

## **TSE resistance in goats in Cyprus - Protocol for additional data collection**

### ***Aim***

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.

### ***Background***

Scrapie is a problem for the sheep and goat industry in Cyprus. Control of the disease in sheep through breeding for resistance is well underway in Cyprus, but breeding for TSE resistance in goats is not established as there is limited knowledge about the genetic resistance of goats to scrapie in Cyprus and worldwide. A pilot study was carried out in 2006/07 in goats in Cyprus which addressed the role that specific prion protein gene (PRNP) alleles have in contributing to the absence of clinical scrapie in a animal/herd. This study was taken into consideration by an European Food Safety Authority (EFSA) working group and led to an EFSA opinion, published in March 2009 (The EFSA Journal 995:1-25).

The EFSA opinion concluded that, while the results are encouraging, it was too early to recommend breeding for the 'resistant' PRNP alleles in goats in Cyprus and further research needs to be done. Six areas of further research were identified and experimental approaches were stipulated (see below).

The European Commission's Directorate General for Health and Consumer Protection (DG SANCO) then convened a consultation meeting with EFSA, the Veterinary Services of Cyprus and the TSE Community Reference Laboratory (CRL) and asked the CRL to draft a protocol for short term research over the next 6 to 12 months in order to supplement the initial findings of the Cypriot study. This protocol will then be sent to the EFSA working group for consideration.

### ***Research suggested by the EFSA opinion***

The published opinion of the EFSA working group identified six areas for further research (chapter 4 of the published opinion).

1. Effect of H154, D146 and S146 PRNP alleles on individual susceptibility to classical scrapie infection.
2. TSE agent diversity and resistance/susceptibility associated to H154, D146 and S146 alleles.
3. Effect of H154, D146 and S146 PRNP alleles on classical scrapie pathogenesis
4. Effect of H154, D146 and S146 PRNP alleles on the dynamics of TSE agent transmission in affected flocks
5. PRNP allele selection and adverse effect on production or health traits.
6. Capacity for selection and diffusion of PRNP allele in Cypriot goat population.

The experiments in these six areas are outlined in the Opinion and it is clear that the collection of relevant data would need a research programme of several years duration. Furthermore, neither the experiments in this protocol nor the more elaborate experiments outlined in the Opinion would eliminate all uncertainty of the outcome of a policy that could be based on these findings, i.e. a selective cull of susceptible animals and the breeding of resistant animals. Research findings may only reduce the uncertainty of the outcome of such policies.

In practical terms, the protocol below focuses on the experiments that can be undertaken in a relatively short time frame, i.e. the selection of herds, genotyping of animals, selection of cases for a screening cull, screening of tissues and some modelling exercises. It will not be possible to get data from challenge studies or via flock monitoring protocols, as this would take several years.

## ***Suggested Protocol***

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

Two types of experiments were suggested by the working group.

- a) oral and intracerebral challenge of goats homozygous and heterozygous for the alleles of interest
- b) production of animals harbouring the genotypes of interest in a number of affected herds and follow up evaluation of these goats.

#### *Suggested Protocol:*

re a) The inoculation experiments would take several years to complete and cannot be done in the timeframe of this protocol. A plan for such an experiment could be devised in the framework of the protocol. However, such a plan has to address, amongst other considerations, the important points of the selection of scrapie free animals (breed/genotype/country of origin/ biosecurity of facility in which experiment would be conducted) and the selection of appropriate inocula (there is currently little information on the diversity of natural isolates in Cyprus, or their potential host genotype tropisms). It would only be possible to cover a few of these variables at a practical level, and it should be anticipated that the group sizes would not be considered statistically robust. The experiment itself, if thought to be necessary, would have to happen in parallel and contemporaneously with the practical measures to control scrapie in Cyprus.

re b) The production of animals harbouring the genotypes of interest is in all likelihood unnecessary as it is our understanding that in most affected herds there are already a considerable number of animals present with these genotypes. The genotypes (codons 146 and 154) of all goats in Cyprus is expected to be determined by the end of 2009 provided that the tender will be awarded without any appeals from other tenderers, with the potential partial funding of the European Commission. The genotypes of selected herds will be available by the end of July 2009. While following up animals with resistant genotypes over several years will not be possible within this protocol, some estimate of true resistance (i.e. absence of PrP<sup>Sc</sup> as opposed to absence of clinical disease) to scrapie could be obtained from the screening of selected animals. These 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. For each resistant selected animal in codon 146 a matched by age and herd goat with susceptible alleles will be selected as control and equally sampled and tested.

Such screening will be restricted to the obex and tonsil (see 3. a) for rationale). It is anticipated that 2000 genotyped animals will be available and the average genotype distribution will be for codon 146 (assuming the distribution is similar to the frequencies in the pilot study, see Annex 1): ~ 75% NN, >10% ND, >10% NS, <1% DD, <1% SS, <1%DS. It is expected that around 20 animals will therefore have each of the rare genotypes DD, SS, and DS out of the 2000 genotyped animals. All animals with DD, SS and DS genotypes, as well as all animals with the genotypes NS and ND (over 200 each) make a minimum total of 460 resistant or semi-resistant animals to be examined, plus an equal number of susceptible NN animals matched by age and herd. For the codon 154 (R or H), it is expected that approximately 10% of animals are heterozygous RH and a small amount (0.5 – 1%) will be homozygous HH (see Annex 1). Therefore, assuming the presence among the 460 NN controls of approximately 46 RH and 4 HH animals, the rest of the homozygous HH (10) and heterozygous RH (108) in the remaining NN cull population (1080) will be sampled and tested. Thus the total number of animals to be sampled and tested to cover the resistant and semi-resistant alleles of codons 146 and 154 independently of each other will be 1,038, distributed as follows (assuming the presence of all possible combination of alleles):.

Codon 154: 154 NN/RH, 14 NN/HH and 410 NN/RR

Codon 146: 200 NS/RH-HH-RR, 200 ND/RH-HH-RR, 20 SS/RH-HH-RR, 20 DS/RH-HH-RR and 20 DD/RH-HH-RR (RH-HH-RR referring to codon 154 genotypes).

The number of controls for codon 154 within the 1038 tested animals will be approximately 820 (animals with the genotype RR at codon 154).

It should be emphasised again that the number of animals tested in each of the available genotypes may vary if the allele frequency in the selected herds for testing is different from the one reported by the pilot study in Annex 1.

As H154 does occur, unlike S146 and D146, on P240 and S240, the codon 240 would need to be determined in each animal that harbours H154. In addition, the coupling of H at codon 154 with P or S at codon 240 would need to be determined in all animals that are heterozygous for both codons. This more extensive genetic analysis cannot be done within the time frame of the protocol, but it could be done in subsequent years. Care has to be taken that genomic DNA or fresh-frozen tissue is kept from all animals.

The analysis of obex and tonsil will be done by immunohistochemistry. Brain tissue of animals that are positive in the obex will be examined by Western blot to allow cross reading of the results in this experiment with the findings under 2.

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

The following experiments were suggested in the Opinion:

- a) rational characterisation (using biochemistry and bioassay) of a panel of Cypriot TSE isolates
- b) Experimental transmission of a panel of TSE isolates (incl BSE and atypical scrapie) in goats or transgenic mice harbouring the resistant alleles.
- c) in vitro conversion assays using a panel of TSE isolates.

### *Suggested protocol:*

re a) A panel of Cypriot TSE isolates should be sourced retrospectively and prospectively.

Around 400 samples of scrapie-affected animals were examined between 2005 and 2009 by discriminatory Western blot and a BSE-specific pattern was excluded for all of them. These cases were sourced from 305 herds and mixed 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). Given the lack of information on the number of field strains present in the Cypriot goat population, the prevalence of strains in scrapie-affected herds and the presence of co-infection with multiple strains, it is not possible to estimate the sample size required to detect strain diversity if present at all. However the number of cases and affected herds widely geographically distributed will maximize the chances to detect strain diversity.

While a BSE-specific pattern was excluded for all of them, one unusual case was however identified and is currently being examined by the Strain-typing Expert Group (STEG) of the CRL. A further case may be unusual as well, but this is currently unverified. The remaining results will be scrutinised and some of the Western blots will be repeated, if necessary. Due to the bad quality of the frozen tissue particularly from the early years of submission, it will be necessary to repeat the analysis of approximately 100 samples. It will be possible to replace them with samples from the same or neighbouring herds from the Cypriot archive. Similarly, fixed tissue of all 400 samples can be retrieved from the Cypriot archive, and analysed by IHC methods. It will be necessary to determine the genotype (determine codons 146 and 154) of all cases from the brain material.

Further analysis of some samples might be necessary, should the primary tests (Western blot and IHC) be unusual. The biochemical evaluation of these samples will depend to some extent on the available amount of tissue. We would anticipate that at most 10% of these samples would be subject to

further analysis, such as PK-titration, guanidine melting, conformation-dependent immunoassay (CDI), and the detection of a C-terminal protease resistant prion protein fragment that distinguishes 'CH1641-like' scrapie from BSE (Baron et al., 2008, PLoS Pathog 4(8)).

Unusual cases will be further analysed alongside established Strain-type expert group (STEG) procedures.

Three samples will be inoculated within the time frame of the protocol (the two that have shown some different characteristics, see above, and one 'ordinary' case for comparison). It is proposed to use the transgenic lines tg338 and TgshpXI for this purpose. These lines are sensitive models for classical scrapie and some preliminary data already exists for UK goat isolates. The completion time of such bioassays is entirely dependent on the incubation period of the disease, and would probably not be available until some time in 2010. Ultimately, a representative panel of up to 10 cases could be selected for bioassays. The bioassays, if thought to be necessary, would have to be undertaken in parallel to the practical measures to control scrapie in Cyprus.

Such analyses would provide good background data for optimising the selection of inocula for any proposed challenge/pathogenesis studies undertaken in response to recommendation 1a.

re b) see 1a regarding goat transmissions. It should be added that transgenic mouse models carrying the various goat alleles are not available and would need to be established. The experiments, if thought to be necessary, may happen in parallel to the practical measures to control scrapie in Cyprus.

re c) These assays need to be established and cannot be done in the framework of this protocol. The experiments, if thought to be necessary, may happen in parallel to the practical measures to control scrapie in Cyprus.

### **3. Effect of H154, D146 and S146 PRNP alleles on classical scrapie pathogenesis**

The following experiments were suggested in the opinion:

- a) A systematic assessment of PrP<sup>Sc</sup> presence in peripheral tissue of clinically healthy and scrapie affected animals from infected herds to look at distribution of TSE agent in resistant animals.
- b) A kinetic study of TSE agent dissemination in organs of orally challenged goats, ie. sequential kill and analysis as above.

*Suggested protocol*

re a) Following discussions with the Veterinary Services in Cyprus the following two strategies for the sampling of animals to examine the presence of PrP<sup>Sc</sup> in peripheral tissue are practical within the time frame of the protocol (although there are some logistical dependencies relating to the seasonality of the culls, and the availability of appropriate staff at the right time for sampling).

(i) a cull of all animals in several highly infected herds.

(ii) the management cull of older animals in a number of highly infected herds (this part of the experiment is already described in 1. b)).

(i) According to our information from the Veterinary Services in Cyprus some flock owners may be willing to submit their whole herd for culling, which would enable screening of CNS and peripheral tissue involvement throughout the age and genotype range. The selection criteria for the whole-herd cull will be:

- herds whose owners are willing to submit the herd for culling,
- herds with the largest prevalence of scrapie cases in the last two years,
- herds with minimum herd size of 200.

Once the sampling frame is extracted from the database, a list will be produced by ranking the herds according to the selection criteria. The Cypriot veterinary services will approach the herd owners in the same order to propose the whole cull. The number of herds will depend on how many herds are needed to complete the target of 1000 animals over 6 months of age. It is expected though that the number of herds culled will range between 3 and 7. The convenient sample of 1000 animals will ensure that enough animals with the rare homozygous genotypes in codons 146 and 154 are available. A clinical assessment will be conducted by official veterinarians in order to ascertain the health status of the animal prior to the cull.

The cull will take place in the one operating culling centre already available, which will serve all 5 districts, with sufficient trained staff and disposal capacity. The sampling rate and incineration of carcasses in these centres is 40 animals per centre and day. Thus the cull and sampling of all animals could be carried out in approximately six weeks.

After the cull a systematic assessment of PrP<sup>Sc</sup> distribution will be carried out using IHC and ELISA. Given the scale of such a cull and the time-limits of the protocol we would propose a restricted sample panel consisting of:

- whole brain,
- thoracic spinal cord (to identify the earliest site of neuroinvasion, and inform on pathogenesis),

- distal ileum to indicate possible oral route of infection and involvement of GALT and/or enteric nervous system (again for the purpose of informing on pathogenesis),
- tonsil (or lateral retropharyngeal lymph node – whichever is the most straightforward to sample) – to represent the lymphoreticular system.

We recognise that this is not an exhaustive list of tissues, but it represents a pragmatic approach to the major systems known to be involved in TSE pathogenesis in small ruminants, given the large number of animals we would wish to sample and the limited time available. Thus selection is also based on a personal communication from Martin Groschup regarding the full PrP<sup>Sc</sup> distribution in Cypriot goat scrapie examined within an EU research project (full data not yet available). We propose to multiblock obex and LN together for IHC. Any animal which screened negative would be considered negative, and no further investigation will be done. Any positive animal would be fully tested. Once the genotypes are known (from the national genotyping study) full screening of animals homozygous or heterozygous for one of the resistant alleles (S146, D146 or H154) will also be performed.

The data collected from the whole-herd cull including genotype, age and disease status will be used to estimate the relative risk of infection/disease by genotype and the incubation periods for the different genotypes present in goats using the back-calculation model (Arnold and Wilesmith, 2003, 2004).

re b) see considerations under 1a).

#### **4. Effect of H154, D146 and S146 PRNP alleles on the dynamics of TSE agent transmission in affected flocks**

The following experiments were suggested within the Opinion:

- a) genetic distribution of alleles in full herds (at least 10)
- b) screening of genetic distribution over few years
- c) include TSE evaluation by testing on more tissues or using ante mortem test

Point a) The genotypes of all goats will be determined by the end of December 2009 as mentioned above.

Points b) and c) The screening of genetic distribution over few years would require the follow-up of infected herds with known genotype distribution, so as to detect significant changes over time due to the theoretical impact of the disease on it. In discussions with the Veterinary Services in Cyprus, it was considered impractical to set up such study given the ongoing genotype



programme as in point a) and the uncertain scientific management for a study of this kind over many years:

(i) Since farmers will have access to full details of the genotypes of their animals in the next six months, it is expected that they will intervene soon after to cull animals of susceptible genotypes interfering with the potential natural changes of genotype distribution of the affected herds.

(ii) The TSE evaluation of these herds would require the identification of all diseased and infected animals from the selected herds in the study in order to relate the transmission dynamics within the herds and the changes in genotype distributions. With the current surveillance of scrapie-affected herds in Cyprus that covers the testing of a small proportion of clinical suspects, it would be insufficient to evaluate the TSE status of these herds over time.

(iii) The follow up time necessary to detect significant changes in genotype distribution is beyond the timeframe of this protocol.

## **5. PRNP allele selection and adverse effect on production or health traits.**

The Opinion suggests the consideration of the risk of 'founder' effect' due to the low frequency of potentially resistant alleles.

### *Suggested protocol:*

In the discussion with the Veterinary Services of Cyprus, it became clear that data from government herds could be used to approach the question about the association between genotype and production and health traits.

Production data from two government herds, the Athalassa farm of the Agricultural Research Institute and the Achelia farm of the Department of Agriculture have been collected for more than 20 years and will be made available for analysis. Apart from individual data like ID, sex, date of birth, mating dates, type of mating etc, production parameters recorded include monthly milk yield, dam, sire, fertility and abortion rates, birth weight, weaning weights (49 days), weight at 120 days. However, the genotypes are only known for two breeding seasons in the Athalassa farm with approximately data from 170 genotyped goats and four breeding seasons in the Achelia farm with approximately 350 genotyped goats. These two farms are currently implementing a breeding programme to increase the prevalence of resistant alleles in the female population and produce homozygous resistant billy goats. Although no accurate information could be obtained from the farm managers at short notice, it is expected that the proportion of animals with susceptible genotypes included in the analysis will be sufficient to detect significant differences if they were present.

Raw data will be extracted and collated by the farm management. Further manipulation of the data might be necessary to produce a final dataset for analysis. Appropriate statistical analyses will be conducted and to test

differences by genotypes in production parameters of goats born and raised in the two governmental herds

Irrespective of future breeding programmes, care should be taken to keep a subpopulation with at least one susceptible allele (such as N146). The goats in Cyprus are genetically fairly unique and it might not be possible to restock from somewhere else in case of a genetic problem linked with the resistant alleles.

The Cypriot authorities have indicated that an embryo and semen cryobank is to be established for which additional funding is being sought. The target set is to extract and store 1,500 embryos, 300 from each of the five selected groups in a two-year period: local Machaeras goats, local Akama goats, local fat-tailed sheep, and goats and sheep with susceptible genotypes.

## **6. Capacity for selection and diffusion of PRNP allele in Cypriot goat population.**

The Opinion suggest the following experiments:

- a) Determine the genetic structure of Cypriot goat population.
- b) to get data on feasibility and duration of selection for resistant alleles
- c) to determine capacity of breeding system to support diffusion of resistant alleles by modelling.

### *Suggested Protocol:*

re a)

This will be done separately as a national genotyping programme to be completed by the end of 2009

re b and c)

A metapopulation model of the British sheep industry, based on that used to predict the progress of the GB NSP's Ram Genotyping Scheme (Arnold et al., 2002), will be adapted to the structure of the Cypriot goat population using the genotype data as in point a) and additional input parameters collected during the genotyping of all animals (breed, age and sex), available in the literature and/or elicited through expert opinion. Different breeding strategies will be discussed and agreed with the Cypriot veterinary authorities, including different rates of introduction of resistant males and mating regimes. The output of the model would be the allele prevalence in the total population per year and the changes in the frequency of alleles in the total population over a 10-year period.

While the model can be adapted to the Cypriot goat population in advance, the simulations cannot be carried out until genotype data is available (i.e. earliest Jan 2010). The subsequent interpretation of the preliminary results, further refinement of the model to produce the final results and sensitivity analysis is predicted to require at least 4 months.

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## ANNEX 1

Current knowledge about allele and genotype distribution in the Cypriot goat population (from the report on the pilot project: 'Polymorphisms of caprine PrP gene and their association with resistance or susceptibility to natural scrapie' by Papasavva-Stylianou, 2006/07)

The frequency of the different alleles for the 146 codon was determined for (i) scrapie-free herds (n=9615), (ii) healthy controls in affected flocks (n=219) and (iii) scrapie-negative controls in affected flocks (n=280) in the pilot study during 2006/07. The allele frequencies were 6.9% (i), 5.7% (ii) and 5.9% (iii) for D146 and 7.1% (i), 7.3% (ii) and 6.1% (iii) for S146. The frequency for H154 was only determined in (ii) and (iii) and was 5.9% and 5.1%, respectively.

The genotype frequencies were the following for codon 146:

- (i) 74.1% NN, 11.7% ND, 12%NS, 0.7% DD, 0.6%SS and 0.9% SD
- (ii) 76.2% NN, 9.6% ND, 11.9% NS, 0.5% DD, 0.9% SS and 0.9% SD
- (iii) 78.2% NN, 9.3% ND, 10.4% NS, 1% DD, 0.7% SS and 0.4% SD

The genotype frequencies were the following for codon 154:

- (ii) 88.5% RR, 11.5% RH and 0.5% HH
- (iii) 90.7% RR, 8.2% RH and 1.1% HH

While D146 and S146 occur most probably only on the wild type allele that carries P at codon 240 (P240), the H154 occurs on P240 and the variant S240 in about similar frequency (regarding this parameter the pilot study provides only limited information).