Preventive Veterinary Medicine 105 (2012) 49–58

Contents lists available at SciVerse ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

Case control study to investigate risk factors for bovine neonatal pancytopenia (BNP) in young calves in southern Germany

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ARTICLE INFO

Article history:
Received 5 April 2011
Received in revised form 8 February 2012
Accepted 9 February 2012

Keywords:
Calves
Haemorrhagic diathesis
Bone marrow depression
Case control
BNP

ABSTRACT

A case control study on farm level was conducted at the Clinic for Ruminants, LMU Munich, to identify possible risk factors associated with the observed increase in numbers of calves showing clinical signs of Bovine Neonatal Pancytopenia (BNP) since 2006 in southern Germany. Interviews were conducted between August 2008 and June 2010.

The characteristics of 56 dairy farms with at least one confirmed case of BNP (thrombocytopenia and leukocytopenia and/or typical findings in post-mortem examination and bone marrow histology) were compared with those of two sets of 50 control dairy farms each, with no history of BNP. The first set of 50 control farms was selected randomly from veterinary practices which had never observed a BNP case on the farms they serviced. The second set of 50 control farms was matched by the veterinary practices which had provided case farms. Two separate analyses were conducted: (1) case farms ($n = 56$) vs. randomly selected control farms ($n = 50$) and (2) case farms ($n = 56$) vs. a matched set of control farms ($n = 50$). All variables with $p < 0.2$ in the univariable analysis were included in stepwise logistic regression models. In the first analysis, only the use of PregSure® BVD vaccine was positively associated with BNP with an odds ratio of 1292 (95% CI: 114–14707). In the second analysis, conditional logistic regression models did not converge, therefore non-conditional logistic regression models were conducted. In the non-conditional analysis five variables remained in the model, three of which were negatively associated with BNP: the use of vitamin E and selenium, the frequent use of mastitis tubes, and the use of stem growth regulators in grain production. The use of prophylactic measures (such as control of parasites or vaccination of calves against respiratory disease) was positively associated with BNP with an odds ratio of 14.3 as well as the use of PregSure® BVD vaccine with an odds ratio of 426 (95% CI: 20–9095).

1. Introduction

Since 2006, a striking increase in the incidence of bleeding disorders in young calves on both conventional and organic dairy farms and cow-calf operations has been reported. The condition occurred sporadically, only rarely were there multiple cases per farm. Cases have been reported in several European countries, such as Belgium, France, Germany, Hungary, Ireland, Italy, Luxembourg, the Netherlands, Poland, Spain, and UK. The disease has caused considerable animal health concern in the western world (ProMed-mail, 2010). Initially, the syndrome was inconsistently reported under several names like "haemorrhagic diathesis" (Friedrich et al., 2009b; Klee, 2009; Pardon

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doi:10.1016/j.prevetmed.2012.02.008

et al., 2010), "bleeding calf syndrome" (Bell et al., 2009), "haemorrhagic diathesis syndrome" or "idiopathic haemorrhagic diathesis" (Corbière et al., 2009; Kappe et al., 2010; Penny et al., 2009; Smolenaars and Mars, 2009), until the syndrome was given the official denomination "bovine neonatal pancytopenia – BNP" at a symposium in Marseille, France, in December 2009. The principal clinical signs of mucosal petechiae, bleeding after injections, various amounts of blood in the faeces, spontaneous cutaneous haemorrhages, and severe secondary infections can be explained by the pronounced thrombocytopenia and leukocytopenia (Bell et al., 2009; Brugère-Picoux, 2009; Corbière et al., 2009; Doll et al., 2009; Friedrich et al., 2008, 2009a,b; Gentile et al., 2009; Kappe et al., 2010; Pardon et al., 2009, 2010; Penny et al., 2009; Sanchez-Miguel et al., 2010; Smolenaars and Mars, 2009). In affected calves bone marrow is profoundly affected with reduction of megakaryocytes, lymphoid and myeloid precursor cells (panmyelophthisis) (Friedrich et al., 2009b; Pardon et al., 2010; Kappe et al., 2010).

Calves of both genders were similarly affected and the disease was observed in several breeds, such as Simmental, Holstein Friesians, Brown Swiss, Belgian Blue, Charolais, Blonde d'Aquitaine, Limousin, Aberdeen Angus, Montbéliard, as well as in various cross breeds. There were no obvious irregularities in the relative frequency of the breed distribution (Bell et al., 2009; Corbière et al., 2009; Doll et al., 2009; Friedrich et al., 2009b; Gentile et al., 2009; Kappe et al., 2010; Pardon et al., 2010; Penny et al., 2009; Smolenaars and Mars, 2009). Data available to the different research groups on the frequency of occurrence of BNP are probably incomplete because BNP is not notifiable in any country and the disease may be overlooked because it is rare and calves may die without farmers realising that it was BNP. Various studies failed to find evidence of specific infections or intoxications (Doll et al., 2009, 2010; Friedrich et al., 2010; Pardon et al., 2010). Kappe et al. (2010) suggested an association between BNP and the presence of porcine circovirus type 2 (PCV-2), as they identified PCV-2-DNA in 5 of 25 BNP calves and in 1 of 8 control calves. However, this finding could not be confirmed by Willoughby et al. (2010). At the beginning of the study the clinical picture of the disease and some information about the occurrence of the disease in some European countries were known. Assumptions about potential causes or involvement of specific agents included vaccines and other prophylactic measures or toxins, as well as chemicals used in agriculture (such as crop stem growth regulators, e.g. chlorocholine chloride, CCC – these are chemicals that reduce the stem growth of crop and thus reduce the risk of stem breakage). The increase of BNP cases and the first cases of Bluetongue (BT) occurred roughly at the same time in Germany. This temporal correlation between BNP and BT and the subsequent legal obligation to vaccinate against BT, led to the assumption of a causal link between either the disease or the vaccination campaign against BT and BNP (unpublished statements). The aim of the present study was to conduct a case control study to investigate different risk factors for BNP within southern Germany. To the best of our knowledge, this is the first such study on this subject.

2. Materials and methods

2.1. Study design

A case control study was designed to investigate a wide range of risk factors potentially associated with the increased occurrence of BNP in young calves. In setting up the study and in reporting it, the STROBE guidelines were followed whenever possible (von Elm et al., 2008). The unit of analysis in this study was farm. All factors hypothesised as associated with BNP at the beginning of the study were included in the questionnaire; hence hypotheses regarding potential risk factors covered a wide range of potential management, health and nutritional factors as applied prior to the study. The case control study was designed with the intention of including 50 case farms (with at least one confirmed case of BNP) and respective control farms (without reported or known cases of BNP in calves). With this sample size an odds ratio of 3.5 for a risk factor prevalent at 20% with 80% power would be significant with $p \leq 0.05$ in an unconditional logistic regression. Another study (unpublished) revealed that several BNP farms were recorded by some veterinary practices, whereas no cases were seen by neighbouring practices, suggesting that differences existed between practices in the occurrence of BNP or in diagnosing or reporting BNP (BNP is not notifiable). Therefore, the study design included a random control set and a control set matched on veterinary practice, each set containing 50 control farms. Interviews with farmers of case farms were conducted between beginning of 2009 and June 2010 and with farmers of control farms between November 2009 and June 2010. In the following, BNP farms are referred to as "cases" and control farms as "controls".

2.2. Selection of case farms

The study was conducted at the Clinic for Ruminants, LMU Munich, Germany. Most cases of BNP were reported from southern Germany to the clinic due to the vicinity, with first cases being reported in 2008 and increasing numbers of reports in 2009 and 2010. Farms were eligible as case farms if they had one or more confirmed cases of BNP in their calves. The post mortem demonstration of bone marrow depletion in calves less than four weeks of age was considered the gold standard even in the absence of information on clinical signs and/or haematological changes. In calves without post mortem examination, clinical signs indicative of BNP plus haematological changes (thrombocytopenia [<200 G/L] (Stöber and Gründer, 1990) and leukocytopenia [<4 G/L] (Kraft et al., 2005)) had to be present for case confirmation. Farms were eligible as control farms if they had never observed any clinical or otherwise confirmed cases of BNP in the past.

Initially (January until October 2009) all farms that had presented BNP calves to the Clinic for Ruminants, LMU Munich, were enrolled ($n = 27$). By that time, more and more cases were confirmed by sending blood samples to the clinic rather than taking the animals themselves to the clinic. Therefore the study also included farms with calves being confirmed by blood samples only. Thus, the remaining farms ($n = 29$) were selected randomly from the subsequently reported farms with confirmed cases ($n = 248$) irrespective of the method of BNP confirmation. Due to time-consuming interviews with the case farms and funding constraints, the study was limited to Bavaria.

2.3. Selection of control farms

The selection of control farms took place from November 2009 until June 2010. Two sets of control farms were selected. The first 50 controls were randomly chosen from veterinary practices in Bavaria that had never observed the disease in their practice. These practices were identified in a related study on the regional distribution of BNP in Bavaria (paper in preparation). In total, 1060 practices were listed in the official database of the Bavarian Veterinary Association and invited to participate. Among 435 (41%) respondents, 322 (74%) reported that they had never seen the disease in the farms they serviced. Of these practices, 50 were chosen randomly and they in turn selected five farms each randomly from their lists of clients. Of the total of 250 farms, 50 were chosen randomly as controls.

For the second set of control farms, matching was done with veterinary practice as the matching factor and practices with one or more case farms in their client database were supplied with as many random numbers as they had case farms. On the basis of these random numbers, the practices selected the control farms using their practice management software. If the farm managers confirmed the absence of any clinical cases and declared their willingness to be interviewed, their farm was enrolled as a control farm. This matching was only feasible for 45 case farms; the remaining five control farms were taken additionally from the veterinary practices with case farms. Thus, in general only one control was chosen for each case farm, except for five case farms that were matched with two control farms.

2.4. Data collection

A specific questionnaire of eight pages was designed which covered numerous details of all case and control farms, a summary of which is listed in Table 1. The questionnaire was developed in consultation with several veterinary professionals from the Clinic for Ruminants, LMU Munich, and external professionals (at Pfizer Animal Health). The first part of the questionnaire (Table 1 except the last row) was completed for all case and control farms and covered information about farm management, nutrition of cows and calves, preventive healthcare and health issues. Special consideration was given to medical treatments and vaccinations applied by veterinarians or farmers as part of the routine health management. With regard to feeding of cows and calves, farmers were asked for all commonly used feed components and feed additives such as propylene glycol, propionate, glycerine, and fermentation additives for silage. Preventive measures taken at the farm were divided into vaccinations (coded as specific variables) and in general preventive measures such as antiparasitic prophylactic measures.

Table 1
Short form of the questionnaire used in a case control study towards BNP in southern Germany (2008–2010).

Parameters	Description
General information on the farm	Zip code, number of cattle, purchase of cattle, other species, housing of cattle (cows, young stock, calves)
Feeding of cows	Components of the cow's ration, feed additives
Feeding of calves	Feeding system, colostrum, whole milk, milk replacer, oral rehydratation solutions, concentrates
Preventive health care	Prophylactic use of halofuginon, vaccinations (vaccination of dams against calf diarrhoea, blue tongue, BVD, calf pneumonia)
Prophylactic and therapeutic interventions in cows	Dry cow mastitis treatment, milk fever prophylaxis, teat dips, mastitis tubes, infertility treatment
Health problems	Diarrhoea, respiratory disease, navel ill, infectious agents
Miscellaneous	Disinfection, vaccination status
Specific questions on case farms relating to BNP cases	Number and time of cases, symptoms, pedigree of cases, information on dams of cases, treatment and progress of cases

The second part of the questionnaire (3 pages; last row of Table 1) requested information about the BNP calves, first signs, treatment, course of the disease and further details about the affected calves and dams.

Telephone interviews were conducted with the herd managers of case and control farms from January 2009 until June 2010. Farms with cases occurring in 2008 were interviewed at the time of occurrence with a preliminary questionnaire first and then interviewed a second time for the missing information with the final questionnaire, in the beginning of 2009. The interviews with farmers of case farms lasted about 1–2 h (depending also on the number of cases on the farm), while the interviews with farmers of control farms lasted around 0.5–1 h. During the interview the answers were recorded on paper. The interviews were conducted in a uniform fashion by three veterinarians (AC; AA; FR). All three interviewers were trained prior to the interviews and instructed to avoid leading questions.

2.5. Statistical analysis

The unit of observation in this study was the farm. Analysis was done using SPSS (version 18.0; SPSS Inc., Chicago, IL, USA) and SAS (version 9.2; SAS Institute Inc., Cary, NC, USA).

Except for the use of vaccines all categorical variables were recorded either as positive ('1') if they had been in place during the previous two years, or negative ('0') if they had never been in place (independent of the years of use) or were used more than two years prior to the interview. The categorisation of the use of vaccines was based on the previous five years because of the possible long-term effect of vaccinations. For the variable vaccination positive/negative, one single use of the vaccine within five years was sufficient to classify it as positive. The only continuous variables were the number of animals present on the farm (total herd size, cows, young stock and calves).

The dependent variable was the farm status with regard to BNP (confirmed BNP calves or no such calves). None of the variables used in the analyses had missing data. Cases were compared to controls in two separate analyses. The first included the comparison between cases ($n = 56$) and the first set of randomly selected controls ($n = 50$). The second analysis compared the same cases ($n = 56$) with the second set of matched controls ($n = 50$).

2.6. Random controls

Each exposure variable was compared to the case/control status in two-by-two contingency tables using chi-square tests, odds ratios (OR), and their 95% confidence intervals. Continuous data were interpreted visually for normality using box plots and QQ plots. If normality could be assumed, an independent *t*-test was used where the continuous variable was the outcome and the case/status formed the groups; else they were compared to the case/control status of a farm using non-parametric Mann–Whitney *U*-tests. Correlations between different risk variables were investigated using Spearman's correlation coefficient and Chi-square tests. Of variables that were highly correlated ($r_s > 0.8$; Chi squared test, $p < 0.001$) and related to one specific factor (e.g. feeding) only one variable was selected for the multivariable analysis. This was also the case for the use of PregSure® BVD vaccine and the variable BVD-vaccination. Only the variable PregSure® BVD vaccine (used yes/no) was used in the multivariable model for all 50 and 56 farms, respectively.

Rare or frequent exposures that were reported by less than 10% or more than 90% of the farmers could not be evaluated by the multivariable analysis because the datasets were small and multivariable logistic regression would either not converge or result in very large standard errors due to covariate combinations with zero observations. All other variables that were associated with the case/control status with $p < 0.2$ were first tested for correlation between each other to explore possible interrelations and to identify potential sources for collinearity, and were then included in a multivariable logistic regression model with automated forward stepwise variable selection. In stepwise selection, independent variables were entered into the model if their *p*-value was less than 0.20, and retained if $p < 0.05$. The linearity assumption of continuous variables was tested by using quartiles as categorical variables, or polynomials as an alternative, and if significant were included as such, else in their original form. Two-way interactions between significant exposure variables were either added to the logistic model or in case of non-convergence tested for homogeneity across strata by the Mantel–Haenszel technique (Rothman and Greenland, 1998).

Quasi complete separation with a very large odds ratio (OR = 1292) occurred when the exposure variable PregSure® BVD vaccine was included in the logistic regression model with the random set of controls, thus only PregSure® BVD vaccine was retained in the final model. To evaluate the effect of other variables, the first model was run after exclusion of PregSure® BVD vaccine. Each significant variable from this model was subsequently subjected to a Mantel–Haenszel analysis to evaluate possible confounding or interaction effects between the variable and PregSure® BVD vaccine with respect to the case/control status. Confounding was defined as a >15% change in the coefficient of any of the other significant variables added to the model. Yates correction (realised in SAS proc freq) was used for tables that included one or more zero counts to obtain odds ratios; a procedure, which adds a correction factor of 0.5 if cells contain zero counts (Yates, 1934; Kleinbaum et al., 2007). This resulted in a series of adjusted ORs for PregSure® BVD vaccine.

2.7. Matched controls

Matching controlled for the possible confounding effect of veterinary practice. A conditional logistic regression analysis including all variables did not converge, due to the non-existence of matched factor occurrences. For example, there were no pairs where the control farm used the factor, but the associated case farm did not. If this was the case, conditional logistic regression models did not converge and stepwise unconditional logistic regression was therefore initially used to select significant variables. In a second step, all significant variables for which all possible pair combinations were available were subjected to conditional logistic regression, omitting variables like PregSure® BVD vaccine, which lacked dis-concordant pairs. Finally, coefficients of significant variables from the conditional and unconditional analysis were compared to evaluate whether matching was a source of confounding. Since matching introduced only minor confounding effects, all inferences about the second control group were based on the unconditional model, which also included PregSure® BVD vaccine.

Herd size and age structure (cows, young stock and calves) of the herd were included in all logistic regression models as they were considered to be potential confounding factors. All unconditional logistic regression models were tested for model fit and outliers according to Hosmer and Lemeshow (2000). The ratio of $-2\text{Log}\,L$ (deviance)/df (overdispersion) was used for the conditional model as measure of goodness of fit. A good fit of the model was assumed when Hosmer–Lemeshow test had a *p*-value >0.1 and overdispersion was <2 (Schukken et al., 2003). Common leverage and influence diagnostics were done as described in the literature (Hosmer and Lemeshow, 2000).

3. Results

A total of 56 case farms had reported 137 confirmed BNP cases. 23 farmers reported only one case calf, the remaining farmers reported up to 9 affected calves on their farm. Half the farmers had detected their affected calves on the basis of haemorrhages, while the calves in the other half of the case farms were affected by other diseases prior to the bleeding disorder. None of the farmers reported any unusual treatments in the dams of the affected calves during pregnancy.

Univariable analysis using the set of randomly selected control farms identified a number of variables that were significantly ($p < 0.05$) associated with the occurrence of

Table 2
Variables with *p*-values less than 0.2 in the univariable analysis comparing 56 BNP-case farms with two sets of 50 control farms in a study on BNP in southern Germany (2008–2010) (*p*-values ≤ 0.05 are indicated in bold).

Variable	Case farms (*n* = 56)	Set 1 control farms (*n* = 50)	Univariate *p*-value (control set 1)	Set 2 control farms (*n* = 50)	Univariate *p*-value (control set 2)
General information on the farm					
Number of breeding young stock	Median 29.5	Median 40.0	**0.039**[a]	Median 30	0.441[a]
Number of calves	Median 20	Median 15	0.166[a]	Median 18.5	0.692[a]
Purchase of cattle	2/56	10/50	**0.018**	7/50	0.054
Free stall (cows)	25/55	33/50	**0.034**	30/50	0.114
Free stall (heifers)	31/55	19/50	0.059	30/50	0.629
Heifers in same barn as lactating cows	20/55	25/50	0.158	21/50	0.507
Dry cows and lactating cows in same barn	39/56	37/50	0.619	41/50	0.140
Calves housed in groups	47/56	39/50	0.436	47/50	0.102
Components of the cows' ration					
Barley	32/56	39/50	**0.022**	29/50	0.929
Rape	32/56	36/50	0.111	42/50	**0.003**
Linseed meal	10/56	0/50	**0.001**	10/50	0.778
Sugar beet pulp	19/56	8/50	**0.034**	12/50	0.190
Grass silage	55/56	45/50	0.098	50/50	0.342
Fresh grass	19/56	9/50	0.063	21/50	0.392
Clover	11/56	4/50	0.086	9/50	0.829
Maize (Corn)	40/56	27/50	0.063	31/50	0.303
Soybean	43/56	36/50	0.057	35/15	0.429
Brewer's grains	6/56	6/50	0.835	10/50	0.182
Hay	53/56	36/50	**0.001**	43/50	0.129
Straw	24/56	39/50	**<0.001**	16/50	0.250
Propylene glycol	21/56	8/50	**0.013**	7/50	**0.006**
Glycerol	9/56	7/50	0.766	3/50	0.102
Feeding of calves					
Hay	49/56	50/50	**0.014**	50/50	0.010
Water free choice	49/56	50/50	**0.014**	49/50	**0.063**
Oral rehydration solutions	50/56	40/50	0.182	46/50	0.746
Colostrum replacer[b]	9/56	2/50	**0.041**	1/50	**0.013**
Concentrates	41/56	43/50	0.105	29/50	0.099
Prophylactic measures					
Prophylactic use of Halofuginone	11/56	3/50	**0.038**	5/50	0.166
Vaccination of dams against calf diarrhoea	32/56	17/50	**0.018**	20/50	**0.052**
Oral vaccination of calves	3/56	3/50	0.886	0/50	0.097
Other prophylactic measures (e.g. parasitic, insect, other vaccinations)	20/56	14/50	0.395	4/50	**0.002**
Vitamin E/selenium	6/56	7/50	0.607	12/49	0.062
Vaccination against BVD	55/56	16/50	**<0.001**	17/50	<**0.001**
PregSure® BVD[c]	55/55	2/16	**<0.001**	13/17	<**0.001**
Prophylactic and therapeutic interventions in cows					
Teat dips	14/56	24/50	0.014	14/50	0.680
Mastitis tubes	29/56	38/50	0.010	45/50	**0.001**
Dry cow mastitis treatment	43/56	45/50	0.070	42/49	0.245
Frequent calf diseases					
Diarrhoea	29/56	14/50	0.013	19/50	0.182
Bronchopneumonia	19/56	6/50	0.008	13//50	0.411
Infectious agents (*E. coli*, rotavirus, coronavirus) identified	15/56	6/50	0.056	15/49	0.665
Inappetence	1/56	1/50	0.935	5/50	0.064
Miscellaneous					
Use of stem growth regulators	12/56	33/50	<0.001	37/50	**<0.001**
BVD status (vaccinated/free/unknown)	55/0/1 (56)	16/10/24 (50)	<0.001	17/2/31	**<0.001**
Disinfection of calf buckets	5/56	10/50	0.102	4/49	0.889
Insect control	36/56	38/50	0.190	27/49	0.338
No access to sweet clover, horse tail and bracken fern	52/56	47/50	0.813	50/50	0.054

[a] Assessed using Mann–Whitney *U*-test.
[b] Some case farms had changed to use colostrums replacer after having had BNP cases, therefore this variable is biased.
[c] Number of farms that used PregSure® BVD of those farms that vaccinated against BVD. Farms were classified as using PregSure® BVD vaccine even if not all animals were vaccinated, as the variable was on herd level. For the multivariable analysis this variable was recoded into a new variable using PregSure® yes/no for all 50 and 56 farms, respectively.

BNP, either as risk or as protective factors (Table 2). Of the 56 case farms, 55 vaccinated against BVD, and all of them were using PregSure® BVD vaccine, whereas 16 of the 50 control farms vaccinated against BVD, of which two used PregSure® BVD vaccine; one farmer did not recall the name of the vaccine that had been used. Linseed meal, which was fed to cows by 10 of 56 case farms, but by none of the control farms, was also a highly associated risk factor.

Univariable analysis using the matched controls, comparing the case farms with controls of matched veterinary practices, bivariate conditional logistic regression models did not converge for the use of PregSure® BVD vaccine and many other variables, due to the non-existence of matched factor occurrences. Of the 45 matched case control pairs, 32 were discordant in that the case farm used PregSure® BVD vaccine, but the control did not; in 12 pairs both, case and control farm used this vaccine; and in one pair, neither case nor control farms used this vaccine. However, there were no pairs where the control farm used PregSure® BVD vaccine, but the case farm did not. Therefore, a non-conditional univariable analysis was used for the comparison between case and control farms of the second set (Table 2). In total, 17 of the 50 control farms in the second set of controls vaccinated against BVD, of which 13 used PregSure® BVD vaccine, which led to an odds ratio of 156.5 (95% CI: 19.6–1248.3) for the use of PregSure® BVD vaccine.

There were no significant differences between case and control farms in other factors known to lead to haemorrhagic diathesis, such as access to bracken fern (*Pteridium aquilinum*), sweet clover (*Melilotus* spp.) or horsetail (*Equisetum* spp.), investigated in the questionnaire (Table 1).

The results of the multivariable logistic regression models are listed in Table 3 for both sets of comparisons.

In the first comparison linseed meal and purchase of cattle were excluded from the multivariable logistic regression, due to their rare occurrence (<10%). Also the variables breed, feeding of hay to cows and feeding of grass silage to cows were excluded, as they occurred in both cases and controls in very high frequencies (Table 2). Only the use of PregSure® BVD vaccine remained as significant risk factor in the model. The analysis of correlations between this factor and all other factors not included in the model revealed several significant high correlations (Chi-squared test $p < 0.001$). In a logistic regression model without the factor PregSure® BVD vaccine five factors remained in the model (Table 3). Two-way interactions were not significant. Two factors were positively associated with the BNP-status of the farm: respiratory health problems in calves and the use of dam vaccination against calf diarrhoea, while three factors were negatively associated with the BNP-status of the farm: the feeding of barley to cows, the use of teat dips and the use of stem growth regulators. These variables were used to estimate adjusted odds ratios for the factor PregSure® BVD vaccine and the presence of BNP on the farm. The resulting odds ratios for PregSure® BVD vaccine ranged between 528 (adjusted for respiratory health problems) and 714 (adjusted for dam vaccination), while the crude odds ratio for PregSure® BVD vaccine was at 1292.

The results of the multivariable logistic regression model for the comparison with the second set of controls are given in Table 3. Variables excluded from the stepwise logistic regression due to their rare occurrence (<10%) were: purchase of cattle, a frequent problem of inappetence in calves, the use of oral vaccination or colostrum replacers in calves and the use of calcium boli in cows. Other factors that were present in most case and control farms, such as the feeding of whole milk or hay to calves, feeding of hay or maize silage to cows, were also excluded from the analysis. Five variables remained in the final non-conditional logistic regression model: positively associated factors were the use of other prophylactic measures in cows, such as parasite control (OR = 14.3; 95% CI: 1.4–151.9) and the use of PregSure® BVD vaccine (OR = 426.0; 95% CI: 19.9–9095.4). Negatively associated with the occurrence of BNP were the use of vitamin E and selenium (OR = 0.113; 95% CI: 0.014–0.905) the frequent use of mastitis tubes (OR = 0.032; 95% CI: 0.002–0.421) and the use of stem growth regulators (OR = 0.083; 95% CI: 0.015–0.448). No significant interactions were found.

The results of the non-conditional logistic regression model using all significant variables of Table 3 – except for PregSure® BVD vaccine – and of a conditional regression model using the same variables are compared in Table 4. The obtained odds ratios and *p*-values differed only slightly.

4. Discussion

The presented case control study is to our knowledge the first of this kind, investigating potential risk factors for the increased occurrence of BNP in young calves. We used a multivariable approach to investigate a large number of putative factors. The study was conducted while the disease emerged and revealed several risk factors, such as the use of prophylactic measures (i.e. antiparasitic treatment) or the use of PregSure® BVD vaccine, which had the highest odds ratio. It was conducted between early 2009 and June 2010, when most cases of BNP emerged in Germany.

The definition of a case farm was that it had at least one confirmed case of BNP in a calf. Since a possible connection between the vaccine PregSure® BVD and BNP had already been discussed in the farming press (agrarheute.com, 2009), farm managers using PregSure® BVD vaccine might have been more likely than farmers not using PregSure® BVD, to bring calves to the clinic or submit blood samples in hope of having the cases documented "officially", with a view of later indemnity claims. But this topic was never brought up by farmers during interviews. Moreover, selecting more than half of case farms at random eliminated the risk of preferential selection of farmers using or not using specific vaccines. Additionally many interviews were conducted before the mentioned press-release. Therefore, we are confident that selection bias was limited. The definition of the control farms was more critical, as BNP could have occurred unnoticed, either in subclinical form or in undiagnosed deaths. In addition, BNP cases could have occurred on control farms after the interview. This potential bias could not be ruled out. However, a misclassification of controls was likely to be independent of the use of PregSure®. Such non-differential misclassification would therefore have reduced but not increased the observed

Table 3
Results of the multivariable logistic regression models in a study on BNP in southern Germany (2008–2010) using parameters statistically significant in the univariate analysis in comparing case farms against the farms of two control groups.

Model	Parameter	OR	Lower 95% confidence interval	Upper 95% confidence interval	Significance
Case farms (n = 56) vs. control farms (set 1) (n = 50)	Use of PregSure® BVD vaccine	1292.5	113.6	14706.9	<0.001
Case farms (n = 56) vs. control farms (set 1) (n = 50) without PregSure® BVD	Feeding of barley to cows	0.319	0.104	0.982	0.046
	Use of teat dips	0.148	0.045	0.483	0.002
	Use of stem growth regulator	0.052	0.015	0.187	<0.001
	Respiratory health problems in calves	4.556	1.26	16.43	0.021
	Dam vaccination against calf-diarrhoea	6.283	1.99	19.84	0.002
Case-farms (n = 56) vs. control farms (set 2) (n = 50)	Vitamin E and selenium	0.113	0.014	0.905	0.040
	Frequent use of mastitis tubes	0.032	0.002	0.421	0.009
	Use of stem growth regulator	0.083	0.015	0.448	0.004
	Use of other prophylactic measures	14.29	1.35	151.87	0.027
	Use of PregSure® BVD vaccine	425.80	19.94	9095.36	<0.001

association with PregSure® BVD vaccine (Rothman and Greenland, 1998).

We acknowledge that bias could have been introduced at the stage of the interviews. One of the measures taken to reduce such bias was instructing interviewers to avoid leading questions. Seven farmers were unwilling to participate, due to time constraints or unknown reasons. If these unknown reasons were associated with the disease, then this might have introduced some bias. However, as the answers of the participating farm managers varied considerably in the use of certain prophylactic measures or other factors, it is considered unlikely that this small number of non-participating farmers had an effect on the outcome of the analysis. Recall bias during the interviews could not be avoided. In general data were asked for the period of two years prior to the interview. Some data, like the use of vaccines, were asked for the period of the last five years. No documentation of vaccination on the level of individual animals was available, as there is no legal obligation to record the data for vaccinations in Germany, which would have indicated the exact time of administration of vaccine for each individual cow. However, many farmers stated they had used vaccines or applied other prophylactic measures five or more years previously, indicating that recall bias was minimal. On most farms vaccinations are applied for several years consecutively, not as a one-year measure. It was not possible to record the vaccination schemes on each farm, as this often has changed over the years and also is variable within the farms. In a further study it should be tried to obtain this data more detailed in order to compare different vaccination schemes. On the other hand, we are confident, that the vaccination-status of the farms (having vaccinated within the last five years: yes/no) is precise, as farmers and veterinarians were asked for vaccinations conducted on the farm. Recall bias was more likely concerning specific details of affected calves but not that cases had occurred at all. These details were not part of the present study. Therefore we believe that recall bias was, if it occurred, equally in case and control farms, and therefore unlikely to have had any considerable effect on the inferences from this study.

The analysis of this study was complex as the data included a greater number of risk variables than observations (cases and controls), and two sets of controls had to be addressed by different types of analysis (matched and non-matched).

In the univariable analysis many variables that were discussed prior to the beginning of the study as being potential risk factors for BNP on farms were not found to be significant in the present study, such as an association with the vaccination against Bluetongue, or intoxications with field melilot (*Melilotus officinalis*) or bracken fern (*Pteridium aquilinum*) (Stöber, 2006). In the questionnaire it was asked explicitly for contact with these plants, either directly or through hay and silage, but only few farm owners (case farms 3/50; control farms 5/100) mentioned that access to these plants could not be excluded completely. In addition, the intake of these toxic agents would have had to be present for a prolonged period to cause clinical disease, while most calves affected were about 2 weeks of age (Friedrich et al., 2009b). Also, only in few case and control farms access to rodenticides could not be excluded completely for animals. But since most frequently used rodenticides are based on dicumarol which does not lead to bone marrow damage (Stöber, 2006; Wang et al., 2007), these poisons can be ruled out as a cause for BNP. Also the

Table 4
Comparison of the results of a non-conditional and a conditional logistic regression analysis of BNP-case farms (n = 56) and control farms of the second set of matched controls (n = 50) excluding the factor PregSure® BVD-vaccine in a case control study on BNP in southern Germany (2008–2010).

Parameter	Non-conditional model		Conditional model	
	OR (95% CI)	Significance	OR (95% CI)	Significance
Vitamin E and selenium	0.248 (0.051–1.191)	0.082	0.118 (0.011–1.299)	0.081
Frequent use of mastitis tubes	0.083 (0.020–0.351)	<0.001	0.089 (0.014–0.551)	0.009
Stem growth regulator	0.090 (0.029–0.276)	<0.001	0.151 (0.039–0.587)	0.006
Use of other prophylactic measures	11.121 (2.362–52.363)	0.002	13.140 (1.751–98.623)	0.012

hypothesized risk factor of using stem growth regulators could not be confirmed; more control than case farmers used these regulators.

There is no evidence that hereditary diseases, such as the factor XI-deficiency (Meydan et al., 2009), the "Simmental hereditary thrombopathy" (Steficek et al., 1993; Weisser et al., 2010) or the Chédiak-Higashi-Syndrom (Shiraishi et al., 2002) were the cause of BNP. It also seems unlikely that a sudden increase of cases with thromobocytopenic purpura could have accounted for the observed number of BNP cases.

In the literature individual cases of bleeding disorders with thrombocytopenia due to damage of bone marrow of unknown causes have been described (Shimada et al., 2007; Braun et al., 2008; Friedrich et al., 2009b; Fukunaka et al., 2010). Even if the signs and post-mortem findings of BNP calves are very similar to these cases described earlier, the striking increase in incidence of BNP cases suggests a new contributing factor.

In the univariable analysis extremely strong associations between PregSure® BVD vaccine and the occurrence of BNP were found. However, this also caused convergence problems as 55 of the 56 case farmers had used this vaccine, while only two of the randomly selected set of 50 control farmers and 13 of the matched set of 50 control farmers used this vaccine. This resulted in very high crude odds ratios of 1292 and 156 for random and matched controls, respectively. The lower odds ratio of the matched controls for the use of PregSure® BVD vaccine was not surprising because veterinary practices tended to use only one or two specific BVD vaccines for client farms due to logistic and economic reasons. Matching for practice therefore reduced the chance of detecting an association with vaccine type.

The present study used a multivariable approach to investigate a large number of putative factors. Including PregSure® BVD vaccine into the multivariable analyses caused quasi-complete separation of the data, and therefore non-convergence of several logistic regression models. This often occurs in small samples with highly predictive variables. Despite the use of a correction recommended by Firth (Webb et al., 2004) for such circumstances, PregSure® BVD vaccine remained the only significant variable in the model with random controls (data not shown). A bias causing an effect size as large as this would have to be huge and should be obvious. We therefore deem it was highly unlikely that the PregSure® BVD vaccine effect was caused by selection bias or confounding. Including such a large number of farm management variables in the analysis was a priori a means to reduce confounding by any type of farm level variables. The exclusion of PregSure® BVD vaccine from the analysis of case and random control farms in a second step was done in order to identify further risk factors.

The use of stem growth regulators was negatively associated in both analyses, matched and non-matched, therefore not confirming the hypothesis that the chemical possibly included in the straw being fed or offered as bedding to cows and calves could cause BNP. Feeding of barley was also negatively associated with the occurrence of BNP. However, there is no obvious reason, why it would reduce the risk of having BNP on the farm. Barley has been used for many years in traditional feeding of cattle in southern Germany (Voigt et al., 1993). Other factors which were negatively associated with the occurrence of BNP were the use of teat dips, the frequent use of mastitis tubes and the supplementation of vitamin E and selenium. The effect of vitamin E and selenium has to be interpreted with caution as it was not statistically significant in the univariable analysis and as it showed a very wide confidence interval in the logistic regression analysis. It is therefore regarded as spurious. The use of teat dips and mastitis tubes as well as the three other factors, which were positively associated with the occurrence of BNP in the two analyses, the use of dam vaccination against calf diarrhoea, the more frequent occurrence of respiratory health problems in calves and applying other prophylactic measures (e.g. vaccination against respiratory diseases in calves, parasite control) all relate to animal health issues.

Respiratory health problems occurred in calves of case farms more frequently than in control farms (19/56 vs. 6/50). It could be speculated that in herds where BNP has been observed, additional calves were affected subclinically, and therefore a reduction in disease resistance resulted in an increase of the incidence of respiratory disease in these herds (Lorenz et al., 2011). This cannot be clarified as the questionnaire did not ask if the frequency of health problems in calves had increased before or after the appearance of BNP. Control farmers used more often teat dips and more frequently mastitis tubes than case farmers. The association of case farms with a more frequent use of dam vaccination agrees with reports of veterinarians that more progressive farms that are trying to achieve a good health status in their animals are affected by BNP. The use of dam vaccination was also positively associated with the use of PregSure® BVD vaccine, but stratifying for it only reduced the strong effect of PregSure® BVD vaccine to a relatively small extent. On the other hand, more investment into animal health, including vaccinations and for example the use of teat dips, could be a farmer response to the occurrence of disease. In the present study it is not possible to state if prophylactic measures have been implemented to maintain good animal health or whether they were implemented to improve animal health, as the study participants were not asked specifically on this. A further study addresses this issue in more detail. Hence, the interpretation of associations between BNP and prophylactic measures in general is equivocal.

It was difficult to adjust the crude effect of PregSure® BVD vaccine for possible confounding effects due to non-converging models. We addressed this dilemma by comparing several adjusted odds ratios for the association between PregSure® BVD and the occurrence of BNP by stratification for other significant variables using the Mantel–Haenszel technique. In the end, the effect of PregSure® BVD remained unequivocally strong for both control sets, random and matched. Adjusted odds ratios were relatively similar, ranging from 485 to 713. Despite the fact that these were lower than the crude effect in the matched set, PregSure® BVD had the strongest association with BNP among all variables studied. Even though other variables may also be associated with BNP, our data strongly suggest that a true effect of PregSure® BVD on

BNP could not be ruled out. We arrived at this conclusion after evaluating a very large number of farm management and farming systems variables using a study design with only few sources of bias (2 control groups) and critical and ambitious multifactorial analysis.

A relatively small scaled study such as this may not easily be extrapolated to populations outside the studied farms. However, the selected case and control farms and animals resembled typical dairy farms in southern Germany. Although the structure of farms in southern Germany differs from the ones in northern Germany, the absence of significant associations between case/control status and factors like herd size or grazing cattle on pasture as well as a large number of farm management variables suggests that the major findings of this study may also be valid for a wider population.

5. Conclusions

In conclusion, while bleeding disorders in calves due to bone marrow damage have occurred sporadically before the introduction of PregSure® BVD, and in herds without vaccinations, the analyses provide strong evidence for an association between the use of PregSure® BVD vaccine in cows and the development of BNP in their offspring. This result is compatible with the results of other authors (Pardon et al., 2009). However, the presented case control study was not designed, and therefore unable to demonstrate a causal relationship. The data were insufficient to evaluate the effect of different schemes of vaccination, e.g. dual priming vaccination plus annual booster vaccination versus sporadic vaccination.

The present case control study had the aim to identify risk factors, which has been achieved. Of all the identified factors, the use of PregSure® BVD on herd level remained associated with a very high risk for BNP on these farms. However, in relation to the estimated number of herds and also animals vaccinated with PregSure® BVD, the incidence of BNP was very low, even if low rates of detection and reporting were taken into account.

Conflict of interest statement

None declared.

Acknowledgements

We acknowledge gratefully the help of all veterinarians in the selection of control farms and in the diagnosis of cases. The time and help of all farmers involved in this study is highly appreciated. Without their help this study would not have been feasible. This study was funded by Pfizer Animal Health GmbH.

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