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Spatio-temporal risk factors for viral haemorrhagic septicaemia (VHS) in Danish aquaculture

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ABSTRACT: Viral haemorrhagic septicaemia (VHS) is an economically very important fish disease in the northern hemisphere. When the VHS virus was first isolated in Denmark 50 yr ago, more than 80% of the 800 Danish fish farms were considered to be infected, but vigilant surveillance and eradication programmes led to a drastic reduction in prevalence, and finally, to complete eradication of VHS. Denmark thus obtained official status as an approved VHS-free member state within the European Union in November 2013. Data on outbreaks within the country have been collected since 1970, and here we combined these data with the geographical coordinates of fish farms to identify clusters of high disease prevalence and other risk factors. Our analyses revealed a statistically significant cluster in the southwestern part of the country, which persisted throughout the study period. Being situated within such a cluster was a significant risk factor for VHS. For freshwater rainbow trout farms situated inland, the number of upstream farms was a determining risk factor for VHS, as was distance to the nearest VHS-infected farm and year. Whether the farm used fresh or marine water in production did not have any influence on the risk of VHS, when accounting for whether the farm was situated inside a cluster of high risk. This information can be used when implementing risk-based surveillance programmes.

KEY WORDS: VHSV · Rainbow trout · *Oncorhynchus mykiss* · Disease surveillance · Spatial analysis · Eradication · Denmark

INTRODUCTION

Viral haemorrhagic septicaemia (VHS) is a notifiable disease caused by a novirhabdovirus (OIE 2013). The agent, VHS virus, was first isolated and identified as the causative agent of VHS in 1962 in 3 fish farms in Jutland, Denmark. In 1965, approximately 400 Danish fish farms were infected with the virus, which at that time constituted about 50% of all farms (Kahns et al. 2012). More than 95% of the fish farms in Denmark produce rainbow trout *Oncorhynchus mykiss* Walbaum. VHS primarily affects this species, and due to high mortality, it causes great economic losses to the farming industry (Olesen & Lorenzen 1999). Transmission of the virus occurs horizontally via water from upstream to downstream farms, with escaped fish migrating upstream, with mechanical vectors such as anglers, birds or rodents moving between farms both within the same river and adjacent river systems, and with trade and movements of infected live fish or eyed eggs between farms (Wolf 1988, Olesen & Korsholm 1989).

In Denmark, the majority of VHS outbreaks used to occur in spring or early summer, when the sudden in-

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crease and high fluctuation in temperature is a stress factor to the fish (Olesen & Korsholm 1989). The incubation period is negatively correlated with temperature and virus concentration. Under field conditions, an incubation period of up to 12 mo at low temperature and low exposure has been suspected (Jørgensen 1992). Likewise, higher temperatures shorten the period of clinical infection and virus shedding (Jørgensen & Olesen 1983a, Wolf 1988). This is one of the reasons why eradication is best carried out when water temperature is high, thereby minimising the occurrence of permanently infected wild fish (Jørgensen & Olesen 1983b).

VHS is endemic in many continental European countries, but other countries have only experienced sporadic outbreaks (e.g. Norway and the UK; Stone et al. 2008, Duesund et al. 2010). Sanitation programmes for VHS are in place in many countries, and so far, 'eradication by stamping out' has been successful in countries with a low prevalence of VHS (Olesen & Korsholm 1997, Olesen et al. 2008). In Denmark, a voluntary disease eradication programme was initiated in 1965 by collaboration between the fish farmer organisation and the National Veterinary Institute. In 1970, management of the control programme was taken over by the veterinary authorities, and from then on, all outbreaks of VHS in Danish fish farms have been recorded. The programme was based on stamping out, i.e. removal of all fish on the infected farm, drainage of all ponds and channels followed by cleansing, disinfection and fallowing for at least 6 wk during the period 1 April to 1 October, after which the farm is restocked with approved virus-free fish. The cost of this programme was borne entirely by the industry (Mellergaard 2013). Within the first 10 yr of the eradication programme, the number of infected fish farms decreased from approximately 400 to 100 (Olesen & Korsholm 1989). By 1990, most of the farms in the northern region of Jutland, the peninsula of Denmark where almost all freshwater fish farms are situated, had successfully freed themselves of infection, which led to this zone being approved in 1997 as a VHS-free zone by the European Commission (Olesen 1998). After 1997, the number of infected farms in the southern region was constant at around 20 to 30 farms yr^{-1}, as eradication efforts had not been successful in all areas of that region. A final eradication programme was initiated in the southern region in 2009. In this programme, the farms which had previously experienced repeated outbreaks of VHS were fallowed for a period of 6 wk each spring during the first 2 yr, and the farms on the isthmus between RingKœbing fjord and the North Sea, which had been permanently infected, were fallowed for 2 yr. In the event of an outbreak, it was mandatory to cull all affected fish stock immediately after VHS diagnosis, without the option to let the fish grow to market size first, to reduce the risk of feral fish maintaining the infection in the river system. The economic losses were in part compensated by the European Fisheries Fund, and partly by the farmers (Mellergaard 2013). The programme was successful, and since 2009, there have not been any VHS-infected fish farms in Denmark.

This successful programme is the first time VHS has been eradicated from a high-prevalence country. Therefore, retrospective studies of the epidemiology of VHS together with information on eradication programmes can provide valuable information to be adopted into sanitation programmes in other high-prevalence areas.

In the last decade, the application of geographical information systems to epidemiology has opened up opportunities for a whole new range of epidemiological analyses, especially for the spatio-temporal analysis of disease patterns amongst farmed animals (Norström et al. 2000, Allepuz et al. 2008, Abatih et al. 2009, Mardones et al. 2009).

The aim of the present study was to analyse spatio-temporal risk factors for outbreaks of VHS in Danish rainbow trout farming, in order to gain knowledge relevant to the control and eradication of the disease. Significant spatio-temporal clusters were identified and included in the analyses as risk factors, along with non-spatial (number of farms in a stream and number of upstream farms) and spatial (distance to nearest VHS-positive farm and number of VHS-positive farms within a distance of 5 km) risk factors.

MATERIALS AND METHODS

Study population and data collection

In Denmark, the production of rainbow trout mostly takes place on freshwater farms, which get their water supply from springs or rivers. These farms are stocked with fingerlings that are normally bought from farms that specialize in the production and hatching of eggs and the raising of fingerlings. This practice changed somewhat during the study period. In the early 1980s, it was more common for farmers to produce their own fingerlings, and especially during the period with high prevalence of VHS, the introduction of fingerlings into farms only happened on upstream farms, if at all. Normally the fish are grown to 'plate-size' (approximately 300 to 500 g), but there

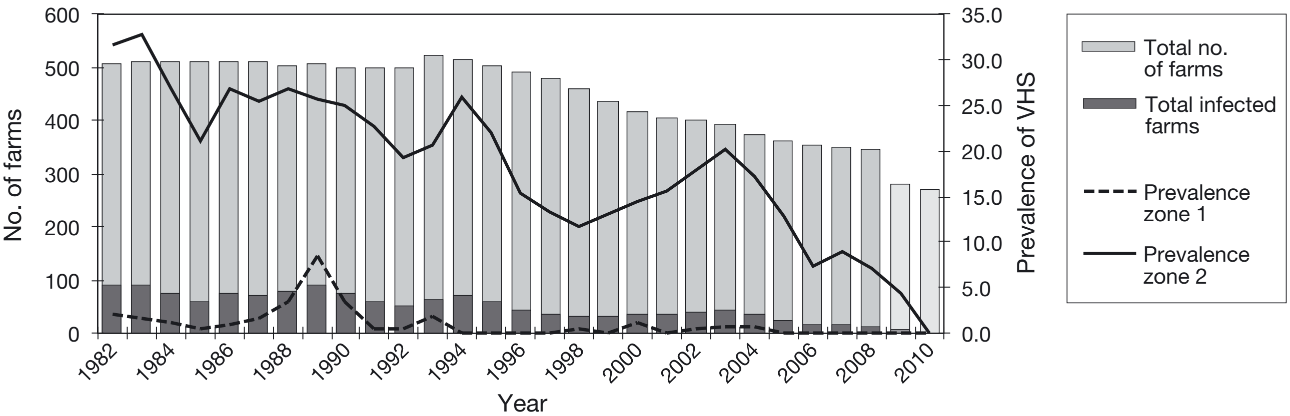

Fig. 1. Number of infected and non-infected fish farms in Denmark from 1982 to 2010 and prevalence of viral haemorrhagic septicaemia (VHS) in Zone 1 (approved free of VHS) and Zone 2 (not approved free of VHS). The years 2009 and 2010 were not included in our analysis, but are given here to show the decrease in prevalence to 0% in 2010

are also a few marine farms who base their production on the transfer of large (600–1000 g) freshwater trout to the sea in the spring. These fish are then harvested in the fall, when they have reached market weights of approximately 2 to 4 kg.

An electronic register of all Danish fish farms was established in 1982. At that time, a total of 509 farms were in operation in Denmark, decreasing to 275 in 2010 (Fig. 1). The present study only included farms cultivating rainbow trout, as this species accounts for more than 90% of the production and is the species most susceptible to VHS. However, as some of the farms occasionally include brown trout *Salmo trutta fario* or brook trout *Salvelinus fontinalis*, these species are also represented, but the percentages are not known.

In total, 496 fish farms were included in the study starting in 1982 and decreasing to 341 by the end of 2008, which marked the end of the study period.

The electronic register holds epidemiologic data on the farms, including a unique identification number, geographical location of the farm (*x*, *y* coordinates in UTM), number of farms in the same stream and number of upstream fish farms in the same stream for each year in production. Farms were only included in the study in those years when fish stock was present, and thus the number of upstream farms and of farms in stream varied with year for each farm.

VHS case definition

The diagnosis of VHS is based on clinical examination and on results from the viral examination performed at the Danish National Veterinary Institute as described in the European Union Commission Decision 2001/83/EC (EU Council 2001). Since 1995, all Danish fish farms have been inspected clinically at least once per year, and samples (30 fish farm^{-1}, pooled in groups of a maximum of 10 fish) have routinely been collected for virological examination in cell culture on no less than half the fish farms each year (as required by the European Union Council Directive 91/67/EEC; EEC 1991). Apart from these inspections, as VHS is a notifiable disease, veterinary officers are called to the farms when an outbreak of VHS is suspected. Data on VHS diagnoses were collected from the Danish Veterinary and Food Administration. All farms contracting VHS during a 1 yr period were registered as VHS-infected that year. For the present study, a farm was considered permanently infected in the following years after infection until an eradication programme had been conducted and approved by the competent authorities.

Non-spatial risk factors

The variables included in this study are described in Table 1. The production of trout mainly occurs in inland freshwater farms, with water intake from a spring or a river. These farms are referred to as 'freshwater farms' throughout the study. A few farms are located on an isthmus between the brackish Ringkøbing Fjord and the North Sea. These farms pump up water from the fjord, which receives water from many streams, including the Skjern Å river system that houses many of the inland freshwater farms. These farms are referred to as 'brackish farms' in the study. In addition, a varying number of marine farms were in operation during the period. These are referred to as 'marine farms'.

Table 1. Descriptive characteristics of variables: type of farm (as defined by water source; see 'Materials and methods'), number of farms in stream system, number of upstream farms and number of farms infected with viral haemorrhagic septicaemia (VHS) within a radius of 5 km for all farms in the endemic zone (Zone 2) in the period 1982 to 2008. N_{Total}: total number of observations during the entire study period, N: number of VHS observations during the entire study period. The significance level of the unadjusted association between VHS status and each of the variables is given by the p-value

Variable	Level	N_{Total}	VHS-positive farms		
			N	%	p
Overall		6362	1341	21.1	
Farm type	Freshwater (N_{farms} = 268)	6027	1170	19.4	<0.001
	Marine (N_{farms} = 12)	170	6	3.5	
	Brackish (N_{farms} = 7)	165	165	100	
No. of farms in stream system[a]	1	181	18	9.9	<0.001
	2–5	658	59	9.0	
	6–9	898	103	11.5	
	10–20	1111	151	13.6	
	≥21	3179	839	26.4	
No. of upstream farms in stream[a]	0	2423	124	5.1	<0.001
	1	857	108	12.6	
	2–3	880	147	16.7	
	4–6	840	267	31.8	
	7–14	519	260	50.1	
	≥15	508	264	52.0	
No. of infected farms within 5 km[a]	0	3490	195	5.6	<0.001
	1	878	240	27.3	
	2	541	204	37.1	
	3	416	178	42.8	
	≥4	702	353	50.3	

[a]Freshwater farms only

For the freshwater farms, the number of farms in each water catchment varies from 1 to 62. For this study, the water catchments were divided into groups according to number of farms along the stream system (Table 1). These farms were also grouped according to the number of upstream farms (Table 1).

Spatial risk factors

Two neighbourhood risk factors were derived to account for the local spread of VHS: (1) distance to nearest VHS-positive farm and (2) number of VHS-positive farms within a distance of 5 km. For some farms, the distance to the nearest VHS-positive farm was more than 25 km. Therefore, the distance to nearest positive farm was truncated at a maximum of 10 km. The mean distance to the nearest infected farm was 6.9 km (SD: 3.35) for farms without VHS and 3.03 (SD: 2.67) for farms with VHS. The number of VHS-positive farms was categorized as 0, 1, 2, 3 and ≥4 positive farms in the neighborhood (within a 5 km radius). In order to evaluate the effect of truncating the distance to nearest VHS-positive farm at 10 km, a sensitivity analysis was performed using truncation at 25 km. Similarly, to evaluate the effect of calculating the number of VHS-positive farms within a distance of 5 km, numbers of VHS-positive farms within a distance of 3 and 10 km, respectively, were evaluated.

Statistical modelling approach

Spatio-temporal cluster analysis

Presence, significance and approximate location of spatio-temporal clusters were evaluated using spatio-temporal scan statistics (Kulldorff 1997). The cluster analysis was carried out using the scan statistic software SaTScan (Ver. 8.0, www.satscan.org).

Space–time cluster analysis searches for approximate locations of space–time clusters and tests for significance (Abatih et al. 2009). The search window was an elliptic cylinder centred on each location (fish farm) with the base representing space (elliptic) and the height of the cylinder representing time. A Bernoulli distribution of VHS-positive (cases) and -negative (controls) fish farms was used. The space–time scan statistics searches for clusters in space and time by using an elliptic cylindrical search window of variable size to detect spatio-temporal clusters. For each location and size of the window, a test is performed evaluating whether the risk of disease within the cylinder is higher than outside the cylinder. The null hypothesis is that the proportion of positive fish farms is the same inside and outside the cylinder and the alternative hypothesis is that the proportion of positive fish farms within the cylinder is higher than outside the cylinder. The significance of identified clusters was tested using a likelihood ratio test. The p-value of the test was obtained using Monte Carlo simulations (999 permutations). It is possible to identify and test significance of secondary clusters in addition to the primary, most likely cluster. The clusters are ordered according to the value of their likelihood. The parameter settings for the space–time cluster analysis were: (1) the maximum percentage of the population at risk to be included in clusters in space was varied from 10 to 50%; (2) a maximum of 50% of the years to be included in clusters; (3) a search for high rates only; and (4) no geographical overlap between clusters was allowed.

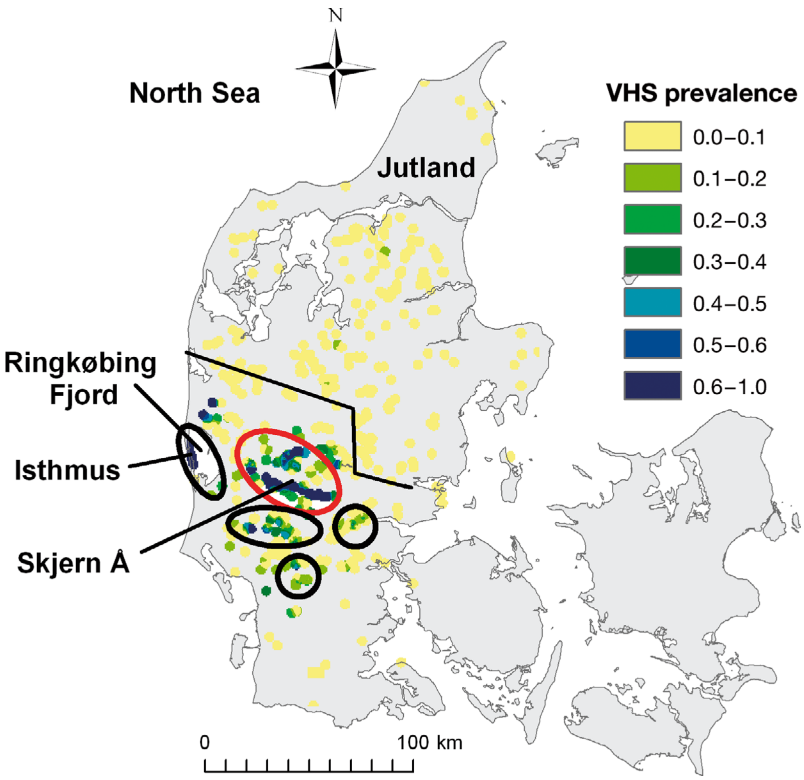

Fig. 2. Spatial distribution of the prevalence of viral haemorrhagic septicaemia (VHS) calculated per fish farm in Denmark for 1982–2010 as number of years with VHS/number of years in use over the entire time period. Also shown are significant primary (red ellipse) and secondary (black ellipses) clusters. Zones 1 (approved as free of VHS in 1997) and 2 (not approved as free of VHS in 1997) are above and below the solid black line, respectively

Multilevel logistic regression analysis with repeated measurements

As described earlier, Denmark was divided into 2 zones in 1997; Zone 1 was approved as free of VHS, and Zone 2 was not approved as free of VHS (Fig. 2). The statistical analyses were performed for the endemic zone (Zone 2) only. VHS (present, absent) each year for each fish farm was the binary outcome. Analyses were performed for (1) the 3 types of farms (freshwater, brackish, marine) to evaluate differences in risk of VHS between farm types, (2) for marine and freshwater farms to evaluate the risk of VHS in relation to type of farm and farm location in clusters, and (3) for freshwater farms to evaluate the effect of spatial and non-spatial explanatory variables only available for freshwater farms.

Initially, the unadjusted association between VHS status of the fish farms and type of farm (freshwater, brackish, marine) was evaluated using Fisher's exact test. The difference in VHS status between freshwater and marine farms without the brackish farms was then evaluated. This was done using a multilevel logistic regression model with repeated measurements (Model 1). The autocorrelation between repeated measurements within the same fish farm was modelled by including an autoregressive correlation structure in the model accounting for non-equidistant time distances. Year was included as a fixed effect. The type of fish farm and the farm being located in a cluster were included as fixed effects. Subsequently, univariable analyses were performed on only freshwater farms to evaluate the unadjusted association between the VHS status of the fish farms and the non-spatial variables (number of farms along the stream system and number of upstream farms) and the spatial risk factors (number of VHS-positive farms within a distance of 5 km regardless of water catchment and distance to nearest VHS-positive farm). The associations were evaluated using univariable logistic regression with VHS status as the outcome.

Finally, in order to further examine the potential effect of risk factors for a positive VHS status of fish farms, multilevel logistic regression analyses with repeated measurements were performed on freshwater farms (Model 2). The autocorrelation between repeated measurements within the same fish farm was modelled by including an autoregressive correlation structure in the model accounting for non-equidistant time distances. Year was included as a fixed effect. The effect of the fish farm being located in a cluster was examined, in addition to other non-spatial and spatial risk factors. Due to collinearity between number of farms in the stream and number of upstream farms and collinearity between number of VHS-positive farms within a distance of 5 km and distance to nearest VHS-positive farm, 4 combinations of the factors were evaluated. Year and farm location in a cluster were included in all 4 combinations. The interaction between being in a cluster and the other variables was also included in the model. The best fitting model was identified using the highest possible coefficient of determination (R^2) (Mittlböck & Schemper 1996) and the dispersion parameter (as close as possible to 1). Backward elimination was used to exclude non-significant effects, starting with the interactions. The odds ratio (OR) was calculated for significant effects. Furthermore, pairwise comparisons between levels within the same effect for significant variables were performed. Because of the many levels for year, pairwise comparisons were not performed for this effect.

The analysis was performed using generalised linear models in the Statistical Analysis System (SAS® Ver. 9.3, SAS Institute) using the GLIMMIX MACRO procedure, and a 5% significance level.

RESULTS

Prevalence of VHS

The number of VHS-positive and -negative farms and the prevalence of VHS by year in the 2 zones are illustrated in Fig. 1. In total, 1417 cases of VHS diagnosis were registered in this time period, with 593 of these being re-infections or new outbreaks, the rest being persistent infections. The overall prevalence of VHS amongst farms in the entire period in Zone 1 was 1.2%, with a yearly min–max of 0–8.5%, and in Zone 2, it was 20.8% with a yearly min–max of 2.2–32.5%.

The number of years with VHS divided by number of years in use over the entire time period for each farm is illustrated in Fig. 2.

Cluster analysis

Spatio-temporal scan statistics were performed varying the maximum percentage of the population at risk in space to be included in a cluster from 10 to 50%. Very little difference was seen between farms included in clusters identified with a maximum percentage of the population at risk in space at 10 and 20% (Table 2). The latter included a few more fish farms and resulted in 4 secondary clusters, whereas a maximum of 10% resulted in 6 secondary clusters. Increasing the maximum percentage of the population at risk in space to be included in a cluster to 30, 40 and 50% resulted in 1 large cluster, which for the 50% included all clusters identified with 10 and 20% maximum percentage of the population. Using a maximum percentage of 30 or 40% resulted in a cluster not including the farms located at the isthmus that were continuously VHS infected. As a spatio-temporal risk factor, we used clusters defined using a maximum percentage of the population in space at risk at 20%. In total, 5 clusters were identified (Fig. 2, Table 2). The primary cluster was centred in the Skjern Å region, with 57 fish farms in the cluster and 379 cases. The duration of the primary cluster was 1982 to 1994, with a relative risk (RR) of 3.2 for being infected within the cluster as compared to outside the cluster.

Risk factors for VHS

Type of farm (freshwater, brackish or marine) was statistically significant for the risk of VHS (Table 1). No marine farms were included in a cluster. Therefore, a new variable was derived as a combination of farm type and fish farm being located in a cluster with the following 3 combinations: (1) marine farm, (2) freshwater farms outside a cluster and (3) freshwater farms inside a cluster. The analysis of marine and inland freshwater farms showed a significantly higher risk of VHS for freshwater farms located in a cluster (Model 1).

For freshwater farms (Model 2), distance to nearest VHS-positive farm, the number of upstream farms combined with whether a farm was included in a cluster and year were all significantly associated with the probability of VHS (Table 3). Fig. 3 illustrates the varying OR associated with year, from each of Models 1 and 2 (including marine and freshwater farms and including only freshwater farms, respectively). The odds for VHS were much higher at the beginning of the study period, and with larger confidence intervals, than during the last half of the period. The odds were consistently higher for the model including both marine and freshwater farms (Model 1) than for the one only including the latter group (Model 2), but both were well within each other's confidence intervals (Fig. 3).

The odds for VHS decreased with increasing distance to the nearest VHS-positive farm (Table 3). The odds for VHS increased with the number of upstream farms regardless of whether farms were situated inside or outside of a cluster (Table 3). However, the odds increased by a maximum of 54.8 for farms inside a cluster, whereas the maximum increase was 7.97 for farms not within a cluster. Number of farms in a stream was not significant, and neither was the interaction between number of farms in a stream and number of upstream farms.

Table 2. Spatio-temporal cluster analysis of Danish fish farms infected with viral haemorrhagic septicaemia (VHS) in 1982 to 2008 using space–time scan statistics (the primary and the secondary clusters with $p < 0.05$ are presented). For the maximum cluster size, the 'Space' factor represents the percentage of the population at risk, and in all maximum cluster sizes, the 'Time' factor in the analysis represented 50% of the years. See 'Materials and methods' for details. O/E: observed divided by expected number of cases in a cluster; p-value: significance level of the cluster; LLR: log likelihood ratio; RR: relative risk of VHS inside compared to outside the cluster

Cluster	Time period	No. of farms in cluster	No. of cases in cluster Observed	Expected	O/E	p	LLR	RR
Maximum cluster size: Space = 10%								
Primary	1983–1995	15	205	49.1	4.2	<0.001	251.5	4.8
Secondary	1990–2002	7	84	17.6	4.8	<0.001	133.3	5.0
	1982–1988	23	109	32.7	3.3	<0.001	88.7	3.5
	1982–1994	11	92	30.0	3.1	<0.001	64.4	3.2
	1989–2001	1	13	2.7	4.8	<0.001	20.4	4.8
	1984–1991	2	15	3.4	4.5	<0.001	20.0	4.5
	1989–1990	13	18	5.0	3.6	0.007	16.1	3.6
Maximum cluster size: Space = 20%								
Primary	1982–1994	57	379	146.0	2.6	<0.001	217.1	3.2
Secondary	1990–2002	7	84	17.6	4.8	<0.001	133.3	5.0
	1982–1994	12	94	32.5	2.9	<0.001	59.1	3.0
	1989–2001	1	13	2.7	4.8	<0.001	20.4	4.8
	1989–1990	13	18	5.0	3.6	0.009	16.1	3.6
Maximum cluster size: Space = 30%								
Primary	1982–1994	77	472	196.0	2.4	<0.001	243.5	3.2
Maximum cluster size: Space = 40%								
Primary	1982–1994	95	552	244.2	2.3	<0.001	260.0	3.1
Maximum cluster size: Space = 50%								
Primary	1982–1994	129	669	331.9	2.0	<0.001	260.5	3.0

DISCUSSION

Our results demonstrated the presence of clusters of VHS in space and time in Danish fish farms producing rainbow trout, and identified that farm type (as defined by the type of water supply), distance to nearest VHS-positive farm and number of farms situated upstream for farms in streams are significant risk factors for outbreaks of VHS, in addition to being situated within a cluster.

The cluster analysis consistently identified a region in the southwest (in Zone 2) as being a high-prevalence area. In this region, most of the fish farms are connected in 1 large water catchment (the Skjern Å river system), with a collecting river that has its outlet into a fjord with brackish water. The Skjern Å river system had long been known to have a high prevalence of VHS, and it proved difficult to eradicate the disease in this region. Different explanations for this have been put forward since the beginning of the 1980s. One such explanation was the high number of fish farms that were situated closely together (Jørgensen & Olesen 1983b). Another explanation was that the virus was probably widespread in escaped free-living rainbow trout (Enzmann et al. 1992), which would make it difficult to contain the disease in that area where many streams connect and where angling, with the accompanying movement of equipment between streams, is popular. Furthermore, fish farms in one of the streams in the region and those that are situated on the isthmus between the fjord and the sea, had consistently been buying survivor fish from VHS outbreaks for outgrowing, thereby maintaining chronic infection in the area. Thus, the main components in the final eradication programme in 2009 to 2012 were auxiliary fallowing of 19 and 16 fish farms known to be at high risk for contracting VHS in 2009 and 2010, respectively, electrofishing of feral rainbow trout in the river systems, immediate culling of fish and fallowing of farms in the event of a VHS-diagnosis on a farm and letting the farms on the isthmus sit empty for 2 yr, in order to eliminate the virus circulating in the water catchment area (Mellergaard 2013).

Diadromous fish migrating from the sea to streams or vice versa can also transport the virus into the freshwater milieu. The Skjern Å system flows into the brackish water of Ringkøbing Fjord, with a large population of salmonid fish, especially whitefish *Coregonus lavaretus*, and this is one plausible expla-

Table 3. Multivariable logistic analysis of risk factors for outbreak of viral haemorrhagic septicaemia (VHS) in Danish fish farms in the endemic zone (Zone 2) in the period 1982 to 2008. Levels within the same effect with the same superscript letters are not significantly different at $p \leq 0.05$. OR: odds ratio, 95% CI: 95% confidence interval for OR. OR for 2 levels within the same fixed effect with different superscript letters are significantly different at a 5% significance level

Variable	Level		Estimate	SE	OR	95% CI	p
		Model 1: Marine and freshwater farms					
Fixed effects							
Intercept			–3.59	0.33			
Type of fish farm combined with fish farm location in clusters							<0.001
	Marine		–0.74	0.65	0.48^{b}	[0.13; 1.71]	
	Freshwater inside clusters		1.88	0.11	6.56^{a}	[5.34; 8.21]	
	Freshwater outside clusters		0^{b}	–	1^{b}	– –	
Year[‡]							<0.001
Random effect							
Autocorrelation			1.31	0.042			
Model fit	Dispersion = 1.001; R^2 = 0.17						
		Model 2: Freshwater farms					
Fixed effects							
Intercept			–0.83	0.48			
Distance to nearest VHS-positive farm			–0.27	0.017	$0.77^{\&}$	[0.74; 0.79]	<0.001
No. of upstream farms × Fish farm location in clusters	Location in cluster	No. of upstream farms					<0.001
	Yes	0	–1.13	0.39	$1^{e\#}$	– –	
		1	–0.11	0.43	2.77^{d}	[1.25; 6.15]	
		2–3	0.70	0.37	6.23^{c}	[3.19; 12.18]	
		4–6	1.41	0.36	12.70^{b}	[6.80; 23.74]	
		7–14	2.09	0.37	25.00^{a}	[13.09; 47.75]	
		≥15	1.83	0.36	19.34^{ab}	[10.28; 36.41]	
	No	0	–1.37	0.34	1^{d}	– –	
		1	–0.33	0.36	2.81^{c}	[1.68; 4.70]	
		2–3	–0.13	0.36	3.43^{bc}	[2.07; 5.72]	
		4–6	0.33	0.35	5.44^{ab}	[3.32; 8.93]	
		7–14	0.61	0.40	7.18^{a}	[3.84; 13.42]	
		≥15	0	–	3.92^{abc}	[2.01; 7.65]	
Year[‡]							0.002
Random effect							
AutoCorrelation			1.13	0.037			
Model fit	Dispersion = 1.109; R^2 = 0.382						

[‡]OR for the variable 'year' is presented in Fig. 3 for the 2 models
[&]OR for the distance to nearest farm infected with VHS is calculated for an increase in distance of 1 km
[#]OR for the interaction is calculated for the comparison of number of upstream farms inside and outside clusters, respectively

nation for the persistent infections in this river system. This putative transmission route was explored by Skall et al. (2004), who concluded that whitefish can be a possible carrier of VHS virus; however, they were unable to isolate the virus from wild whitefish spawners caught in the Skjern Å region.

Different maximum sizes of percentage of population at risk to be included in the clusters were evaluated. There are no guidelines on appropriate selection of the maximum size of the population at risk. Appropriate selection of the maximum size of the population at risk is important, as too small a maximum size can result in overlooking a significant cluster. However, using a large maximum size could hide small significant clusters. Using a maximum size of 50% identifies the most likely cluster in the range 1 to 50% of the population at risk by including a very large proportion of the study unit. This usually results

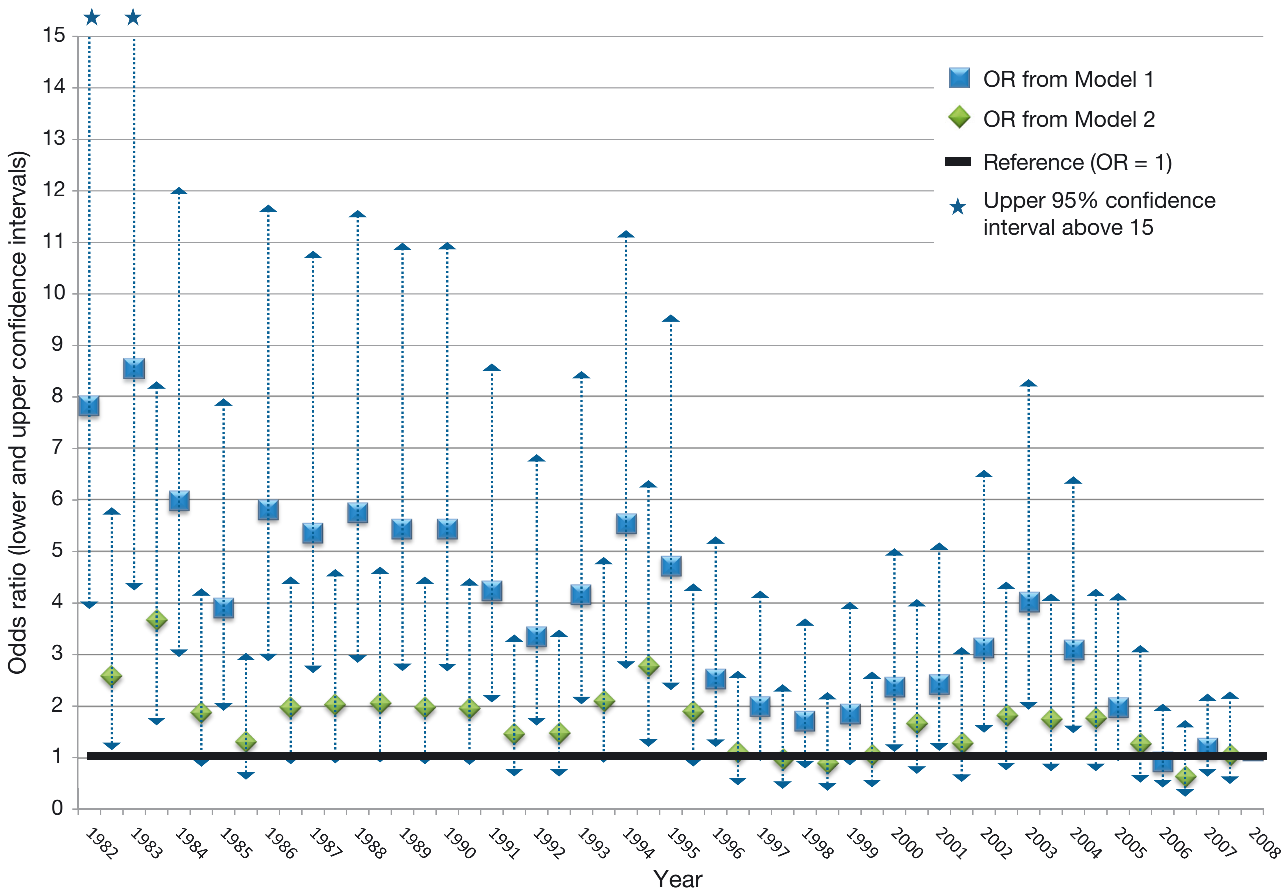

Fig. 3. Odds ratios (OR) of the variable 'year' with 95% confidence intervals from the multivariable logistic analysis including freshwater and marine farms (Model 1) and only freshwater farms (Model 2; see Table 3); 2008 was set as the reference (OR = 1)

in a very large cluster and might not necessarily be very informative (see also Pfeiffer et al. 2008). In the present study, we examined an increasing maximum size from 10 to 50%. We found the best solution to be a maximum size of 20% of the population at risk. At this resolution, all farms identified by including 50% were also included in the clusters, but it was possible to identify several small clusters within the area of high prevalence.

Farm type had a significant influence on the risk of VHS, and it was apparent that farms situated on the isthmus stand out from the rest. These farms pump in brackish water from Ringkøbing fjord, which receives water from the Skjern Å and adjacent water catchments. Moreover, these farms often bought remaining fish from inland freshwater farms which have experienced VHS and wanted to implement 'stamping out'. Therefore, these farms were more or less chronically infected, and were not included in the risk factor studies.

From the analysis including both marine and freshwater farms, we found that the marine farms had a similar risk of VHS as freshwater farms not part of a cluster, whereas being part of a cluster increased the odds of VHS 12-fold. This is an interesting finding that suggests that farm type in itself is not as important as being in a cluster with other infected farms, which is something to be considered when performing risk-ranking of farms for risk-based surveillance. The practice in Denmark has been to categorize the marine farms in category III (unknown status) as per the European Union Council Directive 2006/88/EC (EU Council 2006), since these farms presumably have close contact with wild fish in the marine environment. VHS virus has repeatedly been found in the seas surrounding Denmark (Mortensen et al. 1999), although the isolates have been of different genotypes than the ones normally found in freshwater aquaculture (Skall et al. 2005).

For the freshwater fish farms, we were able to study the additional risk factors of VHS present within the neighbourhood, number of farms in a stream and number of upstream farms.

As the VHS virus is transmitted horizontally both via water and by mechanical vectors, it can be relevant to study spread via river networks and by Euclidian distances. In the present study, we included Euclidian distances using 2 different measures: distance to nearest VHS-positive farm and number of VHS-positive farms within a defined radius. Both factors were significant, but model evaluation identified distance to the nearest VHS-positive farm as being the best predictor for VHS. As would be expected, the odds of getting VHS decreased with increasing distance to VHS-positive farms.

The number of farms in a stream did not have a significant effect on the risk of VHS. Before the study, we had anticipated that the more farms there are connected in a waterway, the greater is the risk for the individual farm for becoming infected, probably via transmission from one of the other farms in the stream. Another reason for higher prevalence of disease in streams with more farms may be that it is more difficult to plan and carry out eradication programmes if many farms are involved, compared to streams with fewer farms. Especially since stamping out and fallowing have not been compulsory or subsidised, meaning that it has been very difficult to get all farmers in the streams to comply with the programme. However, we suspect that the cluster analysis captures the effect of large streams with many farms by already identifying areas with high prevalence of VHS.

The number of farms upstream of a farm has a significant effect on the prevalence of VHS, which was also expected, since the virus will spread via the downstream water current from one farm to another (Wolf 1988). This effect was much higher for farms inside a high-risk cluster than outside. This could be due to the generally lower risk for farms not situated inside a cluster, and accentuates the effect of cluster.

Other studies have identified introductions of live fish and eggs to farms as an important risk factor for VHS (e.g. Oidtmann et al. 2011). As there are no reliable records of live fish movements within Denmark, it was not possible to include this as a risk factor in our study. As explained previously, in Denmark it has been most common to hatch eggs and grow out the fish on the farm, and not to buy fingerlings from other farms. Under the eradication programmes, it was advised to only buy eggs or fingerlings from upstream farms in the same area, in order to avoid accidental introduction of the VHS virus with subclinically infected fish. We therefore expect that the effect of movement of fingerlings has been mostly captured by the cluster analysis.

In recent years, this practice has changed, so that farmers now stock their farms with fingerlings from more geographically distant areas if needed to maintain steady production. This should be taken into consideration in future eradication programmes.

This study aimed at identifying clusters of high prevalence of VHS in Denmark, and at investigating risk factors for occurrence of VHS on fish farms. Previous studies on spatial risk factors associated with disease in aquaculture have been performed by including spatial risk factors in multivariable statistical models (see for example Murray et al. 2003, Saksida 2006, Gustafson et al. 2007), and we are aware of 2 other studies wherein SatScan software was used for diseases in aquaculture (Mardones et al. 2009, Wallace et al. 2012). These studies showed that cluster analysis can be a very useful tool in aquatic epidemiology, in that it can easily identify areas with high prevalence of disease within farms. Further, the analysis made it possible to calculate RR for farms in such clusters as compared to farms outside, something that is in high demand for risk-ranking models (see e.g. Oidtmann et al. 2013).

The SatScan analysis objectively identified areas with a high risk of VHS, without regard to underlying risk factors, and can thus capture many different characteristics specific to a geographical area (e.g. management, owner networks, temperature) that might not be possible to obtain information on to use in risk factor analysis. Thus, Mardones et al. (2009) demonstrated that the RR of clusters were highly confounded by ownership, i.e. farms owned by the same company had comparable risk of disease. The final eradication programme of VHS from Denmark in 2009 to 2013 was centred at the Skjern Å river system, which, as demonstrated here, has been the area with consistently high prevalence. The experiences from the Danish programme and the analysis of the risk factors associated with disease can be used when planning sampling and surveillance for VHS in other countries, especially when applying risk-based surveillance as required, for example, in the European Union Council Directive 2006/88/EC on surveillance of diseases in aquatic animals (EU Council 2006).

LITERATURE CITED

Abatih EN, Ersbøll AK, Lo Fo Wong DMA, Emborg HD (2009) Space-time clustering of ampicillin resistant *Escherichia coli* isolated from Danish pigs at slaughter between 1997 and 2005. Prev Vet Med 89:90–101

Allepuz A, Saez M, Alba A, Napp S, Casal J (2008) Exploratory spatial analysis of Aujeszky's disease during four phases of the eradication programme in Catalonia, Spain (2003-2007). Prev Vet Med 86:164–175

EEC (Council of the European Communities) (1991) Council Directive 91/67/EEC of 28 January 1991 concerning the animal health conditions governing the placing on the market of aquaculture animals and products. Off J Eur Union L 46:1–18

Duesund H, Nylund S, Watanabe K, Ottem KF, Nylund A (2010) Characterization of a VHS virus genotype III isolated from rainbow trout (*Oncorhychus mykiss*) at a marine site on the west coast of Norway. Virol J 7:19

Enzmann PJ, Konrad M, Rapp J (1992) Epizootiological studies on viral haemorrhagic septicaemia in brown trout *Salmo trutta fario.* Dis Aquat Org 12:143–146

EU Council (Council of the European Union) (2001) Commission Decision 2001/183/EC of 22 February 2001 laying down the sampling plans and diagnostic methods for the detection and confirmation of certain fish diseases and repealing Decision 92/532/EC. Off J Eur Union L 67: 65–76

EU Council (2006) Council Directive 2006/88/EC of 24 October 2006 on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals. Replacing CDs 91/67/EEC, 95/53/EC and 95/70/EC. Off J Eur Union L 328:14–56

Gustafson L, Ellis S, Robinson T, Marenghi F and others (2007) Spatial and non-spatial risk factors associated with cage-level distribution of infectious salmon anaemia at three Atlantic salmon, *Salmo salar* L., farms in Maine, USA. J Fish Dis 30:101–109

Jørgensen PEV (1992) Recent advances in surveillance and control of viral haemorrhagic septicaemia (VHS) of trout. In: Kimura T (ed) Proceedings of the OJI International Symposium on Salmonid Diseases. Hokkaido University Press, Sapporo, p 60–71

Jørgensen PEV, Olesen NJ (1983a) Indflydelse af vandtemperatur og smitteintensitet på sygdommens forløb. In: Bekæmpelse af Egtvedsyge (VHS) hos regnbueørreder. Fællesudvalget for det Danske Ørrederhverv, Herning, p 16–21

Jørgensen PEV, Olesen NJ (1983b) Vurdering af mulighederne for bekæmpelse af Egtvedsygen (VHS). In: Bekæmpelse af Egtvedsyge (VHS) hos regnbueørreder. Fællesudvalget for det Danske Ørrederhverv, Herning, p 51–56

Kahns S, Skall HF, Kaas RS, Korsholm H and others (2012) European freshwater VHSV genotype Ia isolates divide into two distinct subpopulations. Dis Aquat Org 99:23–35

Kulldorff M (1997) A spatial scan statistic. Commun Stat Theory Methods 26:1481–1496

Mardones FO, Perez AM, Carpenter TE (2009) Epidemiologic investigation of the re-emergence of infectious salmon anemia virus in Chile. Dis Aquat Org 84:105–114

Mellergaard S (2013) Technical and financial report on the Danish programme for the eradication of viral haemorrhagic septicaemia in accordance with Commission Decision 2008/897/EC, Article 19 (c) for 2009-2012. Danish Veterinary and Food Administration. Available at: www.foedevarestyrelsen.dk/english/SiteCollectionDocuments/25_PDF_word_filer%20til%20download/01kontor/VHS%20report%202010.pdf (accessed 27 March 2014)

Mittlböck M, Schemper M (1996) Explained variation for logistic regression. Stat Med 15:1987–1997

Mortensen HF, Heuer OE, Lorenzen N, Otte L, Olesen NJ (1999) Isolation of viral haemorrhagic septicaemia virus (VHSV) from wild marine fish species in the Baltic sea, Kattegat, Skagerrak and the North Sea. Virus Res 63: 95–106

Murray AG, Busby CD, Bruno DW (2003) Infectious pancreatic necrosis virus in Scottish Atlantic salmon farms, 1996-2001. Emerg Infect Dis 9:455–460

Norström M, Pfeiffer DU, Jarp J (2000) A space-time cluster investigation of an outbreak of acute respiratory disease in Norwegian cattle herds. Prev Vet Med 47:107–119

Oidtmann BC, Crane CN, Thrush MA, Hill BJ, Peeler EJ (2011) Ranking freshwater fish farms for the risk of pathogen introduction and spread. Prev Vet Med 102: 329–340

Oidtmann B, Peeler E, Lyngstad T, Brun E, Bang Jensen B, Stärk KDC (2013) Risk-based methods for fish and terrestrial animal disease surveillance. Prev Vet Med 112:13–26

OIE (World Organisation for Animal Health) (2013) Aquatic animal health code, 15th edn. OIE, Paris. Available at www.oie.int/international-standard-setting/aquatic-code/access-online/ (accessed 27 March 2014)

Olesen NJ (1998) Sanitation of viral haemorrhagic septicaemia (VHS). J Appl Ichtyol 14:173–177

Olesen NJ, Korsholm H (1989) Epidemiologic studies of fish diseases in aquaculture in Denmark with special reference to viral haemorrhagic septicaemia. Proc 1st Annual Meeting of the Nordic Society for Veterinary Epidemiology, Tune, Denmark

Olesen NJ, Korsholm H (1997) Control measures for viral diseases in aquaculture: eradication of VHS and IHN. Bull Eur Assoc Fish Pathol 17:229–233

Olesen NJ, Lorenzen N (1999) Rhabdovirusinfeksjoner. In: Poppe T (ed) Fiskehelse og fiskesykdommer. Universitetsforlaget AS, Oslo, p 142–147

Olesen NJ, Kahns S, Skall HF, Nicolajsen N (2008) Survey and diagnosis of listed fish diseases in the European Community 2007. Report from the 12th Annual Meeting of the National Reference Laboratories for fish diseases, 17-20 June 2008, Aarhus, Denmark. Available at www.eurl-fish.eu/~/media/Sites/EURL-FISH/english/activities/annual%20meetings/12%20am%202008.ashx (accessed 11 October 2013)

Pfeiffer D, Robinson T, Stevenson M, Stevens KB, Rogers DJ, Clements AC (2008) Spatial analysis in epidemiology. Oxford University Press, New York, NY

Saksida SM (2006) Infectious haematopoietic necrosis epidemic (2001 to 2003) in farmed Atlantic salmon *Salmo salar* in British Columbia. Dis Aquat Org 72:213–223

Skall HF, Kjær TE, Olesen NJ (2004) Investigation of wild caught whitefish, *Coregonus lavaretus* (L.), for infection with viral haemorrhagic septicaemia virus (VHSV) and experimental challenge of whitefish with VHSV. J Fish Dis 27:401–408

Skall HF, Olesen NJ, Mellergaard S (2005) Viral haemorrhagic septicaemia virus in marine fish and its implications for fish farming — a review. J Fish Dis 28:509–529

Stone DM, Ferguson HW, Tyson PA, Savage J and others (2008) The first report of viral haemorrhagic septicaemia in farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), in the United Kingdom. J Fish Dis 31:775–784

Wallace IS, Purvis NL, Hall M, Matejusova I (2012) Spatial analysis of a gyrodactylid surveillance data set. Annual meeting of the Society for Veterinary Epidemiology and Preventive Medicine, 28–30 March 2012, Glasgow, Scotland

Wolf K (1988) Fish viruses and fish viral diseases. Cornell University, Ithaca, NY

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