EPIDEMIOLOGICAL STUDIES ON PARATUBERCULOSIS IN SMALL RUMINANTS IN PORTUGAL*

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***SUMMARY** : Preliminary results of a serological survey on paratuberculosis in 66 sheep and goat flocks in the area of Lisbon, Portugal, identified 27% of flocks with seropositive animals. The follow-up on six of these flocks showed from one to nine percent of positive animals per flock. A higher probability of occurrence of seropositive animals was identified in the goats than with the sheep flocks. A higher proportion of positives also occurred in the milk producing when compared with the meat producing flocks. Typical lesions of paratuberculosis were found in 6 animals with intestinal lesions from which* Mycobacterium avium *subsp*. paratuberculosis *had been isolated.*

The adjustment of the methodology for future use in the project and the implementation of adequate prevention and disease control measures are discussed by the authors taking in account the epidemiological risk factors identified in this study.

***Keywords:** Paratuberculosis,* Mycobacterium avium *subsp* paratuberculosis*, Sheep, Goats, Serology, Epidemiology, Risk factors, Control.*

***RESUME :** Les résultats préliminaires d'une enquête sérologique sur la paratuberculose réalisée sur 66 troupeaux ovins-caprins dans la région de Lisbonne, au Portugal, ont mis en évidence 27% de troupeaux à sérologie positive. Le suivi exhaustif de six de ces troupeaux a montré entre un et neuf pour cent d'animaux à sérologie positive par troupeau. Les troupeaux caprins ont présenté une plus forte probabilité d'apparition d'animaux séropositifs que les troupeaux ovins. Les troupeaux laitiers ont présenté une plus forte proportion de séropositifs que les troupeaux producteurs de viande. Des lésions typiques de paratuberculose ont été observées chez six animaux présentant des lésions intestinales à partir desquelles* Mycobacterium avium *subsp* paratuberculosis *a été isolé.*

L'ajustement de la méthodologie pour la suite du projet ainsi que la mise en œuvre de mesures adaptées de prévention et de contrôle de la maladie sont discutés par les auteurs, en prenant en compte des facteurs de risque identifiés dans cette étude.

***Mots-clés:** Paratuberculose,* Mycobacterium avium *subsp* paratuberculosis*, Ovin, Caprin, Sérologie, Epidémiologie, Facteurs de risque, Contrôle.*

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I - INTRODUCTION

Paratuberculosis is a disease caused by *Mycobacterium avium* subspecies *paratuberculosis (Map)* [Twort and Ingram, 1912] that affects mainly small and large ruminants causing chronic enteritis and emaciation [Stabel, 1998 ; Sweeney, 1996 ; Whittington and Sergeant, 2001].

No efficient treatment is known and the disease leads to economic losses [Valentin-Weigand and Goethe, 1999 ; Winterhoff *et al.*, 2002 ; Wiszniewska and Szteyn, 2002] due to relevant decrease in milk production, costs in the diagnosis and in disease control, the early culling of affected animals and the low carcass value at slaughter. Although not yet proven some authors refer the possibility of its potential relationship with Crohn's disease in humans [Bakker *et al.*, 2000 ; El-Zaatari *et al*., 2001 ; Hermon-Taylor *et al*., 2000 ; Kennedy and Benedictus, 2001 ; Lund *et al*., 2002 ; Naser *et al*., 2002 ; Nauta and Giessen, 1998 ; Richter *et al*., 2002].

This disease is specially transmitted through the faeces of adult animals with clinical or subclinical disease, which constitute the greater risk, to young animals that are the most susceptible and that will become diseased when adults [Stabel, 1998 ; Sweeney, 1996 ; Whittington and Sergeant, 2001].

The *Map* can persist for periods of up to one year in the environment [Manning and Collins, 2001], especially if in acid soils rich in iron [Johnson-Ifearulundu and Kaneene, 1999].

This disease is presently distributed throughout the world [Daniels *et al.*, 2003 ; Olsen *et al.*, 2002 ; Stehman, 1996 ; Valentin-Weigand and Goethe, 1999] and is considered an emerging disease as its prevalence has the tendency to increase [Bakker *et al.*, 2000 ; Winterhoff *et al.*, 2002]. In Portugal, since it was first reported in 1983 in small ruminants [Reis and Ferreira, 1988], sporadic cases have been reported in the Region of Trás-os-Montes [Ferreira, 1989] and in the area of Lisbon (Figure 1). An extensive serological survey was carried out in small ruminant flocks in the 1992-94 period, in the area of Vouzela, in the Region of Beira Litoral [Amado *et al.,* 1994] and the sole detailed epidemiological study in Portugal was made in the 1996-98 period in the Region of Alentejo, in an area with large holdings of small ruminants of the extensive type of production [Ferreira *et al.*, 2000] (figure 1).

A 3-year research project was initiated in mid 2002 and involved the Veterinary Diagnostic Reference Laboratory (LNIV), the Veterinary Faculty of Lisbon (FMV) and the local Sanitary Defense Group (ADS SOCLA).

The objectives of the project were the epidemiological study of paratuberculosis in sheep and goats flocks in the Region of Lisbon, and namely, to study the prevalence of the disease, to characterize risk factors for its transmission and to evaluate measures for disease control and also to validate methods and protocols for laboratory diagnosis.

II - MATERIALS AND METHODS

1. AREA OF STUDY

The sheep and goats flocks studied were located in the area of action of the Sanitary Defense Group ADS-SOCLA, i.e. the District of Lisbon and included 5 counties and 21 parishes (table I, figure 1, figure 2).

2. FLOCKS AND ANIMALS

The majority of the flocks in the region are smallholdings of the semi-intensive type of production.

The main criteria for selection of sheep and goats flocks for this study were the larger size flocks in the region and, for one flock, a clinical suspicion of paratuberculosis.

Table I

Results of the serological survey for paratuberculosis of small ruminants flocks according to their location in the parishes and counties on the area studied

Counties	Positive / Surveyed (%)	
	Parishes	**Flocks**
Sintra	10/13	15/ 47 (32%)
Cascais	2/2	2/ 9 (22%)
Amadora	1/3	1 /5 (20%)
Oeiras	0/2	0/4
Lisboa	0/1	0/1
Total	13/21	18/ 66 (24.6%)

Figure 1

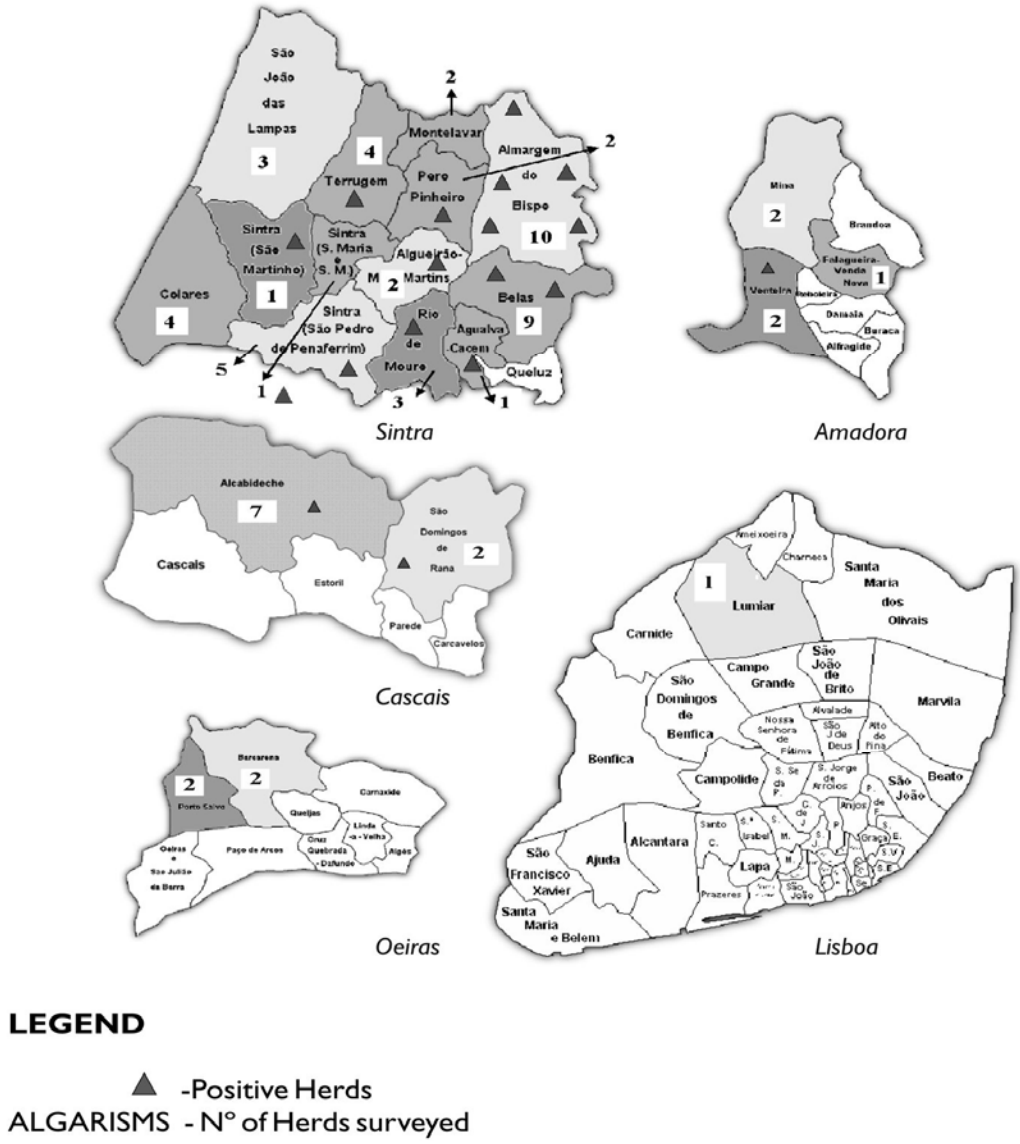
Location of the area of study

The sixty six flocks evaluated constituted 24.6% of the existing flocks (table II), were property of 65 producers and included single species of sheep, goats and mixed flocks, accounting respectively for 20.4%, 12.9% and 40% of the existing flocks.

The flocks sampled had 5,370 heads what was 56.2% of the population in the region (54.8% of existing sheep and 65% of existing goats) (table II).

Figure 2

Location of the flocks surveyed and positive for paratuberculosis



LEGEND

▲ -Positive Herds

ALGARISMS - N° of Herds surveyed

The flocks studied had meat and milk breeds and the size ranged from 32 to 327 heads with an average of 81.4 animals per flock.

3. FIELD SURVEY

Flocks were sampled twice for serology testing over two consecutive years.

The first round included 10% of the animals in the flock with a minimum of 10 sera. The animals selected in the flocks were the older animals that, in general, had more than two years of age. In mixed flocks, the collection included both ovine and caprine species.

If the first results were negative the same sampling criteria were applied to the same flocks in the second round. If the serology was negative in the two rounds the flocks were considered as negative.

Table II

Flocks and animals surveyed for paratuberculosis

Species	Flocks			Animals		
	Existing	Surveyed	%	Existing	Surveyed*	%
Sheep	167	34	20.4	8 187	4 484	54.8
Goats	31	4	12.9	1 364	886	65
Mixed	70	28	40.0	NA	NA	NA
Total	268	66	24.6	9 551	5 370	56.2

NA – Not applicable.
* Total number of animals in the flock sampled

If positive serology occurred in any of the rounds, serology and a comparative intradermal skin test were performed on 100% of the adult animals in the respective flocks. In addition, an epidemiological questionnaire [Toma *et al.*, 2004] was used in farms with positive animals to evaluate for risk factors for paratuberculosis.

4. TESTING PROCEDURE

4.1. SEROLOGICAL TESTING

Blood (5 ml) was collected in the jugular vein to tubes (Lourivet) and sera were separated after clotting by centrifugation at 200 *g* for 10 minutes and frozen at –20º C.

Every 2,351 serum samples collected were analysed for specific serology by two techniques: Agar Gel Immuno Diffusion (AGID) [Albuquerque, 1998] and Indirect ELISA (Paracheck – CSL).

4.2. TUBERCULIN TESTING

Comparative intradermal skin testing used Avian and Bovine PPD tuberculin (LNIV, Lisbon) with the concentration of 25,000 IU/ml. Maclintock syringes (Bar Knigth , Mclintock limited) were used for intradermal inoculation of 0.1ml of each tuberculin in the left and right caudal folds. Evaluation of the skin reaction was made three days after inoculation by palpation of the inoculation site.

4.3. FOLLOW-UP

A sample of the serologically positive animals was followed-up by:

- Necropsy and histopathology using haematoxylin-eosin and Ziehl-Neelsen stains;
- Collection for bacteriological isolation of samples of the target organs, specially the small intestine, ileo-caecal valve and mesenteric lymph nodes;
- Bacteriological isolation of *Map* was performed according to OIE [Thorel, 2000]. The samples were ground up with sterile sand and suspended in PBS and 0.1 ml was inoculated on Herrold's medium supplemented with mycobactin (Allied laboratories, USA) and in Bactec 9000 MB (Becton Dickinson). Observations of growth were made with the periodicity of 45 days for a period of up to eight months.

III - RESULTS

Paratuberculosis seropositive animals were detected in 18 flocks (27% of the flocks surveyed) of which 15 herds were located in 10 of the 13 parishes of the county of Sintra, accounting for 32% of the flocks surveyed in this county (table I, figure 2). The other counties with positive flocks were Cascais with two positive flocks and Amadora with one positive flock. No seropositive animals were identified in the four flocks surveyed in the

county of Oeiras or in the single flock surveyed in the county of Lisbon (table I, figure 2).

The highest seropositivity rate was in the flocks with goats only (50% of the four flocks sampled) followed by the mixed flocks and finally by the sheep flocks (table III).

According to the type of production, 22% of the 50 meat flocks surveyed had seropositive animals while this proportion increased to 44% in the 16 milk flocks surveyed.

Of the 53 positive samples diagnosed, 77.4% were only ELISA positive while 12 were positive in both ELISA and AGID (table IV). This showed a low agreement rate between the tests (k= 0.33) [Martin *et al.*, 1987]. In this study, there were no ELISA negative sera that gave AGID positive results.

Table III

Results of the ELISA survey for paratuberculosis

	Nº of flocks		
	Surveyed	**Seropositive**	**%**
Sheep	34	6	18
Goats	4	2	50
Mixed	28	10	36
Total	66	18	27

Table IV

Serologic results (and *Map* isolation) of seropositive animals

Results of the test	**Sheep**	**Goats**	**Total**
ELISA + & AGID -	21	20 (1+/0-)	41 (77.4%)
ELISA + & AGID +	0	12 (5+/1-)	12
Total seropositives	21	32 (6+/1+)	53

Note – No ELISA negative sera had AGID positive results. The results of *Map* isolation are shown between brackets

Up to the present, six flocks located in six parishes in the county of Sintra were followed-up with 100% serology, and 29 seropositive animals were identified (table V). The number of seropositive animals per flock ranged from 1.0 to 8.9% (with an average of seropositives of 3.5%). The seropositive goats accounted for 5.7% of the surveyed while sheep accounted for 2.6% of the seropositive animals but only in three of the five mixed flocks the rates of seropositives were higher in the goats (table V). When comparing by Chi-square test the seropositivity results in the two species in these flocks they showed significant differences.

Map isolation was attempted in nine animals of which six were positive, one was negative and two are still waiting for completion of laboratory isolation (table VI). *Map* isolation was achieved in six samples of goats: five of the animals were seropositives for the two tests and another was only positive by ELISA. In the six positives and one negative for *Map* isolation, the concordance between the results of the bacteriology and the pathology findings was 100% (k=1) [Martin *et al.*, 1987] (table VI).

Table V

Flocks followed up by 100%

	Nº positives/ nº sampled (% positives)						
Flock ID	139	72	133	120	96	140	Total
Sheep	0/249	3/151 (2.0)	9/152 (5.9)	2/28 (7.1)	2/28 (7.1)	NA	16/608* (2.6)
Goats	3/40 (7.5)	1/10 (10.0)	0/65	1/20 (5.0)	3/38 (7.9)	5/56 (8.9)	13/229* (5.7)
Total	3/289 (1.0)	4/161 (2.5)	9/217 (4.2)	3/48 (6.2)	5/66 (7.6)	5/56 (8.9)	29/837 (3.5)

NA – Not applicable; * Significant difference- $p<0.05$.

Table VI

Results of pathology and microbiology

		Pathology		
		+	-	Total
Map isolation	+	6	0	6
	-	0	1	1
	NC	0	2	2
	Total	6	3	9

NC – Not yet completed, waiting for laboratory confirmation.

The main lesions constantly observed were the thickening of the intestinal wall of the small intestine showing a corrugated appearance, specially in the ileon and in the ileo-caecal valve, the mesenteric lymph nodes showed hypertrophy and congestion and there was effusion of fluids into the body cavities. Microscopically, the intestine showed typical granulomatous lesions with multinucleated cells and syncytia and acid fast rods that were abundant in the intestinal wall and also present, in smaller amounts, in the mesenteric lymph nodes.

The epidemiological questionnaires, filled up to the present, allowed the characterization of the type of husbandry as semi-intensive with two lambing seasons, the offspring milking their mother and weaning at two months of age. The epidemiological links between the flocks included pastures that were sometimes shared between small ruminant flocks and, on two occasions with bovine and sporadic local trading of animals (although auto-replacement of the breeding stock prevailed).

The local evaluation of the possibility of implementation of the main prophylactic and control measures, namely, the testing and culling of seropositives and of bacteriologically positive animals, the acquisition of breeders from paratuberculosis free certified flocks, the quarantine and testing of acquired animals, keeping the potentially infected pastures kept empty for a minimum period of one year, avoidance of feeding from colostrum of the dams and its heat treatment or artificial colostrums, was not considered acceptable by any of the producers enquired.

IV - DISCUSSION AND CONCLUSIONS

Considering that the results here reported are preliminary, as the project is only in the middle of its expected duration, the present evaluation is considered useful as it can provide a general overview of some aspects of the epidemiology of paratuberculosis in small ruminants in the area of study and can allow the assessment of the protocols for testing and their adjustment, to be followed for the rest of the project.

The flocks with seropositive animals were mostly found in the county of Sintra, which has the highest population of small ruminants and flocks of the five counties studied. The higher density of flocks can account for risk factors such as the sharing of pastures and the facilitation of the local trade of animals.

Although not generally reported in the bibliography, in our study, a higher proportion of seropositive animals were diagnosed in the milk producing rather than in the meat producing breeds of sheep. This can be especially important for some agricultural regions of Portugal like Beira Interior, where the milk breeds can account for up to 79% of the ovine population [MADRP, 2001] and the milk is highly valuated for the production of traditional sheep cheese. Lactation can anticipate the beginning of clinical disease [Kennedy and Benedictus, 2001] with increase of excretion. It should, therefore, be considered as a major risk factor in the transmission of disease, mainly to young susceptible milking lambs. The sanitary control measures to stop the cycle of transmission are especially important in those flocks.

In our study, the goats showed a significantly higher seroprevalence rate suggesting a higher sensitivity to paratuberculosis. In the sanitary management of caprine and mixed flocks they should, therefore, be considered as animals with a higher risk of infection and, eventually, of transmission of the disease. Therefore, the future work to be developed by the project would focus on goats' sampling.

An early detection of infected animals was expected with the implementation of a tuberculin test [Thorel, 2000]. The results of the testing were not presented in the present paper, as they were considered not reliable due to the subjectivity resulting from the difficulties in the interpretation of the local reactions. This happened especially in the milk-producing sheep where the management procedure of cutting of the tail did not allow using the caudal folds and the skin of the genital area presented irregularities that confused the interpretation of the results. This testing will therefore be interrupted and replaced by the Gamma Interferon test which might allow the detection of the paratuberculosis infection at earlier stages than by serology [Jones, 2001].

A low agreement of the results was found between the ELISA and the AGID tests but every positive AGID result was also ELISA positive. Due to its high sensitivity the ELISA test [Amado *et al.*, 1995] will become the only screening test used for serology.

Although complete concordance has been verified among the results of *Map* isolation and the occurrence of typical lesions, and even if the pathological studies are a very useful tool in the diagnostic of disease, the confirmation of infection needs to be made by laboratory identification of *Map* [Albuquerque*,* 1998].

Up to the present, the only test used for the confirmation of infection has been the microbiological isolation of *Map* on the target organs of seropositive animals that were necropsied.

Due to the low rate of growth of *Map*, the incubation of the samples for bacteriological isolation is of up to 8 months. On opposition, the technique of PCR provides results in a period of a few hours. This highly sensitive diagnostic technique has already been reported for the genome detection of *Map* in tissues and faeces using the sequence IS 900 [Juste *et al.,* 2002], but, up to the present has not been used for routine diagnosis by the Portuguese Reference Laboratory, which coordinates this research project. Studies are being pursued for its validation, so that it can be used on a routine basis for the diagnosis of paratuberculosis, replacing the bacteriology as a screening test for infection.

As a confirmatory test, the bacteriological isolation is expected to continue, but in the meantime while the results are not yet available, some control measures could be implemented in the flock.

As a cost-effective strategy, the use of the faeces of the seropositive animals, for bacteriology, could also help in confirming the presence of *Map* without the cost paid for compensation to the producer for the animals taken for analysis. As it is known that the excretion of *Map* in the faeces is not always occurring at the same time as antibodies

[Radostitis *et al.,* 2000]. The fact that the collection of samples would be facilitated could also allow that a larger number of samples could be tested. Nevertheless, previous to this change it will be necessary to validate, by agreement studies, the testing results for *Map* isolation from the faeces and from organs of the animals.

There is no universal diagnostic protocol for paratuberculosis as proven by a wide range of results obtained in different trials that are dependent on the stage of evolution of the disease in the animals and on the epidemiological situation. The results here reported can help on the definition of a laboratory protocol and on the development of techniques for use in the project that can be also of application in the Portuguese situation.

The awareness provided by our study of the high prevalence of paratuberculosis in the area raised the importance of this disease in small ruminants and of the need to further evaluate the situation and to establish a local control programme.

Although extension work has been made to the producers on disease management, due to their low technical background and to the small-scale production, it is difficult to adjust the flock management to implement most of the sanitary prophylactic measures involving the young. Thus, the control of the disease is not effective, and paratuberculosis would likely increase in the region along the years.

The use of vaccination as a control method is presently not authorised in Portugal as the vaccinated animals can interfere with the eradication programs for *Mycobacterium bovis* by confounding the diagnosis [Smith and Sherman, 1994]. This can be a problem for bovine in Portugal but not for sheep and goats as tuberculosis is not considered relevant in these species.

The vaccine has been used for the control of the disease in several countries decreasing the positivity rate of flocks and animals but, as a single method, was not enough to achieve the eradication of the disease [Kennedy and Benedictus, 2001 ; Smith and Sherman, 1994]. It should not be used in flocks producing and selling breeding stock as excretion of the bacteria can occur in vaccinated animals [Kennedy and Benedictus, 2001] and, if applied with a proper control in areas without tuberculosis, it is generally considered to be a good tool for disease control.

We expect that, with the results obtained until the end of the project, the present findings can be consolidated.

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