ANALYSING ASSOCIATION OF *TMEM154*, *CCR5* AND *ZNF389* VARIANTS WITH MAEDI-VISNA RESISTANCE IN GERMAN SHEEPS

SHORT TITLE: TMEM154-35K MARKER CAN PREDICTS MAEDI-VISNA RESISTANCE

MARWA A. ELTANANY

Department of Animal Wealth Development,

Faculty of Veterinary Medicine, Benha University, Egypt.

Ovine lentivirus (OvLV) belongs to the family *Retroviridae* and causes maedi-visna (MV) in sheep. The virus is widespread in sheep populations around the world. No European country except Iceland can be considered to be free of OvLV infection (Peterhans *et al*., 2004). This disease is also substantially prevalent among sheep flocks in Germany (Hüttner *et al*., 2010). There is no cure for the chronic disease caused by OvLV, which includes symptoms such as pneumonia, wasting, mastitis, arthritis and progressive paralysis (Leroux *et al*., 2010). A vaccine preventing OvLV infection has not been developed yet (Blacklaws, 2012). Production losses stem from lamb mortality (Arsenault et al. 2003), lower lamb weights and milk production from older infected ewes (Keen *et al.* 1997, Arsenault *et al,*. 2003), early culling (Peterhans *et al*., 2004) and export restrictions (Reina *et al*., 2009). The significant economic losses have led to the development of control programs in Europe and elsewhere which commonly include separation of lambs from dams at birth to prevent virus transmission and test/cull methods. Although they can be successful (Peterhans et al. 2004), these methods are neither cost-effective (Houwers, 1990) nor sustainable, as OvLV-free herds are still susceptible to infection if exposed to other infected sheep or goats (Gjerset *et al*., 2009).

Evidence of the genetic potentiality of sheep to resist OvLV infection which differs between and within breeds (Straub 2004) may develop selective breeding programs for lower OvLV infection susceptibility. The host genetic susceptibility to OvLV varies between breeds in both odds of infection and control of virus once infected (Blacklaws, 2012). Variants in the genes: ovine transmembrane protein (*TMEM154*), chemokine (C-C motif) receptor 5 (*CCR5*) and zinc finger protein (*ZNF389*) have been reported to be associated with the serological status and/or the provirus concentration of ovine lentivirus (OvLV)

Egypt. J. Genet. Cytol., 48:271-294, July, 2019

Web Site (*www.esg.net.eg*)

and can be recommended for marker-assisted selection in U.S. sheep (White *et al*., 2009, Heaton *et al*., 2012, White *et al*., 2013).

It was suggested that *TMEM154* protein can function as a receptor for some strains and/or as a co-receptor for many or all SRLV (White and Knowles, 2013). Based on whole genome association analysis, *TMEM154* was considered as a major MV susceptibility gene on chromosome 17 providing an opportunity to breed MV resistant sheep (Heaton *et al*., 2012). Heaton *et al*., (2013) indicated that most sheep populations around the world have highly-susceptible forms of *TMEM154* (Haplotypes 2, 3). The ancestral *TMEM154* haplotype (designated Haplotype 3) encodes glutamate (E) at position 35 and asparagine (N) at position 70. Haplotype 2 has an isoleucine (I) mutation at position 70 while haplotype 1 has a lysine (K) mutation at position 35. Recently, association of *TMEM154* E/K allelic frequencies with SRLV susceptibility were assessed in some German, Turkish and Iranian sheep flocks (Molaee *et al*., 2018; Molaee *et al*., 2019; Yaman *et al*., 2019).

Larruskain and Jugo, (2013) reviewd that *CCR5* plays a role in leukocyte induction against different pathogens, including ovine lentiviruses and a deletion in this locus showes association with a decreased provirus-burden by reducing leukocyte influx to site of infection. White *et al*., (2009) sequenced the whole ovine *CCR5* gene and determined a 4-base deletion within a binding site for octamer transcription factors in the promoter region. Additionally they found the association of this *CCR5* promoter deletion with proviral MV levels in U.S. sheep. However, *CCR5* deletion allele frequencies did not show a consistent association with serological MV status studied in more than 500 sheep from 17 serologically MV positive German sheep flocks with different breed backgrounds (Molaee *et al*., 2018).

The Zinc finger proteins (*ZNF*) function to regulate gene transcription because of their nucleic acid-binding domains. Jauregui *et al*., (2012) supposed that one or more of the zinc finger genes locating on ovine chromosome 20 may play a transcriptional regulation role for host genes that restrict directly proviral replication of OvLV, such as *TRIM5a*. The genome-wide association approach (GWAS) determined diverse genomic regions associated with proviral concentration including four zinc finger genes on ovine chromosome 20, *ZNF165*, *ZSCAN16*, *ZNF192* and *ZNF389* in one U.S. Rambouillet flock (White *et al*., 2012). Thereafter, White *et al*., (2013) detected a small insertion/deletion variant in the 5′ genomic region of *ZNF389* which showed consistent association with proviral concentration in three animal sets ($P < 0.05$) composed of Rambouillet, Polypay and crossbred sheep from multiple locations and management conditions. Thus the deletion variant in *ZNF389* might have predictive value for OvLV proviral concentration under a range of conditions including breeds, locations and virus strains and selective programs. Therefore, this variant could be useful for marker-assisted selection to reduce proviral concentration and consequently serve in reducing viral transmission.

Hence, the detection of a genetic marker in a population or country should not be generalised to the others, the main aim of current work was to test if the three candidate gene variants (*TMEM154* K, *CCR5* promoter deletion and *ZNF389* insertion) could be valid markers for selection against MV susceptibility in German sheep populations. This association analysis was fulfilled on two different levels: in accordance with the known OvLV susceptibility level in six German sheep breeds and with MV antibody titer in MV-positive German sheep flocks as well.

MATERIALS AND METHODS

ANIMALS AND EXPERIMENTAL DESIGN

Overall, 306 whole blood samples were collected in 9 ml EDTA monovettes. The animals sampled in this study varied according to the exprimental design and the purpose. Thereby, the used animals were classified into set 1 and set 2. Animals of set 1 selected to determine association of the three markers with breed susceptibility, were unrelated regardless of gender and age. A total of 192 samples (32 samples each breed) represented six German sheep breeds with known OvLV susceptibility level (Schaller *et al*., 2000; Straub, 2004; Scherf, 2010). These breeds were German Grey Heath (GGH), Merinoland (ML) and Suffolk (Su) known to be MV less susceptible and Cameroon (Cam), East Friesian Milk (EFM) and Texel (Tex) known to be MV highly susceptible.

Animals of set 2 were selected to assess association of the candidate genes markers with OvLV serological status in German sheep flocks. Totally, 114 blood samples (57 each of negative and positive OvLV-seroprevalence groups) were isolated from naturally infected, nine sheep flocks scattered in Germany and composed mainly from purebred or crossed Texel ewes of at least four years old. For identification of MV negative or positive ewes, plasma samples were separated and serologically tested using an enzyme-linked immunosorbent assay (ELISA) (IDEXX CAEV-MVV Total Ab ELISA, IDEXX GmbH, Ludwigsburg, Germany). Considering ELISA kit, the MV negative samples should have cut-off values equal or lower than 110 % however the MV positive samples have to give estimates of equal or more than 120%. The samples which had values between 110% and 120% were excluded.

GENOTYPING VARIANTS OF CANDIDATE GENES

The genomic DNA was manually extracted from blood samples remained after plasma separation by a modifed salting out method according to Montgomery & Sise, (1990).The two sample sets were genotyped for three sequence variants in regions of candidate genes for MV disease susceptibility.

The four base-pair deletion variant ("aatg") in the *CCR5* promoter (Ovis aries chromosome 19, Oar_v4.0, NC_019476.2:g.52961717_52961714delAATG, minus strand) and the two base-pair deletion variant in the 5′ genomic region of *ZNF389* (Ovis aries chromosome 20, NC_019477.1:g.29500068_29500069delAT, NCBI dbSNP ss748775100) were determined by fragment length analysis. Polymerase chain reactions (PCR) amplifcation was done in a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, München, Germany. Primer sequences and PCR product sizes are given in Table 1. PCR reaction mixes and conditions were performed according to White et al. (2009) and White et al. (2014). Fragment-length analysis of denaturated PCR products was performed by using ABI 3130 automated sequencer and the software GeneMapper version 4.0 as recommended by the manufacturer (Applied Biosystems, Darmstadt, Germany).

The single nucleotide substitution (SNP) in exon 2 of *TMEM154* (Ovis aries chromosome 17, Oar_v4.0, NC_019474.2:g.4860407G>A) causing the substitution of the ancestral glutamic acid (E) with lysine (K) at position 35 of the mature protein was genotyped by allele-specific PCR using KASP technology (LGC, Hoddesdon, UK). The sequences of the two reverse allele-specifc primers and the common forward primer and PCR product size are demonstrated in Table (1). PCR reaction mixes and conditions were carried out according to Molaee *et al*. (2018).

STATISTICAL ANALYSIS

For each candidate locus, the protective and risk allele and genotype frequencies within each breed and each group representing MV status (either positive or negative) and mean frequency in each set were assessed using POPGENE software version 1.3 (http://www.ualberta.ca/~fyeh/popgene.html). Hence the risk allele pro locus (*TMEM154* E, *CCR5* promotor wild type, *ZNF389* deletion) so far has the dominate effect (Molaee *et al*., 2018), the frequencies of risk genotypes including one or two copies were combined. The Fisher's exact test for Hardy-Weinberg Equilibrium (HWE) to evaluate independent segregation of alleles within each breed was additionally implemented. Significant differences (*P* values < 0.05) of mean allele and genotype frequencies between groups of low and high MV breed-susceptibility (sample set 1) as well as between MV-positive and -negative sheep (sample set 2) were measured by chi-square/Fisher's exact test executed in SPSS program (version 23.0) for Windows (IBM SPSS Statistics, Armonk, NY: IBM Corp).

RESULTS AND DISCUSSION

Fundamentally, the present study targeted to investigate the association of sequence variants of three candidate genes for MV susceptibility (*TMEM154*, *CCR5* promotor and *ZNF389*) at two different levels including firstly the known MV German sheep breed-susceptibility level and secondly the MV seroprevalence status in German sheep flocks.

For the first association analysis, the breeds were selected in accordance to their known MV susceptibility based on both seroconversion rates and number of sheep showing MV clinical signs surveyed in previous studies (Schaller *et al.*, 2000, Straub, 2004; Scherf, 2010). Nevertheless, for the second association analysis, the nine sheep flocks were selected to be naturally MV-infected and ewes sampled were serologically tested for MV-infection using MV-ELISA kit which is approved for determination of SRLV infection in sheep and goats by the German licensing authority (German Friedrich-Loeffer-Institut, FLI). All genotyped MV-seropositive sheep showed cut-off values ranged from 201.73% - 313.36%, while all genotyped MV-seronegative sheep had cut-off values in a rate of -6.34% - 57.18%.

Regarding the first analysis, the mean frequencies of protective *TMEM154* 35 K and risk *TMEM154* 35E alleles in all genotyped samples of MV-less suceptible breeds were 75.4% and 24.6%, respectively and of MV-high suceptible breeds were 21.1% and 78.9%, respectively (Table 2). The mean frequencies of homozygous protective genotype (KK) and hemi- and homozygous risk genotypes (KE & EE) in MV-less suceptible breeds were 62.6% and 37.4%, respectively and in MV-high suceptible breeds were 5.2% and 94.8%, respectively (Table 2). The differences of *TMEM154* protective and risk allele and genotype frequencies between the two groups of breed-susceptibility were highly significant *(P* value < 0.000*)* showing strong association of such a marker with the knowen breed-susceptibility level (Table 2). Observably, there was no devaition from HWE at this locus in all studied breeds indicating no intended selection may be practiced toward resistant genotype of that locus. Interestingly, the frequency of *TMEM154* 35K allele in Suffolk (44 %) was lower than expected from the published frequency (69 %) in U.S. Suffolks (Heaton *et al*., 2012) and the known lower OvLV susceptibility of this breed. This may be compensated by the highest frequency for K at position 35 (98 %) in GGH which had no EE genotype. The found low proportion of KK genotype (16.1%) in studied German Suffolk may be attributed to viral risk factors such as the prevalent SRLV serogroups (Sider *et al*., 2013). Up to date, the data on virus subgroups circulating in the German sheep population is hardly available. Further contribution could be the conditional properity of host genetic resistance overcome by some circumstances like exposure to high viral dose, repeated viral exposures, viral genetic adaptation to host defenses, multiple routes of infection, other breed-specifc genetic factors, managmental impacts (e.g. overcrowding) and coinfection with another disease at the same time (*Heaton et al*., 2012).

Considering the second association analysis at *TMEM154* E35K locus, the protective allele (K) and genotype (KK) frequencies in genotyped MV-seropositive sheep were 25.5% and 5.5% whereas, in genotyped MV-seronegative sheep were 63.4% and 46.4%, respectively (Table 3). Hereby, the detected highly significant differences (*P* vlaue < 0.000) of both protective and risk allele and genotype frequencies between MV-seropositive and -seronegative sheep can point to strong association of the *TMEM154* E35K locus with MVV

seroprevalence status in the studied 9 German sheep flocks of Texel pure- and cross-breed background. These results are in consistence with results of previous study on 17 MV positive German sheep flocks of different breed background (Molaee *et al.*, 2018).

In regard to the first analysis, the results given by the protective *CCR5* promotor four-fold deletion varaint (Table 4) were in consistence with the known breed differences in OvLV susceptibility. However, the protective *CCR5* promotor deletion allele and homozygous genotype were seen in low frequencies in all samples of set 1 (n = 192). The mean allele frequency of *CCR5* deletion in MV-less suceptible breeds was 14.9% and in MV-high suceptible breeds was 5.8%. The mean genotype frequency of homozygous *CCR5* deletion in MV-less suceptible breeds was 4.4% and in MV-high suceptible breeds was 0.0%. Moreover, mean frequencies of the protective *CCR5* promotor deletion allele and genotype varied significantly between groups of sheep breeds with low and high susceptibility to MV disease ($P < 0.05$) refering to a consistent association with the breed-susceptibility level. Supposing random allele segregation at *CCR5* locus, there was no deviation from HWE found at this locus. Interestingly, the frequency of the *CCR5* deletion in the less susceptible breed German Grey Heath (7 %) was in a similar range with the highly MV disease susceptible breeds East Friesian Milk and Texel. However, the highest frequency for the *CCR5* deletion (22 %) was seen in Suffolk.

In the second analysis, the deletion in the *CCR5* promotor did not exhibit an expected protection and consequently no consistent association with serological MV status (Table 5). However, the allele frequency of the protective *CCR5* deletion was more in MV-seronegative sheep (20.2%) than in MV-seropositive sheep (15.2%) but did not varied significantly ($P > 0.05$). This result was consistent with the result of Molaee *et al.*, (2018). Inconsistently, White *et al.*, (2009) and White *et al.*, (2014) observed that deletion homozygotes in promotor of *CCR5* displayed significant associations with OvLV provirus load in naturally infected U.S. Rambouillet, Polypay and Columbia sheep. The current result could be mainly contributed to the very low number of sheep carrying homozygous genotype of the CCR5 promoter deletion (only 4 out of 114 genotyped sheep of set 2). In addition, the known mode of action of the four-fold deletion variant in CCR5 promotor is to reduce influx of monocytes/macrophages, the main target cells of MVV, to the site of infection which could decrease the cellular infection and consequently diminish the proviral concentration (Larruskain and Jugo, 2013). Probably, this activity can act indirectly on serological MV status and affected by other unknown causal host genes or internal metabolic pathways that can vary between breeds and populations.

In both association analyses of this study, it was observed that the putatively protective wild type of *ZNF389* locus (insertion) had no assocaiton neither with the known MV breed-susceptibility level nor with MV seroprevalence status in studied German sheep populations (Tables 6 &7). However, White *et al.*, (2013) concluded that the small inser-

tion/deletion variants in *ZNF389* gene showed consistent association with proviral concentration in three animal sets of multiple origins ($P < 0.05$). Hence, the *ZNF389* may act alone or together with other zinc finger genes directly or indirectly via transcriptional regulation of other genes in restricting proviral replication (Jauregui *et al.*, 2012), the potentiality of this regulatory activity could differ obviously among various sheep populations in different countries and circumstances. In general, it is reasonable to found different susceptibility phenotypes as antibody response and provirus load to be controlled in part or completely by different host genes. However, it is precious to mention that the *TMEM154* E35K displayed strong association with known MV breed-susceptibility level, the MV serological status as well as the MVV proviral load in sheep as noticed in present study and study of Heaton *et al.*, (2012). A more suggestion that, the diagnostic power of a MV ELISA can be limited by the virus subgroup(s) present in a flock giving false results if other subgroups are circulating.

Altogether, the amino acid substitution E35K in exon 2 of TMEM154 currently is the most promising marker for selection towards OvLV resistance in German sheep populations. However, these results will be validated in a larger number of breeds and samples, also considering other susceptibility parameters (e. g. provirus load) and different virus phylogroups. Additionally, the K35 allele will have to be tested for possible correlations with other important production and functional traits.

SUMMARY

Maedi-visna disease considerably spreads among German sheep flocks. Variants in or near the genes *TMEM154, CCR5*, and *ZNF389* have been reported to be associated with the serological status or the provirus concentration of ovine lentivirus (OvLV) in U.S. sheep. The first goal of present study was to assess these markers for association with SRLV susceptibility in German sheep breeds. The second goal was to evaluate associations of those markers with MV antibody titer in MV-positive German sheep flocks. A total of 306 DNA samples were divided in two sets according to the purpose in this study. In the set 1 of less and highly susceptible sheep breeds, mean frequencies of putatively protective alleles were 75.4 and 21.1 % (*TMEM154* 35K, $P < 0.000$), 14.9 and 5.8 % (*CCR5* deletion, $P < 0.05$) and 46.3 and 54,7 % (*ZNF389* insertion, $P > 0.05$), respectively. In the set 2 of serologically (ELISA) MV-positive and -negative sheep, mean frequencies of putatively protective alleles were 25.5 and 63.4 % (*TMEM154* 35K, $P < 0.000$), 15.2 and 20.2 % (*CCR5* deletion, $P > 0.05$), and 53.6 and 49.1 % (*ZNF389* insertion, $P > 0.05$), respectively. According to these results, *TMEM154* (E35K) seems to be a more promising marker for selection against MV susceptibility in the German sheep population. These findings warrant further study.

ACKNOWLEDGMENTS

The author acknowledged so much for Professor Dr. Gesine Luehken, the direcktor

of Pathogenetic Department, Institute for Animal Breeding and Genetics, Justus-Liebig Unive

REFERENCES

Arsenault, J., Dubreuil, P., Girard, C., Simard, C. & Belanger, D. (2003). Maedi-visna impact on productivity in Quebec sheep flocks (Canada). *Prev Vet Med.,* 59: 125-137.

Blacklaws, B. A. (2012). Small ruminant lentiviruses: immunopathogenesis of visna-maedi and caprine arthritis and encephalitis virus. *Comp Immunol Microbiol Infect Dis,* 35. 259-269.

Gjerset, B., Rimstad, E., Teige, J., Soetaert, K. & Jonassen, C. M. (2009). Impact of natural sheep-goat transmission on detection and control of small ruminant lentivirus group C infections. *Vet Microbiol.,* 135. 231-238.

Heaton, M. P., Clawson, M. L., Chitko-Mckown, C. G., Leymaster, K. A., Smith, T. P., Harhay, G. P., White, S. N., Herrmann-Hoesing, L. M., Mousel, M. R., Lewis, G. S., Kalbfleisch, T. S., Keen, J. E. & Laegreid, W. W. (2012). Reduced lentivirus susceptibility in sheep with TMEM154 mutations. *PLoS Genet.,* 8. e1002467.

Heaton M.P., Kalbflfleisch T.S., Petrik D.T., Simpson B., Kijas J.W., Clawson M.L., Chitko-McKown C.G., Harhay G.P. & Leymaster K.A. (2013). Genetic testing for TMEM154 mutations associated with lentivirus susceptibility in sheep. *PLoS ONE* **8**: 55490

Houwers, D. J. (1990). Economic importance, epidemiology and control. Maedi-visna and related diseases. G. Petursson and R. Hoff-Jorgensen. Boston, MA., Kluwer Academic Press. 83-117.

Hüttner, K., Seelmann, M. & Feldhusen, F. (2010). Prevalence and risk factors for Maedi-Visna in sheep farms in Mecklenburg-Western-Pomerania. *Berl Münch Tierarztl Wochenschr* 123: 463-467.

Jauregui P., Crespo H., Glaria I., Lujan L., Contreras A., Rosati S., de Andres D., Amorena B., Towers G.J. & Reina R. (2012): Ovine TRIM5alpha can restrict visna/maedi virus. *Journal of Virology,* 86. 9504-9.

Keen, J. E., Hungerford, L. L., Littledike, E. T., Wittum, T. E. & Kwang, J. (1997). Effect of ewe ovine lentivirus infection on ewe and lamb productivity. *Prev Vet Med.,* 30: 155-169.

Larruskain Amaia and Jugo M. Begoña (2013). Retroviral Infections in Sheep and Goats: Small Ruminant Lentiviruses and Host Interaction. *Viruses.,* 5: 2043-2061.

Leroux, C., Cruz, J. C. & Mornex, J. F. (2010). SRLVs: a genetic continuum of lentiviral species in sheep and goats with cumulative evidence of cross species transmission. *Curr HIV Res.,* 8: 94-100.

Molaee Vahid, Eltanany Marwa & Luehken Gesine (2018). First survey on association of TMEM154 and CCR5 variants with serological maedi-visna status of sheep in German focks.*Veterinary Research.,* 49: 36-46.

Molaee Vahid , Otarod Vahid , Abdollahi Darab & Luehken Gesine (2019): Lentivirus Susceptibility in Iranian and German Sheep Assessed by Determination of TMEM154 E35K. *Animals.,* 9. 685.

Montgomery G.W. and Sise J.A. (1990): Extraction of DNA from sheep white blood-cells. *N Z J Agric Res.,* 33: 437-441.

Peterhans, E., Greenland, T., Badiola, J., Harkiss, G., Bertoni, G., Amorena, B., Eliaszewicz, M., Juste, R. A., Krassnig, R., Lafont, J. P., Lenihan, P., Petursson, G., Pritchard, G., Thorley, J., Vitu, C., Mornex, J. F. & Pepin, M. (2004). Routes of transmission and consequences of small ruminant lentiviruses (SRLVs) infection and eradication schemes. *Vet Res.,* 35: 257-274.

Reina, R., Berriatua, E., Lujan, L., Juste, R., Sanchez, A., de Andres, D. & Amorena, B. (2009). Prevention strategies against small ruminant lentiviruses: an update. *Vet J.,* 182: 31-37.

Schaller P, Vogt HR, Strasser M, Nettleton PF, Peterhans E & Zanoni R (2000). Seroprevalence of maedi-visna and border disease in Switzerland. *Schweiz Arch Tierheilkd.,* 142:145-153 (in German).

Scherf Claudia (2010). Untersuchungen zum Einfluß einer Maedi-Infektion auf die Funktion und die Morphologie der Lungen verschiedener Schafrassen. PhD thesis, University of Veterinary Medicine Hannover, Germany.

Straub, O. C. (2004). Maedi-Visna virus infection in sheep. History and present knowledge. *Comp Immunol Microbiol Infect Dis.,* 27: 1-5.

White, S. N., Mousel, M. R., Reynolds, J. O., Lewis, G. S. & Herrmann-Hoesing, L. M. (2009). Common promoter deletion is associated with 3.9-fold differential transcription of ovine CCR5 and reduced proviral level of ovine progressive pneumonia virus. *Anim Genet.,* 40: 583-589.

White S. N. & Knowles D. P. (2013). Expanding Possibilities for Intervention against Small Ruminant Lentiviruses through Genetic Marker-Assisted Selective Breeding. *Viruses.,* 5:

1466-1499.

White, S. N., Mousel, M. R., Reynolds, J. O., Herrmann-Hoesing, L. M. & Knowles, D. P. (2013). Deletion variant near ZNF389 is associated with control of ovine lentivirus in multiple sheep flocks. *Anim Genet.,* 45: 297-300.

Yaman Y., Keles M., Aymaz R., Sevim S., Sezenler T., Önaldi A.T., Kaptan C., Ba¸skurt A., Koncagül S., Öner Y., *et al*. (2019). Association of TMEM154 variants with visna/maedi virus infection in Turkish sheep. *Small Rumin. Res.,* 177: 61-67.

Arsenault, J., Dubreuil, P., Girard, C., Simard, C. & Belanger, D. (2003): Maedi-visna impact on productivity in Quebec sheep flocks (Canada). *Prev Vet Med* **59**(3): 125-137.

Blacklaws, B. A. (2012): Small ruminant lentiviruses: immunopathogenesis of visna-maedi and caprine arthritis and encephalitis virus. *Comp Immunol Microbiol Infect Dis* **35**(3): 259-269.

Gjerset, B., Rimstad, E., Teige, J., Soetaert, K. & Jonassen, C. M. (2009): Impact of natural sheep-goat transmission on detection and control of small ruminant lentivirus group C infections. *Vet Microbiol* **135**(3-4): 231-238.

Heaton, M. P., Clawson, M. L., Chitko-Mckown, C. G., Leymaster, K. A., Smith, T. P., Harhay, G. P., White, S. N., Herrmann-Hoesing, L. M., Mousel, M. R., Lewis, G. S., Kalbfleisch, T. S., Keen, J. E. & Laegreid, W. W. (2012): Reduced lentivirus susceptibility in sheep with TMEM154 mutations. *PLoS Genet* **8**(1): e1002467.

Heaton M.P., Kalbflfleisch T.S., Petrik D.T., Simpson B., Kijas J.W., Clawson M.L., Chitko-McKown C.G., Harhay G.P. & Leymaster K.A. (2013): Genetic testing for TMEM154 mutations associated with lentivirus susceptibility in sheep. *PLoS ONE* **8**: 55490

Houwers, D. J. (1990): Economic importance, epidemiology and control. Maedi-visna and related diseases. G. Petursson and R. Hoff-Jorgensen. Boston, MA., Kluwer Academic Press**:** 83-117.

Hüttner, K., Seelmann, M. & Feldhusen, F. (2010): Prevalence and risk factors for Maedi-Visna in sheep farms in Mecklenburg-Western-Pomerania. *Berl Münch Tierarztl Wochenschr* **123**(11-12): 463-467.

Jauregui P., Crespo H., Glaria I., Lujan L., Contreras A., Rosati S., de Andres D., Amorena B., Towers G.J. & Reina R. (2012): Ovine TRIM5alpha can restrict visna/maedi virus. *Journal of Virology* **86**: 9504–9.

Keen, J. E., Hungerford, L. L., Littledike, E. T., Wittum, T. E. & Kwang, J. (1997): Effect of ewe ovine lentivirus infection on ewe and lamb productivity. *Prev Vet Med* **30**(2): 155-169.

Larruskain Amaia & Jugo M. Begoña (2013): Retroviral Infections in Sheep and Goats: Small Ruminant Lentiviruses and Host Interaction. *Viruses* **5**: 2043-2061.

Leroux, C., Cruz, J. C. & Mornex, J. F. (2010): SRLVs: a genetic continuum of lentiviral species in sheep and goats with cumulative evidence of cross species transmission. *Curr HIV Res* **8**(1): 94-100.

Molaee Vahid, Eltanany Marwa & Luehken Gesine (2018): First survey on association of TMEM154 and CCR5 variants with serological maedi-visna status of sheep in German focks.*Veterinary Research* **49**: 36-46.

Molaee Vahid , Otarod Vahid , Abdollahi Darab & Luehken Gesine (2019): Lentivirus Susceptibility in Iranian and German Sheep Assessed by Determination of TMEM154 E35K. *Animals* **9**: 685.

Montgomery G.W. & Sise J.A. (1990): Extraction of DNA from sheep white blood-cells. *N Z J Agric Res* **33**: 437–441.

Peterhans, E., Greenland, T., Badiola, J., Harkiss, G., Bertoni, G., Amorena, B., Eliaszewicz, M., Juste, R. A., Krassnig, R., Lafont, J. P., Lenihan, P., Petursson, G., Pritchard, G., Thorley, J., Vitu, C., Mornex, J. F. & Pepin, M. (2004): Routes of transmission and consequences of small ruminant lentiviruses (SRLVs) infection and eradication schemes. *Vet Res* **35**(3): 257-274.

Reina, R., Berriatua, E., Lujan, L., Juste, R., Sanchez, A., de Andres, D. & Amorena, B. (2009): Prevention strategies against small ruminant lentiviruses: an update. *Vet J* **182**(1): 31-37.

Schaller P, Vogt HR, Strasser M, Nettleton PF, Peterhans E & Zanoni R (2000): Seroprevalence of maedi-visna and border disease in Switzerland. *Schweiz Arch Tierheilkd* **142**:145–153 (in German).

Scherf Claudia (2010): Untersuchungen zum Einfluß einer Maedi-Infektion auf die Funktion und die Morphologie der Lungen verschiedener Schafrassen. PhD thesis, University of Veterinary Medicine Hannover, Germany.

Straub, O. C. (2004): Maedi-Visna virus infection in sheep. History and present knowledge. *Comp Immunol Microbiol Infect Dis* **27**(1): 1-5.

White, S. N., Mousel, M. R., Reynolds, J. O., Lewis, G. S. & Herrmann-Hoesing, L. M. (2009): Common promoter deletion is associated with 3.9-fold differential transcription of ovine CCR5 and reduced proviral level of ovine progressive pneumonia virus. *Anim Genet* **40**(5): 583-589.

White S. N. & Knowles D. P. (2013): Expanding Possibilities for Intervention against Small Ruminant Lentiviruses through Genetic Marker-Assisted Selective Breeding. *Viruses* **5**: 1466-1499

White, S. N., Mousel, M. R., Reynolds, J. O., Herrmann-Hoesing, L. M. & Knowles, D. P. (2013): Deletion variant near ZNF389 is associated with control of ovine lentivirus in multiple sheep flocks. *Anim Genet* **45**(2): 297-300.

Yaman Y., Keles M., Aymaz R., Sevim S., Sezenler T., Önaldi A.T., Kaptan C., Ba¸skurt A., Koncagül S., Öner Y., et al. (2019): Association of TMEM154 variants with visna/maedi virus infection in Turkish sheep. *Small Rumin. Res.* **177**: 61–67.

this study.

Table (1): Primer sequences and PCR product sizes used for analysis of variaion in or near the candidate genes. .

Candidate gene	primer sequences	product size	Sequence information
TMEM154	5'- CCACAGGAGAGGAGRACACA-3' (forward)	40/41 bp	Determination of nucleotide substitution NC_019474.2:g.4860407G>A, resulting in amino acid substitution E35K, using KASP technology (LGC, Hoddesdon, UK) NB: Bases written in bold are sites for primer binding
	5'-GGGCACGTCTCCTGACAGTT**T**-3' (reverse, FAM-labeled, K allele)		
	5'-GGCACGTCTCCTGACAGTT**C**-3' (reverse, HEX-labeled, E allele)		
CCR5	5'-CCCCATTGATAAGCCCTACA-3' (forward)	160/156 bp	Determination of promotor region deletion NC_019476.2:g.52961717_52961714delAATG (minus strand); amplification and sequencing for verification of genotyping results
	5'-CACCCAACTACCCAAATGGT-3' (reverse, FAM-labeled or unlabeled)		
ZNF389	5'-CGAATGGATCTTCAAGGCTTA-3' (forward)	148/150 bp	Detection of a two base-pair deletion variant in the 5′ genomic region of Exon 4 NC_019477.1:g.29500068_29500069delAT ovine chromosome 20, NCBI dbSNP ss748775100
	5'-CAGCTTTTCCATGCAGAGTC -3' (reverse, FAM-labeled or unlabeled		

Table (2): Allele and genotype frequencies of *TMEM154* putative protective and risk variants (K and E, respectively) in animal set 1 considering association to breed susceptiblity and behavior of allele segregation in each breed using HWE (Hardy-Weinberg Equilibrium) test.

Animal set 1	**Breed (n)**	***TMEM154* allele frequency**		***P* value for HWE**	***P* value**	***TMEM154* genotype frequency**				***P* value** (*P* value when genotypes of risk allele combined)
		K	E			KK	KE	EE	KE-EE	
MV-low susceptible breeds	GGH (32)	0.984	0.016	-	0.000	0.968	0.032	0.000	0.032	0.000 (0.000)
	ML (32)	0.844	0.156	0.137		0.750	0.188	0.062	0.250	
	Su (32)	0.435	0.565	0.718		0.161	0.548	0.290	0.838	
	Mean values (SD)	0.754 (0.285)	0.246 (0.285)			0.626 (0.417)	0.256 (0.265)	0.117 (0.153)	0.374 (0.417)	
MV-high susceptible	Cam (32)	0.071	0.929	1.000		0.000	0.143	0.857	1.000	
	EFM (32)	0.266	0.734	1.000		0.062	0.406	0.531	0.937	

breeds	Tex (32)	0.297	0.703	1.000		0.094	0.406	0.500	0.906	
	Mean values (SD)	0.211 (0.123)	0.789 (0.123)			0.052 (0.048)	0.318 (0.152)	0.629 (0.198)	0.948 (0.048)	

n: No of genotyped individuals; GGH: German Grey Heath; ML: Merinoland; Su: Suffolk; Cam: Cameroon; EFM: East Friesian Milk; Tex: Texel; - : Not estimated HWE Fisher's test (Monomorphic population at *TMEM154* locus).

Table (3): Allele and genotype frequencies of *TMEM154* putative protective and risk variants (K and E, respectively) in animal set 2 considering association to serological MV status.

n: No of genotyped individuals

Animal set 2 (n)	*TMEM154* allele frequency		*P* value	*TMEM154* genotype frequency				*P* value (*P* value when genotypes of risk allele combined)
	K	E		KK	KE	EE	KE-EE	
MV-positive sheep (57)	0.255	0.745	0.000 (0.000)	0.055	0.400	0.545	0.945	0.000 (0.000)
MV-negative sheep (57)	0.634	0.366		0.464	0.339	0.196	0.535	

Table (4): Allele and genotype frequencies of *CCR5* promotor putative protective and risk variants (deletion, del and wild type, wt, respectively) in animal set 1 considering association to breed susceptiblity and behavior of allele segregation in each breed using HWE (Hardy-Weinberg Equilibrium) test.

Animal set 1	Breed (n)	*CCR5* allele frequency		*P* value for HWE	*P* value	*CCR5* genotype frequency				*P* value (*P* value when genotypes of risk allele combined)
		del	wt			deldel	delwt	wtwt	del-wt	
MV-low susceptible breeds	GGH (32)	0.067	0.933	1.000	0.004	0.000	0.133	0.867	1.000	0.023 (0.044)
	ML (32)	0.161	0.839	1.000		0.032	0.258	0.710	0.968	
	Su (32)	0.219	0.781	0.133		0.094	0.250	0.656	0.906	
	Mean values (SD)	0.149 (0.077)	0.851 (0.077)			0.043 (0.048)	0.215 (0.070)	0.742 (0.104)	0.957 (0.048)	
MV-high susceptible	Cam (32)	0.000	1.000	-		0.000	0.000	1.000	1.000	
	EFM (32)	0.081	0.919	1.000		0.000	0.161	0.839	1.000	

breeds	Tex (32)	0.094	0.906	1.000		0.000	0.188	0.812	1.000	
	Mean values (SD)	0.058 (0.051)	0.942 (0.051)			0.000 (0.000)	0.120 (0.102)	0.880 (0.102)	1.000 (0.000)	

n: No of genotyped individuals; GGH: German Grey Heath; ML: Merinoland; Su: Suffolk; Cam: Cameroon; EFM: East Friesian Milk; Tex: Texel; - : Not estimated HWE Fisher's test (Monomorphic population at *CCR5* locus).

Table (5): Allele and genotype frequencies of *CCR5* putative protective and risk variants (deletion, del and wild type, wt, respectively) in animal set 2 considering association to serological MV status.

Animal set 2 (n)	*CCR5* allele frequency		*P* value	*CCR5* genotype frequency				*P* value (*P* value when genotypes of risk allele combined)
	del	wt		deldel	delwt	wtwt	del-wt	
MV-positive sheep (57)	0.152	0.848	0.325	0.036	0.232	0.732	0.964	0.487 (0.986)
MV-negative sheep (57)	0.202	0.798		0.035	0.333	0.632	0.965	

n: No of genotyped individuals.

Table (6): Allele and genotype frequencies of *ZNF389* putative protective and risk variants (wild type, wt and deletion, del, respectively) in animal set 1 considering association to breed susceptiblity and behavior of allele segregation in each breed using HWE (Hardy-Weinberg Equilibrium) test.

Animal set 1	Breed (n)	*ZNF389* allele frequency		*P* value for HWE	*P* value	*ZNF389* genotype frequency				*P* value (*P* value when genotypes of risk allele combined)
		wt	del			wtwt	wtdel	deldel	wt-del	
MV-low susceptible breeds	GGH (32)	0.409	0.591	1.000	0.070	0.200	0.433	0.367	0.800	0.115 (0.037)
	ML (32)	0.432	0.568	0.724		0.200	0.467	0.333	0.800	
	Su (32)	0.550	0.450	0.689		0.241	0.621	0.138	0.759	
	Mean values	0.464 (0.076)	0.536 (0.076)			0.214 (0.024)	0.507 (0.100)	0.279 (0.124)	0.786 (0.024)	
MV-high susceptible	Cam (32)	0.451	0.549	1.000		0.190	0.524	0.286	0.810	
	EFM (32)	0.790	0.210	0.287		0.586	0.414	0.000	0.414	

breeds	Tex (32)	0.400	0.600	0.233		0.240	0.320	0.440	0.760	
	Mean values	0.547 (0.212)	0.453 (0.212)			0.339 (0.213)	0.419 (0.102)	0.242 (0.223)	0.661 (0.213)	

n: No of genotyped individuals; GGH: German Grey Heath; ML: Merinoland; Su: Suffolk; Cam: Cameroon; EFM: East Friesian Milk; Tex: Texel.

Table (7): Allele and genotype frequencies of *ZNF389* putative protective and risk variants (wild type, wt and deletion, del, respectively) in animal set 2 considering association to serological MV status.

nimal set 2 (n)	*ZNF389* allele frequency		*P* value	*ZNF389* genotype frequency				*P* value (*P* value whe genotypes of allele combin
	wt	del		wtwt	wtdel	deldel	wt-del	
/-positive sheep (57)	0.536	0.464	0.500	0.291	0.491	0.218	0.709	0.769 (0.483)
/-negative sheep (57)	0.491	0.536		0.232	0.518	0.250	0.768	

n: No of genotyped individuals