however, that if insect-borne transmission plays a role in the spread of the disease, then persistence, latency or maintenance of the virus in biotic or abiotic substrates may occur during winter, followed by reactivation of the mechanisms of infection in the next warm season. Thus, a possible explanation for the geographical and genetic association detected here could be the selective presence of such factors or forces in the geographical regions where spatial clusters were detected. Noteworthy is the observation that genetically similar VSV were identified from outbreaks that affected Colorado and New Mexico in 1995 and 1997 (Rodriguez et al., 2000), supporting the hypothesis that this particular region may offer conditions that favor over-wintering of the VSV.

VSV epidemics cause alarm and concern in the United States because the disease is clinically indistinguishable from FMD and because of the impact that VS has on producers operations and trade. The results presented here may have impact on the development and establishment of prevention and control strategies for the disease. Control of VSV epidemics is based on the quarantine of infected premises and the recommendation to apply measures aimed at reducing the exposure of susceptible animals to biting insects. Identification of areas where over-wintering may be more likely to occur will help to establish early control and prevention measures in order to prevent the occurrence of new outbreaks in following years. For example, herds in areas at high risk of over-wintering may be tested at the beginning of the summer in order to early detect and prevent the possible re-emergence and spread of the virus and as part of a risk-based surveillance program. Although nine southwestern states of the United States have suffered VSV outbreaks between 2004 and 2006, the evidence for VSV over-wintering offered here affected only the states of Colorado, New Mexico, and Wyoming. This finding suggests that there may be factors in most of the regions affected by the epidemic that limit the spread of the disease to one single season, whereas some specific regions potentially allow over-wintering of the virus. Assessment of the factors or conditions associated with disease over-wintering will help to design and apply measures aimed at preventing or controlling the presence or frequency of such factors in those regions where over-wintering is more likely to occur.

## 5. Conclusion

This study presents evidence that is consistent with the hypothesis that VSV over-wintering may have occurred in certain regions of the Southwestern United States affected by the VSV epidemic in 2004–2006. These results will inform the development and application of disease prevention strategies in the event of future VS epidemics. The joint application of techniques for the identification of spatial clusters of disease and phylogenetic analysis in a web-based framework such as the one presented here may be easily applied to modeling and surveillance of other animal diseases and regions of the world.

## Conflict of interest

None declared.

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