Small ruminant lentivirus and *Mycobacterium avium* subsp. *paratuberculosis*: co-infection prevalence and preliminary investigation on genetic resistance to both infections in a Garfagnina goat flock

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SUMMARY

Small ruminant lentiviruses (SRLVs) are a heterogeneous group of viruses of sheep, goats and wild ruminants, causing persistent infection and responsible of chronic degenerative disease of joints, lungs, udder and central nervous system in small ruminants. Mycobacterium avium subsp. paratuberculosis (MAP) is also a major production-limiting disease, which causes a chronic infection of ruminants. The disease causes persistent diarrhea, progressive weight loss, debilitation, anemia, and eventually death. Both SRLVs and MAP are widespread in small ruminants in many countries. The aim of this study was to estimate the prevalence of SRLVs and MAP co-infection in a Garfagnina goat flock consisting of 269 females and 20 males and located in Garfagnana district (Tuscany, Italy). All adult females were tested for MAP and SRLVs infection. Thirty-six out of 269 animals in total resulted positive for one or both infections. A total of 27 goats (10%) were positive to MAP and 21 goats (7.8%) were positive to SRLV. The apparent prevalence of co-infection was 4.5%, counting 12 goats positive for both infections. No significant association was found between subjects seropositive to SRLV and MAP. To investigate possible genetic influences on susceptibility or resistance of goats for both disease, all co-infected animals were compared with no infected animals (control group, 12 goats). Blood samples were collected and 12 STR markers (MAF65, SRCRSP5, INRA023, MCM527, CSRD247, SRCRSP23 OarFCB20, TGLA53, INRA005, INRA063, ETH10, ILSTS87) were investigated. For each marker, allele and genotypes frequencies between the two groups of animals were compared using the chi-square test and Fisher's exact tests. No statistical differences in STR alleles or genotypes frequency were observed between healthy animals and co-infected animals. Future works may include replication of this study with a larger number of animals to try to identify candidates genes for the genetic resistance to both infections.

KEY WORDS

Garfagnina goats, Paratuberculosis, small ruminant lentivirus, co-infection, STR, genetic resistance.

INTRODUCTION

Maedi/Visna Virus (MVV) and Caprine Arthritis Encephalitis Virus (CAEV) are members of the *Retrovidae* family, genus *Lentivirus*. Both viruses are unequivocal closely related lentiviruses and thus designated as Small Ruminant Lentiviruses (SRLV)[1]. The majority of SRLV isolates can be classified in 3 main genotypes: A and B including isolates originally isolate from sheep and goats, respectively; and genotype E firstly identified in certain Italian caprine isolates[2]. SRLVs in small ruminants are responsible of persistent infection causing for a slow and progressive clinical disease involving lungs, mammary glands, joints and in young kids' central nervous system. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the bacterial etiologic agent which causes a chronic progressive granulomatous enteritis known as Paratuberculosis or Johne's disease and it primarily affects wild and domestic ruminants[3]. Nowadays, Paratuberculosis represents not only an important animal disease but also a questioned human health problem, since MAP has been isolated from intestinal tissues of Crohn's disease patients, giving rise to a hypothesis of there being a possible link between these two diseases[4]. In addition, several reports of MAP isolation from sheep and goat cheeses both in Europe[5,6] and also in our country[7] have been published. Both SRLV and MAP are widespread in small ruminants in many countries[8,9,10,11,12].

Presently, for both disease, there are no effective vaccines neither treatments so an alternative strategy to reduce their incidence could be the selection of the most genetic resistant animals. Several attempts to locate loci associated with resistance to MAP and to SRLV have been made during the last 15 years[13,14,15,16]. In particular, on Garfagnina goats, association studies between STR markers and susceptibility/resistance to both SRLV and MAP infections have been made[17,18]. Moreover, attempt to identify genomic regions associated to the infection using a genome-wide scan[19,20] have been carried out.

The aims of this study were to estimate the prevalence of SRLV and MAP co-infection in a Garfagnina goat flock located in Garfagnana district (Tuscany, Italy), and to investigate the association between both infection-resistant or both infection-susceptible goats and STR markers.

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MATERIALS AND METHODS

The study was performed in a Garfagnina goat breed flock. Garfagnina is an Italian native goat population registered on the Tuscan regional repertory of genetic resources at risk of extinction, with about 745 animals belonging to 17 flocks. The origin of this population is still uncertain, even if it seems to derive from crossings between native goats from Alpine Arc and from the Tuscan-Emilian Apennines; local breeders refer that the population was reared for generations for its milk and meat production. The flock under study consisted of 269 females and 20 males. Age ranged from 2 to 9 years. All animals were recorded in the herdbook, but genealogical information was not available. The flock was located in the Garfagnana district (Media Valle del Serchio, Lucca, Italy) and a semi-extensive farming was practiced. The goats grazed during the morning (feed supplements are given mainly over the winter), and were housed overnight, when they received an integration of forage and feed. Flock management was of a family farm type. Milking was practiced twice a day using a trolley milking and the milk was conveyed in refrigerated tanks.

From 269 female goats, 5 mL of blood were collected into tubes (Vacutainer, BD Biosciences) without anticoagulant; the samples were transported, under refrigeration conditions, to the laboratories (Department of Veterinary Sciences, University of Pisa) and sera were separated from the clot by centrifugation at 2000 g for 10 min and frozen at −20 °C until performing serological analyses.

Serum samples were analyzed by ELISA ID screen® Paratuberculosis Indirect screening test (ID.VET, Montpellier, France) and the positive samples were subsequently tested with ELISA ID screen® Paratuberculosis Indirect confirmation test (ID.VET, Montpellier, France).

To evaluate the SRLV seroprevalence and characterize the SRLV antibody response sera samples were analyzed by a genotype specific ELISA test (Eradikit™ SRLV Genotyping kit, In3diagnostic, Torino, Italy). The genotyping ELISA plates were coated with a mix of gag and env peptides belonging to the three most divergent SRLV viral genotypes: genotype A, B, and E. The serum samples were examined according to the manufacturer's instructions. Specific genotype was assigned when the OD value of a single well was >40% when compared to the OD value of the same sera in other wells coated with different antigens. Sera were considered as SRLV positive when positive on at least one genotype.

Blood were collected from 48 goats (including all MAP and SRLV positive goats and twelve healthy goats as control groups). Blood samples were collected in Vacutainer tubes with K-EDTA as anticoagulant and stored at −20 °C until genomic DNA was extracted using Qiagen QIAamp DNA blood mini/midi kit (Qiagen, San Diego, CA, USA).

Twelve microsatellites were considered: MAF65, SRCRSP5, INRA023, MCM527, CSRD247, SRCRSP23 OarFCB20, TGLA53, INRA005, INRA063, ETH10, ILSTS87; some of these markers belonged to a panel validated by the International Society of Animal Genetics (ISAG) and others routinely were used by the facilities of the Laboratorio di Genetica e Servizi (Associazione Italiana Allevatori, Migliaro, Italy). The markers were located in 12 chromosomes and amplified in one multiplex PCR reactions[21].

The association for each single animal between MAP and SRLV serological results were verified by Chi-square test[22]. For each marker, the following parameters were computed using the Molkin v2.0 program[23]: number of alleles, effective allele size, observed heterozygosity and polymorphism information content (PIC). Allelic and genotype frequencies were estimated by direct counting. To investigate possible genetic influences on susceptibility or resistance of goats to both infections, for each marker, alleles and genotypes frequencies between co-infected animals and negative animals were compared using the Chi-square test and Fisher's exact tests.

RESULTS

Serological analysis revealed that 36 out of 269 animals were positive for one or both infections. Twenty-seven goats (10%) were positive to MAP and twenty-one goats (7.8%) were positive to SRLV. Genotype analysis for SRLV could classify 15 out of 21 seropositive sera into the 3 genotypes, in details four sera reacted specifically to genotype A antigens, five to genotype B and six to genotype E. Genotype characterization was not determined in six sera, although reactive to SRLV (Table 1).

Table 2 reports the number of positive animals for MAP and SRLV or both infections according to the animals' age. The greatest number of infected animals were aged between 5 and 7 years. The highest number of co-infected animals were aged between 5 and 6 years (50%).

Analysis of the goat MAP/SRLV data revealed no significant associations between the two infections (Table 3).

Detailed information of the used markers and the results of

Table 1 - Serological results.

Goat	OD vs A	OD vs B	OD vs E	Genotype
POS1	0.835	0.387	0.463	A
POS2	0.414	0.182	0.237	A
POS3	1.078	0.352	0.615	A
POS4	0.554	0.277	0.184	A
POS5	0.19	0.422	0.187	B
POS6	1.043	2.656	0.63	B
POS7	0.446	2.134	0.204	B
POS8	0.378	1.855	0.26	B
POS9	0.337	1.254	0.279	B
POS10	0.2	0.162	1.923	E
POS11	0.142	0.169	1.774	E
POS12	0.44	0.456	1.457	E
POS13	0.523	0.352	0.956	E
POS14	0.144	0.321	0.456	E
POS15	0.144	0.18	1.532	E
POS16	0.663	0.595	0.238	Ind
POS17	0.333	0.613	0.485	Ind
POS18	0.126	0.476	0.411	Ind
POS19	0.375	0.371	0.434	Ind
POS20	0.497	0.349	0.504	Ind
POS21	0.456	0.177	0.574	Ind

the microsatellite analysis in term of number of alleles observed, alleles size, PIC and observed heterozygosity of the total analyzed Garfagnina goat population were reported in a previous paper[21]. No statistical differences in STR alleles or genotypes frequency were observed between healthy animals and co-infected animals.

Since in previous papers SRCRSP05[18] and ETH10 markers[17,18] showed significant association with the infections in this paper we report the percentage of each of the most frequent alleles (>15%) for both SRCRSP05 and ETH10 markers in co-infected group (SRLV and MAP seropositive) and control group (SRLV and MAP Seronegative) (Table 4).

The co-infected group showed a higher frequency of allele 205bp of ETH10 marker (0.583 vs 0.458 in control group) and of allele 203 bp (0.250 vs. 0.125 in control group) and a less frequency of allele 207bp (0.167 vs 0.0.417 in control group). Consequently, the frequencies of the six genotypes of this marker were different in the two groups. Co-infected group presented a higher frequency of homozygous genotype 205205 of ETH10 marker (41.7% vs 16.7%) and a low frequency of heterozygous 205207 and heterozygous 207207 genotypes (8.30% vs 33.30% for the first genotype and 8.30% vs 25% for the second one).

Table 2 - Positive records according to the animals' age.

Age (years)	PosMAP	PosLentivirus	PosBoth	Tot
2	2	2	1	5
3	3	0	2	5
4	0	0	2	2
5	2	1	3	6
6	4	1	3	8
7	4	3	0	7
8-9	0	2	1	3
Tot	15	9	12	36

Table 3 - Representation of SRLV and MAP infection in goats.

	SRLV positive	SRLV negative	Total
MAP negative	9	233	242
MAP positive	12	15	27
Total	21	248	269

Table 4 - Percentage of each alleles for SRCRSP05 and ETH10 markers in Sero-Positive (SP) and Sero-Negative (SN) group.

Group	N°			
Marker		SRCRSP05		
		163	173	179
SN	12	0.375	0.375	0.167
SP	12	0.250	0.250	0.250
Marker		ETH10		
		203	205	207
SN	12	0.125	0.458	0.417
SP	12	0.250	0.583	0.167

Regarding SRCRSP05 marker six different alleles were observed but only three alleles had a frequency higher than 15%. Co-infected animals had a lower frequency of 163bp (0.25 vs. 0.375) allele and 173bp (0.25 vs. 0.375) allele and a higher frequency of allele 179 (0.25 vs. 0.167). For this reasons differences were observed for 163173 genotype with a frequency of 41.7% in control group vs 16.7% in co-infected group.

DISCUSSION

To our knowledge only a single research carried out by Stones et al.[24] discussed SRLV and MAP co-infection considering 28 Ontario goats flock (about 20 goats per flock) recording a prevalence of 14.3%. Our co-infection prevalence of 4.5% was lower than that reported by these Authors. Moreover, the lack of association between the two infections highlighted in this work seems to be in contrast with the results obtained by Stones et al.[24]. Nevertheless, these Authors observed a significant association only when considering the total data deriving from 558 goats irrespectively to flock origin, while the associations carried out in each single flock was not significant, given the small number of subjects.

Finally, despite the possible implication of the ETH10 marker in the resistance to the two infections, previously highlighted by the studies of association analysis between microsatellites and each infections[17,18], it is possible to suppose that there is no correlation between genetic resistance to the two infections, in consideration of the evidence that the candidate genes for genetic resistance to MAP[19] and to SRLV[20] carried out with SNPs, were different and located on different chromosomes.

CONCLUSIONS

The observed results were obtained from the analysis of animals from a single farm, indicating that few animals are co-infected and that there is not association between these two infections.

For this reason, our results must be supposed completely preliminary. Future works may include replication of this study involving different flocks to increase the number of observations to better understand the role of the ETH10 marker and to try to reveal and identify candidates genes implicated in genetic resistance to both infections.

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References

1. Zanoni R.G. (1998). Phylogenetic analysis of small ruminant lentiviruses. J Gen Virol, 79: 1951-1961.
2. Grego E., Bertolotti L., Quasso A., Profiti M., Lacerenza D., Muz D., Rosati S. (2007). Genetic characterization of small ruminant lentivirus in Italian mixed flocks: evidence for a novel genotype circulating in a local goat population. J Gen Virol, 88: 3423-3427.

3. OIE (2014). Paratuberculosis (Johne's disease). OIE Terrestrial Manual; Chapter 2.1.11.
4. Chiodini R.J., Chamberlin W.M., Sarosiek J., McCallum R.W. (2012) Crohn's disease and the mycobacterioses: a quarter century later. Causation or simple association? Crit Rev Microbiol, 38: 52-93.
5. Ikonomopoulos J., Pavlik I., Bartos M., Svastova P., Ayele W.Y., Roubal P., Lukas J., Cook N., Gazouli M. (2005). Detection of Mycobacterium avium subsp. paratuberculosis in retail cheeses from Greece and the Czech Republic. Appl Environ Microbiol, 71: 8934-8936.
6. Williams A.G., Withers S.E. (2010). Microbiological characterisation of artisanal farmhouse cheeses manufactured in Scotland. Int J Dairy Technol, 63: 356-369.
7. Galiero A., Fratini F., Mataragka A., Turchi B., Nuvoloni R., Ikonomopoulos J., Cerri D. (2016) Detection of Mycobacterium avium subsp. paratuberculosis in cheeses from small ruminants in Tuscany. Int J Food Microbiol, 217: 195-199.
8. Windsor P.A. (2015). Paratuberculosis in sheep and goats. Vet Microbiol, http://dx.doi.org/10.1016/j.vetmic.2015.07.019.
9. Angelidou E., Kostoulas P., Leontides L. (2014). Flock-level factors associated with the risk of Mycobacterium avium subsp. paratuberculosis (MAP) infection in Greek dairy goat flocks. Prev Vet Med, 117: 233-241.
10. Liapi M., Botsaris G., Slana I., Moravkova M., Babak V., Avraam M., Di Provvido A., Georgiadou S., Pavlik I. (2015). Mycobacterium avium subsp. paratuberculosis Sheep Strains Isolated from Cyprus Sheep and Goats. Transbound Emerg Dis 62: 223-227.
11. Galiero A., Turchi B., Pedonese F., Nuvoloni R., Cantile C., Colombani G., Forzan M., Cerri D., Fratini F. (2017). Serological, culture and molecular survey of Mycobacterium avium paratuberculosis in a goat flock in Tuscany. Folia Microbiol, 62: 6, 471-477.
12. Minguijón E., Reina R., Pérez M., Polledo L., Villoria M., Ramírez H., Leginagoikoa I., Badiola J.J., García-Marín J.F., de Andrés D., Luján L., Amorena B., Juste RA. (2015). Small ruminant lentivirus infections and diseases. Vet Microbiol, 181:75-89.
13. Zare Y., Shook G.E., Collins M.T., Kirkpatrick B.W. (2014). Genome-wide association analysis and genomic prediction of Mycobacterium avium subspecies paratuberculosis infection in US Jersey cattle. PLoS ONE, 9, e88380.
14. Moioli B., D'Andrea S., De Grossi L.C., Sezzi E., De Sanctis B., Catillo G., Steri R., Valentini A., Pilla F. (2016). Genomic scan for identifying candidate genes for paratuberculosis resistance in sheep. Animl Prod Sci, 56: 1046-1055.
15. Clawson M.L., Redden R., Schuller G., Heaton M.P., Workman A., Chitko-McKown C.G., Smith T.P., Leymaster K.A. (2015). Genetic subgroup of small ruminant lentiviruses that infects sheep homozygous for TMEM154 frameshift deletion mutation A4 53. Vet Res, 46: 22.
16. Wang W., Ye C., Di Zhang J.L., Kimata J.T., Zhou P. (2014). CCR5 Gene Disruption via Lentiviral Vectors Expressing Cas9 and Single Guided RNA Renders Cells Resistant to HIV-1 Infection. PLOS Published: December 26, https://doi.org/10.1371/journal.pone.0115987.
17. Cecchi F., Russo C., Fratini F., Turchi B., Preziuso G., Cantile C.(2018). Preliminary association analysis of microsatellites and Mycobacterium avium subspecies paratuberculosis infection in the native Garfagnina goats. J Applied Anim Res, 46: 879-882.
18. Cecchi F., Fratini F., Bandecchi P., Cantile C., Mazzei M. (2018). Investigation on goat lentiviral infections and preliminary association analysis with microsatellites in the native Garfagnina goats breed. Rend Lincei Sci Fis Nat, 29: 885-889.
19. Cecchi F., Russo C., Iamartino D., Galiero A., Turchi B., Fratini F., Degl'Innocenti S., Mazza R., Biffani S., Preziuso G., Cantile C.(2017). Identification of candidate genes for paratuberculosis resistance in the native Italian Garfagnina goat breed. Trop anim health and prod, 49: 1135-1142.
20. Cecchi F., Dadousis C., Bozzi R., Fratini F., Russo C., Bandecchi P., Cantile C., Mazzei M. (2019). Genome scan for the possibility of identifying candidate resistance genes for goat lentiviral infections in the Italian Garfagnina goat breed. Trop Anim Health and Prod, 51: 729-733.
21. Cecchi F., Russo C., Cantile C., Preziuso G. (2017). Preliminary genetic variability analysis of the native Garfagnina goats based on microsatellite polymorphism. Large Animal Review 23: 191-193.
22. SAS, JMP.User's Guide, (2007). ver. 7.0 SAS Inst. Cary, NC, U.S.A.
23. Gutièrrez J.P., Royo L.J., Alvarez I., Goyache F.(2005). MolKin v2.0:a computer program for genetic analysis of populations using molecular coancestry information. J Hered, 96: 718-721.
24. Stonos N., Bauman C., Menzies P., Wooton S.K., Karrow N.A. (2017). Prevalence of small ruminant Lentivirus and Mycobacterium avium subsp. Paratuberculosis co-infection in Ontario dairy sheep and dairy goats. Can J Vet Res, 81: 155-159.