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SCIENTIFIC REPORT OF EFSA

Scientific and technical assistance on the provisional results of the study on genetic resistance to Classical scrapie in goats in Cyprus[1]

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ABSTRACT

This EFSA Scientific Report reviews and discusses the provisional results of a study (EURL/Cypriot study) on genetic resistance to Classical scrapie in goats in Cyprus. It is concluded that the provisional results obtained in the study further support the lower susceptibility to Classical scrapie in goats carrying the D146 and S146 alleles compared to wild type (N146N) goats. The results from intracerebral challenge are not compatible with a level of resistance as high as the one observed in sheep carrying the ARR allele or in goats carrying the K222 allele. Final results from the oral challenge will be crucial in determining the level of resistance associated with the D146 and S146 alleles. Furthermore, it is concluded that the provisional results obtained in the study are compatible with the possibility to use the D146 and S146 alleles to build a genetic strategy to control and eradicate Classical scrapie in goats in Cyprus. However, the success of such a strategy will be determined by the level of resistance associated with the D146 and S146 alleles against infection with all the different TSE agents proved to be circulating in Cyprus, which at this stage of the EURL/Cypriot study remains to be definitively assessed. In addition, as compared to the results of the model developed in the study, it is concluded that the efficiency of the implementation in the field of a breeding strategy selecting for the D146 and S146 alleles may be lower due to potential practical constraints related to the management of genetic diversity, to the selection for production and health traits and to the need of moving animals for breeding purposes in Cyprus. Recommendations on aspects that may be considered when completing the study are formulated.

KEY WORDS

TSE, Classical scrapie, goat, genetic resistance, breeding programme, Cyprus.

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SUMMARY

Following a request from the European Commission, EFSA was asked to provide scientific and technical assistance on the provisional results of a study on genetic resistance to Classical scrapie in goats in Cyprus. The study was performed by the European Union Reference Laboratory for Transmissible Spongiform Encephalopathies (TSE EURL) and the Cypriot Veterinary Services (CVS). In 2006/2007 a pilot project on this topic was undertaken in Cyprus. In 2009 EFSA was asked by the European Commission to evaluate the results from the pilot project and, later, a draft protocol for additional studies. A final protocol for new studies to be carried out in Cyprus was subsequently agreed by the European Commission, the TSE EURL and the CVS. Implementation of the protocol started at the end of 2009 (EURL/Cypriot study).

This EFSA Scientific Report reviews and discusses the provisional results of the EURL/Cypriot study as concerns the four research areas implemented: i) effect of D146 and S146 *PRNP* alleles on the individual susceptibility to Classical scrapie infection; ii) TSE agent diversity and resistance/susceptibility associated with the D146 and S146 alleles; iii) effect of D146 and S146 *PRNP* alleles on Classical scrapie pathogenesis; and iv) capacity for selection and diffusion of the *PRNP* allele in the Cypriot goat population.

It is concluded that the provisional results obtained in the study further support the lower susceptibility to Classical scrapie in goats carrying the D146 and S146 alleles compared to wild type (N146N) goats. The results from intracerebral challenge are not compatible with a level of resistance as high as the one observed in sheep carrying the ARR allele or in goats carrying the K222 allele. Final results from the oral challenge will be crucial in determining the level of resistance associated with the D146 and S146 alleles.

Furthermore, it is concluded that the provisional results obtained in the study are compatible with the possibility to use the D146 and S146 alleles to build a genetic strategy to control and eradicate Classical scrapie in goats in Cyprus. However, the success of such a strategy will be determined by the level of resistance associated with the D146 and S146 alleles against infection with all the different TSE agents proved to be circulating in Cyprus, which at this stage of the EURL/Cypriot study remains to be definitively assessed. In addition, as compared to the results of the model developed in the study, it is concluded that the efficiency of the implementation in the field of a breeding strategy selecting for the D146 and S146 alleles may be lower due to potential practical constraints related to the management of genetic diversity, to the selection for production and health traits and to the need of moving animals for breeding purposes in Cyprus.

The Scientific Report formulates recommendations on aspects that may be considered when completing the study. In particular, approaches are suggested for characterising TSE isolates from Cypriot goats and estimating the potential level of resistance towards them associated to the D146 and S146 alleles. Suggestions are made about possible issues to be considered in the analysis of the capacity for selection and diffusion of the alleles of interest. Finally, it is indicated that introgression of the K222 allele into the Cypriot goat Damascus breed could be considered as a possible alternative to selection for the D146 and S146 alleles in case this solution was not suitable.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

On 24 March 2009 and following a request from the Commission, EFSA published a scientific opinion on genetic resistance to scrapie in goats in consideration of a pilot project study carried out in Cyprus[4]. The report of the Cypriot pilot study presented a case control study in Cypriot goat herds aiming at the identification of the effect of *PRNP* polymorphisms on TSE susceptibility. According to the author of the report, the results obtained would indicate a higher resistance against clinical disease in goats bearing the H154, D146 or S146 *PRNP* polymorphism than in goats bearing R154 or N146.

The BIOHAZ Panel was requested to assess the scientific validity of the study and to indicate to what extent, based on this study, genetic breeding can be used as a control program for Classical scrapie in goats in Cyprus.

The BIOHAZ Panel concluded that the study conducted in Cyprus brings additional proof of a potential lower susceptibility to Classical scrapie in goats in H154, D146 and S146 *PRNP* allele carriers and can be considered as encouraging information on the path for identifying *PRNP* polymorphisms that could be used as part of a genetic strategy to control and eradicate TSE agents in goats. However, the BIOHAZ Panel concluded at the same time that the study on its own was an insufficient basis to evaluate accurately and reliably the efficacy and the potential adverse consequence of the large-scale breeding for H154, D146 and S146 *PRNP* alleles as a tool to control and eradicate Classical scrapie in Cyprus and recommended new investigations in order to assess the efficacy of breeding for the *PRNP* alleles in goat populations in Cyprus. In addition, the operational possibility to conduct these alleles selection in the Cypriot goat population and the potential adverse effect of such selection on genetic variability in the Cypriot goat breeds should be evaluated.

Following the publication of this opinion, the Commission tasked the EU Reference Laboratory for TSEs (EURL-TSE) to draft a new protocol for additional studies in order to adequately supplement the initial findings of the Cypriot pilot study and following the six areas of research as given in the EFSA Scientific Opinion.

EFSA was tasked by the Commission on 4 June 2009 to evaluate this amended protocol and published a statement on 24 July 2009[5]. The BIOHAZ Panel concluded that the protocol is an extension and an improvement of the earlier pilot project. The BIOHAZ Panel made a series of detailed conclusions and recommendations for further development of the EURL protocol.

The EURL-TSE and the Cypriot veterinary services launched the implementation of the protocol at the end of 2009. The EURL-TSE produced in May 2012 the 5th progress report of the study.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

EFSA is requested to provide scientific and technical assistance to the Commission on the following questions, based on the 5th progress report published by the EURL-TSE in May 2012:

1. Are the provisional results of the study supportive of a significantly lower susceptibility to Classical scrapie in goats carrying the D146 and S146 alleles?

2. Are the provisional results of the study consistent with the possibility to use this *PRNP* polymorphism in the future to build a genetic strategy to control and eradicate Classical scrapie in goats in Cyprus?

[4] doi:10.2903/j.efsa.2009.995. Genetic TSE resistance in goats.
[5] doi:10.2903/j.efsa.2009.1203. Statement on a protocol for additional data collection based on the EFSA recommendations about resistance to scrapie in goats in Cyprus.

Clarification of the Terms of Reference

After discussion with the requestor of the mandate (European Commission), the Terms of Reference were further clarified and it was agreed to delete the word "significantly" from the first Term of Reference, which would read as follows: "*Are the provisional results of the study supportive of a lower susceptibility to Classical scrapie in goats carrying the D146 and S146 alleles*?".

CONTEXT OF THE SCIENTIFIC OUTPUT

As introduced in the Background section above, after the evaluation by EFSA (EFSA, 2009) of the results of a study conducted in Cyprus to investigate the association of polymorphisms of the *PRNP* gene at codons 146 and 154 in goats with susceptibility/resistance to Classical scrapie in Cyprus, the protocol for a new study was submitted by the European Commission to EFSA, with the request to indicate whether the protocol was appropriate to adequately supplement the initial findings of the Cypriot study and was addressing the recommendations of the previous EFSA Opinion (EFSA, 2009). Following to this request, EFSA published a Statement (EFSA Panel on Biological Hazards (BIOHAZ), 2009), concluding that the protocol was an improvement of the earlier pilot project, and providing a series of conclusions and recommendations for its further development. Detailed comments were provided for the six different research areas originally recommended for research (EFSA, 2009) and developed by the protocol.

In its overall conclusions, the BIOHAZ Panel (2009) indicated that the low frequencies of the *PRNP* alleles of interest in the population compromise the statistical power of the proposed protocol. Since the potential diversity of TSE agents in Cypriot goats could not be foreseen, the systematic screening of all samples available by high throughput biochemical test was advised rather than the proposed reduced panel of isolates. It was further indicated that in vitro conversion assays were available and could be used to document potential resistance associated to the D146 and S146 *PRNP* alleles, and that standardised bioassays of TSE isolates of interest, as used by the Strain Typing Expert Group (STEG), were necessary to document the biodiversity of TSE strains in goats in Cyprus. Considering the limited frequencies of the *PRNP* alleles of interest and the assumed prevalence of Classical scrapie in infected herds, it was concluded that the proposed experiments were unlikely to document the distribution of PrP^{Sc} in homozygous goats but could document, within some limits, the distribution of PrP^{Sc} in heterozygous goats. It was also indicated that the modelling approach on feasibility and duration of selection for resistant alleles was subject to the availability of input data. Finally, it was concluded that the proposed protocol could not substitute the experiments advised in the earlier EFSA Opinion EFSA (2009) on cohort follow up and experimental inoculation, which remained crucial for definitive assessment of D146 and S146 *PRNP* alleles' resistance.

Following to the publication of the EFSA Statement (EFSA Panel on Biological Hazards (BIOHAZ), 2009), a final protocol for the studies to be carried out in Cyprus was agreed in October 2009 by the European Commission, the European Union Reference Laboratory for TSE (TSE EURL) and the Cyprus Veterinary Services. Implementation of the protocol started at the end of 2009 (EURL/Cypriot study).

Since then additional scientific information has become available in relation to genetic resistance to Classical scrapie in goats. In particular, some studies support a potential high level of resistance for goats carrying the S146 and K222 alleles (Acutis et al., 2012; White et al., 2012). Additional experiments are ongoing in the framework of European research projects (*European goatBSE* project, *EMIDA goat TSE free* project), which are providing useful information in relation to breeding for TSE resistance in goats.

It should be noted that the present report addresses only Classical scrapie and no conclusions can be drawn with respect to other TSEs in goats. Practical definitions of the different TSEs in small ruminants can be found in a previous EFSA Opinion (2005).

ASSESSMENT

1. EURL/Cypriot study, Research area 1

1.1. Brief overview of the study

The objective of this research area was to study the effect of D146 and S146 alleles on individual susceptibility to Classical scrapie infection. The study was composed of different parts:

- Intracerebral (i.c.) challenge of goats homozygous and heterozygous for the alleles of interest.

  Thirty-seven goats were included in the study: eight animals for each of the D146D, S146S, N146S and N146D putative resistant genotypes, and five animals of the N146N putative susceptible genotype (wild type). All recipient and donor animals in the inoculation experiments were homozygous R at codon 154. The inoculum used for the experiment consisted of a pool of brain material from 12 field cases of scrapie in N146N goats from Cyprus. All the brains were tested for abnormal PrP presence by Western blot, and BSE was excluded on the basis of PrP^{Sc} biochemical properties. Recipient animals were killed as soon as they reached a clinical disease endpoint and PrP^{Sc} distribution and amount were determined in their nervous, lymphoid, muscular tissues and other tissues by ELISA and immunohistochemistry.

  Four of the animals challenged died from intercurrent diseases, but all of them tested negative for TSE. All the other animals were culled following clinical disease, and tested positive. The mean incubation times observed in the different genotype groups are reported in Table 1.

  While all N146N animals were central nervous system (CNS) and lymphoreticular system (LRS) positive, the heterozygous and homozygous animals for both 146D and 146S allele remained LRS negative.

Table 1: Mean incubation times of intracerebrally challenged goats according to their genotype.

Genotype	Mean incubation time (days)
D146D	489
S146S	556
N146S	564
N146D	425
N146N	427

- Oral challenge of goats homozygous and heterozygous for the alleles of interest.

  For this experiment 125 goats were inoculated orally with the inoculum described above. The age of animals at challenge ranged from 2 to 15 months, with about 72% of the animals being below 6 months of age, and 28% above. Groups of five inoculated animals of different genotypes (D146D, S146S, N146S, N146D, N146N) were killed at 6, 12 and 24 months post inoculation (mpi), and several tissues tested for PrP^{Sc} presence by ELISA and immunohistochemistry. The remaining animals are currently alive and kept under observation until clinical signs are observed or the animals have to be culled for welfare reasons.

  According to the provisional results of the study, positive findings have been so far described only in goats carrying the N146N genotype. In particular, 1 out of 5 tested positive at 6 mpi (palatine tonsil and retropharyngeal lymph node), 4 out of 5 at 12 mpi (lymphoreticular system, with one of them also positive in the brainstem), and 4 out of 4 at 24 mpi (both lymphoreticular and CNS system). One additional goat carrying the N146N genotype died at 23 mpi and also tested positive. No positive findings were described in animals of the other genotypes under investigation.

- Genotyping of the entire Cypriot goat population.

  The aim of the study was to perform the PrP genotyping (codons 146 and 154) of the entire Cypriot goat population.

  So far, over 318,000 goats were genotyped. The large majority of animals showed to carry the R154R genotype (91.5%). Among the R154R goats, for which more detailed information is available, the following frequency was reported for the different genotypes: 71.16% for the N146N, 12.74% for the N146D, 11.90% for the N146S, 1.52% for the S146D, 1.39% for the D146D, and 1.30% for the S146S genotypes. Data show a low frequency of non-N146 goats.

- Management cull.

  For this experiment animals from scrapie-infected herds were selected according to pre-specified criteria (i.e. animals included in the study to be over 6 years of age, homozygous and heterozygous for the alleles of interest, originating from herds with confirmed cases of scrapie for at least 6 years). The experiment is still ongoing, and so far samples from 423 goats (obex and retropharyngeal lymph node) from 37 scrapie infected herds were collected and tested by means of ELISA and immunohistochemistry.

  Among the tested samples, all the 109 positive cases were identified in animals carrying the N146N genotype (109 over 273 N146N animals tested). No positive case was reported among the remaining 150 animals (69 N146S, 72 N146D, 4 D146D, 4 S146D, 1 S146S).

More details on the provisional results of the study can be found in the EURL/Cypriot 5th report on progress (see Appendix A).

1.2. Considerations on the provisional results of the study

1.2.1. Intracerebral challenge experiment

Intracerebral challenge of animals harbouring different genotypes is used to obtain an estimate of the ability/efficacy of TSE agents to propagate in a given PrP substrate. Lack of transmission in a particular PrP variant compared to a 100% attack rate in wild type PrP qualifies for a low susceptibility/strong resistance to infection. Longer incubation periods of the disease in inoculated animals without a reduction of the attack rate are also compatible with a lower susceptibility/higher resistance to the infection.

The results obtained by the EURL/Cypriot study do not indicate an association between the tested PrP polymorphism and the transmission attack rate. A statistical analysis of the difference in incubation period between the different genotype groups remains necessary to assess its significance and the potential impact of PrP genotype on the incubation period.

In previous experiments in Classical scrapie i.c. challenged homozygous ARR sheep (Foster et al., 2001; Goldmann et al., 1994) and heterozygous K222 goats (Acutis et al., 2012), no clinical signs or PrP^{Sc} accumulation in brain or lymphoid tissue were observed. These results were compatible with very high resistance to infection.

The results obtained by the EURL/Cypriot study after i.c. challenge of S146 and D146 allele carriers are not compatible with a level of resistance as high as the one observed in ARR sheep or in K222 goats.

The absence of PrP^{Sc} accumulation in lymphoid tissue from i.c. challenged homozygous and heterozygous S146 and D146 animals, as obtained by the EURL/Cypriot study, cannot be considered as an indicator of a potential resistance. In heterozygous Q211 goats, PrP^{Sc} was observed in lymphoid tissue after i.c. challenge with a Classical scrapie isolate, whereas hardly any deposit was identified in

the lymphoreticular system of the homozygous Q211 challenged animals[6]. However, in another study (Corbière et al., 2012) both genotypes showed a similar degree of resistance to the infection compared to baseline goats (homozygous $I_{142}R_{154}R_{211}Q_{222}S_{240}$ goats) under natural exposure conditions.

1.2.2. Oral challenge experiment

Oral inoculation of animals is considered as the best proxy for natural contamination with TSE agents in ruminants. It provides an estimate of the level of resistance associated to particular PrP alleles to the infection with a TSE agent. It encompasses not only the capacity of the agents to replicate in a PrP substrate, as assessed in i.c. challenge experiments, but also the ability of the agent to infect and disseminate efficiently in hosts harbouring a particular PrP genotype. Nevertheless, oral experimental exposure cannot be considered to perfectly reproduce the natural disease (Tabouret et al., 2010).

Recent results obtained in sheep of a fully susceptible genotype after oral challenge with Classical BSE demonstrated the strong influence of the age of the recipient at inoculation on the efficacy of disease transmission (Hunter et al., 2012). A significant reduction in disease transmission was observed in animals challenged after weaning. This phenomenon was more marked as the age of the animals increased. These results are fully consistent with those reported for naturally scrapie affected flocks, where disease is more rarely observed in animals introduced at adult age by comparison to animals introduced at younger age (Hourrigan et al., 1979).

The provisional results obtained by the EURL/Cypriot study indicate that none of the orally challenged homozygous/heterozygous S146 and D146 allele carriers has so far developed clinical disease or accumulated PrP^{Sc} in either central nervous system (CNS) or lymphoid tissue. These results are in line with those recently reported in orally challenged N146S goats by White et al. (2012). They are potentially compatible with a low susceptibility/high resistance to infection following oral exposure to the pool of Classical scrapie isolates used in the experiment. Alternatively, as reported by Gonzalez et al. (2010; 2009) for some animals carrying methionine at codon 142, and observed in the EURL/Cypriot study (Research area 3), these observations could be the consequence of an efficient infection in the absence of replication in lymphoid tissue before invasion of the CNS and onset of clinical signs (which may happen at a later stage).

Final results from the oral challenge, including long-term observations of the last groups of challenged goats, will be crucial in determining the level of resistance associated with the S146 and D146 alleles. However, the age at challenge over six months of some of the animals belonging to these groups may interfere with the final interpretation of the results.

1.2.3. Management cull experiment

The management cull experiment brought additional data indicating a lower susceptibility to Classical scrapie infection in Cypriot goats carrying the S146 and D146 alleles compared to homozygous N146 goats. Because of the limited frequency of homozygous D146 and S146 and heterozygous S146D animals in the population under investigation, this part of the experiment does not provide elements in relation to these genotypes.

The results obtained are fully consistent with those reported in the 2006/2007 pilot project (EFSA, 2009). A statistical analysis will be needed to compare odds of infection in the different genotype groups.

[6] Results obtained in the framework of the *European goatBSE* project (FOOD-CT-2006-36353). Details on the results are available upon request to the project coordinator (see www.goatbse.eu).

2. EURL/Cypriot study, Research area 2

2.1. Brief overview of the study

The objective of this research area was to study the TSE agent diversity and the resistance/susceptibility associated with the D146 and S146 alleles.

Samples from scrapie affected animals examined between 2005 and 2009 were selected, with the aim to cover all geographical areas in Cyprus where scrapie cases had been confirmed, and thus maximizing the chances to include different TSE strains. Overall, a panel of 400 Cyprus goat isolates with matched fixed and fresh brain tissue were subjected to discriminatory immunohistochemistry, discriminatory Western blot and genotyping. Following scrutiny of the initial results, a panel of samples was chosen for further examination by discriminatory ELISA and a small subset of samples was ultimately selected for characterisation by mouse bioassay (Tg338 and TgShXI).

According to the provisional results of the study the vast majority of cases were biochemically and histopathologically identical and were classified as Classical scrapie cases. Seven cases, which differed in some test parameters, are still undergoing bioassay. More details on the provisional results of the study can be found in the EURL/Cypriot 5th report on progress (see Appendix A).

2.2. Considerations on the provisional results of the study

The EURL/Cypriot study applied state of the art biochemical PrP^{Sc} screening to a large Panel of goat TSE isolates and bioassayed seven isolates that displayed apparently divergent phenotypes in ovine transgenic mice expressing the VRQ (Tg338) and the ARQ (TgShXI) PrP allele. A second passage of the seven selected scrapie isolates in Tg338 and TgShXI would allow a comparison of these isolates with a representative panel of goat isolates from continental Europe that are being characterised, using the same approach, under the framework of the *European goatBSE* and the *EMIDAgoat TSE free* projects[7].

Whereas limitations in the current methodology to study TSE agents diversity preclude concluding that the EURL study describes the whole diversity of TSE agents circulating in Cyprus, this experiment identified several phenotypically different strains. Like in the rest of Europe, this diversity might represent a limitation to the eradication of Classical scrapie through breeding for resistance and to the final interpretation of the experimental inoculations.

As previously indicated (EFSA, 2009), in vitro conversion assay could be considered as an approach to solve this issue. This methodology could investigate the ability of TSE agents to convert PrP^{C} in PrP^{Sc} within goat PrP substrates of different genotypes. For the purpose of this study, it would allow estimating the potential level of resistance associated to the D146 and S146 alleles against the different TSE agents proved to be circulating in Cyprus.

3. EURL/Cypriot study, Research area 3

3.1. Brief overview of the study

The study aimed at describing PrP^{Sc} accumulation patterns in CNS and lymphoreticular system of healthy and scrapie infected wild type (N146N) and heterozygous or homozygous S146 and D146 allele carriers from infected herds in Cyprus.

A total of 1,075 goats aged from 1.1 to 11.2 years (average 3.9 years) originating from 4 herds were culled and tested in the last quarter of 2009. Brain, thoracic spinal cord, distal ileum and lateral retropharyngeal lymph nodes were tested using immunohistochemistry and ELISA.

[7] See www.goatbse.eu and www.emida-era.net

As for the other surveys, in the four herds the N146N genotype was the most frequent one (78.9%) followed by N146S (11.4%) and N146D (9.0%). Very few animals bearing the other genotypes were identified (3 S146D, 3 D146D and 1 S146S goats). All 234 positive cases were of the N146N genotype, except one, which was of an unknown genotype. More details on the provisional results of the study can be found in the EURL/Cypriot 5th report on progress (see Appendix A).

3.2. Considerations on the provisional results of the study

Due to the low frequency of S146 and D146 allele carriers in the goat population, the design of the protocol allowed to study only a very limited number of homozygous S146 (1 goat) and D146 (3 goats) allele carriers. None of the S146 and D146 allele carriers appeared to be infected. Therefore no information is available on the accumulation of PrP^{Sc} in lymphoid tissue of goats carrying these alleles. Nevertheless, the study provided further evidence on the lower susceptibility of heterozygous N146S and N146D goats compared to N146N goats.

The study also documents the occurrence of CNS-positive infected goats in which no deposition of PrP^{Sc} in lymphoid tissue was observed, in line with findings reported in a previous study carried out in the UK (Gonzalez et al., 2010; Gonzalez et al., 2009). As indicated above, these results may (or may not) limit the significance of the absence of PrP^{Sc} in the lymphoreticular system of S146 and D146 allele carriers orally challenged with Classical scrapie for predicting the level of resistance associated to these alleles.

4. EURL/Cypriot study, Research area 4

4.1. Brief overview of the study

A stochastic model was developed to predict the evolution of the frequency of genotypes in male and female Cypriot goats under five different scenarios. Differences between the scenarios included the extent of routine genotyping in the population (genotyping of males only vs genotyping of both males and females), the genotypes of males accepted for use as sires, and the breeding technique employed (natural mating vs artificial insemination).

In all scenarios, the model predicted a rapid increase of the frequency of the desired alleles in the population, with almost a disappearance of the N146 allele from the male population and of homozygous N146N male and female goats within 10 breeding cycles. According to the model, natural mating would be more efficient than artificial insemination, as a consequence of a much lower fertility rate in artificially inseminated goats.

More details on the provisional results of the study can be found in the EURL/Cypriot 5th report on progress (see Appendix A).

4.2. Considerations on the provisional results of the study

The genotyping of the entire Cypriot goat population (see Chapter 1.1) clearly indicated a low frequency of non-N146 carriers. This implies that the removal of N146 allele carriers from the population will require a considerable period of time.

Some of the assumptions of the model may limit the pertinence of its outputs as compared to the real situation:

- The herd structure of the population was not taken into account. The whole population was considered as a single flock, without accounting for the sanitary implications of animals' movements. For example, movement restrictions based on the scrapie herd status could have a considerable impact on the availability of animals carrying favourable genotypes.

- Control of genetic diversity or selection for other health and production traits were not considered. This may be a crucial point because the frequencies of non-N146 carriers (S146S, S146D, D146D) are presently very low, suggesting that non-N146 carriers could be closely related.

- No complementary use of artificial insemination with natural mating was modelled, a situation normally encountered in real conditions. Accounting for such a possibility may change the outcome of the scenarios and their ranking, and may limit the sanitary problems potentially caused by animals' movements.

It must be also emphasized that the scenarios foreseeing selection of females are based on huge efforts in terms of genotyping over many years.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Answer to Term of Reference 1

Are the provisional results of the study supportive of a lower susceptibility to Classical scrapie in goats carrying the D146 and S146 alleles?

- The provisional results obtained in the study further support the lower susceptibility to Classical scrapie in goats carrying the D146 and S146 alleles compared to wild type (N146N) goats.

- The results from intracerebral challenge are not compatible with a level of resistance as high as the one observed in sheep carrying the ARR allele or in goats carrying the K222 allele.

- Final results from the oral challenge will be crucial in determining the level of resistance associated with the D146 and S146 alleles.

Answer to Term of Reference 2

Are the provisional results of the study consistent with the possibility to use this PRNP polymorphism in the future to build a genetic strategy to control and eradicate Classical scrapie in goats in Cyprus?

- The provisional results obtained in the study are compatible with the possibility to use the D146 and S146 alleles to build a genetic strategy to control and eradicate Classical scrapie in goats in Cyprus.

- However:

    - The success of such a strategy will be determined by the level of resistance associated with the D146 and S146 alleles against infection with all the different TSE agents proved to be circulating in Cyprus, which at this stage of the EURL/Cypriot study remains to be definitively assessed.

    - As compared to the results of the model developed in the study, the efficiency of the implementation in the field of a breeding strategy selecting for the D146 and S146 alleles may be lower due to potential practical constraints related to the management of genetic diversity, to the selection for production and health traits and to the need of moving animals for breeding purposes in Cyprus.

RECOMMENDATIONS

- A second passage of the seven selected scrapie isolates in Tg338 and TgShXI would allow a comparison of these isolates with a representative panel of goat isolates from continental Europe that are being characterised using the same approach.

- In vitro conversion assay could be considered as an approach to estimate the potential level of resistance associated to the D146 and S146 alleles against the different TSE agents proved to be circulating in Cyprus.

- The genetic diversity in D146 and S146 allele carriers should be assessed in order to allow the estimation of possible side effects of the selection in the Cypriot goat population, such as excessive inbreeding.

- Inclusion in the model of variables like herd structure, preservation of genetic diversity in the population, combined use of artificial insemination and natural mating, and sanitary constraints linked to movements of animals should be considered.

- The introgression of the K222 allele into the Cypriot goat Damascus breed could be considered as a possible alternative to selection for the D146 and S146 alleles in case this solution was not suitable. The use of genetic molecular markers would allow this process to be completed in about four years.

DOCUMENTATION PROVIDED TO EFSA

1. TSE Resistance in goats in Cyprus - Protocol for additional data collection including experimental challenging studies. 5th Report on progress, May 2012. The TSE European Union Reference Laboratory at the Animal Health and Veterinary Laboratory Agency New Haw, UK and the Government Veterinary Services, Cyprus. Submitted by the European Commission.

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APPENDIX

A. TSE RESISTANCE IN GOATS IN CYPRUS - PROTOCOL FOR ADDITIONAL DATA COLLECTION INCLUDING EXPERIMENTAL CHALLENGING STUDIES. 5TH REPORT ON PROGRESS, MAY 2012.

See next page.

TSE resistance in goats in Cyprus - Protocol for additional data collection including experimental challenging studies

5th Report on progress, May 2012.

Executive Summary

The aim of this project is to provide further data on the genetic resistance to scrapie in goats carrying certain polymorphisms (particularly at codon 146) in the prion protein (PrP) gene with the ultimate aim of using this information to control scrapie in goats in Cyprus, and possibly beyond, similar to the well-established control of scrapie in sheep through genetic selection and controlled breeding (relative to codon 171 in the sheep gene). The experiments are being performed by the Veterinary Services in Cyprus and by the TSE EU Reference Laboratory (EURL) at AHVLA in the UK. This report summarises the progress made to date in the various parts of the project. Details of all data are given in the Annexes.

Central to the project are two inoculation studies in goats of various codon 146 PrP genotypes (Part 1a). The intracerebral (ic) inoculations started in spring 2010 and animals of the susceptible genotype (NN) succumbed to scrapie in summer 2011. Animals of the other, putatively resistant genotypes (ND, NS, SS and DD) also developed clinical disease or showed deposition of PrP^{Sc} in the brain when culled. The ic experiment is now complete, although some examinations are still outstanding. A prominent outcome based on the current examinations is that animals of the NN-genotype showed widespread PrP^{Sc} in the CNS and the lymphoid tissue, while animals with all other examined genotypes showed PrP^{Sc} only in the CNS.

The oral inoculations of goats of the various genotypes also started during 2010. Clinical scrapie has developed in some of the animals of the susceptible genotype (NN), but none of the animals of the putatively resistant genotypes (ND, NS, SS and DD) have developed any indication of successful infection with scrapie so far. Two timed culls of a subset of animals (6 and 12 months post inoculation) have been completed and the examinations showed some deposition of PrP^{Sc} in the lymphoid tissue of the susceptible NN-genotype animals only. A third cull took place 24 months post inoculation in March 2012 and only animals of the NN-genotype showed deposition of PrP^{Sc} in the CNS and the lymphoid tissue, while animals with all other examined genotypes were completely negative.

Two additional lines of study are investigating the susceptibility of goats of various genotypes in the field situation. The 'whole-herd cull' (part 3a) has been finalised, including the diagnosis from lymphoid tissue of the culled animals, and it confirms and strengthens the findings from earlier case-control studies. Four scrapie-affected herds were culled and a total of 1075 goats were genotyped and tested. All positive cases of known genotypes (233) were of the susceptible genotype (NN). The analysis showed that the odds of having the NN genotype among scrapie cases was over 100 times higher than having any other or unknown genotype at codon 146, adjusted by herd and age. The 'management cull' (part 1b), while not finalised, strongly points in the same direction. So far 422 animals, from 37 herds which have lived throughout their productive lives in a scrapie-infected environment, have been culled and tested and all the scrapie positive animals identified (n=109) were of the susceptible NN-genotype.

Another component of this study (part 2a) examined the diversity of the TSE agent in 400 caprine scrapie cases sampled over 5 years and from many different locations in Cyprus. The vast majority of cases were biochemically and histopathologically identical and indistinguishable from classical scrapie. A small subset of cases (n = 7), which differed in one or more criteria, are currently undergoing bioassay in transgenic mice for a more in depth characterization. Preliminary results indicate that two of those isolates differ from the prevalent Cypriot caprine scrapie strain, based on biological phenotype.

The final investigation developed and employed a mathematical model in an attempt to simulate several possible scenarios for a breeding program to reduce the number of fully susceptible NN genotype goats in the Cypriot national goat herd (part 4). The results of the model showed that the most effective program is to genotype all offspring at a young age and use this information to select only the most resistant animals as replacements in a natural breeding programme. Assisted reproduction did not offer a significant advantage with regard to the rate of allele dissemination.

Protocol

Overall Aim of the Study

To provide a protocol for additional data collection based on the EFSA opinion on 'Genetic TSE Resistance in Goats' to progress knowledge about resistance to scrapie in goats in Cyprus.

The protocol represents the final approaches proposed following the discussion between representatives of the Commission, the Cypriot Veterinary Services, the CRL (now EURL) and EFSA in Brussels on 15th September 2009.

This report details the progress made to date, and indicates the timeline necessary for completion of these studies.

Objective 1. Effect of H154, D146 and S146 PRNP alleles on individual susceptibility to classical scrapie infection.

Background

Two types of experiments were undertaken:

1a) Oral and intracerebral challenge of goats homozygous and heterozygous for the alleles of interest

Experimental challenges (intracerebral and oral) are the most rigorous tests for the absolute susceptibility of an animal to TSE. While the intracerebral challenge is the most effective and rapid mode of infection, the oral challenge most likely mimics the natural mode of infection. Goats carrying susceptible and possibly resistant genotypes were challenged with an inoculum prepared from a pool of scrapie-affected brains from Cypriot goats. By determining the survival times, degree of neuroinvasion and peripheral pathogenesis patterns of TSE infection in these genotypes it will be possible to assess the potential value of strategies for breeding for resistance in this population. Data from this study will complement the data collected from the screening of animals from the whole herd cull (see objective 3a) and management cull (Objective 1b), which represent animals with natural field exposure, as proposed below.

1b) Production of animals harbouring the genotypes of interest in a number of affected herds and follow up evaluation of these goats.

The production of animals harbouring the genotypes of interest is in all likelihood unnecessary as in most affected herds there are already a considerable number of animals present with these genotypes. The *PRNP* genotypes of all goats in Cyprus will be determined in a parallel study.
While following up animals with resistant genotypes over several years was not be possible within this study, it was hypothesised that some estimate of true resistance (i.e. absence of PrP^{Sc} as opposed to absence of clinical disease) to scrapie could be obtained from the screening of selected animals (the 'management cull'). The animals examined will be adult animals with homozygous and heterozygous resistant alleles, at least 6 years old, culled for management purposes from herds with confirmed cases of scrapie for at least the lifetime of the selected animals for testing, i.e., 6 years. This will ensure that culled animals have been exposed to scrapie throughout their lifetime. A number of animals with susceptible alleles, matched by age and herd, will be selected as controls and equally sampled and tested.

Results

a) Oral and intracerebral challenge of goats homozygous and heterozygous for the alleles of interest

The programme of work and the results can be divided into three phases.

Phase 1: Sourcing animals, facilities and challenge inocula

Goats of the relevant PrP genotypes were sourced in order that sufficient numbers of kids of appropriate age were available for the planned challenges. Kids were identified by ruminal bolus at around 2 months of age (dependent on the physical size of the kid), and genotyped.
There are no sources of goats in Cyprus which could definitely be considered to be scrapie free. However, there are government herds, and some commercial herds, in which there have been no recorded cases of scrapie recently, and animals to be challenged were purchased from these herds.

For intra-cerebral challenge, animals up to the age of 15 months were used. For oral challenge, the influence of age on susceptibility to scrapie in sheep has been inversely correlated with the regression of gut lymphoid tissue and therefore younger animals (3-6 months of age) were used whenever possible. However, thirty two animals were older (7-12 months of age) as not enough animals of the right genotype and age were available.

Tables 1 and 2 summarise the requirements for goats and infected material for challenge. Thirty-seven goats were required for the intra-cerebral (ic) experiments (table 1 for summary Annexe A1 for individual animal details). For homozygous and heterozygous resistant groups, 8 animals were challenged, with 5 in the non-resistant positive control group. This experimental design allows for the culling of 3 animals from each non-NN challenge group at the point when the positive control (NN) group develop disease. All other animals were allowed to progress to clinical end-point, should it occur.
For the oral challenge experiment 125 goats were used (table 2 for summary, Annexe B1 for individual animal details). Groups of 5 inoculated animals were killed 6 months, 12 months and 24 months after exposure. A last group of animals will be observed until clinical signs are observed or the animals have to be culled for welfare reasons.
Table 1

Intracerebral: 1ml of 10% brain homogenate in saline	
Recipient genotype	Number of goats
D146D	8
S146S	8
S146N	8
D146N	8
N146N	5

Table 2

Oral : 5g brain homogenate				
Recipient genotype	Survival time			
	180 dpi	360 dpi	720 dpi	Clinical End-point
D146D	5*	5	5	5
S146S	5	5	5	5
S146N	5	5	5	5
D146N	5	5	5	5
N146N	5	5	5	5

* 5 extra animals of each genotype (i.e. and additional 25 animals) were challenged to help maintain group sizes in the event of intercurrent disease problems.

For the ic and oral challenges approximately 1000g of brain tissue from field cases of scrapie in Cyprus were used to prepare the inoculum. Brains from 12 positive goats (genotype N146N; all recipient and donor animals in the inoculation experiments were homozygous R at codon 154) were collected to make the inoculum, and sent to the EURL. All the brains were Western blotted, and BSE was excluded. There was some bacterial contamination, so the inoculum intended for ic challenges was treated and re-tested before being sent back to Cyprus in time for the inoculations**.** The inoculum used for these challenges has also been inoculated into Tg338 and tgShpXI mice (see Annexe D7 for full details). This inoculum is considered representative of classical scrapie in Cypriot goats, and will be used as the comparator for 'unusual' cases identified within objective 2, which have also been put into bioassay.

The goats are being maintained for the duration of the experiments in isolation (i.e. fenced in such a way that no direct animal to animal contact is possible between challenged and unchallenged animals, and with segregation of equipment and staff). Buildings at the Government farm at Athalassa have been refurbished by the Cypriot authorities in order to accommodate the animals for this challenge study.

Phase 2: TSE challenge in goats by intracerebral inoculations

In the week from 17-21 May 2010, two staff-members from the EURL, experienced in intracerebral inoculation techniques visited Cyprus in order to carry out the intracerebral inoculations. As laid down in table 1, a total of 38 goats were successfully challenged. All goats were between 12 and 18 months of age and they were inoculated intracerebrally with 1ml of a 10% brain homogenate from the prepared brain pool (see phase 1).

The development of clinical signs was assessed daily by a veterinary officer. A staff member from the EURL, experienced in the clinical assessment process visited Cyprus in Spring 2011 in order teach the veterinary officer in charge the clinical assessment process. As soon as the animals reached an endpoint based on clinical disease, as defined based on observations in naturally-occurring field cases, and taking account of ethical requirements, they were killed. Nervous system, lymphoid tissue, muscle and various other tissues were sampled aseptically. PrP^{Sc} distribution and amounts in those tissues sampled were established using ELISA and IHC.

The individual animal details and test results for these ic challenged animals is shown in the spreadsheet in Annexe A1.

Two animals of the ND group died three months post inoculation from intercurrent diseases and a restricted number of tissues were sampled (obex, medial retropharyngeal lymph node and mesenteric lymph node). All samples were tested by IHC and were found negative. Two further goats (one SS and one DD) died in November 2010, due to intercurrent diseases. Both of them were sampled and tested by IHC and ELISA (TeSeE Biorad rapid test), with negative results.

In May 2011, approximately 12 months post inoculation, the first clinical signs were seen in the NN animals. During June /July 2011 all ND animals developed severe clinical signs and in the meantime some of the DD, NS and SS animals were also affected but with moderate clinical signs. Animals that reached clinical endpoint were culled and tissues were examined. By August 2011 there were no animals alive in the NN (mean incubation period 427d) and the ND group (mean ip 425d) and by mid of November 2011 no animals of the DD group were alive (mean ip 489d). By April 2012, there were no animals alive of the NS (mean ip 564d) and SS group (mean ip 556d).

The results of the biochemical and histological examinations are complex and the examinations and their interpretation are far from finalised. However, a few general statements can be made:

- Immunohistochemistry detected PrP^{Sc} in the brains of all the culled animals irrespective of genotype. The pattern of the IHC labeling was similar in all animals regardless of the genotype, although intra neuronal and intra glial labeling was more prominent in the non-NN animals, and neuropil labeling was much more widespread and intense in the NN group (see Annexe A2)
- Immunohistochemistry detected PrP^{Sc} in lymphoid tissue *only* in animals of the NN-genotype.
- The amounts of PrP^{Sc} detected in the obex by IHC and rapid test do not necessarily correlate with each other. While in NN and ND animals the amounts of PrP^{Sc} detected by IHC were similar, the results from the rapid tests digressed enormously. NN-animals were strongly positive in the rapid test, while ND-animals were either only weakly positive or negative. Further discrepancies between IHC and rapid tests were found in the DD, which were clearly positive in the IHC examinations but negative in rapid tests.
- Western immunoblotting is still underway. Analysis and interpretation will be carried out when all cases are completed.

In conclusion, it currently seems that animals of all examined genotypes can develop scrapie-like clinical signs following intracerebral inoculation and that animals of all genotypes have detectable amounts of PrP^{Sc} in the CNS, with the S allele appearing to slightly prolong the incubation period. The most notable difference between the genotypes is that only animals of the NN-genotype have shown PrP^{Sc} in lymphoid tissue. The biochemistry of the accumulated PrP may also be different (eg more PK sensitive), since it is not detected in IHC using MAb P4. This may also account to some extent for the very low ELISA readings in the positive animals of non-NN genotype. This hypothesis is still to be tested by a PK digestion study which is being planned at the moment.

Phase 3: Scrapie dynamics in orally exposed goats.

On 9th and 10th March 2010 a member of the EURL staff experienced in oral inoculation techniques visited Cyprus in order to perform the oral challenges on the animals available by that time, together with the staff of the Veterinary Services. All animals (weaned goat kids, approximately 3-6 months old) were challenged orally by the administration of 5g of brain homogenate into the oropharynx. In total, 85 goats of different genotypes were inoculated and Cypriot staff were trained in the inoculation methods. The full panel of oral challenges was completed in August 2010. A number of animals died a few days after the inoculation and they have been replaced by new animals which were challenged in the first week of January 2011.

Six months after the oral challenge, 25 animals (5 from each genotype group) were culled and sampled (central nervous system, gut associated lymphoid tissue, secondary lymphoid organs, muscle and blood). All samples were tested by IHC. The brainstems of all 25 animals were also tested by the TeSeE Biorad rapid test ELISA. Only one NN animal, 9 months old, was found positive by IHC, in palatine tonsil and medial retropharyngeal lymph node.

In the 12 month post-inoculation cull, 4/5 NN animals were identified as positive in the lymphoreticular system, with evidence of neuroinvasion (brainstem) in one of the four, identified by both ELISA and immunohistochemistry. The other 21 animals were negative in all examined tissues (a summary of all orally challenged animals culled to date including the results of the examinations of the various tissues is given in Annexe B1).Due to intercurrent diseases three further animals with the genotypes SS, NN and DD were culled and sampled 8, 12 and 16 months, post oral inoculation, respectively. All animals were negative.

In February 2012 (23 months post inoculation) two animals, one NN and one NS, that died were examined. The NN animal was positive in CNS and lymphoid tissue, while the NS was negative.

The 24 month post inoculation cull was performed in March 2012. During this cull 17 animals, 5 of each of the genotypes, NS, ND, 3 of NN and 4 of DD genotypes, were culled and sampled. For the remaining 8 animals (5 SS, 2 NN and 1 DD) the 24 month post inoculation cull will be in August 2012 due to the later inoculation dates for these animals,

The results of the biochemical and histological examinations shown PrP^{Sc} deposition both in CNS and lymphoid tissue only in animals with the susceptible NN-genotype. Four of these animals were showing clinical signs at this time, which gives a mean incubation time for oral challenge in NN as 725d, more than 50% longer than the incubation time in the ic challenged animals of the same genotype. The animals with all other examined genotypes were all negative.

By May 2012 51 animals are still alive in the experimental Government farm of Athalassa : 9 NN, 9 ND, 8 NS, 10 DD and 15 SS.

All individual animal status and test results are given in the spreadsheet in Annexe B1.

The pattern of the IHC labeling in the oral challenge animals resembles that seen in natural infection, withintra-neuronal, intra-glial, punctuate, coalescing or granular and particulate immunolabelling in the neuropil or around neuronal perikarya (see Annexe B2).

1b) Production of animals harbouring the genotypes of interest in a number of affected herds and follow up evaluation of these goats.

The programme of work and the results can be divided into two phases.

Phase 1: Genotyping of the entire Cypriot goat population

The PrP genotyping of the entire Cypriot goat population is co-financed by DG-SANCO in the framework of the national programme of eradication and monitoring of TSE. As its results were valuable and necessary for various parts of this project, the results of the comprehensive genotyping are included here.

During the period of September 2010, and until mid of May 2012 about 318,000 goats were genotyped in three laboratories abroad. The PrP genotyping results are as follows (codons 146 and 154 were determined; the genotypes are given e.g. as NR/NR in case of homozygosity at both codons 146 (N/N) and 154 (R/R)):

Table 4: PrP genotyping results for the national Cypriot goat herd

PrP genotype	2010 – 2012 (until mid May)	
	No of animals	Percentage (%)
NR/NR	207,119	65,12
DR/DR	4,053	1,28
SR/SR	3,776	1,19
DR/SR	4,432	1,40
NR/SR	34,628	10,88
NR/DR	37,073	11,65
OTHER (NR/NH, NH/NH, DR/DH, DR/NH, SR/SH and SR/NH)	26,987	8,48
TOTAL	318,068	100

Phase 2: The 'management cull'

For the 'management cull' an initial list of approximately 50 herds fulfilling the requirement criteria was pre-selected. As per initiative of the Cypriot Veterinary Services, herd owners were contacted and after obtaining their consent, the adult animals selected for management cull, towards a planned total of 2000, were sampled for determination of their genotype at codon 146. The 'management cull' screening has not taken place according to the original timescale. Following genotyping and selection of animals, many owners opted to keep these animals for further breeding to capitalise on their possible lower risk status as indicated by the genotype data.

Since August 2009, samples from 423 goats (obex and retropharyngeal lymph node) from animals of different ages and genotypes have been collected from 37 scrapie infected herds

allocated in four districts (for full details see Annexe C). These samples were tested by IHC and 109 of them were found positive. The 109 positive cases were all homozygous NN at 146 (Table 5). Further testing of samples from 386 of these animals using TeSeE Biorad rapid test ELISA identified only 53 of them as positive.

Table 5: The results of animals tested between August 2009 - April 2012 of the "management cull" per genotype and age. For breakdown of this data by herd of origin, please refer to Annexe C

Age (years)	**No of animals per genotype**																**Total**
	NR/NR		**NR/SR**		**NR/DR**		**DR/DR**		**SR/SR**		**DR/SR**		**NH/NR**		**NH/SR**		
	Neg	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	Pos	**Neg**	Pos	
3	**1**	**0**	2	0	0	0	0	0	0	0	0	0	1	0	0	0	**4**
4	**1**	**1**	2	0	1	0	1	0	0	0	1	0	0	0	0	0	**7**
5	**7**	**5**	1	0	2	0	0	0	0	0	0	0	0	0	0	0	**15**
6	**21**	**18**	11	0	13	0	1	0	0	0	0	0	5	1	1	0	**71**
7	**32**	**26**	13	0	15	0	1	0	0	0	0	0	11	2	1	0	**101**
8	**29**	**29**	19	0	20	0	0	0	0	0	2	0	8	0	0	0	**107**
9	**22**	**16**	12	0	10	0	0	0	1	0	1	0	5	0	1	0	**68**
10	**8**	**7**	1	0	6	0	1	0	0	0	0	0	5	0	1	0	**29**
11	**4**	**3**	3	0	3	0	0	0	0	0	0	0	1	0	0	0	**14**
12	**2**	**1**	1	0	1	0	0	0	0	0	0	0	0	0	0	0	**5**
13	**1**	**0**	0	0	1	0	0	0	0	0	0	0	0	0	0	0	**2**
TOTAL	**128**	**106**	**65**	**0**	**72**	**0**	**4**	**0**	**1**	**0**	**4**	**0**	**6**	**3**	**4**	**0**	**423**

The management cull is still ongoing, and to date all positive animals have been of the susceptible NN genotype. More than 100 animals heterozygous or homozygous for putatively resistant alleles have been screened with no positive results.

Objective 2. TSE agent diversity and resistance/susceptibility associated to H154, D146 and S146 alleles.

Background

2a) characterisation (using biochemistry and bioassay) of a panel of Cypriot TSE isolates

While the available data at the time of the design of this project were not suggestive of heterogeneity of scrapie strains in Cyprus, they were not able to exclude this possibility. It was therefore decided to scrutinize a representative panel of cases using a range of tests. As part of routine surveillance procedures, brain samples from scrapie-affected animals were examined between 2005 and 2009 by discriminatory Western blot, and BSE was excluded for all of them, based on the Western blotting approach detailed in regulation 36/2005. For the purpose of this objective, a selection of cases from this population were sourced from 305 herds and mixed herds and flocks covering all geographical areas in Cyprus where scrapie cases had been confirmed and represented approximately half of all infected herds (e.g. 62.6% of all infected goat herds in 2007 and 42.5% of the all infected herds in 2009). Further material from these cases, included fixed brainstem, was subjected to discriminatory IHC, and repeat WB. The number of cases and the wide geographical distribution of affected herds were intended to maximize the chances of detecting strain diversity. Following scrutiny of the initial results, a panel of samples was chosen for further examination by discriminatory ELISA (as outlined in the regulation) and a small subset of samples was ultimately selected for characterisation by mouse bioassay.

Results

A panel of 400 Cypriot goat TSE isolates with matched fixed and fresh tissue were sourced retrospectively (approximately 280 cases from 2005 to 2009, see Background above) and prospectively. All cases were subjected to discriminatory Western blot (WB), discriminatory immunohistochemistry (IHC) and genotyping (to cover codons 143, 146, 151, 154 and 168 of the PrP gene).

All examined cases were homozygous NN at codon 146 and the large majority of cases (371) showed uniform results in IHC and WB in agreement with classical scrapie in sheep and goats and were consistent with the phenotype observed in NN cases from the whole herd cull (see part 3.a below). These are considered to be ‘typical’ Cypriot caprine scrapie, and have not been investigated further.

Individual test results for the cases selected for further investigation, including the repeat tests, are tabulated in Annexe D1, and are summarised below.

Five cases were considered unusual by either WB and/or IHC. They all differ from the predominant form of Cypriot scrapie and from each other (see Annexes D2 and D3), therefore were selected on this basis to go forward for bioassay (ongoing). They were also subjected to further investigation (see below).

Thirteen cases showed some generalised drop off in the level of P4 labelling with IHC (likely to be attributable to the technical processing) and five of these were randomly selected for further investigation.

An additional group of 11 cases were considered to be scrapie by both WB and IHC but were noted to be heterozygous (RH) at codon 151, and five of these were randomly selected for further investigation.

Two positive cases from the 'whole herd cull' (see also part 3.a) that were considered to be classical scrapie by both WB and IHC were also included with the panel for further investigation, one of which was selected for bioassay (ongoing).

The panel to be investigated further (total 17, plus additional controls) was sent to the CEA laboratory in Paris for testing by the discriminatory ELISA (Simon et al[1]) and was also Western blotted at the EURL using a method to determine if a 14kDa band was present, which, if so, distinguishes CH1641-like scrapie from classical and L-type BSE (Baron et al[2]).

As the discriminatory ELISA gave some unexpected results with certain control cases (Annexe D4), a second panel containing repeat aliquots of anomalous cases plus additional controls was sent for testing (Annexe D5). The controls included five samples from experimentally transmitted BSE in goats (inoculated intracerebrally with bovine classical BSE in the UK). These were also subjected to further WB analysis.

WB images for a representative selection of the uniform samples, together with those selected for further investigation are shown in Annexe D2. Details and raw data for the CEA discriminatory ELISA testing for the 1st panel are attached in Annexe D4, and the second panel in Annexe D5. WB images and details of WB analysis for the experimental BSE in goats are attached in Annexe D6.

The results from the further investigation using the CEA-ELISA appear to indicate that discrimination between bovine BSE, ovine scrapie and experimental BSE in sheep is clear and consistent, but more variability is observed with caprine samples. The WB analysis for experimentally transmitted BSE in goats also indicates some samples showing an increase in the N-terminal antibody (P4) affinity which is generally considered a characteristic for scrapie.
The discriminatory tests have been developed and optimised using predominantly ovine samples to determine cut off values. The CEA-ELISA had use only of a single French goat that was a confirmed as a natural BSE case as a control reference, otherwise caprine BSE analysis is limited to the 5 UK experimental goat BSE cases. At this stage we are unable to determine whether further optimisation of the test parameters would resolve the apparent issues or whether it is a problem inherent with caprine tissues. It is planned to submit this data to the next Strain Typing Expert Groups (STEG) meeting for consideration of the impact of these observations on the robustness of current discriminatory procedures for small ruminant when attempting to characterise caprine isolates.

Further screening of a subset of cases using mass spectroscopy is being considered, but the method has not yet been fully optimised for caprine material.

Currently there are six cases that are undergoing bioassay plus one case from the whole herd cull (part 3.a), which vary in at least one test parameter (the sample from the whole herd cull has given a consistent BSE-like result with the discriminatory CEA-ELISA,) and these assays will be compared with the bioassay data generated using the challenge inoculum from objective 1 (See Annexe D7). In addition, two of the experimental BSE in goat cases are undergoing bioassay (one giving 'intermediate' characteristics by WB and CEA ELISA and the other being consistently BSE-like by both tests). All samples were inoculated in the transgenic mouse lines tg338 and TgshpXI. Some of the bioassays are still ongoing, but the data emerging so far indicates that the isolates form two distinct groups, the majority being similar to the inoculum used for the ic and oral challenges. Two of the isolates (PG52/10 and PG358/10) differ however from the prevalent Cypriot caprine scrapie strain. Immunohistochemical analysis of the mouse tissue is planned, to elucidate which differences reflect possible strain differences, rather than possible titre effects.

[1] Simon et al. (2008) Rapid typing of transmissible spongiform encephalopathy strains with differential ELISA. *Emerging Infectious Diseases;* 14: 608–616.

[2] Baron et al (2008) A C-terminal protease-resistant prion fragment distinguishes ovine 'CH1641-like' scrapie from bovine classical and L-type BSE in ovine transgenic mice. PLoS Pathogens 4;8:e1000137

Objective 3. Effect of D146 and S146 *PRNP* alleles on classical scrapie pathogenesis

Summary

The objective of this study was the systematic assessment of PrP^{Sc} presence in peripheral tissue of clinically healthy and scrapie affected animals from infected herds. This was achieved by looking at the distribution of PrP^{Sc} in all animals over 12 month of age from four affected herds selected for culling due to their high prevalence of scrapie observed in previous years

A total of 1075 goats from the four herds were culled and tested in the last quarter of 2009: 257, 37, 394 and 387, respectively. The average age of the culled population was 3.9 years, with a range from 1.1 to 11.2 years.

Overall in the four herds NN was the most frequent genotype at the 146 codon (78.8%) followed by NS (11.3%), DN (9%). The other genotypes were present at a very low frequency: DS (0.3%), DD (0.3%) and SS (0.1%).

There were 234 positive cases which correspond to an average herd prevalence of 21.7%. The prevalence in each of the four herds was: 28.8%, 56.7%, 20.3% and 15.2%, respectively.

All positive cases were of the NN genotype except one. The only non-NN positive case was of unknown genotype and had the following profile: positive to the rapid test and to IHC in both obex and LRT

The immunolabelling observed in the positive cases was consistent with that of the prevailing field case classical scrapie pathology (data not shown). One case from this cull, initially identified erroneously as heterozygous as codon 146, was included in the panel for further investigation. This case (PG 52/10) subsequently proceeded to bioassay because of its CEA-ELISA signature being BSE-like, and is one of two isolates which behave differently to the rest in transgenic mice.

The odds of having the NN genotype among scrapie cases was over 100 times higher than having any other or unknown genotype at codon 146, adjusted by herd and age (95% CI: 16-866). Positive cases were more likely to occur in goats of, or older than, 27 months than in goats younger than 27 months. There were significant differences in the prevalence of scrapie between the four herds

When assessing the effect of imperfect test sensitivity by the use of Bayesian model, there was no evidence in the data that a significant number of positive animals were missed by the imperfect diagnostic tests. In particular, the model estimated a high sensitivity for the IHC test at the LRT.

The details of this study are given in Annexe E.

Objective 4. Capacity for selection and diffusion of the PRNP allele in the Cypriot goat population.

Summary

This model attempts to represent several possible scenarios for a breeding program to reduce the number of fully susceptible NN genotype goats in the Cypriot national goat herd. The age, breed, sex and genotype structure of the current national goat herd is derived from the results of the current national genotyping program and provide baseline data for the model. Subsequently the model simulates 10 years of breeding and culling within the herd, applying several possible intervention strategies.

The possible interventions involve the genotyping of replacement animals and the use of artificial insemination programs. Genotyping replacement animals involves testing males and possibly females at an age young enough for the genotype to be considered when selecting replacements. The use of artificial insemination provides an opportunity for increasing the numbers of female animals that a male can serve in one season. Since there is a lack of data on the conception rates associated with such programs, a range of possible values have been used.

There are several assumptions in the model that should be considered with the results. With replacement animals it is assumed that any offspring can be considered, however it is unlikely that all animals will have the desired production traits. The impact of this assumption is that there may be an over-estimation in the rate of production of fully resistant animals.

The results of the model show that the most effective program is to genotype all offspring at a young age and use this information to select only the most resistant animals as replacements. The potential drop in production (number of weaned kids per doe) associated with the use of artificial breeding reduces the numbers of offspring to such an extent that it provides little advantage over a natural breeding program. Within all the scenarios NN male animals have almost disappeared from the population within two generations and fully resistant male animals make up the majority of the male population within five or six generations. For the female population the change is slower with most of the NN female animals being eliminated from the population within six generations. The proportions of DD, SS, DS, DN and SN animals is largely dependent on the intervention scenario being applied and the use of AI.

The details of this study are given in Annexe F.

Interim Conclusions

The oral challenge studies, the management cull and the whole herd cull data all support the previously published field observations that on one side the genotype N146N is particularly associated with scrapie susceptibility and on the other side the genotypes N146D, N146S, D146D, D146S and S146S are associated with a degree of resistance.

All genotypes can succumb to challenge by the intracerebral route, but the resulting phenotype is different when comparing the animals carrying genotype N146N with all the others (most notably, no detection of PrP^{Sc} in the periphery of infected animals with all the non-N146N-genotypes).

It is important that the oral challenges are continued to endpoint to establish the relative resistance of other genotypes to challenge by this more natural route

All components of the study reinforce previously published UK caprine data[3] which indicates that the current ELISA rapid test screen has considerably lower sensitivity (approx 50%) than immunohistochemistry.

The wider issue of discriminatory testing for BSE vs scrapie may need to be reviewed (regardless of genotype), since data from these studies suggest that direct extrapolation from ovine data may not be appropriate for all caprine isolates.

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[3] González et al (2009) High prevalence of scrapie in a dairy goat herd: tissue distribution of disease-associated PrP and effect of PRNP genotype and age. Vet Res. 40:65