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Impact of *B. melitensis* Rev-1 vaccination on brucellosis prevalence

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Abstract: Trás-os-Montes e Alto Douro, in the northeastern region of Portugal, previously exhibited the highest brucellosis prevalence in the entire small ruminant population of Portugal. Consequently, a vaccination program of the whole population with *Brucella melitensis* Rev-1 was carried out from 2001 to 2004, and further compulsory Rev-1 vaccination of 3- to 6-month-old lambs and kids was carried out between 2005 and 2007. The prevalence of brucellosis decreased by 71.42% in 2004, with a 2-fold decrease occurring from 2005 to 2007. The reduction of brucellosis prevalence was statistically significant ($P < 0.001$) according to flock size. By species, brucellosis prevalence decreased 14.5-fold in sheep and 5.5-fold in goats in 2004. Regarding flock constitution, it decreased by 77% for pure flocks and 73% in mixed flocks in 2004. Regarding the animals involved in production, brucellosis prevalence decreased during the 7-year period. It was concluded that Rev-1 vaccination of the whole population was effective in decreasing brucellosis prevalence. These results contribute to the scarce information available regarding the effect of Rev-1 vaccination on different characteristics of flocks. They may be used to improve the efficiency of brucellosis eradication programs within livestock management.

Key words: Brucellosis, *Brucella melitensis* Rev-1 vaccination, prevalence, sheep, goat

1. Introduction

Brucellosis is an important contagious zoonotic disease responsible for reproductive failure, with profound public health significance due to its zoonotic character (1). Although there is still no vaccine available for humans, the vaccination of animals against brucellosis is a cost-effective measure used to control the disease (2) as well as an essential tool to achieve its eradication (3).

The *Brucella melitensis* Rev-1 vaccine is the considered the best available vaccine against brucellosis, although it is not the ideal vaccine due to its adverse effects (4). Rev-1 can infect humans; it may interfere with both Rose Bengal (RBT) and complement fixation (CFT) tests, the classic serodiagnostic tests; and it is excreted in milk when adult animals are vaccinated (5).

Mass vaccination programs have been described as the unique and first basic strategy to be applied in countries with high animal and/or farm brucellosis prevalence in order to control the disease (6).

The animal and farm prevalence of brucellosis in the region of Trás-os-Montes e Alto Douro (in northeastern Portugal) was 5.5% and 26.7%, respectively, in 1991 (7). From 1991 to 2001, the principal measures to control brucellosis in small ruminants were based on both animal identification and test-and-slaughter of brucellosis-positive animals. However, after 10 years of culling positives, in 2001 the prevalence of animal brucellosis was 5.9%, while the farm prevalence reached 34.9%. Due to the sanitary conditions of sheep and goats in relation with brucellosis, and due to the elevated cases of human brucellosis in the region (8), a Rev-1 vaccination program of young and adult animals was carried out from 2001 until 2004, where all flocks required vaccination (7).

The study of the progress of sheep and goat brucellosis prevalence in the region of Trás-os-Montes e Alto Douro during the young and adult vaccination program (from 2001 to 2004) and the study of its changes in the following years (from 2005 to 2007) is an essential step to assess the impact of the vaccination program on the sanitary status of the sheep and goat population. The aim of this study was to evaluate brucellosis prevalence in the sheep and goat population in Trás-os-Montes e Alto Douro from 2001 to 2007 according to several characteristics, such as flock size, species, flock composition, and main animal production.

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2. Materials and methods

2.1. Control of brucellosis at the farm level

Sheep and goats over 3 months old were conjunctivally vaccinated with *B. melitensis* strain Rev-1, and all of them were identified with both a tattoo on the left ear and special ear tags that included the vaccination date. The small ruminants were vaccinated by a single conjunctival administration. Moreover, all animal data were recorded in the national animal health software (Pisa.net). Blood samples of adult and young animals were collected at the same time as the vaccination. Animals with positive results in both RBT and CFT were culled (7). After 12 months, new blood samples were collected in the animals vaccinated as youths; seropositive animals were culled. In animals vaccinated as adults, a blood sample was collected after 30 months to assess the behavior of the vaccine. As additional measures, animal replacement was only allowed in the group vaccinated as youths, animal movement restriction was enforced for 21 days after Rev-1 vaccination, and the movement of positive flocks by veterinary official services was restricted (9).

From 2005 until the present, the whole population of small ruminants was sampled by blood collection for brucellosis screening once a year. Lambs and kids from 3 to 6 months old were compulsorily vaccinated, identified with a tattoo and special ear tag as previously described, and tested after 12 months. Positive animals were culled as described above.

In infected flocks, the special measures carried out at farms consisted of the study of the source of infection by an epidemiological survey, small ruminant movement restriction, and a minimum of four serological tests in a 240-day period (9).

2.2. Data collection

The study was carried out in all flocks registered in the national animal health software (Pisa.net) from 2001 to 2007 in the Trás-os-Montes e Alto Douro region. The data available consisted of farm identification, species, flock size, main animal production, birth date, sex, breed, blood sampling date, Rev-1 vaccination date, RBT and CFT results, and the culling dates of brucellosis-positive animals. According to the data of the national animal health software, the Rev-1 vaccination coverage of the small ruminant population of the study area was over 98%.

The animal population of the 3-month study included the entire small ruminant population (young and adult animals) subjected to blood sampling and Rev-1 (Ocurev-Shering and Plough) conjunctival vaccination from February 2001 to July 2004. Blood was taken from the jugular vein using sterile tubes and allowed to clot at ambient temperature. The vaccination was carried out by the utilization of a commercial live freeze-dried vaccine against brucellosis for active immunization of sheep and goats to reduce infection and clinical signs caused by *Brucella melitensis* that contains *B. melitensis* strain Rev-1 at $1–2 \times 10^9$ cfu/dose. The vaccine was transported to the field at proper refrigerated temperature. The reconstitution of the vaccines was carried out by mixing the live freeze-dried vaccine with the manufacturer's solvent in the field prior to vaccine administration. The vaccine was then administered into the conjunctival sac of the left or right eye by a dropper that delivered a volume of approximately 35 μL.

Blood sampling and Rev-1 vaccination were carried out by veterinarians belonging to the local livestock production organizations. The application of the vaccine was carried out strictly according to the manufacturer's recommendations. Moreover, the collection of blood samples and all manipulations of the animals were performed according to the ethics and the rules in the EU's legislation for animal welfare (10). From August 2004 to December 2007, the whole population was screened for brucellosis by blood sampling (young and adult animals) and young animals over 3 months of age that were subjected to Rev-1 vaccination were considered for study.

After collection, blood samples were stored at ambient temperature and processed in the next 24 h at the local official veterinary laboratory where RBT and CFT were performed. The antigens used were standardized according to instructions in the Manual of Standards for Diagnostic Tests and Vaccines and EU legislation (11)

Flocks with incomplete and/or lacking data in the national animal health software were excluded. In the case of flocks with two or more blood samplings per year, only the data of the first blood sampling was considered. A flock was considered brucellosis-positive when at least one animal had positive results in both RBT and CFT.

According to the number of heads, flocks were classified into three categories: small flocks (≤30 animals), medium flocks (>30 and ≤150 animals), and large flocks (>150 animals). According to the species, flocks were classified as a "sheep flock" or "goat flock" when the predominant species was up to 50% of the flock size. Moreover, the flocks were considered "pure" if they contained only one species (sheep or goat) and "mixed" if they contained both of them. The animal production was classified as "dairy" or "meat" if more than 50% of the flock produced milk or meat, respectively.

2.3. Data analysis

Animal prevalence was calculated in brucellosis-positive flocks. The chi-square test was used to compare prevalence values from 2001 to 2007 according to the characteristics of the flocks as described above (herd size, species, herd constitution, and main animal production). All data were entered into Access 2003 (Microsoft Inc.) and SPSS 15.0 (SPSS Inc.), with $P < 0.05$ considered statistically significant. Confidence limits for the proportions were established by

exact binomial test with a 95% confidence interval. The prevalence differences were calculated for each variable by subtracting the value of 2007 from the value of 2001. The relative decrease was calculated by dividing the value of each variable from 2007 by the corresponding 2001 value.

3. Results

3.1. Flock characterization

The results of the characterization of flocks revealed that the main animal production was meat (77%). Almost 61% of the flocks were medium-sized and over 80% of them consisted of sheep as the main species. In addition, over 80% of the flocks consisted of only one species.

3.2. Brucellosis prevalence by animals

From 2001 to 2007, an average of 198,466 small ruminants (Table 1) were tested for brucellosis with RBT and CFT. The number of small ruminants sampled varied in each year. For example, the number of samples collected in 2002 and 2003 were 38.65% and 47.07% lower, respectively, compared to the number of samples collected in 2001, and an increase of approximately 28% was observed between 2004 until 2007, compared to 2001.

However, in contrast to the number of small ruminants sampled, brucellosis prevalence decreased during the study period. The largest decrease occurred in 2003 with a 2.63-fold decrease compared to the previous year. At the end of the Rev-1 vaccination program (2004), brucellosis prevalence in animals was 1.6% (71.42% lower than in 2001) and during the 3-year period of Rev-1 vaccination of ewes and 3- to 6-month-old lambs and kids, brucellosis prevalence decreased by 50% (2007). The reduction in the prevalence of sheep and goat brucellosis was found to be statistically significant ($P < 0.001$). Regarding human brucellosis, the national health authority registered 52 cases of human brucellosis in the region of study in 2000. However, in 2004 and 2007, the number of cases decreased to 16 and 8 cases, respectively, according to the official data, showing the same reduction tendency observed in small ruminant brucellosis.

3.3. Prevalence of brucellosis by flock size

The decrease in the prevalence of brucellosis was also statistically significant ($P < 0.001$) according to flock size (Table 2). Brucellosis prevalence decreased progressively from 2001 to 2007, with a slightly higher reduction in small and medium flocks than in large flocks. In the case of large flocks, an increase in the animal prevalence of brucellosis was observed between 2004 and 2005, although the prevalence then decreased until 2007.

The largest reduction in the prevalence of brucellosis for all three flock sizes was observed in 2003, when the prevalence decreased almost 2.5-fold for small flocks and over 2-fold for medium flocks; however, a reduction of almost 6-fold was achieved in large flocks in the same year. By 2004, when the Rev-1 vaccination program of the whole population ended, brucellosis prevalence was similar to what it was in 2003. In addition, the prevalence in large flocks was half of that in small and medium flocks, in spite of the fact that large flocks presented the highest animal prevalence of brucellosis in 2001.

In 2007, the maintenance of the Rev-1 vaccination of lambs and kids between 3 and 6 months old reduced brucellosis prevalence by up to 0.4% for small and large flocks and 0.5% for medium flocks.

Despite the fact that brucellosis prevalence was similar for the three flock sizes in 2007, the overall reductions rates from 2001 to 2007 were higher for large and medium flocks (5.7% and 5.1%, respectively) than for small flocks (2.7%)

3.4. Prevalence of brucellosis by species

Results showed that sheep was the predominant species of the study area (Table 3) and the changes in the number of sheep and goats from 2001 to 2007 exhibited similar patterns in both species. A reduction in numbers for both species was observed up until 2004. Numbers then increased from 2005 to 2007. In addition, the population of sheep and goats increased by 30% and 22%, respectively, compared to 2001.

Table 1. Animal brucellosis prevalence from 2001 to 2007 ($P < 0.001$).

Year	2001	2002	2003	2004	2005	2006	2007
Animals	217,491	133,437	115,115	149,990	240,810	254,325	278,097
Positive animals	12,073	5658	1833	2002	2900	2083	1236
Prevalence (%)	5.6	4.2	1.6	1.3	1.2	0.8	0.4
CI 95%	5.4–5.7	4.1–4.4	1.5–1.7	1.3–1.4	1.2–1.2	0.8–0.9	0.4–0.5

Animal mean: 198,466; SD: 64,733.3; CI: confidence interval.
Reduction of prevalence (%) = 92.85%.
Prevalence 2001 / prevalence 2007 = 14-fold.

Table 2. Animal brucellosis prevalence by flock size (P < 0.001).

Year	Small flock (P < 0.001)						
	2001	2002	2003	2004	2005	2006	2007
Animals	17,685	24,102	29,000	29,273	28,943	30,428	33,849
Positive animals	535	896	425	405	330	195	126
Positive flocks	221	370	245	266	254	152	72
Ind Prev (%)	3.0	3.7	1.5	1.4	1.1	0.6	0.4
CI 95%	2.8–3.3	3.5–4.0	1.3–1.6	1.3–1.5	1.0–1.3	0.6–0.7	0.3–0.4
Herd Prev (%)	13.9	16.5	9.2	10.1	9.6	5.5	2.4
CI 95%	12.2–15.6	15.0–18.1	8.1–10.3	9.0–11.3	8.5–10.7	4.7–6.4	1.9–3.0

Reduction of prevalence (%) = 86.67. Prevalence 2001 / prevalence 2007 = 7.5-fold
Ind Prev (%): individual prevalence. Herd Prev (%): herd prevalence.

Year	Medium flock (P < 0.001)						
	2001	2002	2003	2004	2005	2006	2007
Animals	140,256	82,515	67,428	90,576	149,550	153,083	159,328
Positive animals	7911	3651	1279	1391	1878	1226	793
Positive flocks	736	485	284	498	865	531	323
Ind Prev (%)	5.6	4.4	1.9	1.5	1.3	0.8	0.5
CI 95%	5.5–5.8	4.3–4.6	1.8–2.0	1.5–1.6	1.2–1.3	0.8–0.8	0.5–0.5
Herd Prev (%)	41.9	41.0	27.8	36.8	43.5	26.6	15.5
CI 95%	39.6–44.2	39.8–43.8	25.0–30.5	34.2–39.6	41.3–45.7	24.6–28.5	14.0–17.1

Reduction of prevalence (%) = 91.07. Prevalence 2001 / prevalence 2007 = 11.2-fold.
Ind Prev (%): individual prevalence. Herd Prev (%): herd prevalence.

Year	Large flock (P < 0.001)						
	2001	2002	2003	2004	2005	2006	2007
Animals	59,550	26,820	18,687	30,141	62,317	70,814	84,920
Positive animals	3627	1111	129	206	692	662	317
Positive flocks	159	49	20	75	182	154	92
Ind Prev (%)	6.1	4.1	0.7	0.7	1.1	0.9	0.4
CI 95%	5.9–6.3	3.9–4.4	0.6–0.8	0.6–0.8	1.0–1.2	0.9–1.0	0.3–0.4
Herd Prev (%)	54.8	37.7	22.0	51.0	59.5	44.9	22.8
CI 95%	49.1–60.6	29.4–46.0	13.5–30.5	42.9–59.1	54.0–65.0	39.6–50.2	18.7–26.9

Reduction of prevalence (%) = 93.44. Prevalence 2001 / prevalence 2007 = 15.25-fold.
Ind Prev (%): individual prevalence. Herd Prev (%): herd prevalence.

Table 3. Animal brucellosis prevalence by species (P < 0.001).

Year	Sheep (P < 0.001)						
	2001	2002	2003	2004	2005	2006	2007
Animals	175,666	11,0275	93,331	121,005	197,667	206,628	226,799
Positive animals	10,251	4911	1529	1604	2355	1599	852
Prevalence (%)	5.8	4.5	1.6	1.3	1.2	0.8	0.4
C.I. 95%	5.7–6.0	4.3–4.6	1.6–1.7	1.3–1.4	1.1–1.2	0.7–0.8	0.4–0.4
Reduction of prevalence (%) = 93.10. Prevalence 2001 / prevalence 2007 = 14.50-fold.							
Year	Goat (P < 0.001)						
	2001	2002	2003	2004	2005	2006	2007
Animals	41,825	23,162	21,784	28,985	43,143	47,697	51,298
Positive animals	1822	747	304	398	545	484	384
Prevalence (%)	4.4	3.2	1.4	1.4	1.3	1.0	0.8
CI 95%	4.2–4.6	3.0–3.5	1.2–1.6	1.2–1.5	1.2–1.4	0.9–1.1	0.7–0.8
Reduction of prevalence (%) = 81.82. Prevalence 2001 / prevalence 2007 = 5.5-fold.							

Brucellosis prevalence was higher in sheep than in goats in 2001; however, from 2001 to 2004, prevalence decreased for both species, although it was slightly lower for sheep. Brucellosis prevalence further decreased to 0.4% for sheep and 0.8% for goats from 2005 to 2007. Overall, brucellosis prevalence decreased 14.5-fold for sheep and 5.5-fold for goats from 2001 to 2007.

3.5. Prevalence of brucellosis by flock constitution

The study of flock composition (Table 4) revealed that the numbers of pure flocks were 4-fold higher than mixed flocks. During the 7-year period, the number of pure flocks increased by 30%, whereas mixed flocks increased by 18%. The prevalence of brucellosis decreased by 77% in pure flocks and 73% in mixed flocks from 2001 to 2004.

Table 4. Animal brucellosis prevalence by flock composition (P < 0.001).

	Pure (P < 0.001)						
Year	2001	2002	2003	2004	2005	2006	2007
Animals	166,773	106,486	95,727	119,986	189,866	202,944	218,191
Positive animals	8741	4623	1357	1464	2273	1645	810
Prevalence (%)	5.2	4.3	1.4	1.2	1.2	0.8	0.4
CI 95%	5.1–5.4	4.2–4.5	1.4–1.5	1.2–1.3	1.2–1.3	0.8–0.9	0.3–0.4
Reduction of prevalence (%) = 92.30. Ratio of [Prevalence 2001 / prevalence 2007] = 13.00-fold.							
	Mixed (P < 0.001)						
Year	2001	2002	2003	2004	2005	2006	2007
Animals	50,718	26,951	19,388	30,004	50,944	51,381	59,906
Positive animals	3332	1035	476	538	627	438	426
Prevalence (%)	6.6	3.8	2.5	1.8	1.2	0.9	0.7
CI 95%	6.4–6.8	3.6–4.1	2.2–2.7	1.6–1.9	1.1–1.2	0.8–0.9	0.6–0.8
Reduction of prevalence (%) = 89.39. Prevalence 2001 / prevalence 2007 = 9.43-fold.							

Brucellosis prevalence further decreased between 2005 until 2007; however, the prevalence of mixed flocks almost doubled compared to pure flocks in 2007.

3.6. Prevalence of brucellosis by animal production

The main animal production of the farms of the study area was meat. From 2001 to 2007, the number of small ruminants raised for meat production increased by 45% while the number of dairy small ruminants decreased by 20%. The prevalence of brucellosis decreased progressively from 2001 until 2007 for both meat and dairy. However, an approximately 8% increase of the prevalence in dairy small ruminants in 2005 compared to 2004 was observed. From 2005 to 2007, brucellosis prevalence further decreased, being slightly lower in small ruminants intended for dairy production than those intended for meat production. In addition, the decrease in brucellosis prevalence by animal production was statistically significant ($P < 0.001$).

4. Discussion

Trás-os-Montes e Alto Douro region was the area of Portugal with the highest prevalence of brucellosis for sheep and goats. The application of a Rev-1 vaccination program to the whole small ruminant population (young and mature) was aimed to control and decrease this expensive zoonotic disease.

The advantages of a mass conjunctival Rev-1 vaccination program in areas with high brucellosis prevalences have been widely described (5,9). The main characteristics of small ruminant flocks of the region of study were the extensive management, low head number per flock, and meat as the main form of animal production. The highly significant associations and similar patterns of brucellosis prevalence in all the flock characteristics in the study indicated that the Rev-1 vaccination program was the main factor in the decrease of brucellosis prevalence.

The decrease of brucellosis prevalence from 5.6% to 0.4% indicated the effectiveness of the Rev-1 mass vaccination program in young and mature sheep and goats, together with a test-and-slaughter program of brucellosis-positive animals and movement restriction of positive flocks (5). Although the Rev-1 program was enforced in the entire small ruminant population, adequate protection was only possible if the vaccines were applied to at least 80% of the animals at risk (12).

The official brucellosis prevalence data (7) in small ruminants in the region of Trás-os-Montes e Alto Douro was 4.63% in 1999 and increased to 6.93% in 2000. Although the prevalence further decreased in 2002, it was lower compared to 2001, achieving a final prevalence of 4.2%. This result may be considered compatible with the bacteremia caused by Rev-1 vaccination that lasts from the first day of vaccination until day 60, with maximum presence in the second week. Thus, the potential bacteremia caused by Rev-1 vaccination due to abortions in pregnant adult females as described by Banai (13) may have contributed to the disease dissemination and permanence

Table 5. Animal brucellosis prevalence by main animal production ($P < 0.001$).

	Meat ($P < 0.001$)						
Year	2001	2002	2003	2004	2005	2006	2007
Animals	159,031	94,006	85,760	121,616	181,037	198,147	231,141
Positive animals	9503	3912	1322	1670	2129	1691	1068
Prevalence (%)	6.0	4.2	1.5	1.4	1.2	0.9	0.5
CI 95%	5.8–6.1	4.0–4.3	1.5–1.6	1.3–1.4	1.1–1.2	0.8–0.9	0.4–0.5
Reduction of prevalence (%) = 91.66. Prevalence 2001 / prevalence 2007 = 12.00-fold.							
	Milk ($P < 0.001$)						
Year	2001	2002	2003	2004	2005	2006	2007
Animals	58,460	39,431	29,355	28,374	59,773	56,178	46,956
Positive animals	2570	1746	511	332	771	392	168
Prevalence (%)	4.4	4.4	1.7	1.2	1.3	0.7	0.4
CI 95%	4.2–4.6	4.2–4.6	1.6–1.9	1.0–1.3	1.2–1.4	0.6–0.8	0.3–0.4
Reduction of prevalence (%) = 90.91. Prevalence 2001 / prevalence 2007 = 11.00-fold.							

in the study area (14). Excretion in milk has been also described in pregnant vaccinated adult females; however, its dissemination has been considered weak and irregular, with lower epidemiological importance compared to the excretion observed in fetal or vaginal discharges (13,15). The largest reduction in the prevalence of brucellosis was observed from 2002 to 2003 and can probably be explained by the increase of the immunity due to Rev-1 vaccination in 2001 and 2002.

At the beginning of the compulsory vaccination period in 2001, the test-and-slaughter program had a "cleaning effect" according to the high number of positive animals. In 2002, the prevalence of brucellosis decreased compared to 2001; however, this lower reduction, about 25%, was probably related to the persistence of the etiological agent in the environment or to the contamination caused by the abortions triggered by *B. melitensis* Rev-1 vaccine (16). On the other hand, the protective effect of Rev-1 on adult animals and the increase of the immune status of the new generation, both by vertical transmission or by Rev-1 vaccination before reproductive age, reflected the decrease observed in the number of positive animal slaughtered in 2001. Consequently, the further decrease of brucellosis prevalence that occurred in 2003 may be due to the increased immunity status of the new generations achieved by Rev-1 vaccination, maternal transference, and the Rev-1 vaccination of 3- to 6-month-old lambs and kids (before reproductive age) carried out between 2001 until 2002, as well as the decreased persistence of brucellosis in the environment. In addition, the reduced brucellosis prevalence in animals may also be explained by the more voluntary adhesion of farmers to the Rev-1 vaccination program as well as the enforcement of control policy in nonsampled/unvaccinated flocks.

The brucellosis prevalence recorded in 2007 was the lowest in the study period; however, brucellosis-positive animals were still present, presumably due to a lack of collaboration by farmers in blood sampling, absence of communication of the presence of young animals eligible for Rev-1 vaccination, and/or uncontrolled animal trade and/or movements by veterinary official services from nonsampled flocks. In addition, there are variations of the replacement rate of the flock where, sometimes, animals originally destined for slaughter and left unvaccinated are kept in the flock, despite vaccination being compulsory (17). The progressive reduction of the prevalence of brucellosis from 2004 up to 2007 was a result of the protective effect of Rev-1 vaccination of lambs and kids, the immunity status of the small ruminants population, and the test-and-slaughter program.

Regarding human brucellosis in the study area, the criteria used to determine success or failure of a vaccination plan were mostly linked with a reduced incidence of human brucellosis in the treated area (13). Thus, in the study area, human cases of brucellosis from 2002 to 2007 were reduced by about 83% (8). This reduction may be related to the success of the Rev-1 mass vaccination program of the small ruminant population.

Brucellosis prevalence related to flock size was similar for the three flock sizes in 2007; however, from 2001 until 2004, it varied each year. The prevalence of brucellosis in large flocks was half of that in small and medium flocks in 2004. These results are difficult to explain and may be associated with the scarce application of biosecurity measures and inadequate farm practices. The application of a biosecurity plan is essential to the control of brucellosis, as in other diseases (18). These plans include measures like movement control, cleaning and disinfection, reproductive management, and veterinary treatments, among others; however, the implementation of a biosecurity plan is not compulsory (19). Small flocks usually graze on pastures near or contiguous to the farm, avoiding contact with other flocks or utilization of common paths and/or roads. Because the flocks' premises are small, cleaning, disinfection, and manure removal procedures are easier and less time-consuming for the farmer. The disinfection is also facilitated by the low resistance of *B. melitensis* to most disinfectant agents (20) and by the low cost of this operation. Farmers of small flocks may have an easier time controlling the partum period and can usually keep dams away from the flock during parturition. This measure is very important in the case of abortions to avoid pasture contamination. Moreover, communication of abortions to the veterinary official services is compulsory. The lower prevalence of brucellosis in 2001 and 2002 in small flocks may be associated with animal movement. In these kinds of flocks, replacement is usually done by repositioning; economic trade is not frequent. Factors like presence of nomadic flocks (13) or elevated rate of animal movement (5) have been described as brucellosis control failures due to the lack of Rev-1 vaccination coverage. The health status of the flock may influence the predisposition to brucellosis infection. Thus, farmers can easily identify sick animals, and veterinary and preventive treatments are usually carried out due to the low costs. Regarding the official control of brucellosis by the veterinary official authority, small flocks are easily controlled. In case of a brucellosis-positive animal, most farmers agree to cull the whole flock to maintain the brucellosis-free status and also to avoid a zoonotic infection (21).

The higher prevalence of brucellosis observed in 2001 in medium and large flocks may be associated with the utilization of communal pasture areas, utilization of common paths and/or roads, and contact with others flocks (22); however (23), proximity to an infected flock is not considered a risk factor for brucellosis. Cleaning

and disinfecting the premises and manure removal in large flocks is more difficult than in medium or small flocks, because it requires the availability of mechanical equipment and consequently a higher economic cost. In addition, an increase in brucellosis prevalence when there was a decrease in proper manure removal and cleaning and disinfection procedures has been described (24). The control of reproductive management is difficult in large flocks, where parturitions on grazing areas are frequent. Thus, abortions are a source of pasture contamination. In addition, the animal movement in a large flock is frequent for both replacement and/or trade, increasing the risk of infection by brucellosis. Due to the high cost of veterinary treatments and/or application of preventive programs, small ruminants in large flocks may be more susceptible to brucellosis infection. Moreover, unvaccinated and/or untested animals may occur in large flocks, remaining unprotected and susceptible to infection. In addition, these animals act as a source of brucellosis contamination for the rest of the flock (5); in the case of brucellosis-positive animals, farmers hesitate to slaughter the entire flock.

The prevalence of brucellosis in large flocks observed in 2003 was already half of that in medium and small flocks, probably due to flock management. Small and medium flocks had family-type management and the owners usually also had another economic activity, while owners of large flocks based their principal income on livestock-based meat and/or milk production. As a result, the presence of brucellosis implies great economic losses due to abortions, culling of positive animals, and interdiction of sheep and/or goat trade due to movement restrictions. Moreover, high brucellosis prevalence in the flock, or the absence of a progressive reduction along with multiple blood samplings, leads to the compulsory slaughter of the whole flock and the end of economic activity for a minimum of 6 months (9).

To avoid these problems, farmers are especially interested in protecting their animals against brucellosis. Thus, the increased immunity against brucellosis achieved by the mass vaccination program may explain the drop in brucellosis prevalence in 2003, as well as its maintenance up to 2004.

After the mass vaccination program, the prevalence of brucellosis in small and medium flocks further decreased from 2005 to 2006; however, the increase of brucellosis prevalence observed in large flocks over this 2-year period can be associated with the purchase of new animals to increase and/or maintain the economic performance after the compulsory slaughter of brucellosis-positive animals in the previous years. In 2007, the level of protection achieved by vaccination of the whole population of small ruminants supports the similar prevalence value, regardless of flock size.

Brucellosis prevalence decreased from 2001 until 2007, according to the animal species. Prevalence was higher in sheep in 2001; however, sheep and goats exhibited similar prevalences in 2004. During a 3-year period, the prevalence of brucellosis in 3- to 6-month-old lambs and kids was twice as high in goats as in sheep (25), although other authors noted otherwise (26). The information available about differences of brucellosis infection by species is scarce. Pregnant dams did not have *Brucella* spp. in vaginal discharges, contrary to goats (27), where excretion may extend over 2 or 3 months (28). This may explain why brucellosis prevalence was higher in goats than in sheep from 2005 to 2007.

Despite brucellosis-related abortions, some authors (27) observed that transmission during pregnancy was lower than transmission observed in nursing; lambs born from infected females were resistant to brucellosis; after a few hours, they were negative for both the RBT and CFT. Thus, the natural resistance of the lambs in association with the Rev-1 vaccination supports the lower prevalence observed in sheep from 2005–2007.

The change in brucellosis prevalence according to the flock constitution was similar as observed previously for species; however, the prevalence was higher in mixed flocks than in pure flocks. No evidence was found to explain this result; however, other authors (29) reported that keeping sheep and goats together has been identified as a risk factor for brucellosis infection. This may be due to brucellosis shedding from vaginal discharges from infected pregnant females as previously described. Moreover, sheep parturition usually happens at night, while it happens during the day in goats; daytime parturition leads to a higher probability of pasture contamination, increasing the risk of transmission.

Brucellosis prevalence was higher in flocks raised for meat production. Dairy flocks use mainly pure breeds to increase the milk yield; this characteristic has been described as a risk factor for brucellosis infection (22,24).

The higher prevalence observed in flocks for meat production is compatible with the main animal production of the study area. The largest reduction of brucellosis prevalence occurred from 2001 to 2004 in flocks for meat production; however, at the end of this 4-year period and also in 2007, brucellosis prevalence was lower in dairy flocks. These results are compatible with the maintenance of the brucellosis-free status by dairy farmers to avoid economic losses due to lower sale price of the milk in addition to the abortion, neonatal losses, increased birth intervals, reduced fertility, decreased milk production, increased culling rates, and emergency slaughtering of the infected animals (30).

In conclusion, the Rev-1 vaccination of the whole small ruminants' population was an effective measure

to decrease brucellosis prevalence in Trás-os-Montes e Alto Douro. However, the evolution and the behavior of the *B. melitensis* Rev-1 was different according to the characteristics of the flocks. Brucellosis prevalence was similar among the different flock sizes in 2007, but the differences observed between 2001 and 2004 may be related to the scarce application of biosecurity measures and/or improper farm practices. Brucellosis prevalence was higher in goats than in sheep, due to the different behavior of *Brucella* spp. in each species. The change in brucellosis prevalence according to the flock composition was similar to that previously described for animal species, although mixed flocks presented a higher prevalence. A higher prevalence was observed in meat production flocks than in dairy production flocks, which was compatible with the main animal production of the study area. In addition, the lower prevalence observed in dairy production was due to the maintenance of the brucellosis-free status to avoid economic losses. These results contribute to the scarce information available regarding the effect of Rev-1 vaccination on the different characteristics of flocks and they can be used to improve the efficiency of brucellosis eradication programs within livestock management.

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