Survey of the seroprevalence of brucellosis in ruminants in Kosovo

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A cross-sectional survey of the seroprevalence of brucellosis in sheep, goats and cattle in Kosovo was made in January 2001. A total of 12,000 serum samples, from 7941 cattle, 3548 sheep and 511 goats, were screened using the Rose Bengal test. Doubtful and positive results were further tested with competitive and indirect ELISAs. The overall serological prevalences derived from the samples positive to all three tests, were 6·26 per cent (95 per cent confidence intervals [CI] 5·5 to 7·1 per cent) for sheep, 7·24 per cent (5·3 to 9·8 per cent) for goats and 0·58 per cent (0·43 to 0·77 per cent) for cattle. The survey covered 26 of the 29 municipalities and showed that brucellosis was widely but unevenly distributed throughout the province. Seropositive animals were found in 25 per cent (19 to 32 per cent) of 162 villages surveyed. The risk of cattle being infected on holdings where both cattle and sheep were kept was greater, with a risk ratio of 4·6 (2·2 to 9·6), than on holdings where only cattle were kept. *Brucella melitensis* probably predominates as the cause of brucellosis in ruminants in the province of Kosovo.

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BRUCELLOSIS due to *Brucella melitensis* causes reproductive wastage and reduced milk production in affected livestock and is an important zoonosis. The disease in human beings is serious and long lasting and often results in chronic and disabling symptoms. Brucellosis was endemic in livestock in Kosovo before 1992 (Taleski and others 2002) and a state-mandated control programme, based on test and slaughter, was in operation, but few details are available about its conduct or efficacy.

Records from the Prishtina Institute of Public Health indicate an increasing incidence of human cases of the disease in the Balkan countries over the past 10 years, with approximately 1000 confirmed cases of brucellosis reported in Kosovo since 1990. The overall incidence is about four cases per 100,000 population per year but levels of 18 to 45 cases per 100,000 population per year were reported in some municipalities. The true incidence is likely to be much higher because many cases are not reported and the condition is easily misdiagnosed.

The objective of this survey was to improve the understanding of the epidemiology of brucellosis in cattle, sheep and goats in Kosovo and to provide information that could be used in human and animal disease control programmes.

MATERIALS AND METHODS

Survey design

A cross-sectional study was made to investigate the seroprevalence of brucellosis in cattle, sheep and goats in Kosovo. The survey involved 26 municipalities and excluded four small municipalities (Fushe Kosovo, Obiliq, Zvecan and Novo Berde) (Fig 1). In Kacaniku, where there were very small populations of sheep and goats, only cattle were sampled. Livestock census data for the municipalities had been collected during a vaccination campaign against anaerobes, anthrax, rabies and classical swine fever organised in early 2001 by the Food and Agriculture Organization (FAO) as part of an emergency farm reconstruction aid project.

Subsistence farming is widely practised in Kosovo and individual holdings are small with families generally keeping one or two dual-purpose cattle. During the summer, many sheep are grazed under extensive conditions in the southern mountainous regions. Flock sizes generally range from about 20 to 100 animals although there are a few larger flocks of 500 to 700 animals. In most villages, small separately owned flocks or herds are sometimes allowed to graze together and they can be regarded as single management units.

A two-stage sampling strategy was used in which villages were the primary sampling units and the livestock holdings in each village were the secondary sampling units.

In the first stage, five villages, separated by roughly the same distance and located in the centre, north, south, east and west of each municipality were selected for sampling from the 1200 villages identified in the 26 municipalities. This sample size gives about 95 per cent confidence for detecting a 40 per cent (± 8 per cent) prevalence of infected villages.

In the second stage, sheep and goats of breeding age were sampled from mixed herds or single farm herds or flocks. Single farm herds or flocks were defined as single units.

Ideally, each village had a large mixed herd or flock, 10 per cent of which could be sampled. The balance of samples for that village was then obtained by systematic random selection of all the animals on single units. Two courses of action were followed when a farm with a larger number of animals was encountered in the systematic selection process. When there were less than 10 animals, all of them were sampled, and when there were more than 10 animals, 10 per cent of them were sampled.

If the required sample size could not be met at one sampling location, then the next closest village was chosen for the remaining samples.

All the animals sampled were identified individually with ear tags, and details of the owner, species and location were recorded. Blood samples were collected in vacutainers and allowed to clot; the serum was removed at the Central Veterinary Diagnostic Laboratory in Prishtina and tested for brucella antibodies with the Rose Bengal test (RBT) and stored at –20°C. All RBT-positive or suspicious sera were further tested with indirect and competitive ELISAs (iELISA and cELISA, respectively) at the Veterinary Laboratories Agency – Weybridge. Poor quality sera were also tested by ELISA (Stack and others 1999).

Serology

The tests were carried out according to the Office International des Epizooties (OIE) Manual of Standards for Diagnostic Tests and Vaccines (MacMillan and Stack 2000) and all the antigens were prepared and standardised at Weybridge according to the OIE Manual. The negative control serum used for all the tests was produced from a collection of known brucellosis-free cattle in Great Britain.

RBT

Sufficient antigen, test sera and positive and negative control sera for a day's testing were removed from refrigeration and

brought to room temperature (22±4°C). The test was validated at the start of each day by using known positive and negative control sera. For testing, 30 µl of test serum was mixed on an enamel white tile base with an equal volume of Rose Bengal-stained antigen to produce a circular zone 2 cm in diameter. Positive sera agglutinated after gentle agitation for four minutes at room temperature. The tests were read under conditions of good lighting under a window to detect the agglutination which varied from being relatively coarse and easily detected to a very fine floccular form. Positive control serum was collected from a cow naturally infected with *Brucella abortus* biovar 1 (Weybridge) and calibrated against the OIE ELISA standard serum (MacMillan and Stack 2000).

iELISA

For the sheep and goat samples, the iELISA tests were made by using a lipopolysaccharide (LPS) antigen derived from *B melitensis* 16M (Weybridge) which was checkerboard titrated against rabbit anti-goat immunoglobulin IgG conjugated to horseradish peroxidase (Dako). The working concentrations of these reagents were chosen as those which provided the optical density (OD) with the greatest difference between a positive and a negative control. The substrate was 40mM azino-bis-(3-ethylbenziazoline-6-sulphonic acid) (ABTS; Sigma) and 300 µl was added to every 12 ml of substrate buffer and 60 µl of 3 per cent hydrogen peroxide. The substrate buffer was pH 4 buffer tablets, and 1mM sodium azide was used to stop the reaction. The results were read at 405 nm. Samples with an OD 10 per cent or more of that of the positive control serum were considered positive.

Samples from cattle were treated as above, using titrated LPS *B abortus* S99 antigen and anti-bovine IgG peroxidase conjugate. The positive control serum used in this test was from a cow naturally infected with *B abortus* biovar 1 and calibrated against the OIE ELISA standard serum (MacMillan and Stack 2000).

cELISA

A checkerboard titrated LPS extraction of *B melitensis* 16M was used as the antigen for all the animal species. The monoclonal antibody used was BM40 (Weybridge) (Greiser-Wilke and others 1991). The substrate used was o-phenylenediamine dihydrochloride (Sigma). One 30 µl tablet was dissolved in 75 ml distilled water and 300 µl 3 per cent hydrogen peroxide. Citric acid at 0·25M was used to stop the reaction which was read at 450 nm. Any sample with an OD less than 60 per cent of that of the buffer control was considered positive. The positive control serum was prepared from a pool of sera from two goats infected experimentally with *B melitensis* biovar 1 strain H38 calibrated against the OIE ELISA standard serum (MacMillan and Stack 2000).

Statistical analysis

The results were analysed by using SPSS and Win Episcope 2.0, and graphics were constructed by using Excel (Microsoft Corporation) and Arcview GIS (Environmental Systems Research). The point estimates of seroprevalence are reported as percentages with 95 per cent confidence intervals in brackets.

TABLE 1: Rose Bengal test (RBT) results for 3548 sheep, 511 goats and 7941 cattle and the seroprevalences for each species and all species combined

	Sheep	Goats	Cattle	Total
Number tested	3548	511	7941	12,000
RBT-positive	231	40	59	330
RBT seroprevalence (%)	6·51	7·83	0·74	2·75

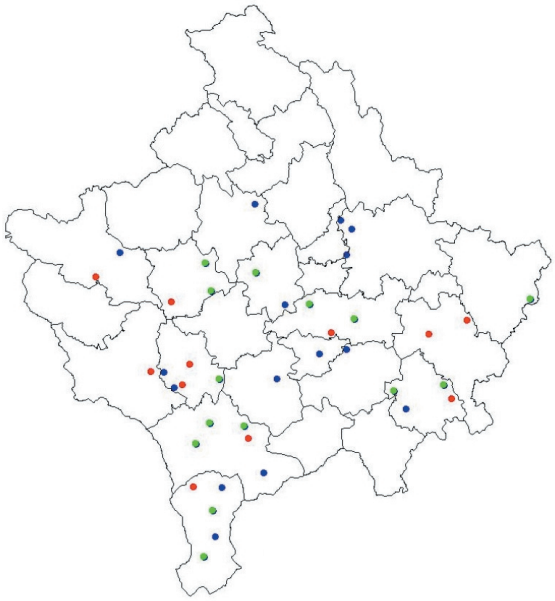

FIG 1: Map of Kosovo showing the borders of the municipalities and the locations of villages where infection was found in cattle only (●), in sheep or goats only (●), or in cattle, sheep and goats (●)

RESULTS

Serum samples were collected from 12,000 animals (3548 sheep, 511 goats and 7941 cattle) in 162 villages in 26 municipalities; they belonged to 4492 livestock farmers of whom 349 owned sheep, 224 owned goats and 4114 owned cattle.

Only three cattle were designated as single animals from a farm holding, and 5705 belonged to herds with more than one animal; no data were available for 2233 cattle. Ten sheep and 88 goats belonged to flocks or herds of unknown size but 3548 sheep and 511 goats were from herds with more than one animal.

The average number of cattle sampled in each municipality was 305 (median 306, range 106 to 477), and the average number of cattle sampled per livestock farmer was two (median one, range one to 20). The average number of sheep sampled per municipality was 141 (median 83, range 18 to 470), and the average number of sheep sampled per livestock farmer was 10 (median nine, range one to 100). The average number of goats sampled per municipality was 32 (median 12, range one to 235), and the average number of goats sampled per livestock farmer was two (median two, range one to 38).

Seroprevalences

The results of the RBTs and the seroprevalences are shown in Table 1; 15 positive sera (eight from sheep, three from goats and four from cattle) were mislaid or otherwise lost to follow-up ELISA testing, and these animals and any which were positive to either or both ELISAs were considered to be infected.

The overall percentage seroprevalence for cattle (0·58 [0·43 to 0·77]) was much lower than for sheep (6·26 [5·5 to 7·1])

TABLE 2: Results of the competitive ELISA (cELISA) and indirect ELISA (iELISA) and the overall seroprevalences for each species and all species combined

	Sheep	Goats	Cattle	Total
Number tested	223	37	54	314
cELISA-positive	210	33	39	282
iELISA-positive	212	34	40	286
RBT and ELISA positive	214	34	42	290
Overall seroprevalence	6·26	7·24	0·58	2·55

RBT Rose Bengal test

TABLE 3: Overall results of the Rose Bengal Test and ELISA brucellosis tests and prevalences of seropositive sheep stratified by sex and age for 105 villages in 25 municipalities in Kosovo

Sex	Result of tests	Number tested	Age of sheep (years) Unspecified	<1	1	2	3	4	5	≥6
Female	–ve	3191	119	320	912	1210	344	74	130	82
Female	+ve	209	7	18	63	95	15	2	6	3
Male	–ve	143	2	35	50	52	7		3	4
Male	+ve	5			3	1			1	
Prevalence (% F)		6·1	5·6	5·3	6·5	7·3	4·2	2·6	4·4	3·5
Prevalence (% M)		3·4	0·0	0·0	5·7	2·3	0·0		25·0	0·0
Prevalence (% M+F)		6·0	5·5	4·8	6·4	7·1	4·1	2·6	5·0	3·4

M Male, F Female

TABLE 4: Overall results of the Rose bengal test and ELISA brucellosis tests and prevalences of seropositive goats stratified by sex and age for 37 villages in Kosovo

Sex	Result of tests	Number tested	Age of goats (years) Unspecified	<1	1	2	3	4	5	≥6
Female	–ve	460	1	47	128	193	54	4	18	15
Female	+ve	31		14	8	6	1		1	1
Male	–ve	17		4	6	3	4			
Male	+ve	3		1	1	1				
Prevalence (% F)		6·3	0·0	23·0	5·9	3·0	1·8	0·0	5·3	6·3
Prevalence (% M)		15·0		20·0	14·3	25·0	0·0			
Prevalence (% M+F)		6·7	0·0	22·7	6·3	3·4	1·7	0·0	5·3	6·3

M Male, F Female

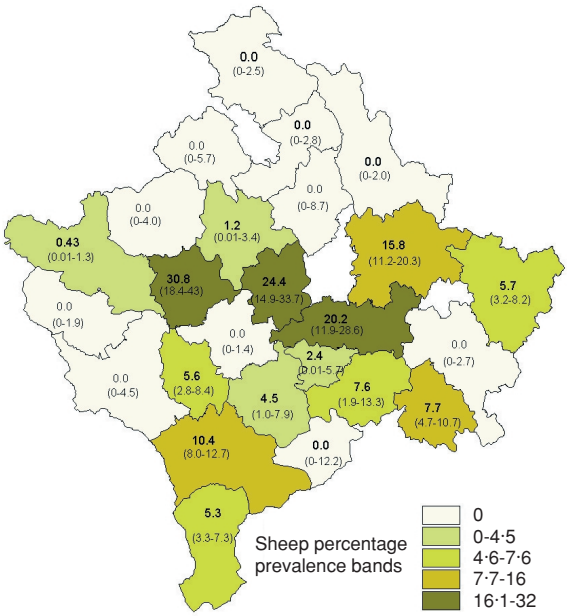

FIG 2: Map showing the estimated percentage prevalences (95 per cent confidence intervals) of brucellosis in sheep in municipalities in Kosovo

and goats (7·24 [5·3 to 9·82]) (Table 2). No male cattle were serologically positive. The seroprevalence in female sheep was higher than in males (Table 3) but overall the seroprevalences in male and female sheep and goats were roughly similar (Tables 3, 4). The prevalence of holdings with positive cattle (0·95 per cent) was significantly lower than the prevalence of holdings with positive sheep (18·3 per cent) or goats (5·8 per cent).

The borders of the municipalities in Kosovo and the locations of villages with positive animals are shown in Fig 1.

Fig 2 shows the positive prevalences and their 95 per cent confidence intervals in sheep and goats in each municipality and Fig 3 shows similar data for cattle. The prevalence of villages with seropositive animals of any of the species was 25 per cent (19 to 32 per cent); the prevalences of villages with seropositive cattle, seropositive sheep, and seropositive goats were 16 per cent (11 to 22 per cent), 25 per cent (19 to 32 per cent), and 8 per cent (5 to 13 per cent), respectively.

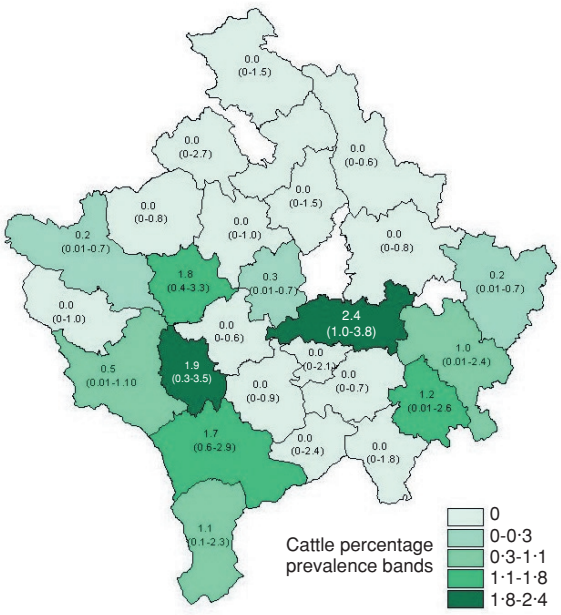

FIG 3: Map showing the estimated percentage prevalences (95 per cent confidence intervals) of brucellosis in cattle in municipalities in Kosovo

Seroprevalence, age and species of animals

The seroprevalences stratified by sex and age for sheep, goats and cattle are shown in Tables 3, 4 and 5, respectively. In the sheep and cattle the prevalences were remarkably similar in all age groups, with no statistically significant differences between them. Similarly, there were no statistically significant differences between the seroprevalences in five age classes of sheep and goats less than or more than two years old (Table 6).

Relationships between seroprevalences in cattle, sheep and goats

There was a poor correlation (r=0·35) between the seroprevalences in cattle, sheep and goats across municipalities, but cattle on farms which also had sheep or goats were more likely to be positive than cattle on holdings with cattle only (relative risk [RR]=4·6 [2·22 to 9·56]). Conversely, there was no evidence of an increased risk for sheep on farms that also had cattle compared with farms with sheep only.

Differences between the results of the RBT and the overall results of all the tests

The prevalences derived from the RBT and the RBT combined with the iELISA and cELISA were compared for each municipality and species. There were no statistically significant differences between the respective seroprevalences in the different municipalities that suggested that the results of the combined tests might have given more positive results than the RBT alone in any particular municipality.

Brucellosis in human beings

Human cases of brucellosis in 2000 were more likely to have occurred in municipalities which recorded test-positive animals in the survey (RR=2·6 [2·2 to 4·0]) than in municipalities where no positive animals were recorded. The distribution of villages where human cases were diagnosed in 2000 is shown in Fig 4.

TABLE 5: Overall results of the Rose Bengal test and ELISA brucellosis tests and prevalences of seropositive cattle stratified by sex and age for 155 villages in 26 municipalities in Kosovo

Sex	Result of tests	Number tested	Age of cattle (years) Unspecified	<1	1	2	3	4	5	6	7	8	9	≥10
Male	–ve	283	1	95	137	23	12	6	5	2			1	1
Female	–ve	7616	32	270	760	1085	1401	1399	988	657	468	143	285	128
Female	+ve	42			1	8	9	10	5	6		1	2	
Prevalence (% F)		0·5	0·0	0·0	0·1	0·7	0·6	0·7	0·5	0·9	0·0	0·7	0·7	0·0
Prevalence (% M+F)		0·5	0·0	0·0	0·1	0·7	0·6	0·7	0·5	0·9	0·0	0·7	0·7	0·0

M Male, F Female

Culture examinations

B melitensis biovar 3 was isolated from cow's milk on four occasions and from sheep blood on one occasion. *B abortus* biovar 2 was isolated from cow's milk on one occasion, and *B abortus* biovar 1 was isolated from sheep on two occasions; an unspecified strain of *B abortus* was isolated on one occasion from sheep blood (L. Pite, personal communication).

DISCUSSION

The objectives of the survey were to improve the understanding of the epidemiology of brucellosis in cattle, sheep and goats in Kosovo and to provide information for disease control in livestock and human beings. Seropositivity was considered to be due to natural infection because vaccination had never been practised in Kosovo. The results showed that brucellosis was endemic in sheep and goats and widely but unevenly distributed throughout Kosovo. Brucellosis in cattle was less widespread and occurred at much lower prevalences than in sheep and goats.

Reasonable estimates of prevalence were made possible through the use of animal census data collected during the vaccination programme, carried out after the 1999 conflict, by FAO and Kosovo veterinarians. Animal census data can be difficult and expensive to collect but the results presented here are an example of how census information can be collected at marginal cost under difficult conditions. The effectiveness of the survey was greatly enhanced by two factors, first the population-at-risk census data, and secondly the geographical information provided by the Prishtina Humanitarian Community Information Centre, administered by the United Nations Office for the Coordination of Humanitarian Affairs, which made it possible to produce descriptive statistics.

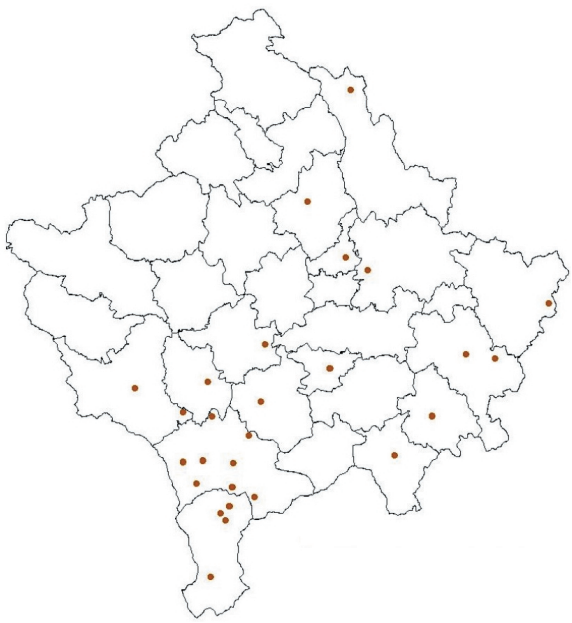

FIG 4: Locations of villages where cases of human brucellosis were diagnosed during 2000

Apparent prevalences are reported in the results but the true prevalences would have been higher (7·1, 7·8 and 0·62 per cent, respectively, for sheep, goats and cattle) given a sensitivity of about 85 per cent for the RBT (MacMillan 1997). ELISA testing of all the RBT-suspicious and positive sera ensured a very high specificity because the specificity of each ELISA was already high. The results of the further testing of the suspicious and positive RBT samples gives some guidance about the likely costs and benefits of using ELISAs on positive animals in the late stages of an eradication programme. Thus nine of 223 positive sheep, three of 37 positive goats and 12 of 54 positive cattle were classed as false positives by the RBT (Table 2). However, the gain in specificity is accompanied by a serious loss of sensitivity and the use of ELISA testing for disease control cannot be recommended in flocks where the disease status is endemic or unknown. The sensitivity of the RBT is similar to that of the ELISAs (MacMillan 1997) and it is to be preferred as a screening test, largely because it does not require expensive equipment and is cheaper in countries where labour costs are low.

The low prevalence detected in cattle was encouraging in terms of future disease control programmes. It suggests that brucellosis is transmitted only rarely from sheep and goats to cattle, or among cattle, under the type of cattle management practised in Kosovo. However, the higher risk for cattle on farms which also had sheep or goats suggests that some of the cattle infections may have originated from sheep. *B melitensis* biovar 3 was isolated from cow's milk on only four occasions, and *B abortus* biovar 2 was isolated from cow's milk only once.

The high prevalence of infected villages (25 per cent) is disturbing in a province where subsistence farming is common. Brucellosis is an occupational disease and its incidence in people is likely to increase in the absence of national controls of the disease in animals. Scalding of milk is a common household practice in rural Kosovo and provides good protection for milk and yoghurt, but the disease may be transmitted through contact with animals and via unpasteurised soft cheese made from the milk of sheep and goats. At the village level the mixing of flocks of animals for grazing and the

TABLE 6: Distribution of overall seroprevalences in sheep and goats less than two years old stratified into five age classes

Results of tests	Age of sheep and goats (months) ≤9	10-12	13-18	19-24	>25
Negative	176	230	167	929	2187
Positive	15	18	16	59	133
Prevalence % (95% CI)	7·8 (4·0-11·6)	7·3 (4·0-10·5)	8·7 (4·7-12·8)	6·0 (4·5-7·5)	5·7 (4·8-6·7)

CI Confidence interval

movement of breeding animals between flocks provide opportunities for the spread of the disease, and in the region as a whole the trading of livestock between communities would help to spread the disease.

Several species of brucella were isolated but the study was not designed to determine the extent to which different species were involved. However, the much higher prevalences in sheep and goats than in cattle strongly suggest that *B melitensis* infection predominates because they are its natural host and infection due to *B abortus* is rare in sheep and goats. The situation with regard to *Brucella ovis* is uncertain because no cultural evidence was available and veterinarians do not routinely palpate testicles for clinical evidence of the disease.

Brucellosis has a long history in people in the Balkans and Kosovo and *B melitensis* is suspected to be the most common cause. The disease occurs commonly in children and young adults (10 to 30 years old) and most patients come from rural areas. The disease tends to cluster within families and several family members are often clinically affected at about the same time. New cases occur most commonly during the summer months and peak in July. The extent to which the disease is under-reported is not known but is thought to be considerable. The disease is often severe and advanced before patients seek treatment and prolonged treatment is often necessary.

Records held at the Prishtina-based Institute of Public Health indicate that before 1985 less than 10 cases were reported annually; the trend for few reported cases continued between 1985 and 1989, with most of them occurring in the Prizren and Lipjan municipalities. In 1991, 241 cases were reported in the province and the annual incidence throughout the 1990s varied between three and five cases per 100,000 inhabitants.

It is uncertain why the disease in livestock is distributed unevenly throughout Kosovo, and further investigations, such as case-control studies at farm and village levels, are warranted to identify factors which may be responsible. Other investigations, such as the identification of strains of brucella and case studies of affected people would improve the understanding of transmission pathways and provide technical support for control programmes. Despite the importance of brucellosis for human and animal health and its widespread distribution throughout the Mediterranean countries, the Middle East and Asia, the epidemiology of the disease is still poorly understood and progress in its control has been generally disappointing. On a global scale, research efforts are often repetitive and tend to focus on the development of diagnostic tests and vaccines. In contrast, epidemiological studies of the transmission pathways and risk factors for the disease that could provide valuable guidance for integrated control programmes have been largely neglected.

The even distribution of prevalences in groups of sheep and goats of different ages was unexpected, because the prevalence of an infectious disease such as brucellosis would have been expected to increase with age. Brucella serological titres decline over time but repeated challenge in an infected flock should maintain titres in infected animals. A lack of precision in recording the ages of the sheep is one possible explanation because there was no audit of the accuracy of the age recording when the blood samples were collected. However, reasonable accuracy should have been possible for sheep up to the full-mouth stage. An alternative explanation is that farmers sell animals that abort, either for slaughter or as replacements elsewhere. The movement of such animals from municipalities with well established sheep industries to the central part of the province where the trading of livestock is common might explain the high prevalences encountered there.

The occurrence of the disease in people, and the distribution of the prevalences among livestock, appeared to be spatially related. A better understanding of the transmission pathways from animals to people, through the animal products, husbandry systems and human behaviour that affect the occurrence of the disease, could be exploited in integrated control programmes aimed at reducing the exposure of people to the disease. Its high prevalence, the ease with which it can be transmitted and the persistence of the infection all contribute to the seriousness of the problem of brucellosis. The movement of animals between herds and throughout the municipalities is uncontrolled and encourages the spread of the disease.

This study has provided useful information for future recommendations for the control of brucellosis. A vaccination programme for sheep and goats in all the municipalities of Kosovo has been agreed and its progress will be monitored continuously.

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