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Effect of Q_{211} and K_{222} *PRNP* polymorphic variants in the susceptibility of goats to oral infection with Goat-BSE

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Abstract

Background. The prion protein-encoding gene (*PRNP*) is one of the major determinants for scrapie occurrence in sheep and goats. However, its effect on bovine spongiform encephalopathy (BSE) transmission to goats is not clear.

Methods. Goats harboring wild type (WT), R/Q_{211} or Q/K_{222} *PRNP* genotypes were orally inoculated with a goat-BSE isolate to assess their relative susceptibility to BSE infection. Goats were culled at different time points during the incubation period and after the onset of clinical signs, and their brains as well as several peripheral tissues were analyzed for the accumulation of pathological prion protein (PrP^{Sc}) and prion infectivity by mouse bioassay.

Results. R/Q_{211} goats displayed delayed clinical signs compared to WT goats. PrP^{Sc} was detected only in brain whereas infectivity was present in peripheral tissues too. In contrast, none of the Q/K_{222} goats showed any evidence of clinical prion disease. No PrP^{Sc} accumulation was observed in their brains or peripheral tissues but very low infectivity was detected in some tissues after very long post-inoculation times (44-45 months).

Conclusions. These results demonstrate that transmission of goat-BSE is genotype dependent and highlight the pivotal protective effect of the K_{222} *PRNP* variant in the oral susceptibility of goats to BSE.

INTRODUCTION

Bovine spongiform encephalopathy (BSE) is a fatal neurodegenerative disease, belonging to the group of prion diseases or transmissible spongiform encephalopathies (TSEs). TSEs are characterized by the post-translational conversion of a normal cellular prion protein (PrP^{C}, which is encoded by the prion protein-encoding gene *PRNP*) into an abnormal isoform (PrP^{Sc}) which accumulates in the tissues of infected humans and animals [1].

Although it was firstly recognized as a cattle prion disease [2], the BSE agent has demonstrated a high capacity to cross species barriers, spreading to humans, with the emergence of variant Creutzfeldt-Jakob disease (vCJD) [3, 4], cats and a variety of zoo animals during the BSE epidemic of 1980s [5]. Sheep and goats can be experimentally infected with this TSE too [6-8]. Moreover, two BSE "natural" cases have been reported in goats [9, 10].

Scrapie is a TSE affecting small ruminants all around the world. Its occurrence is strongly modulated by the prion protein-encoding gene (*PRNP*) and for goats, several *PRNP* polymorphisms have been linked with lower risk of developing classical scrapie: I/M_{142}, N/S_{146} or N/D_{146}, R/Q_{211} and Q/K_{222}. Among them, the K_{222} *PRNP* variant yielded the most promising results, being associated with resistance to scrapie not only in epidemiological studies [11-18] but also in experimental inoculations in goats [19, 20] and transgenic mice [21].

In contrast, little is known about the factors determining the susceptibility of goats to BSE although prolonged incubation times in BSE-infected goats were associated with the I/M_{142} polymorphism [7]. More recently, we showed that transgenic mice expressing the K_{222} *PRNP* variant are resistant to cattle-BSE but not to sheep or goat-BSE [21].

In the present work, we studied the effect of certain *PRNP* genotypes in the oral transmission of goat-BSE agent to goats. For that purpose, WT, R/Q_{211} and Q/K_{222} goats were orally challenged with a goat-BSE isolate and their brain and some peripheral tissues were analyzed for the presence of either the accumulation of PrP^{Sc} by immunohistochemistry (IHC) or the presence of PrP^{res} and prion infectivity by Western-blot (WB) and mouse bioassay, respectively.

MATERIALS AND METHODS

Ethics statements

Animal experiments were carried out in strict compliance with the recommendations of the Code for Methods and Welfare Considerations in Behavioral Research with Animals (Directive 86/609EC). The challenge experiments in goats described in this manuscript were approved by the competent authority of the Federal State of Mecklenburg-Western Pommerania, Germany (Permit Number: 7221.3-2.5-001/05). Experiments in mice were carried out in CISA-INIA (Madrid) and authorized by the Committee on the Ethics of Animal Experiments (CEEA) of the Spanish Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA); Permit Number: CEEA2009/003.

Goat-BSE oral transmissions to goats

Alpine-Saanen cross breed goats used in these transmission experiments were obtained by natural mating and sequenced as previously described [7]. At six to seven months of age goats harboring WT ($R_{211}Q_{222}$/RQ), R/Q_{211} or Q/K_{222} genotypes received a total dose of approximately 1g brain equivalent from BSE first-passaged in goat. This goat-BSE material (gBSE-P12) was obtained by pooling brainstems from three WT goats experimentally inoculated with a mixture of four cattle-BSE field cases [6, 7]. Inoculum was orally administered at 1:5 (w/v) dilution in sterile 0.9 NaCl and each goat got 5ml of pooled goat brain homogenate. Goats were regularly monitored for the presence of

clinical signs typical of BSE (i.e. neurological disorders like abnormalities in sensation and movement). Goats were euthanatized at clinical stage or at scheduled times (Table 1) using Ketaminhydrochlorid (10-15 mg/kg) and Xylazinhydrochlorid for anesthesia followed by euthanasia with T61 (4-6ml/50 kg body weight). During necropsy, a set of tissues was collected under TSE sterile conditions (among others brain, lymph node poplitealis, psoas major and retractor bulbi muscles) and harvested at -80ºC. A part of each sample was immediately fixed in neutral-buffered 10% formalin (4% formaldehyde). Subsequently these samples were treated for 1 h with 98% formic acid and rinsed in tap water for 40 min before dehydration and embedding in paraffin. After rehydration, 3 µm-thick brain slices were stained with hematoxylin and eosin (HE) and a histopathological examination was applied. For detecting PrP^{Sc} deposits, immunohistochemistry (IHC) analysis were performed as previously described [22], using 6C2 monoclonal antibody (mAb) (Central Veterinary Institute of Wageningen UR, Lelystad, Netherlands) and F99 mAb (VMRD, Pullman, USA). Shortly the sections were pretreated by an incubation with 98% formic acid (15 min), followed by autoclaving in citrate buffer for 20 min at 121°C. The endogenous peroxidase was inhibited by using 3% H_2O_2 in methanol for 30 min. The primary antibodies were diluted in goat serum, the 6C2 depending on the charge from 1:50 up to 1:150, the F99 1:4000 and incubated for 2 h at room temperature. Negative control sections were treated with goat serum alone. As a secondary antibody we used the EnVision™ reagent (Dako, Hamburg, Germany) containing a peroxidase-conjugated polymer backbone. Incubation time on these sections was 30 min at room temperature. The slides were finally developed in diaminobenzidine tetrahydrochloride (Fluka, Steinheim, Germany) and counterstained with Mayer's haematoxylin. All sections were examined by light microscopy.

Another part of each sample was homogenized as 10% (w/v) in sterile 0.9 NaCl for both determining the presence of PrP^{res} by phosphotungstic acid Western-blot (PTA-WB) and/or standard Western-blot (WB), and studying the prion infectivity by mouse bioassay (see below). For TSE discriminatory testing PTA-WB of the brain stem samples were performed by using the mabs L42 and P4 while Sha31 mAb [23] was employed in WB analysis following previously published procedures[22, 24, 25].

Mouse bioassay

The sensitivity of BoPrP-Tg110 mice expressing the bovine-PrP^{C} [26] and GowtPrP-Tg501 mice expressing the goat-PrP^{C} [21] for detecting goat-BSE prion infection was determined in a first experiment. BoPrP-Tg110 mice were selected for detecting the prion infectivity in goat tissues because of its higher sensitivity to goat-BSE prion infection than GowtPrP-Tg501 mice (Table S1 and Figure S1). Groups of 6-9 individually identified (six to seven weeks old) BoPrP-Tg110 mice were anesthetized with isoflurane and intracerebrally inoculated with 20 µl of the inocula (10% goat tissue homogenate) in the right parietal lobe using a 25 gauge disposable hypodermic needle. Mice were examined biweekly for the development of neurological signs of the prion disease and humanely euthanized by cervical dislocation when progression of the disease was evident or at the end of the study: 650 days post-inoculation (dpi) (Table 2). A mouse was considered positive for neurological disease when it showed two or three out of the 10 signs of neurological dysfunction previously described [27, 28]. Definitive diagnosis was made on observation of one confirmatory sign of prion disease (ataxia, generalized tremor, loss of righting reflex, limb paralysis, extensive pilo-erection or sustained hunched posture) and animals were culled at this point. Once euthanized, brain was removed and harvested at -20ºC for determining the presence of PrP^{res} by Western-blot using Sha31 mAb, as previously described [25]. Survival time and attack

rate were calculated for each inoculum. Survival time was expressed as the mean of the survival dpi of all the mice scored positive for brain PrP^{res}, with its correspondent standard error of the mean. Attack rate was determined as the proportion of mice scored positive for brain PrP^{res} from all the inoculated mice. Mice that died due to intercurrent diseases were excluded from the study. The criteria for exclusion were as follow: i) animals that died before the first animal scored as positive for brain PrP^{res} within the group of mice inoculated with the same inocula; ii) animals that died before 650 dpi when no positive mice were found in the group.

The relative infectivity in the different goat tissues was calculated as a function of the survival times observed after their inoculation in BoPrP-Tg110 mice. For that purpose, the goat-BSE isolate formerly used in goat oral inoculations (gBSE-P12) [6, 7] was titrated by ten-fold serial dilution inoculation in BoPrP-Tg110 mice and its infectious dose (ID) was subsequently assessed by the Reed-Muench method [29] (Table S1). From these data, a standard dose response curve was established and the relative infectivity of each tissue was extrapolated by comparing their transmission results with those obtained in the goat-BSE (gBSE-P12) titration (Figure S1).

RESULTS

To study the influence of *PRNP* polymorphisms in the susceptibility of goats to BSE, goats harboring wild type (WT), R/Q_{211} or Q/K_{222} *PRNP* genotypes were orally inoculated with a goat-BSE isolate (gBSE-P12) [6, 7] and monitored for the development of prion disease. Goats were euthanized at clinical stage or at scheduled time points during the incubation period to follow neurological lesions, PrP^{Sc} deposition and prion infectivity in brain and/or peripheral tissues.

PrP^{Sc} accumulation in BSE orally inoculated goats

None of the WT goats killed at 6 or 12 months post-inoculation (mpi) exhibited any signs associated with BSE disease in the brain or the peripheral tissues examined here, neither by histopathology, and immunohistochemistry (IHC) nor by Western-blot (WB) and/or phosphotungstic acid Western-blot (PTA-WB) (Table 1). Clinical signs of the disease were first observed in a WT goat (ZG01) at 24 mpi (Table 1). This animal showed pathological alterations and accumulation of PrP^{Sc} in the brain stem as demonstrated by hematoxylin and eosin (HE) and IHC. WB (Figure 1) and PTA-WB confirmed the presence of BSE derived PrP^{res} in this goat brain. However, in the popliteal lymph node (LN), retractor bulbi muscle or psoas major muscle all the applied methods failed to detect any amount of PrP^{Sc} (Table 1).

In contrast to WT goats, WB, IHC and HE analysis of one of the R/Q_{211} goats, killed at 24 mpi, revealed the absence of PrP^{Sc} deposits and neurodegenerative alterations in its brain (Table 1). However, three R/Q_{211} goats reached the clinical phase of BSE disease at 33 mpi (ZG28), 34 mpi (ZG05) or 36 mpi (ZG20) (Table 1) associated with clear spongiform encephalopathy and accumulation of PrP^{Sc} as shown in IHC. Additionally, all three R/Q_{211} goats (33-36 mpi) were scored PrP^{res} positive in their brains by WB (Figure 1) and PTA-WB. As described for the WT goat, neither the muscles nor the popliteal LN revealed any signs of PrP^{Sc} by IHC or WB.

None of the Q/K_{222} goats, which were killed at 24, 44 and 45 mpi, showed any evidence of prion disease (Table 1). These goats were scored as PrP^{res} negative for all of their analized tissues by IHC, PTA-WB and/or WB (Table 1).

WB analysis of the brain PrP^{Sc} in both WT and R/Q_{211} infected goats showed a typical BSE PrP^{Sc} banding pattern characterized by a 19-kDa aglycosyl band and prominent

diglycosylated species. The glycoprofiles were indistinguishable from those produced by the goat-BSE isolate originally used for goat challenges (gBSE-P12) (Figure 1).

Infectivity in tissues from BSE-infected goats

PrP^{Sc} is a specific and widely used TSE marker but its sensitivity can be lower than infectivity measures by mouse bioassay. Therefore, PrP^{Sc} negative goat tissues were inoculated in a very sensitive mouse model (BoPrP-Tg110) to evaluate the presence of prion infectivity (Table 2).

No prion infectivity was detected in any tissue from WT goats killed at 6 or 12 mpi. When their brains and peripheral tissues homogenates were inoculated into BoPrP-Tg110 mice, none of the mice succumbed to a prion disease; they were euthanized at 650 dpi showing neither clinical signs nor PrP^{res} in their brains by WB. Prion infectivity was detected in the brain from the WT goat killed at 24 mpi (ZG01 goat). After its inoculation, BoPrP-Tg110 mice showed 100% attack rates and short survival times (261±20 dpi) (Table 2), thus corresponding to a high infectious titer (>5 x 10^6 ID/g). Interestingly, BoPrP-Tg110 mice also succumbed to the inoculation of PrP^{Sc} negative popliteal LN, psoas major muscle and retractor bulbi muscle homogenates from this goat, displaying longer survival times and lower attack rates than those obtained with the brain homogenate (Table2). At this 24 mpi stage, no infectivity was detected in brain homogenate from R/Q_{211} goat (ZG13) (Table2). However, 100% BoPrP-Tg110 mice succumbed to the inoculation of the pooled brain homogenates from ZG28, ZG05 and ZG20 R/Q_{211} goats killed at 33-36 mpi. These mice showed similar survival times (258±15 dpi) to those previously obtained from the inoculation of the ZG01 WT goat brain and therefore, their infectious titers was comparable (>5 x 10^6 ID/g in both cases). Similarly to WT goat, R/Q_{211} goats also displayed prion infectivity in their PrP^{Sc} negative peripheral tissues. Pooled homogenates from popliteal LN, psoas major muscle

and retractor bulbi muscle infected BoPrP-Tg110 mice with higher attack rates and lower survival times than peripheral tissues from ZG01 WT goat (Table2).

With regard to the Q/K_{222} goat tissues, only the brain from one goat killed at 45 mpi (ZG11) showed low level of infectivity. This was not the case in the other Q/K_{222} goat killed at 44 mpi (ZG25). 100% of the BoPrP-Tg110 mice succumbed to the brain inoculation from goat ZG11 although with considerably longer survival times (400±50 dpi) than those obtained with brains from WT (261±20 dpi) and R/Q_{211} (258±15 dpi) goats. Traces of infectivity were also detected in PrP^{Sc} negative muscle psoas major from both Q/K_{222} goats, producing very low attack rate (1/6) and very long survival times (550 and 522 dpi, respectively) in BoPrP-Tg110 mice.

In all cases, brain WB PrP^{res} patterns from infected BoPrP-Tg110 mice were similar to that observed in the original goat-BSE isolate [6, 7] as well as to those seen in goat tissue homogenates, independently of the goat *PRNP* genotype (WT, R/Q_{211} or Q/K_{222}) or the inoculated tissue (Figure 2).

DISCUSSION

In this study, we analyzed the effect of the prion protein-encoding gene (*PRNP*) in the transmission of BSE to goats. For that purpose, goats harboring different *PRNP* genotypes (WT, R/Q_{211} and Q/K_{222}) were orally inoculated with a goat-BSE isolate, monitored for the development of clinical signs and culled at different time points. A panel of tissue samples was collected and studied for both the presence of PrP^{Sc} and infectivity by mouse bioassay.

WT goats developed first clinical signs of TSE at 24 months after inoculation (mpi). Signs were consistent to previous BSE inoculations in sheep and goats [30, 31]. PrP^{Sc} deposits in the brain and neurodegenerative lesions were also in agreement with those formerly observed in BSE inoculated sheep and goats[31-33], being mainly detected at

the level of the obex. Additionally prion infectivity was also present in muscles and LN tissues as detected by mouse bioassay although with smaller titers than in brain. These results are consistent with the PrP^{Sc} spread from central nervous system (CNS) to peripheral tissues proposed in BSE oral transmissions in sheep and cattle [34, 35]. R/Q_{211} goats firstly developed the BSE disease after 33 mpi. Prion infectivity in R/Q_{211} goats was higher in brain than in peripheral tissues, well in agreement with WT goat results. Interestingly, for the R/Q_{211} goats the onset of the disease was delayed by 9 to 12 months suggesting a genotype dependent transmission of BSE to goats in which the Q_{211} *PRNP* polymorphic variant might have an effect on incubation period but not susceptibility. Therefore, R/Q_{211} polymorphism could be associated with low resistance against goat-BSE, contrasting the high resistance to scrapie associated to this polymorphism in field [14, 18] and experimental [20] studies of goats. These differential results in the association of the Q_{211} *PRNP* variant with TSEs transmission is likely to be linked to the prion strain as a determinant factor for TSEs occurrence [36].

In contrast to the susceptibility of WT and R/Q_{211} goats to goat-BSE, none of the Q/K_{222} goats examined here displayed any evidence of disease. Neither clinical signs nor PrP^{res} were observed in any of these Q/K_{222} goats; histopathological analysis did not reveal any PrP^{Sc} deposits or neurological lesions. However, very low infectivity was detected in the brain from only one of the two Q/K_{222} goats euthanized at 44-45mpi. Traces of infectivity were also detected in psoas major muscles from both goats, thus suggesting that K_{222} *PRNP* variant drastically decreases the susceptibility of goats to goat-BSE. Remarkably, our group described that transgenic mice expressing the goat K_{222}-PrP^{C} variant are 100% susceptible to the intracerebral transmission of goat-BSE, displaying similar survival times than goat wt-PrP transgenic mice [21]. This result suggests that

the K_{222}-PrP^C variant is as able to replicate the goat adapted BSE agent as the goat WT-PrP^C. Hence, the resistance to goat-BSE might not be an intrinsic molecular property of K_{222}-PrP^C variant and the low susceptibility of Q/K_{222} goats against oral infection with goat-BSE might be additionally influenced by other factors than the host *PRNP* genotype.

A plausible reason for the discrepancy between the results of goat-BSE transmissions to Q/K_{222} goats and K_{222}-Tg516 mice could be the route of infection. Intracerebral inoculation (performed for transmission studies in Tg mice) provides the best route for prion replication since the inoculum is directly placed in the target tissue. Whereas by oral transmission (used for goat inoculation) prions must overcome several barriers prior to reach the target tissues: i) crossing the mucosal barrier, ii) amplification in gut-associated lymphoid tissues, iii) lymphatic/haematogenic dissemination, iv) neuroinvasion via the peripheral nervous system (PNS) v) to finally reach the CNS [30]. One or more of these steps might be affected in some way by the *PRNP* genotype, thus modulating the BSE capacity to replicate and/or spread through the organism. In line with this view, some authors have proposed that the ability of peripherally injected prions to replicate in extra neural tissues such as Peyer's patches in the intestinal tract, spleen, tonsils, appendix or lymph nodes may be critical for prions to persist in the host before spreading to the PNS and then to the CNS [37]. Lymphotropism seems to play an important role in prion infection. While BSE-PrP^{Sc} in cattle remained mainly distributed through the CNS and the vegetative nervous system [22, 34], the BSE agent becomes lymphotropic in sheep and goats with PrP^{Sc} accumulation in several lymphoid tissues [8, 38, 39]. BSE orally infected sheep showed PrP^{Sc} replication in lymphoid tissues such as tonsil and ileal Peyer's patches before the infection of some areas of the ENS [40, 41]. On this basis, we hypothesize that the resistance of Q/K_{222} goats to oral

goat-BSE infection could be linked to a lower capability of goat-BSE to convert K_{222}-PrP^C in peripheral organs. Lower levels of expression of K_{222}-PrP^C together with a regional variability of the K_{222}-PrP^C isoforms in these organs of primary transmission might not be ruled out [42].

Discrepancies in prion transmission efficiency linked to the inoculation route were previously described in R/H_{154}, R/Q_{211} or Q/K_{222} goats which were completely resistant to scrapie inoculation by oral route but susceptible by IC route [20]. Interestingly, while 100% of the R/H_{154} and R/Q_{211} goats succumbed to the scrapie IC inoculation, only few Q/K_{222} goats developed the disease and with 4 to 5 times prolonged incubation periods than WT goats; supporting the view that the K_{222} variant renders goats less susceptible to scrapie infection than the Q_{211} variant. This statement might be extended to BSE infection too, as supported by transmission experiments presented here. Moreover, these results together with data collected from numerous experimental [19-21, 43] and epidemiological studies [11-18] indicate the low susceptibility of Q/K_{222} polymorphism to prion infection.

We recently described that K_{222} *PRNP* variant interferes with the WT allele replication of the scrapie agent, thus resulting in prolonged incubation times and/or lower attack rates in heterozygous Q/K_{222} Tg mice compared to goat wt-PrP Tg mice [21]. In the present work, heterozygous Q/K_{222} goats were considerably less susceptible to the transmission of goat-BSE than WT goats, indicating that the K_{222} *PRNP* variant would protect also against goat-BSE oral infection. Since homozygous K/K_{222} goats were not challenged in this study, we cannot conclude on the level of resistance provided by K_{222} *PRNP* variant and its ability to replicate goat-BSE.

In summary, the results presented in this study demonstrate a genotype dependent transmission of the goat-BSE agent to goats where the K_{222} *PRNP* variant plays a

determinant role. The protective effect provided by the K_{222} *PRNP* variant against both goat-BSE (demonstrated here) and scrapie (previously reported) support the view that the K_{222} *PRNP* variant is a good candidate for improving breeding programs to control and eradicate TSEs in goats. IC inoculations of prions in Tg mouse models expressing different PRNP polymorphic variants are highly valuable to determine the role of PRNP amino acid exchanges in the conversion capacity of host PrP^C although this kind of procedure doesn´t always predict the genotype-dependent susceptibility of host species to natural prion infection. Therefore, prion transmission studies in the natural host using the natural route of infection (as presented in this study) are necessary to conclude at this point.

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We declare no competing financial interests.

FOOTNOTES

The authors declare no conflict of interest.

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REFERENCES

1. Prusiner SB. Molecular biology of prion diseases. Science 1991;252:1515-22.

2. Wilesmith JW. Bovine spongiform encephalopathy. Vet Rec 1988;122:614

3. Bruce ME, Will RG, Ironside JW, et al. Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. Nature 1997;389:498-501.

4. Hill AF, Desbruslais M, Joiner S, et al. The same prion strain causes vCJD and BSE. Nature 1997;389:448-50, 526.

5. Sigurdson CJ, Miller MW. Other animal prion diseases. Br Med Bull 2003;66:199-212

6. Foster JD, Hope J and Fraser H. Transmission of bovine spongiform encephalopathy to sheep and goats. Vet Rec 1993;133:339-41.

7. Goldmann W, Martin T, Foster J, et al. Novel polymorphisms in the caprine PrP gene: a codon 142 mutation associated with scrapie incubation period. J Gen Virol 1996;77:2885-91.

8. Foster JD, Parnham DW, Hunter N and Bruce M. Distribution of the prion protein in sheep terminally affected with BSE following experimental oral transmission. J Gen Virol 2001;82:2319-26.

9. Eloit M, Adjou K, Coulpier M, et al. BSE agent signatures in a goat. Vet Rec 2005;156:523-4

10. Jeffrey M, Gonzalez L, Espenes A, et al. Transportation of prion protein across the intestinal mucosa of scrapie-susceptible and scrapie-resistant sheep. J Pathol 2006;209:4-14

11. Acutis PL, Bossers A, Priem J, et al. Identification of prion protein gene polymorphisms in goats from Italian scrapie outbreaks. J Gen Virol 2006;87:1029-33

12. Vaccari G, Di Bari MA, Morelli L, et al. Identification of an allelic variant of the goat PrP gene associated with resistance to scrapie. J Gen Virol 2006;87:1395-402

13. Barillet F, Mariat D, Amigues Y, et al. Identification of seven haplotypes of the caprine PrP gene at codons 127, 142, 154, 211, 222 and 240 in French Alpine and Saanen breeds and their association with classical scrapie. J Gen Virol 2009;90:769-76

14. Bouzalas IG, Dovas CI, Banos G, et al. Caprine PRNP polymorphisms at codons 171, 211, 222 and 240 in a Greek herd and their association with classical scrapie. J Gen Virol 2010;91:1629-34

15. Fragkiadaki EG, Vaccari G, Ekateriniadou LV, et al. PRNP genetic variability and molecular typing of natural goat scrapie isolates in a high number of infected flocks. Vet Res 2011;42:104

16. Papasavva-Stylianou P, Windl O, Saunders G, Mavrikiou P, Toumazos P and Kakoyiannis C. PrP gene polymorphisms in Cyprus goats and their association with resistance or susceptibility to natural scrapie. Vet J 2011

17. Acin C, Martin-Burriel I, Monleon E, et al. Prion Protein Gene Variability in Spanish Goats. Inference through Susceptibility to Classical Scrapie Strains and Pathogenic Distribution of Peripheral PrP(sc.). PLoS One 2013;8:e61118

18. Corbiere F, Perrin-Chauvineau C, Lacroux C, et al. PrP-associated resistance to scrapie in five highly infected goat herds. J Gen Virol 2013;94:241-5

19. Acutis PL, Martucci F, D'Angelo A, et al. Resistance to classical scrapie in experimentally challenged goats carrying mutation K222 of the prion protein gene. Vet Res 2012;43:8

20. Lacroux C, Perrin-Chauvineau C, Corbiere F, et al. Genetic resistance to Scrapie infection in experimentally challenged goats. J Virol 2014

21. Aguilar-Calvo P, Espinosa JC, Pintado B, et al. Role of the Goat K222-PrPC Polymorphic Variant in Prion Infection Resistance. J Virol 2014;88:2670-6

22. Kaatz M, Fast C, Ziegler U, et al. Spread of classic BSE prions from the gut via the peripheral nervous system to the brain. Am J Pathol 2012;181:515-24

23. Feraudet C, Morel N, Simon S, et al. Screening of 145 Anti-PrP Monoclonal Antibodies for Their Capacity to Inhibit PrPSc Replication in Infected Cells. J. Biol. Chem. 2005;280:11247-11258

24. Monleon E, Monzon M, Hortells P, Vargas A and Badiola JJ. Detection of PrP(sc) in samples presenting a very advanced degree of autolysis (BSE liquid state) by immunocytochemistry. J Histochem Cytochem 2003;51:15-8

25. Padilla D, Béringue V, Espinosa JC, et al. Sheep and Goat BSE Propagate More Efficiently than Cattle BSE in Human PrP Transgenic Mice. PLoS Pathog 2011;7:e1001319

26. Castilla J, Gutierrez Adan A, Brun A, et al. Early detection of PrP(res) in BSE-infected bovine PrP transgenic mice. Arch Virol 2003;148:677-91

27. Scott M, Foster D, Mirenda C, et al. Transgenic mice expressing hamster prion protein produce species- specific scrapie infectivity and amyloid plaques. Cell 1989;59:847-57.

28. Scott M, Groth D, Foster D, et al. Propagation of prions with artificial properties in transgenic mice expressing chimeric PrP genes. Cell 1993;73:979-88.

29. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. Am. J. Epidemiol. 1938;27:493-497

30. van Keulen LJ, Bossers A and van Zijderveld F. TSE pathogenesis in cattle and sheep. Vet Res 2008;39:24

31. Foster JD, Parnham D, Chong A, Goldmann W and Hunter N. Clinical signs, histopathology and genetics of experimental transmission of BSE and natural scrapie to sheep and goats. Vet Rec 2001;148:165-71

32. Gonzalez L, Martin S, Houston FE, et al. Phenotype of disease-associated PrP accumulation in the brain of bovine spongiform encephalopathy experimentally infected sheep. J Gen Virol 2005;86:827-38

33. Lezmi S, Seuberlich T, Oevermann A, Baron T and Bencsik A. Comparison of brain PrPd distribution in ovine BSE and scrapie. Vet Pathol 2011;48:1101-8

34. Buschmann A, Groschup MH. Highly bovine spongiform encephalopathy-sensitive transgenic mice confirm the essential restriction of infectivity to the nervous system in clinically diseased cattle. J Infect Dis 2005;192:934-42

35. van Keulen LJ, Vromans ME, Dolstra CH, Bossers A and van Zijderveld FG. Pathogenesis of bovine spongiform encephalopathy in sheep. Arch Virol 2008;153:445-53

36. Aguzzi A, Heikenwalder M and Polymenidou M. Insights into prion strains and neurotoxicity. Nat Rev Mol Cell Biol 2007;8:552-61

37. Beringue V, Vilotte JL and Laude H. Prion agent diversity and species barrier. Vet Res 2008;39:47

38. Foster JD, Bruce M, McConnell I, Chree A and Fraser H. Detection of BSE infectivity in brain and spleen of experimentally infected sheep. Vet Rec 1996;138:546-8.

39. Bellworthy SJ, Hawkins SA, Green RB, et al. Tissue distribution of bovine spongiform encephalopathy infectivity in Romney sheep up to the onset of clinical disease after oral challenge. Vet Rec 2005;156:197-202

40. Andreoletti O, Berthon P, Marc D, et al. Early accumulation of PrP(Sc) in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie. J Gen Virol 2000;81:3115-26

41. Kujala P, Raymond CR, Romeijn M, et al. Prion uptake in the gut: identification of the first uptake and replication sites. PLoS Pathog 2011;7:e1002449

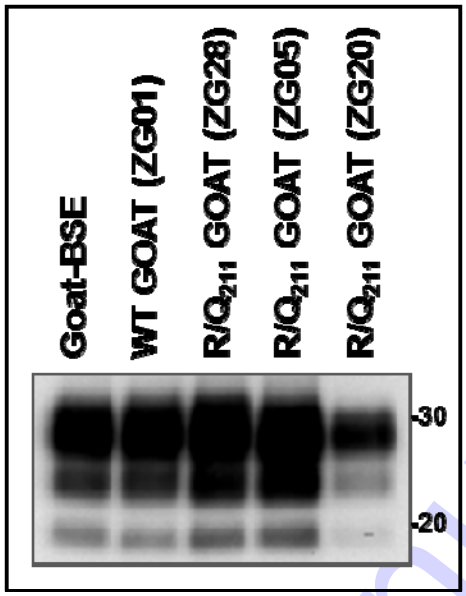
42. Beringue V, Mallinson G, Kaisar M, et al. Regional heterogeneity of cellular prion protein isoforms in the mouse brain. Brain 2003;126:2065-73

43. White SN, Reynolds JO, Waldron DF, Schneider DA and O'Rourke KI. Extended scrapie incubation time in goats singly heterozygous for PRNP S146 or K222. Gene 2012;501:49-51

FIGURE LEGENDS

Figure 1. Brain PrP^{res} from wild type (WT) and R/Q_{211} goats orally inoculated with a goat-BSE isolate. Immunoblots of brain PrP^{res} detected with Sha31 mAb. Identical amounts of 10% brain homogenate were loaded in each lane. The original Goat-BSE isolate (gBSE-P12) used for goat inoculations was also included in the blot (Goat-BSE). Molecular weights (in kDa) are shown at the right side of the blot.

Figure 2. Brain PrP^{res} detected in BoPrP-Tg110 mice inoculated with different goat tissues homogenates. Immunoblots of PrP^{res} in BoPrP-Tg110 mice inoculated with different tissue homogenates from ZG01 WT ($R_{211}Q_{222}$/RQ) goat; ZG28, ZG05 and ZG20 R/Q_{211} goats (pooled homogenates) and ZG11 Q/K_{222} goat. Similar quantities of PrP^{res} were loaded for adequate comparison and immunoblots were detected with Sha31 mAb. The original goat-BSE isolate (gBSE-P12) used for goat inoculations was also included in the blot (Goat-BSE). Molecular weights (in kDa) are shown at the right side of the blot.



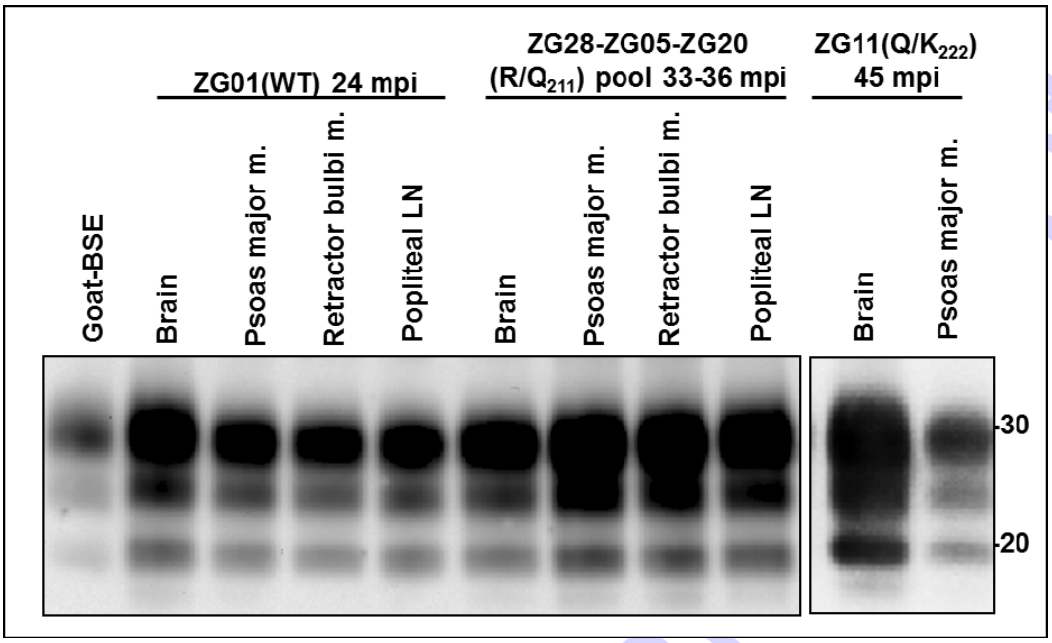


Table 1: Clinical sign evaluation and PrP^{Sc} detection in tissues of wild type ($R_{211}Q_{222}$/RQ), R/Q_{211} and Q/K_{222} *PRNP* goats orally challenged with a Goat-BSE isolate and sequentially culled

PRNP genotype	Goat code	Endpoint (mpi)*	Clinical signs	Goat tissue	PrP^{Sc} ** WB	IHC	PTA-WB
WT^{+}	ZG26	6	-	Brain	-	-	-
				Psoas major muscle	-	ND***	ND
				Retractor bulbi muscle	-	ND	ND
				Popliteal lymphonode	-	ND	ND
	ZG32	6	-	Brain	-	-	-
				Psoas major muscle	-	ND	ND
				Retractor bulbi muscle	-	ND	ND
				Popliteal lymphonode	-	ND	ND
	ZG35	6	-	Brain	-	-	-
				Psoas major muscle	-	ND	ND
				Retractor bulbi muscle	-	ND	ND
				Popliteal lymphonode	-	ND	ND
	ZG19	12	-	Brain	-	-	-
				Psoas major muscle	-	ND	ND
				Retractor bulbi muscle	-	ND	ND
				Popliteal lymphonode	-	ND	ND
	ZG24	12	-	Brain	-	-	-
				Psoas major muscle	-	ND	ND
				Retractor bulbi muscle	-	ND	ND
				Popliteal lymphonode	-	ND	ND
	ZG30	12	-	Brain	-	-	-
				Psoas major muscle	-	ND	ND
				Retractor bulbi muscle	-	ND	ND
				Popliteal lymphonode	-	ND	ND
	ZG01	24	+	Brain	+	+	+
				Psoas major muscle	-	-	ND

				Retractor bulbi muscle	-	-	ND
				Popliteal lymphonode	-	-	ND
R/Q_{211}	ZG13	24	-	Brain	-	-	-
	ZG28	33	+	Brain	+	+	+
				Psoas major muscle	-	-	ND
				Retractor bulbi muscle	-	-	ND
				Popliteal lymphonode	-	-	ND
	ZG05	34	+	Brain	+	+	+
				Psoas major muscle	-	-	ND
				Retractor bulbi muscle	-	-	ND
				Popliteal lymphonode	-	-	ND
	ZG20	36	+	Brain	+	+	+
				Psoas major muscle	-	-	ND
				Retractor bulbi muscle	-	-	ND
				Popliteal lymphonode	-	-	ND
Q/K_{222}	ZG10	24	-	Brain	-	-	-
	ZG25	44	-	Brain	-	-	-
				Psoas major muscle	-	-	ND
				Popliteal lymphonode	-	-	ND
	ZG11	45		Brain	-	-	-
				Psoas major muscle	-	-	ND
				Popliteal lymphonode	-	-	ND

[+] Goat wild type *PRNP* genotype: $R_{211}Q_{222}/RQ$

[*] mpi: months post-inoculation

[**] Abnormal prion protein (PrP^{Sc}) determined by Western-blot (WB), immunohistochemistry (IHC) or phosphotungstic acid Western-blot (PTA-WB)

[***] Not determined

Table 2: Infectious titer in tissues from Goat-BSE orally inoculated WT, R/Q_{211} and Q/K_{222} *PRNP* goats as determined by mouse bioassay

PRNP genotype	Goat codes of pooled tissues	Endpoint (mpi)*	Goat tissue	Mouse bioassay in BoPrP-Tg110 mice	
				MST±SEM (n/n_0)**	Infectious titer***
WT+	ZG26-ZG32-ZG35	6	Brain	650 (0/6)	-****
			Psoas major muscle	650 (0/6)	-
			Popliteal lymphonode	650 (0/6)	-
	ZG19-ZG24-ZG30	12	Brain	650 (0/6)	-
			Psoas major muscle	650 (0/6)	-
			Popliteal lymphonode	650 (0/6)	-
	ZG01	24	Brain	261±20 (6/6)	+++
			Psoas major muscle	596, 598 (2/6)	-
			Retractor bulbi muscle	469±29 (3/6)	-
			Popliteal lymphonode	526 (1/6)	-
R/Q_{211}	ZG13	24	Brain	650 (0/6)	-
	ZG28-ZG05-ZG20	33-34-36	Brain	258±15 (6/6)	+++
			Psoas major muscle	334±24 (6/6)	++
			Retractor bulbi muscle	307±84 (5/5)	++
			Popliteal lymphonode	406±50 (2/4)	+
Q/K_{222}	ZG10	24	Brain	650 (0/6)	-
	ZG25	44	Brain	650 (0/6)	-
			Psoas major muscle	550 (1/6)	-
			Popliteal lymphonode	650 (0/6)	-
	ZG11	45	Brain	400±50 (6/6)	+
			Psoas major muscle	522 (1/6)	-
			Popliteal lymphonode	650 (0/6)	-

+ Goat wild type *PRNP* genotype: $R_{211}Q_{222}/RQ$.

* mpi:months post-inoculation.

** Mean Survival Time in days ± Standard error of the mean (n/n0: diseased, PrP^{res} positive/inoculated animals).

*** Infectious titer of each goat tissue was calculated as a function of the survival times obtained after their inoculation in BoPrP-Tg110 mice and expressed as ID per grams of the inoculated tissue (ID/g) (see supplemental data). Infectious titers of the goat tissues were scored as - when they were lower than the limit of detection of the mouse bioassay calculated by the Reed-Muench method [29] (5x 10^2 ID/g); + when they ranged from 5x 10^2 ID/g to 5x 10^4 ID/g; ++ when they ranged from >5x 10^4 ID/g to 5x 10^6 ID/g, and +++ when they were higher than 5x 10^6 ID/g.