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Serology as an Epidemiological Tool for *Salmonella* Abortusovis Surveillance in the Wild-Domestic Ruminant Interface

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1. Introduction

Salmonella sp, are opportunistic pathogens that can infect a wide range of hosts, including man (Murray, 1991). The increasing numbers of *Salmonella* infections reported in the last decades reveal an important health problem of considerable socio-economic impact (Kapperud et al., 1998). Salmonellosis has been reported in 85% of food-borne bacterial enteritis in humans from Spain (Pérez-Ciordia, et al; 2002), and *Salmonella* sp, are increasingly recorded in animals (Echeita et al.; 2005).

Unlike other *Salmonella* species, *Salmonella enterica* subspecies *enterica* serovar Abortusovis (S. Abortusovis) is adapted to sheep, and considered to be host specific (Jack, 1971). Discarded as a zoonotic pathogen, its importance lies in the economic losses that occur in ovine production systems in regions that depend on sheepherding (Pardon et al., 1988; Sojka, et al., 1983). It has been most frequently associated with ovine salmonellosis in ovine flocks from Europe and the Middle East, causing abortion outbreaks, stillbirths, and illness in lambs infected at birth (Jack, 1968; Pardon et al., 1988). These mainly result from the epidemic behavior of the disease, which is most recognized when the organism is newly introduced into a flock, because abortion storms reach high proportions. In endemic scenarios it also causes abortions in up to 50% of the ewes in a flock, usually during the first pregnancy, as in newly introduced (González 2000).

Available epidemiological data show a limited distribution for Abortusovis serovar. It is considered rare in most countries and regions of the world except in Europe, where it is particularly common, with reported cases in France, Spain, Germany, Cyprus, Italy, Switzerland, Russia, and Bulgaria, southwest England and Wales and also in Western Asia (Jack., 1968; Echeita et al., 2005; Valdezate et al., 2007). In northern Spain it has been considered to be among the major etiological agents of ovine abortion (González, 2000) , but it is also spread through 11 Spanish provinces, where 20 different clones have been identified in fifty-five field strains collected from epidemic abortions or neonatal mortality episodes affecting different ovine flocks during the period 1996–2001 (Valdezate et al., 2007).

The infection can appear in naive flocks by means of animal carriers such as new sheep replacements, contact with other animals in seasonal migration, wild and carrion birds, or

rodents (Valdezate et al., 2007). Sensitive animals acquire the infection by ingestion of food and water contaminated by vaginal discharges, placenta, aborted foetus (liver and stomach contents), and infected newborn. Furthermore, in some conditions, faeces, milk and respiratory secretions can correspond to infectious material. Other routes of acquisition include respiratory and conjunctival routes (Jack., 1971).

From the third month of pregnancy, this pathogen induces abortion, in the absence of other clinical symptoms (Jack, 1971), but it is sometimes preceded by depression, uncertain walking, mucous vaginal discharge and diarrhoea. Following this, ewes seem to be healthy or show transient fever, but sometimes ewe mortality occurs from septicaemic complications like anorexia, acute metritis, enteritidis and peritonitis that result from placental retention (5–7% of cases) (Astorga et al., 2000), differing from infection causes by Dublin and Typhimurium serovars. In addition, neonatal mortality of lambs is frequent with living muttons at term which are non-viable and die within a few hours of birth from septicaemia. Occasionally, lambs appear to be healthy but die during the first month, showing signs of enteritis, pneumonia or polyarthritis. Conversely, the infection is asymptomatic in non-pregnant ewes and rams (Uzzau et al., 2001).

Within a flock, *S.* Abortusovis is maintained by effective transmission from infected to susceptible sheep through the oral, conjunctival, or respiratory routes, while venereal infections appear to be of minor importance (Uzzau et al., 2001). Spread to other susceptible populations is mostly the consequence of commercial translocations of asymptomatic carriers. The dissemination of *S.* Abortusovis by food, water, birds or other mammals, has been traditionally considered as negligible. But as well as the host-specificity to sheep of *Salmonella* Abortusovis, its adaptation to other mammals can be discussed: mice and rabbits can be experimentally infected, and it has occasionally been isolated from goats and rabbits.

Wild ruminants can act as asymptomatic carriers of pathogenic *Salmonella* serovars (Cubero et al., 2002; Renter et al., 2006), and some serovars can also cause clinical disease in deer species (Foreyt et al., 2001; McAllum et al., 1978). However, this bacteria has not been related to abortion in non domestic species.

Many European countries face difficulties in controlling *S.* Abortusovis disease because there are no ways to diagnose all infected animals (Lantier et al., 1983). In this sense, it is essential to consider sampling procedures (e.g. type of samples, sampling frequency) according to the objectives of the testing program, clinical findings, level of detection or precision of prevalence estimates required, cost and availability of sampling resources and laboratory facilities. In recent years a standard method for detecting *Salmonella* from primary animal production has been developed and evaluated, and an ISO-method (ISO 6579:2002 Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp., Annex D) has now been adopted), and diagnosis procedures have been well defined (OIE, 2010).

The identification of the *S.* Abortusovis is based on the isolation of the organism; when infection of the reproductive system, abortion or conceptus occurs, it is necessary to culture fetal stomach contents, placenta and vaginal swabs. Nevertheless, few epidemiological surveys have been able to be carried out owing to the low number of available *S.* Abortusovis isolates (Valdezate et al., 2007).

In this sense, serology can improve the study of natural infections through identification of infected herds, rather than to confirm infected individual animals; although repeated herd tests can be used as an aid to detect chronic carriers. Antibodies to *S.* Abortusovis may become undetectable in some sheep 2–3 months after abortion. Flock diagnosis of *S.* Abortusovis in sheep can be performed by serological tests conducted on a statistically representative sample of the population, but results are not always indicative of active infection (OIE, 2010). These include serum agglutination test (SAT), hemagglutination inhibition, complement fixation, indirect immunofluorescence, gel immunodiffusion, and enzyme–linked immunosorbent assay (ELISA) (Davies, 2004).

In Spanish mountain rural areas extensive-grazing farming and game management practices favor the establishment of multispecies assemblies with a high diversity of herbivore species that share pastures and forests. There, sheep can be considered as primary hosts of *Salmonella* Abortusovis, but the potential dissemination of this agent to other possible sensible ruminant species has not been considered in epidemiological studies, even though specific antibodies have been found in Spanish ibex (*Capra pyrenaica*), fallow deer (*Dama dama*), European mouflon (*Ovis aries*) and red deer (*Cervus elaphus*) from Southern Spain (Pérez, 2007; León et al., 2002). In the Serranía de Cuenca domestic ruminant flocks find sympatric conditions for grazing with wild populations of red deer, fallow deer, roe deer (*Capreolus capreolus*), European mouflon and Spanish ibex, were introduced for big game hunting purposes from 1960 to 1979, and have successfully colonized the territory 30 years later.

2. Objectives

The aim of this study was to identify antibody responses against *Salmonella* Abortusovis in sheep flocks by means of the serum agglutination test (SAT), and evaluate the possibility of adapting it to official veterinary programs sampling efforts. A further aim was to evaluate the potential application of this test for screening *Salmonella* infections in cattle herds and wild ruminant populations. We therefore estimated the risk factors of sero-conversion against specific antigens of *Salmonella* Abortusovis in wild and domestic ruminants, and analyzed the statistical association between the sero-epidemiological indexes of each population and the related environmental conditions in the grazing pastures of the "Serranía de Cuenca Regional Game Reserve", where traditional shepherding constituted the core of the economic activity as in other many regions of Castile La-Mancha, Spain.

3. Material and methods

3.1 Study area

The Serranía de Cuenca Natural Park is one of the best conserved mountainous areas of Castile-La Mancha, Spain. The study area is situated in the centre-east of the Iberian Peninsula (40º 12´- 40º 28´ N / 1º 51´- 2º 03´ W), attached to the limits of the "Serranía de Cuenca Regional Game Reserve". It has a Mediterranean climate tempered by the altitude and barrier effect from the local orography to the wet winds from the west that confer a temporal semi-damp hydrologic pattern (annual rainfall varies between 600 and 1.000 mm, which is more intense from November to January), with long periods of drought during the hottest months (normally July and August). The climatic conditions that occur are typical for the Mediterranean Mountains, with temperate weather (Mean temperatures between 7'5 and

12'5ºC), cold winters (Mean temperatures fall below the 0ºC between 60 to 120 days) and dry, but not excessively hot, summers (law-ranking decree 99/2006). Vegetation is dominated by forests: Special importance is given to those constituted primarily by *Pinus nigra* Subsp. *Sazlmanii*, and the Oromediterranean forests of *Pynus sylvestris*. The latest, integrate the climax vegetal community at altitudes that limit the Mediterranean forests (*Quercus faginea* and *Q. ilex*), or on not suitable soils for the Sabin (*Juniperus sabina*), while Mediterranean scrubland (*Crataegus monogyna*, *Ligustrum vulgare*, *Viburnum lantana*, *Rhamnus saxatilis*) and pastures (*Poo-Festucetum istricis* association) present limited extensions (Peinado et al., 1985).

The "Serranía de Cuenca Regional Game Reserve"is an open area of 25.850'9 hectares covering eleven public mountains with free access to people, an open private property (El Maillo) and a fenced private property (Valsalobre), where human activities are restricted to traditional non-intensive use, such as eco-tourism, wood production, extensive farming, and hunting. In the core of this territory the human activities, including farming, are not allowed in a valley of 910 hectares, known as "El Hosquillo", which is partially fenced for its administration as Experimental Game Park. In contrast, game ungulates share the forest's pasturelands with cattle and small-ruminants herds in the surrounding protected areas.

3.2 Ruminant populations

The bio-climatic conditions in the region traditionally favor extensive ruminant farming system, specially focused on summer-grazing migratory merino-sheep flocks, for wool production (Cava, 1994). During recent decades this system has been modified by different social and economic influences that have led to a current orientation of the production to lamb meat, employing mixed raced animals for such purpose. On the other, goats have always played a self-sufficient role in the human communities of the Serranía de Cuenca and are usually maintained in the sheep flocks as guides or stepmothers, but are not of relevant economic importance. In contrast, beef productions are gaining importance, and the traditional local use of bovines as working elements is no longer practised. During the years 2.003-2.005 permissions for grazing in the "Serranía de Cuenca Regional Game Reserve" allowed the herding of 12.881 domestic ruminants in flocks classified by species composition in: 19 ovine, 14 ovine-caprine, and 6 bovine (Martín Atance, 2.009).

Big game hunting was historically practised in the area until the autochthonous wild ruminant populations became extinct, at the end of the 19th century. In order to encourage this activity, red and fallow deer were introduced in the "Serranía de Cuenca Regional Game Reserve" from 1960 to 1966, the European mouflon from 1974 to 1977, and Spanish ibex from 1976 to 1979 (Rojo Arribas, 2007). In 2001 the free ranging population sizes estimated in the Game Program of the "Serranía de Cuenca Regional Game Reserve" were: 850 red deer, 617 fallow deer, 300 mouflon and 230 Spanish ibex, in 25,724 hectares; and in semi-captivity: 209 red deer, 32 fallow deer, 91 mouflon, and more than 11 Spanish ibex at the fenced hectares in "El Hosquillo" Game Park (Martínez & Verona, 2002).

3.3 Collection and preparation of serum samples

The sampling of domestic ruminants herds that grazed at the study area were collected in two annual Livestock Sanitary Surveys: In 2,004, 241 blood samples were collected from cattle (5 herds), and 1,196 from sheep (13 herds), and in 2.005 samples were taken from 166

cattle (3 herds), and 2,543 from sheep (27 herds) in the course of Official Veterinary Programs. Sampling of wild ruminants was performed from 2,003 to 2,006 on 885 free ranging animals hunted in the "Serranía de Cuenca Regional Game Reserve", and from 225 live animals captured in the Game Park.

Blood samples were taken from the jugular veins in live animals, and from the heart in hunter-harvested wild ruminants. The samples were placed in test tubes and sent to the laboratory under refrigeration (4ºC). They were then centrifuged and the serum was frozen at –80ºC.

Animals were classified into four age groups: young (under 1 year), juveniles (1–2 years), adults (2-9 years), and old (over 9 years).

3.4 Serological procedures

We employed the Serum agglutination test (SAT) to evidence specific antibodies to *Salmonella* Abortusovis O antigen in 5,256 sera from 7 ruminant species: 3,739 from sheep, 556 from fallow deer, 407 from cattle, 314 from red deer, 211 from European mouflon, 21 from Spanish ibex and 8 from roe deer.

SAT were performed according to standard procedures (Pardon et al., 1983; Lindberg, & Le Minor, 1984; Sanchis et al., 1985; Sanchis et al., 1991; OIE, 2010). They were adapted to the microtitre format and used to determine somatic and flagellar titres of specific antibodies induced by *Salmonella* enteritidis subsp. enteritidis serovar Abortusovis natural infections. Antigens used were a serogroup B (O) antigen (Salmonella O Antigen -2840-56-3 Difco®) and a serogroup C (H) antigen (Salmonella Flagelar H Antigen -2846-56-7 Difco®). Negative (serum and normal saline) and positive (Standard sera Salmonella O Anti-sera Grupo B, Difco®, and Salmonella Flagellar Poli Anti-sera Difco®, respectively) controls were included in each test run as confirmatory method for quality control of SAT. Normal saline solution (0'85%) and bi-distilled water Mili-Q were also employed in test procedures.

Sera were screened at dilutions of 1:10 to 1:1.280; 50 µl ml of (O) antigen and added 100 µl of serum pre-diluted to 1:10. The plates were covered by a film and shacked automatically (40-50 rpm) for 5 minutes at 37º C. They were then incubated at the same temperature, without movement, for 18 hours. Sera that presented a positive reaction (from 1:20) were retested with (H) antigen.

To interpret the results of each test run the control wells were examined first, in order to confirm absence of agglutination in the negative controls, and agglutination in the positive controls. Agglutinations appeared as a "matt" or "carpet" at the bottom of the positive O antigen control wells and loose, woolly or cottony in the case of H antigens. In each sample, the highest dilution of serum that produced a positive agglutination was taken as titre.

Samples that held titres over 1:20 to both antigens were considered as sero-positive to *Salmonella* Abortusovis. The sera that reacted at 1:10 against any of the antigens were considered as doubtful, and as negative when agglutination was not observed.

3.5 Statistical tools and definition of epidemiological indexes

In order to describe the immune reactions observed, interpreted as previous exposure to *Salmonella* Abortusovis, and analyze the factors that could contribute to explain the

variations observed between animals and populations, we performed statistical analysis (Caughley, 1977; Crawley, 1993; Petrie & Watson, 1999; Siegel, 1956; Daniel, 1993) suited to the methodological principles of Epidemiology (Thrusfield, 1990; Goldberg, 1994).

As a descriptive study, the main objective was to determine the patterns of sero-conversion presentations. The information obtained from every sample was registered in qualitative terms (presence or absence of sero-conversion), according to the evidence of previous exposure to *Salmonella* Abortusovis; and in quantitative terms related to the intensity of immune recognitions (titre values). The intensity of immune responses in animal groups was expressed by means of the Geometric Mean Titre (G.M.T.), calculated as described by Thrusfield (1995), and in order to measure the variability within groups we have used the standard deviations of the Inverse function of the titres.

The temporal relationships between host, agent, and environmental factors influence the risk of disease. They have been considered here in association to the Prevalence term, defined as the proportion of a population affected by a disease at a given point in time, and interpreted as the probability of a subject in a defined population being diseased at a particular annual sampling campaign. Prevalence is a function of both the incidence (frequency of new cases in a population) and duration (time to recovery of a disease). Also, the relationship between prevalence and incidence will be greatly influenced by the persistence of a detectable antibody following infection, where sero-positivity defines cases of disease.

As an analytical study, our objective was to understand the "natural history" of *Salmonella* Abortusovis. For this purpose we investigated the interrelatedness of the humoral response phenomenon within the biological system, selecting the segments of the chain of infection where the interactions performed betwen hosts and the environment could be measured.

We have used the risk to express the probability of sero-conversion occurrence following a particular exposure to S. Abortusovis. The differences in immune recognition between groups were respected to the frequency of the presence or absence of potential risk factors by means of a cohort study to evaluate the hosts' risk factors. Here, we considered those endogenous characteristics that could influence the immune response of individual ruminants: species and breed (genetic constitution); age and gender (Goldberg, 1994).

Our field study in th e"Serranía de Cuenca Natural Park" measured sero-positive and sero-negative animals considering their exposure to all the known and unknown environmental factors present in their natural environment. The quantification of sero-conversion cases occurrence was performed by counts of sero-positive individuals and expressed as a fraction of the number of animals that held a similar condition (= population at risk). From a mathematical perspective, frequencies were expressed through static measures as proportions and ratios (Thrusfield, 1990; Goldberg, 1994).

Finally,to obtain further data to understand the epidemiology of this infectious disease we evaluated the distribution of the agent in the ecosystem and the factors that could influence the modes of transmission between its compartments (Scott & Smith, 1994). As environmental risk factors we considered those not identifiable with the host or agent but related to the animals comprising individual populations, where they live under similar

conditions of clime, management and nutrition. As empirical research, it involved the measurement of variables, estimation of population parameters and statistical testing of hypotheses by comparisons between groups.

In order to estimate the magnitude of an association between a putatively causal factor and sero-conversion and to assess if there was potential for a cause-effect relationship between a single or multiple risk factors and sero-conversion, we used Generalized linear models (GLM; McCullagh & Nelder 1989) to measure and represent the statistical interaction of response variables (dependents), such as abundance of sero-conversion (prevalences and frequencies),with environmental predictors (independent variables).

Statistical analysis were performed with Microsoft EXCEL 2000© (1985-1999, Microsoft Corporation, USA), Statistica 6.0® (1984-2001 Statsoft, EE.UU.), and EpiInfo 3.3.2 (Center for Disease Control, USA, 2005) integrated epidemiological statistics package. The analysis of the risk factors for the infection was calculated by the Pearson Chi-square test without correction and the Fisher exact test. We considered the value of two tailed P in all analyses. The level of significance was set at $P \leq 0.05$. The association of risk factors and infection were quantified by the analysis of the odds ratio (OR) using Cornfield 95 % confidence limits. Finally, we included the statistically significant factors in General Linear Models to establish which variables act as predisposing factors.

4. Results

Antibodies to *Salmonella enterica* Serogorup B Somatic (O) were not found in 4,318 (82.1 %) samples and were classified as sero-negative SAT reactions. We observed coloured films on the surfaces of the well at 1/10 dilutions in 716 (13.6 %) sera, that were considered as doubtful and, at higher titres 222 (4.2 %) were identified as sero-positives.

Results showed that frequency of immune reactions (titres ≥ 1:10) against somatic antigen of *Salmonella* Abortusovis (applicable to others included in B serogroup) were higher bovines (RF = 36.8%) than in sheep (RF = 19.73%), fallow deer (RF = 7.55%), mouflon (RF = 9.47%), or red deer (RF = 1.59%) (Table 1; Figure 1).

Moreover, specific antibodies to *Salmonella* Abortusovis H antigen were revealed by SAT among the 939 sera that showed titres of 1:10 or higher to the O antigen (Cattle = 150; sheep = 738; fallow deer = 42; red deer = 5; mouflon = 4). Results showed that frequency of immune reactions (titres ≥ 1:10) against flagellar antigen of *Salmonella* Abortusovis were higher in mouflon (RF = 75%) and domestic ovine (RF = 74.39%), than in fallow deer (RF = 54.76%), cattle (RF = 30-66%), or red deer (RF = 0%) (Table 1; Figure 2).

The relative intensity of reactions in this species against each antigen was estimated by the Mean Geometric Titer, and only considering sero-positives (titers ≥ 1:20). Immune responses to O antigen were higher in cattle (MGT = 1:39.2), than in fallow deer (MGT = 1:23.7), sheep (MGT =1:22), or red deer and European mouflon (MGT =1:20). Other ways, responses to H antigen were higher in ovines (MGT = 1:30.6) and mouflon (MGT= 1:25.2), than cattle or fallow deer (MGT = 1:20), and were not detected in red deer (Table 1).

The interpretation of serological results obtained in both techniques for *Salmonella* Abortusovis serological diagnosis was performed with attention to positive reactions (titres

≥ 1:20) to both antigens O and H. This condition was only found in 150 (2.8%) serum samples: 145 (3.85%) ovine sera, 3 mouflon sera (0.95%), and 2 fallow deer sera (0.35%) (Table 1; Figure 3).

Species	Somatic antigen "O"(group B)					Flagellar antigen H (group C)				
		Titre					Titre			
	n	1:10	> 1:10			n	1:10	> 1:10		
		AF	AF	%	MGT		AF	AF	%	MGT
C	407	111	39	9,58	39,2	150	0	46	30,6	20
S	3739	567	171	4,57	22,0	738	128	421	74,3	30,68
RD	314	4	1	0,32	20	5	0	0	0	-
FD	556	34	8	1,44	23,7	42	21	2	54,7	20
RoD	8	0	0	0,00	-	0	0	0	-	-
SI	21	0	0	0,00	-	0	0	0	-	-
M	211	1	3	1,42	20	4	0	3	75,0	25,19
T	5256	716	222	4,22	-	939	149	490	51'7	-

AF: absolute frequency; GMT: mean geometric titre; C: cattle; S: sheep; RD: red deer; FD: fallow deer; RoD: roe deer; SI: Spanish ibex; M: mouflon; T: Total.

Table 1. Frequencies of sero-positives and Mean Geometric Titres aganist S Abortusovis Somatic and flagellar antigens obtained by Serum Agglutination Test

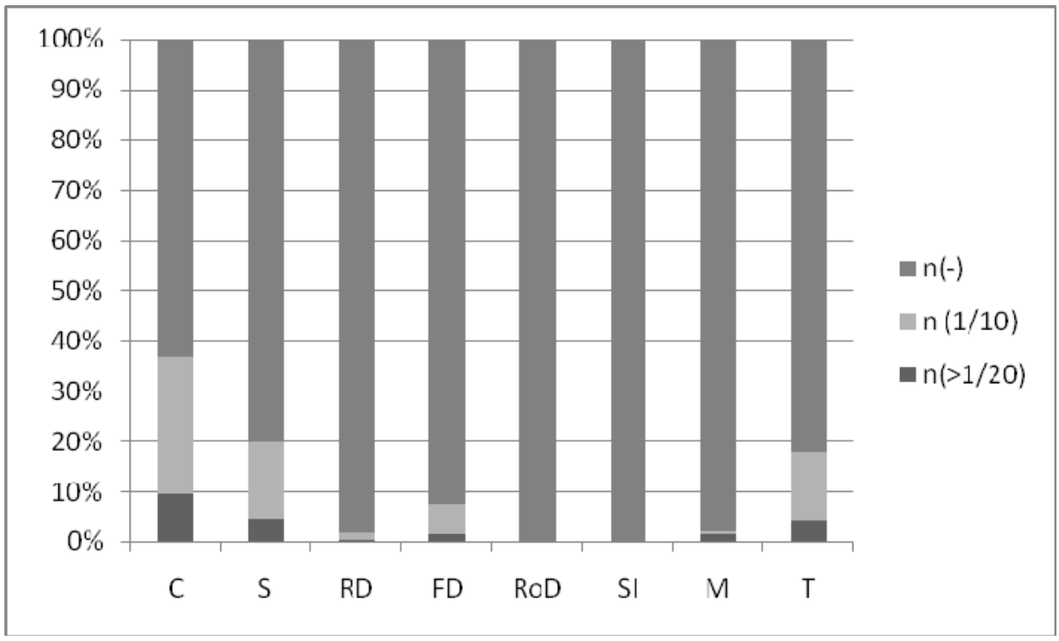

Fig. 1. Percentage of sero-positive, doubtful and sero-negative reactions against O Antigen

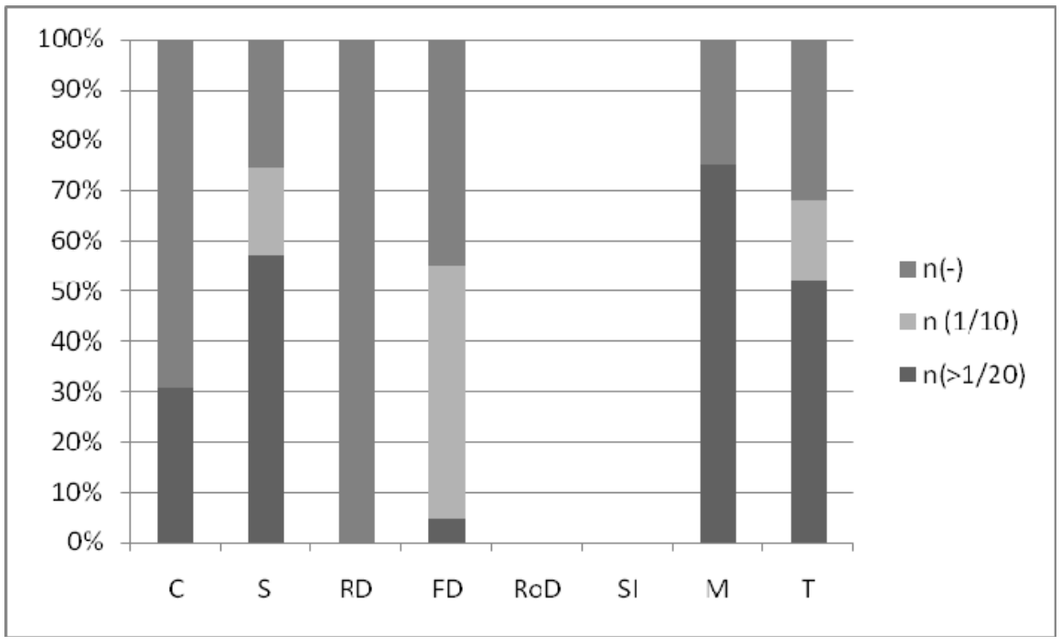

Fig. 2. Percentage of sero-positive, doubtful and sero-negative reactions against F Antigen

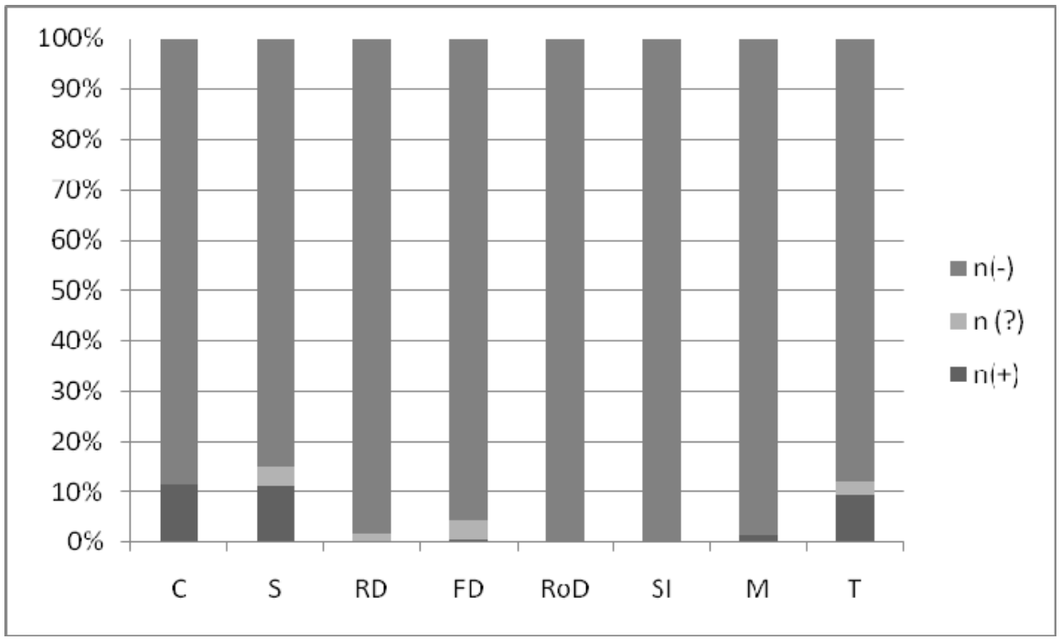

Fig. 3. Percentage of sera catalogued as sero-positive, doubtful and sero-negative against *Salmonella* Abortusovis

Statistical relationship between the sero-conversion cases and individual factors of the animals was explored by means of risk factors analysis. Statistical risk to sero-conversion was observed between sexes in domestic sheep ($X^2 = 42.37$; $p < 0.001$), that was higher for males compared to females (Relative risk = 3.85; IC95%: 2.5-5.8 vs. RR = 0,26; IC95%: 0.1-0.3) (Table 2).

Species	TOTAL			♀		♂		φ (IC 95%)		X^2	p
	Nº	(+)	(-)	(+)	(-)	(+)	(-)	♀			
C	361	0	361	0	290	0	71	-	-	-	-
S	3249	139	3110	116	2974	23	136	0,26 (0,1-0,3)	3,85 (2,5-5,8)	42,37	0,00
RD	314	0	314	0	184	0	130	-	-	-	-
FD	533	2	531	2	307	0	224	-	-	-	-
RoD	8	0	8	0	5	0	3	-	-	-	-
SI	21	0	21	0	9	0	12	-	-	-	-
M	211	3	208	1	141	2	67	0,24 (0,02-2,6)	2,12 0,3-44,6)	1,60	0,20

(+) Positive. (-) Negative. Risk ***φ.-*** *Odds ratio.* AF: absolute frequency; GMT: mean geometric titre; C: cattle; S: sheep; RD: red deer; FD: fallow deer; RoD: roe deer; SI: Spanish ibex; M: mouflon; T: Total.

Table 2. Risk associated to sex in the studied species.

Among domestic ruminants, yearling sheep showed a high risk of sero-conversion to *S.* Abortusovis (p=0'01, φ=2'27) respect to older animals (p=0'14, φ=0'6). In the same way. the results for mouflon sheep, revealed a significantly high *Odds ratio* in yearling animals (φ = 7'58), that was followed by a decreasing value (φ = 3'12 in the next age group (2 to 4 years); estimates in the rest of the cohorts considered were not able to be performed due to the scarce absolute number of sero-positive animals. (Table 3).

By the same way, the juvenile age group seems to be an important risk factor for sero-conversion in domestic sheep (OR = 1'39; $IC_{95\%}$:1'13-2'71) and mouflon (OR = 7,6; $IC_{95\%}$:0,73-77,18), compared to other age categories (X^2 = 5'6; *p* = 0'01 and X^2 = 3,8; *p* = 0,049, respectively) (Table 3).

Group age	Specie	Cases			Probability		Risk	
		+	-	RF (%)	X^2	p	φ	IC 95%
Young	Sheep	8	77	9'4	5'6	0'01	2'27	1'15-4'49
	Cattle	0	66	0'0	-	-	-	-
Adult	Sheep	131	3004	4'3	2'16	0'14	0'6	0'3-1'19
	Cattle	0	177	0	-	-	-	-
Old	Sheep	0	29	0	-	-	-	-
	Cattle	0	118	0	-	-	-	-

RF: Relative frquency. *Odds ratio*

Table 3. *Odds ratio* (φ) for sero-conversion to *S.* Abortusovis in the different age groups.

Sero-prevalence values in sheep flocks were estimated in 2004 and 2005. The frequency of affected flocks in the first year reached 100% (13/13), with sero-prevalences ranging from 4.3% to 22.0% (12-33± 5.9%) and also low MGT (1:23.25 ± 1:5.57). The following year sero-positive sheep were found only in the 33.33% of the sampled flocks (9/27), and sero-prevalence values in the same flocks ranged from 1.06% to 7.9% (3.0±2.14%) with very low MGT (1:0.99±7.91). The results obtained in the Serranía de Cuenca show a decline phase of an epidemic outbreak.

Mean sero-prevalence values in each flock and grazing area were estimated and used as indexes to evaluate association of sero-conversion with management factors, and spatial relationships with environmental conditions, respectively. In the correlation matrix analysis built to evaluate management factors in sheep flocks a significant trend to seroconversión was observed in association with lambing intensities of 3 parturitions each 2 years ($r = 0.4013$; $p = 0\text{-}028$), but this could not be confirmed by ANOVA ($F_{(7,23)} = 1.19$; $p = 0.34$).

The spatial distribution of infected flocks in the "Serranía de Cuenca Regional Game Reserve" Pasture Areas conditioned a wide distribution of sero-positive sheep, restricted only by their own flock permissions, with mean sero-prevalences of 4.2 ± 3.04% (Range: 0 to 9.57%). These sero-prevalence values were associated with the relative extension of *Pinus nigra* within each area, by means of Lineal Regression Models: "PRV OA Salm" = 1.852 + 0.07 * "Pn" ($R^2 = 0.33$; $p = 0.039$).

The scarce evidence of specific antibodies against *Salmonella* Abortusovis in other ruminant populations prevents evaluating the potential host-pathogen interactions in the lesser sampled species (roe deer and Spanish ibex). Furthermore, frequencies of sero-positives in mouflon (3/211, 1.4 %) and fallow deer (2/555. 0.3%) populations were low and not intense (Range of titres: 1:20-1:40), and immune responses were absent in red deer, as in cattle (Table 4).

RD (n = 314)	**FD** (n = 555)	**RoD** (n= 8)	**SI** (n = 21)	**M** (n = 211)
0	2 (0.36)	0	0	3 (1.4)

RD: red deer; FD: fallow deer; RoD: roe deer; SI: Spanish ibex; M: mouflon.

Table 4. Frequency of sero-positives in wild ruminant species.

5. Discussion

Different *Salmonella* serovars have been isolated from a wide variety of vertebrates, including European wild ungulates, but bacterial isolation is not sufficient, in itself, for a diagnosis of salmonellosis (Mörner, 2001; Nielsen et al, 1981).

Diagnosis of salmonellosis should be based on culture and identification of the bacteria, together with clinical and pathological evidence (Olsen et al, 2003; Threlfall & Frost, 1990; Valdezate et al., 2007; Van der Zee & Huis In't Veld, 2000). In the case of paratyphoid abortion diagnosis, this can be compromised, because the use of common bacteriological procedures is not an advisable option, and clinical signs or lesions are not specific enough (González, 2000; Beuzón et al., 1997; Linklater, 1983). In such situations, serological methods, such as SAT on microplates, may also be used to identify infected flocks or herds, rather than to identify individual infected animals (Pardon et al., 1988; González, 2000; OIE, 2010).

For detailed epidemiological investigations strain identifications are necessary (OIE, 2010). Traditionally, the identification of S. Abortusovis has relied on the use of antisera against O and H antigens (Brenner, 1984; Vodas and Martinov, 1986), and currently molecular characterization techniques like ribotyping, plasmid profiling, and IS*200* fingerprinting can be performed successfully to identify different and predominant Abortusovis genotypes (Nikbakht, et al.2002; Schiaffino et al. 1996; Nastasi, et al. 1992). The use of this method led to the identification of two different strains in sheep flocks from Cuenca province between

1996 and 2001, but this epidemiological surveillance system is limited by the low number of Abortusovis isolates available (Valdezate et al., 2007).

On the other hand, detection of specific immune responses to S. Abortusovis can provide further evidence of infection, but little is known about the duration of effective immunity following *Salmonella* infections, and positive results cannot always be interpreted as indicative of active infections (OIE, 2010; Brennan et al., 1994; 1995). Animals that have been infected recently would, in all probability, eventually be detected serologically by an appropriate monitoring programme throughout the life of the flock/herd, but there are often cost limitations to the application of effective monitoring programmes (OIE, 2010).

The agglutination test is considered the preferred method in export and diagnostic purposes for samples from all species of farm animals (Davies, 2004; OIE, 2010). Our research demonstrated the possibility of performing it alongside the analysis scheme established in the brucellosis official eradication program. SAT proved to be easily adaptable to the routine diagnostic procedures in "Albaladejito" Veterinary Laboratory as well as being an economical method of performing simultaneous analyses of large numbers of samples. In this sense, we must argue for the potential adoption of this method by official veterinary programs if further studies are to be carried on.

In order to define the level of detection or accuracy of prevalence in the testing program we must consider the lower sensitivity of SAT in comparison with ELISA tests (Berthon, et al., 1994, Sting et al., 1997; Veling et al., 2000), and the conditions relating to sampling procedures regarding the dynamics of immune responses aganist *Salmonella* Abortusovis (OIE, 2010). For most animals, a significant increase in agglutination titres could be observed from day 5 after inoculation (Lantier, 1987), but care in interpreting the serological results has to be taken if this test is performed after abortion, as antibody levels fall and may become undetectable 2-3 months later (Davies, 2004; González, 2000). Thus, our serological results should be interpreted as punctual sero-frequencies or sero-prevalences.

The wide frequency of O-agglutinating sera found among the domestic (bovines RF = 39.2%; ovines RF=22 %) and wild ruminants (fallow deer RF =23.7%; mouflon RF = 20%: red deer RF = 20%), sampled in the "Serranía de Cuenca Regional Game Reserve" indicated wide immune recognition of *Salmonella* between 2003 and 2006, but these immune responses may respond to other group B *Salmonella* infections, whatever the O antigen (Bernard et al., 2002). In fact, results obtained in cattle and red deer against group C H antigen were not correlated, in any case, to the respecting anti-O titres. In this sense, although serological responses can be demonstrated against both flagellar and somatic antigens, it has been stated that it is advisable to restrict the search to anti-H agglutinins, as these are more specific, reach higher titres and have a precocity and persistence similar to those against O antigen (Pardon et al., 1988; González, 2000).

The frequencies obtained in the group C H-antigen SAT showed very high values among the ovines (mouflon RF = 75%; sheep RF = 74.39%), but were also found in a high percentage of fallow deer (RF = 54.76 %), and cattle (RF= 30.66%). However, many samples presented a low titre to group B O antigen (1:10), but reached high agglutination titres to H antigen, and were not considered as specific responses against *S.* Abortusovis. These were recorded in a limited percentage of the sheep analysed (3.85%), mouflon (0.95%), and fallow

deer (0.35%), thus restricting the potential host range. In order to improve the knowledge of *Salmonella* infections in each species it would be advisable to include other antigens in the serological screening.

The presence of humoral immune specific recognition of *S.* Abortusovis in sheep from the "Serranía de Cuenca Regional Game Reserve" is further evidence of paratyphoid abortion in the province of Cuenca (Valdezate et al., 2007). Besides possible controversial discussions about prevalence values estimated under different methods (Jack., 1968; Uzzau et al., 2000; Valdezate et al., 2007), the characteristic epidemic behaviour of paratyphoid abortions leads to differences of incidence and importance of S. Abortusovis infection between sheep flocks, countries and periods of time (González, 2000; Giannati-Stefanou et al., 1997). In this sense, the use of SAT can improve sheep abortion surveillance by identifying infected flocks (OIE, 2010), but also by indicating epidemiological trends, as may be suggested by the significant differences found among sero-epidemiological indexes (frequency of affected flocks, MGT, and sheep sero-prevalence) estimated in consecutive sampling campaigns, which clearly indicate a decreased epidemic phase of infection.

In addition, the main affection of yearlings and recently purchased sheep is usual after paratyphoid abortion storms (González, 2000). This situation is also suggested by the results obtained in the risk factor analysis. The odds ratio for sero-conversion in sheep showed a statistical association to host factors such as sex or age, with higher risk in males and yearlings. These findings could respond to different predisposing physiological conditions of lambs, ewes, and rams, but also to environmental factors related to management practices. At this point, it is necessary to consider that in the "Serranía de Cuenca Regional Game Reserve" extensive grazing system these sheep groups maintain different farming conditions, such as transport, herding, housing or nutrition, that respond to the conditions of the reproduction scheme in each flock (Martín Atance, 2009).

The communal grazing practices in the "Serranía de Cuenca Regional Game Reserve" may allow a wide dissemination of infection between flocks, but differences in the extension of Pinus nigra forests within each grazing area can modulate the variance of the mean herd-sero-prevalence values estimated ($R^2 = 0.33$; $p = 0.039$). In our opinion, this finding could indicate the effect of the environment on the host conditions, such as nutritional stress in carrier hosts, but this hypothesis needs to be confirmed by specific research (González, 2000; Linklater et al., 1991).

The detection of SAT specific responses in mouflon and fallow deer allows us to hypothesize about the possible sensitivity to infection of both species, or even a potential adaptation of similar strains to this hosts. In this sense, it would be recommendable to favour research into the genetic and physiological differences of *Salmonella* isolates from sheep and compare them to others of wildlife origin in order to test the basis for *Salmonella* enterica evolution in relation to hosts (Bäumler et al., 1997; Hensel, 2004; Popoff & Le Minor, 2005). Nevertheless, the scarce amount of evidence suggests that wild ruminants play a minor role in the epidemiological cycle of paratyphoid abortion in the "Serranía de Cuenca Regional Game Reserve", as has been suggested in Andalusia, where sero-positive Spanish ibex, mouflon, fallow deer and red deer have been detected at higher rates (Arenas et al., 1993; Cubero et al., 2002; Pérez, 2007; León Vizcaíno et al., 1980; León Vizcaíno et al., 1992; León Vizcaíno et al., 1994; León Vizcaíno et al., 2002).

These results indicate the epidemiological role of sheep as primary hosts of S. Abortusovis, and the absence of natural niches of infection among other ruminant populations. However, the presence of specific antibodies in European mouflon and fallow deer could be indicative of the infection in this populations and the presence of chronic carriers that might help to disperse this agent.

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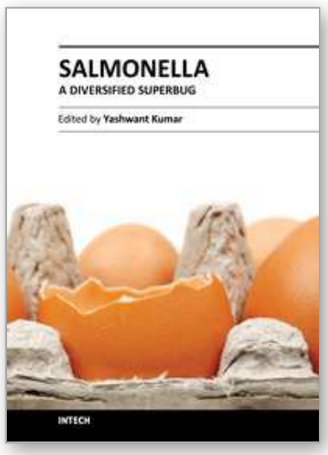

Salmonella - A Diversified Superbug
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Salmonella is an extremely diversified genus, infecting a range of hosts, and comprised of two species: enterica and bongori. This group is made up of 2579 serovars, making it versatile and fascinating for researchers drawing their attention towards different properties of this microorganism. Salmonella related diseases are a major problem in developed and developing countries resulting in economic losses, as well as problems of zoonoses and food borne illness. Moreover, the emergence of an ever increasing problem of antimicrobial resistance in salmonella makes it prudent to unveil different mechanisms involved. This book is the outcome of a collaboration between various researchers from all over the world. The recent advancements in the field of salmonella research are compiled and presented.

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