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Association between *Mycobacterium avium* subsp. *paratuberculosis* infection and culling in dairy cattle herds

Asociación entre la infección por *Mycobacterium avium* subsp. *paratuberculosis* y las causas de eliminación en rebaños de ganado lechero

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RESUMEN

El presente estudio fue diseñado para analizar las causas de eliminación en ganaderías lecheras con diferente *status* de infección relativo a *Mycobacterium avium* subsp. *paratuberculosis* y comparar estas causas con lo observado en la población general de ganado lechero. Durante 2009, las causas de baja fueron registradas en dos grupos diferentes de rebaños: (1) rebaños con vacas seropositivas durante tres años consecutivos (2007-2009) y donde *Mycobacterium avium* subsp. *paratuberculosis* no pudo ser aislado de ninguna de las muestras fecales recogidas y (2) rebaños con vacas seropositivas a *Mycobacterium avium* subsp. *paratuberculosis* durante tres años consecutivos (2007-2009) y donde la bacteria ha sido aislada por lo menos de una muestra fecal. Las causas de eliminación fueron comparadas entre ambos grupos y entre estos y la población general de ganado lechero a través de análisis de regresión. La distribución de causas de eliminación era diferente entre rebaños infectados (bacteriológicamente positivos y negativos) y la población general. El porcentaje de pérdidas parecía ser superior en rebaños infectados desde el primer parto. La diferencia más notable entre grupos fue observada en las bajas debidas “muerte/sacrificio urgente”.

Palabras clave: bovinos lecheros, paratuberculosis, causas de eliminación, control lechero.

SUMMARY

The present study was designed to analyse the causes for culling in dairy herds with different *Mycobacterium avium* subsp. *paratuberculosis* infection status and to compare these causes with those observed over the general dairy cattle population. During 2009, causes for culling were registered in two different groups of farms: (1) farms with seropositive cows for three consecutive years (2007-2009) but where *Mycobacterium avium* subsp. *paratuberculosis* has not been isolated from any of the fecal samples collected and (2) farms with *Mycobacterium avium* subsp. *paratuberculosis* seropositive cows for three consecutive years (2007-2009) and where the bacteria has been isolated from at least one fecal sample. Causes for animal loss were compared between both groups and between them and the general dairy cattle population by means of regression analysis. The distribution of culling reasons was different between infected herds (both bacteriologically positive and negative) and the general population. The percentage of losses seemed to be higher in infected herds from the first parity on. The most remarkable difference among groups was observed in losses due to “death/urgent slaughter”.

Key words: dairy cattle, paratuberculosis, reasons for culling, milk control.

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of paratuberculosis (Johne's disease), a chronic granulomatous enteric disease of domestic and wild ruminants. Johne's disease causes serious economic losses in dairy farming, mainly as a result of reduced milk production (Benedictus *et al* 1987, Ott *et al* 1999, Hendrick *et al* 2005, Tiwari *et al* 2005), increased susceptibility to other diseases, especially mammary infections (Buerge and Duncan 1978, Tiwari *et al* 2005), loss of body weight (Johnson-Ifearu-

lundu *et al* 2001), and consequential premature culling and increased replacement costs. Paratuberculosis has also been related to reduce fertility rates (Buerge and Duncan 1978, Johnson-Ifearu *et al* 2000, Tiwari *et al* 2005, Dieguez *et al* 2008). Raizman *et al* (2007^a) indicated that MAP was also associated with pneumonia.

Johne's disease has become a widespread infectious disease problem for cattle herds in developed countries (Manning and Collins 2001).

Premature culling associated with MAP infection is one of the major economic burdens of this disease. Without management changes designed to reduce the farm-level prevalence of MAP infection, it will continue to reduce farm income by increasing premature removal from the herd (Lombard *et al* 2005).

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MAP is usually introduced into dairy herds through the purchase of infected but clinically normal cattle (Sweeney 1996); other routes, such as the introduction of contaminated feces by vehicles or equipment are less common but may be involved (Wells and Wagner 2000). In a herd, younger animals are the most susceptible to MAP infection, especially shortly after birth. The main routes of transmission are the fecal-oral route and through colostrum or milk from infected dams (Sweeney 1996). The organism has also been isolated from the reproductive tract of both clinically and subclinically infected cows, so calves born from infected cows could be infected *in utero* (Rohde and Shulaw 1990, Sweeney 1996, Bielanski *et al* 2006). The disease has a long incubation period and slow course, and the clinical signs typically appear only after two to five years. The main signs of the disease include progressive weight loss and chronic watery diarrhea; however, infected animals can be identified before the clinical stage by their reduced milk yield.

The aim of this study was to analyse the causes for culling in dairy herds with different MAP infection status and to compare the distribution of causes with that observed over the general dairy cattle population.

MATERIAL AND METHODS

STUDIED AREA AND PARATUBERCULOSIS CONTROL PROGRAM DESCRIPTION

The study was carried out in Galicia. Galicia is the major cattle-farming region of Spain. It is responsible for 35% of the milk and 12% of the beef produced in Spain, constituting approximately 1.7% of the milk and 1.3% of the beef produced in the European Union.

Galicia is the first region in Spain to be establishing a voluntary paratuberculosis control program that started in 2004. The percentage of Galician herds involved increased from 4.6% in 2004 to 33% in 2009. Vaccination against MAP is not permitted, because this interferes with the obligatory screening for tuberculosis.

The control program consists of antibody testing by means of an ELISA of animals older than 12 months at 1 year intervals to determine the serological profile of the herds and to identify cows most likely shedding the organism. Faecal samples of all ELISA-positives were cultured for the presence of MAP.

LABORATORY ANALYSIS

The study was carried out from 2007 to 2009. During the period, following the control program schedule, blood was collected by tail vein venopuncture into anticoagulant-free Vacutainer tubes from all animals over one year belonging to herds under MAP control program. Serum was separated by centrifugation (5,000 g, 5 min)

and aspiration. It was stored at -70° C until analysis by ELISA. The commercial ELISA used was "Paratuberculosis Antibody Screening" (Institute Pourquier, France). False-positive results were reduced by pre-absorbing the samples with sonicates of the environmental mycobacterium *Mycobacterium phlei*. Samples were considered positive at a % sample: positive ratio of 55% or more. According to the manufacturer's validation report, the sensitivity of the test was 40.8 per cent and its specificity 99.8 per cent.

Faecal samples from seropositive cows were taken from the rectum using disposable gloves and stored also at -70°C. Bacterial culture was performed as described by the World Organization for Animal Health (OIE) (2008). Briefly, 1g of feces was added to 20 mL of sterile distilled water, and tubes were shaken for 30 min and then allowed to stand undisturbed for 30 min. Five mL of the supernatant were added to 20 mL of 0.75% hexadecylpyridinium chloride (HPC) (Sigma). Tubes were inverted several times and allowed to stand undisturbed for 18 h at room temperature for decontamination. Triplicate Herrold's egg yolk medium (HEYM) culture slopes containing amphotericin B, vancomycin and nalidixic acid were inoculated with 0.1 mL of the undisturbed sediment, incubated at 37°C and observed at 2-week intervals for 16 weeks. Suspect colonies were evaluated for mycobactin dependence along with morphology and acid-fast staining. Samples with overgrowth were repeated.

Positive-staining colonies were confirmed by PCR. DNA from the fecal samples was extracted and purified using the Adiapure kit (Adiagene). Amplification of the DNA segment IS900 specific to *M. avium* subsp. *paratuberculosis* was performed with the PCR Adia-vet PARATB Real Time kit (Adiagene), following the manufacturer's instructions. For every five samples a positive control (a positive field sample) was included.

SAMPLES

For the study, the same testing strategy described for herds in the control program was used. During 2009, causes for culling were registered in two different groups of farms enrolled in the control program since at least 2007 and under dairy herd improvement program (DHIP): (1) farms with seropositive cows for three consecutive years (2007-2009) but where MAP had not been isolated from any of the faecal samples collected (Ab+/MAP-) and (2) farms with MAP seropositive cows for three consecutive years (2007-2009) and where MAP had been isolated from at least one of the faecal samples per year (Ab+/MAP+). The number of herds that fulfilled these criteria comprised 12 in group 1 and 20 in group 2, with 849 and 1424 cows, respectively.

Causes for culling were compared between both groups and between them and the general population (GP) of dairy cattle in Galicia. The GP includes mortality

records from 2,036 dairy farms and 108,811 cows that were controlled under DHIP in 2009.

Causes for disposal were obtained from the monthly visits by the DHIP, during which the supervising technician asked the farmer about the reason for the animals' losses since the previous visit. The reasons for losses were coded according to the Royal Decree 368/2005 (BOE 2005), which regulates the program in according to specific rules:

- Death/Urgent slaughter: discarded because it was found prostrate or dead on the farm/animal sent to emergency slaughter (as metabolic disorders, accident, toxemia, peritonitis, pericarditis, systemic infection).
- Lack of productivity: discarded because of low production.
- Mastitis: discarded because of udder problems (as mastitis, loss of quarters of the udder, sagging udder).
- Infertility: discarded because of reproductive problems (as abortions, metritis, infertility, sterility, mummified fetuses).
- Loss in official disease eradication programs (zoonoses).
- Other: discarded because of reasons not mentioned in the classifications above or for multiple causes.
- Lameness: discarded because of musculoskeletal problems (as lameness, hoof infection).

For all animals, the parity number, the date of culling and, if the cow were in lactation, production level (L) and days in milk (DIM) at the time of culling, were gathered.

STATISTICAL ANALYSIS

Data were analyzed with Stata 11.1. Initially, to study variations in reasons for culling according to DIM a plot of cumulative incidence, stratified per herd infection status (GP, Ab+/MAP- or Ab+/MAP+), was performed for every reason, based on Kaplan-Meier method.

A contingency table was constructed to analyse the distribution of causes for culling in the different groups of farms.

Herd infection status and herd size (herd-level variables) and parity (animal-level variable) were tested for their association with each cause for culling by using a univariate analysis with $k \times 2$ tables (taking non-culled animals as reference).

Spatial analyses, based on the Bernoulli model, were also run with SatScan® 6.1 to assess the possible existence of spatial clustering. The following case/control definitions were used:

- Farm status: Farm with at least one seropositive animal (by antibody ELISA)
- Farm status: Farm with at least one fecal culture positive animal.

All the factors with moderate statistical significance ($P < 0.15$) in the univariate analysis were incorporated into a multilevel mixed effects regression model.

RESULTS

For the considered groups, in lactating cows, it was observed that the culling by "death/urgent slaughter" was more frequent in the 60 days post-calving (this was more prominent in Ab+/MAP- and Ab+/MAP+ than the GP) and were reduced with advancing lactation. As expected, culling due to infertility was more common at the end of lactation in all groups. Mastitis represented a growing cause of culling as lactation progressed until day 240. From day 240 on, a reduction was observed in the percentage of disposal due to this cause. Culling due to lack of productivity and "other causes" did not seem to show a variable trend throughout lactation in any of the three groups. With regard to lameness, there was a tendency for this factor to represent a greater percentage of culling at an increased age and lactation time up 240 days, after which it was reduced.

The spatial scan statistic did not identify any significant cluster of seropositive or culture positive farms. Causes for culling, in the different infection groups, are presented in Table 1. The overall distribution of causes in 2009 differed among the three groups. The results indicated that the higher the infection status the higher the proportion of cows culled (19.3% in the GP, 21.3% in Ab+/MAP- farms and 24.1% in Ab+/MAP+ farms).

"Death/urgent slaughter" was the most frequent reason for loss in Ab+/MAP- and Ab+/MAP+ farms, whereas in the GP "other causes" and infertility listed at the top.

The percentage of cows discarded by "death/urgent slaughter" increased with the infection status. The multivariate approach leads to similar conclusions (the percentage of cows culled for this reason was 3.49 and 3.91 times higher in Ab+/MAP- and Ab+/MAP+, respectively, than the GP). Besides, this cause was reduced with parity number (Table 2). Conversely, for the other considered causes the percentage of culling increased with increased parity (Table 2). Herd size was not included in the multivariate analysis since it was not related to any of the causes for culling in the univariate approach.

With respect to culling by lack of productivity there was a tendency to be higher in Ab+/MAP- herds than Ab+/MAP+ herds and the GP. In line, regression indicated that the culling for unproductivity was 2.46 times higher in Ab+/MAP- farms compared to the GP. There were no significant differences when comparing the GP to Ab+/MAP+ farms (Table 2).

Culling due to mastitis were more often in Ab+/MAP+ herds and the general population than in Ab+/MAP- farms. The multivariate analysis provided an odd ratio (OR) of 1.30 when comparing this GP to Ab+/MAP+ farms (not significant at 0.05 but close to the significance level) (Table 2).

With regard to infertility, there was a tendency for this factor to represent a higher risk of losses with infection status. However, once corrected by means of regression, significant differences were only observed when the

Table 1. Distribution of causes for mortality in cows in relation to the *Mycobacterium avium* subsp. *paratuberculosis* status of their farms (general population, cows from farms with antibody positive but not fecal culture positive animals (Ab+/MAP-) and cows from farms with both antibody and fecal culture positive animals (Ab+/MAP+).

Distribución de causas de mortalidad de vacas en relación al status de *Mycobacterium avium* subsp. *paratuberculosis* de sus predios (población general, vacas de predios con animales de cultivo anticuerpo positivo pero cultivo fecal no positivo (Ab+/MAP-) y vacas de predios con animales de cultivo positivo tanto de anticuerpos como fecal (Ab+/MAP+).

Cause for culling		General population	Ab+/MAP-	Ab+/MAP+
Death/Urgent sacrifice	Cows (herds)*	4332 (2036)	56 (12)	108 (20)
	% eliminated**	4.0%	6.6%	7.6%
Lack of productivity	Cows (herds)	1595 (1044)	20 (13)	20 (15)
	% eliminated	1.5%	2.3%	1.4%
Mastitis	Cows (herds)	3425 (1813)	22 (12)	46 (17)
	% eliminated	3.1%	2.6%	3.2%
Infertility	Cows (herds)	4443 (2036)	36 (19)	68 (20)
	% eliminated	4.1%	4.2%	4.8%
Official disease eradication program	Cows (herds)	14 (10)	0 (0)	0 (0)
	% eliminated	0.0%	0.0%	0.0%
Others	Cows (herds)	5495 (2036)	37 (16)	72 (19)
	% eliminated	5.0%	4.4%	5.1%
Lameness	Cows (herds)	1768 (1129)	10 (8)	29 (17)
	% eliminated	1.6%	1.2%	2.0%
Total	Cows	21072	181	343
	% eliminated	19.3%	21.3%	24.1%

* N° of cows culled for this cause (n° of herds in which the events occurred) and ** percentage that this N° represents in each population (general population, Ab+/MAP- or Ab+/MAP+).

Table 2. Multivariate mixed effects regression for evaluating factors associated with each cause for culling in Holstein dairy cows.

Regresión de efectos combinados multivariados para evaluar factores asociados con cada causa para sacrificio en vacas lecheras Holstein.

Cause for culling		OR	P-value	95% CI for OR	
Death/Urgent sacrifice	Parity	0.97	0.008	0.95	0.99
	GP* vs Ab+/MAP-	3.49	0.000	2.70	4.51
	GP vs Ab+/MAP+	3.91	0.000	2.78	5.51
Lack of productivity	Parity	1.19	0.000	1.15	1.23
	GP* vs Ab+/MAP-	2.46	0.002	1.41	4.30
	GP vs Ab+/MAP+	1.35	0.134	0.91	2.01
Mastitis	Parity	1.17	0.000	1.14	1.19
	GP* vs Ab+/MAP-	1.17	0.399	0.80	1.71
	GP vs Ab+/MAP+	1.30	0.064	0.98	1.71
Infertility	Parity	0.84	0.000	0.82	0.86
	GP* vs Ab+/MAP-	1.09	0.509	0.83	1.43
	GP vs Ab+/MAP+	1.24	0.039	1.01	1.52
Others	Parity	1.19	0.000	1.17	1.21
	GP* vs Ab+/MAP-	0.68	0.832	0.19	1.18
	GP vs Ab+/MAP+	2.16	0.000	1.61	2.90
Lameness	Parity	1.12	0.000	1.09	1.16
	GP* vs Ab+/MAP-	1.26	0.451	0.68	2.35
	GP vs Ab+/MAP+	2.45	0.000	1.55	3.87

* General population.

GP was compared to Ab+/MAP+ (OR=1.24) but not to Ab+/MAP- farms (Table 2).

The percentage of animals culled due to “other causes” was lower in Ab+/MAP- farms than Ab+/MAP+ farms and the GP. The percentage of culling for “other causes” was 0.68 times lower (although not significant) in Ab+/MAP- farms and 2.16 times higher in Ab+/MAP+ farms, in relation to the GP (Table 2).

In the case of lameness, compared to the GP, the percentage of cows eliminated for this reason decreased in Ab+/MAP- farms and it increased in Ab+/MAP+ farms. According to the multivariate analysis, differences between the general population and Ab+/MAP- farms were not significant. The OR in Ab+/MAP+ farms compared to the general population was estimated 2.16 (Table 2).

DISCUSSION

The low sensitivity of the test for MAP diagnosis, especially at individual but also at herd level (the sensitivity affects the detected intra-herd prevalence), could lead to misclassification of some herds (false negative herds). To avoid this inconvenience, all the GP were used as reference. As samples were pre-absorbed with *Mycobacterium phlei* and as only farms in which positives results were consistent during three consecutive years were included and the specificity of the ELISA is rather high (98%, according to manufacturer specifications) we think that the number of these false positives was low.

Spatial clustering of paratuberculosis infected herds has been mainly associated with soil characteristics. Galicia is a relatively small region where the soil features in the main dairy cattle areas are similar. This could explain the lack of geographical association.

Results indicated that losses by “death/urgent slaughter” were mainly observed during early lactation. These results agree with those presented by Pinedo *et al* (2010). There were no records that give the reasons for these deaths; we estimate that hyperacute infections, lack of therapeutic attention or lack of response to treatment and, mainly, metabolic post-partum disorders constituted the majority of causes. On the other hand, primiparous cows have the higher incidence of post-partum metabolic disorders (Dechow and Goodling 2008).

Data indicated that paratuberculosis was related to losses for “death/urgent slaughter”. The mechanism could be related to the impaired immunological and gastrointestinal absorptive capacity and the accentuated negative energy balance sustained by infected cattle (Johnson-Ifeorunlu *et al* 2000) since “death/urgent slaughter” occurred mainly in the post-partum period. Stabel *et al* (2003) has looked at the effect of negative energy balance on the metabolic and immune status of MAP infected cows around the time of parturition.

A drop in milk production is a common early sign of paratuberculosis; some farmers can note this and may

prematurely cull the animal without requesting further diagnosis (Chiodini *et al* 1984). It is possible that in herds with bacteriologically positive cows, animals were more likely to be culled in later stages of the disease and, therefore, recorded as having chronic diarrhea or weight loss rather than lack of productivity. The association between MAP and milk production was not confounded by mastitis, elevated somatic cell counts, or uterine or metabolic cow conditions (Aly *et al* 2010).

Previous studies (Tiwari *et al* 2005, Dieguez *et al* 2008) detected relationships between the infection status of the herds and the incidence of mammary infections (both in terms of somatic cell count and clinical mastitis incidence). The present paper indicated higher mortality related to udder problems (that included mammary infections, mainly) but only in bacteriologically positive farms (not significant but close to the significance level in the regression model). This finding was in line with previous papers; Dieguez *et al* (2008) detected higher risk of clinical mammary infections only in highly infected farms but not in those that showed lower levels of infection or, where most of the cows, seemed to be in early stages of the disease.

High-shedding animals had also lower calving rates in comparison with low-shedding or ELISA-positive animals, which tended to have higher calving rates than test-negative animals (Smith *et al* 2010). The difference could be explained as a result of reduced oestrus expression or a longer postpartum anoestrous period due to the marked negative energy balance in infected animals (Johnson-Ifeorunlu *et al* 2000). This situation decreases the probability the cow had return to equilibrium allowing normal follicular growth and maturation. This result also could reflect differences in management decisions made on test-positive or negative cows.

Regarding to “other causes”, results are difficult to interpret. Using the official codes from the DHIP makes animals culled both for multiple causes and for less frequent reasons are assimilated into the same code as “other causes”. Thus, it could not be determined whether the increase observed in Ab+/MAP+ farms is due to the culling for multiple causes, for less frequent or both.

There are very few literatures documenting the relationship between MAP and lameness. Raizman *et al* (2007^b) reported lameness as the most common clinical diseases among fecal culture positive cows, in a univariate approach. In the present study differences were only observed in Ab+/MAP+ cows but considering into lameness all the culling related to hoof problems.

In most of the previous trials, to estimate the cost of MAP infections in dairy herds, traits were calculated for cows in each stage of the disease of MAP infection relative to uninfected cows. However, for our purpose, paratuberculosis should be considered a herd, not just an individual animal problem due to difficulties in di-

agnosing pre-clinical cases (especially in terms of sensitivity). For every diagnosis case there are likely to be 5-10 pre-clinical non diagnosis cases; these cows may be culled for other reasons indirectly related to MAP (Collins 1994).

In conclusion, the distribution of culling reasons differed between infected herds (both bacteriologically positive and negative) and the general population and the percentage of losses seemed to be higher in infected herds from the first parity on. The most remarkable difference among infection groups was observed in losses due to “death/urgent slaughter”.

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