The United States of America (USA) appreciates the Terrestrial Animal Health Standards Commission (TAHSC) decision to reprioritize the revision of Chapter 14.8. Scrapie. Given the mounting scientific evidence that supports new methods to mitigate risk while promoting trade, we encourage WOAH to consider certain additions to the Chapter. These would include the use of genetic resistance and/or live animal testing as valid methods for allowing the safe trade of sheep and goats between countries not recognized as free of scrapie, provided the country has the infrastructure to effectively identify and mitigate scrapie risk.

Current science supports allowing the trade of sheep and goats between countries not free of scrapie under the specific provisions outlined below. The supporting science is summarized in this document. Trade of animals under these provisions would not impact a country’s ability to self-certify freedom provided the country also has a robust animal health infrastructure, an effective scrapie control program and a low prevalence of scrapie:

1. Sheep with resistant genotypes. Specifically:
	* Male and female sheep homozygous for alanine (A) at codon 136 and arginine (R) at codon 171 of the prion protein gene – AA136/RR171
	* Male sheep homozygous for alanine at codon 136 and heterozygous at codon 171 where one allele must be arginine (AA136/QR171; AA136/RH171; AA136/RK171)
2. Goats with genotypes selected for increased resistance to classical scrapie. The importing country could determine acceptable risk based on the scrapie prevalence and the affected genotypes in the goat population of the exporting country.
3. Sheep and goats based on antemortem testing that achieves a predictive value of a negative test that meets the risk tolerance of the importing county as is done for other widespread diseases of concern. The risk of importing scrapie can be reduced to a negligible level by combining a low prevalence in the exporting country with flock/herd level and/or individual animal testing. The importing country could determine acceptable risk based on the country’s scrapie prevalence and other factors.
4. Consideration should be given to allowing the use of genetics and/or live animal testing as methods to qualify an establishment as free from scrapie.

**Summary of Scientific Research**

**Genetic Resistance Proposal:**

1. Allow sheep with resistant genotypes to be traded between countries not recognized free of scrapie. Specifically:
	1. Male and female sheep homozygous for alanine (A) at codon 136 and arginine (R) at codon 171 of the prion protein gene – AA136/RR171
	2. Male sheep homozygous for alanine at codon 136 and heterozygous at codon 171 where one allele must be arginine (AA136/QR171; AA136/RH171; AA136/RK171)
2. Allow the trade of goats with genotypes selected for increased resistance to classical scrapie between countries not recognized as free of scrapie. The importing country could determine acceptable risk based on the scrapie prevalence and the affected genotypes in the goat population of the exporting country.

**Scientific Justification**

Genetics in Sheep

**Background**

Multiple studies have shown that the transmission of classical scrapie to sheep is dependent upon genetically susceptible sheep being exposed to the agent (Westaway 1994; Hunter 1996; Hunter et. al., 1997; Moore 1998). The prion protein gene (*Prnp*) has been shown to be a predominant factor in scrapie susceptibility. Ovine prion protein (PrP) polymorphisms influence susceptibility and play a role in the modulation of incubation period and clinical disease manifestation. Polymorphisms at codons 136, 154 and 171 are linked to susceptibility for classical scrapie in sheep (Goldmann, 2008) with 136 and 171 having the most influence.

The abbreviations associated with the alleles for codons 136, 154 and 171 are as follows:

**Codon 136** codes for either the amino acid valine (V) associated with increased susceptibility or alanine (A) (Goldmann et. al., 1991; Hunter et. al. 1994).

**Codon 154** codes for either histidine (H) or arginine (R) (Goldmann et. al., 1991; Laplanche et. al., 1993).

**Codon 171** codes for glutamine (Q), arginine (R), lysine (K), or histidine (H). Glutamine (Q) is associated with susceptibility and arginine (R) is associated with resistance (Westaway et. al., 1994; Clouscard et. al.1995). The presence of H at 171 is presently thought to be equivalent to Q for scrapie susceptibility. K at 171 has not been fully characterized.

There are 15 different genotypes that have a worldwide distribution. These result from 5 different alleles (ARQ, VRQ, AHQ, ARR, ARH) (Belt et. al., 1995; Goldmann, 2008). Other genotypes have been published but most have a low frequency, and their significance is not fully known.

Arginine at codon 171 of the ovine prion protein gene confers resistance to classical scrapie

The allele A136/R154/R171 is known to confer resistance to strains of classical scrapie, with the homozygote genotype ARR/ ARR being extremely resistant, and the heterozygote ARQ/ARR and AHQ/ARR genotypes being rarely affected by classical scrapie (Westaway et. al., 1994; Goldmann et. al., 1994; Bossers et. al., 1997; Baylis et. al., 2004; González et. al. 2014; Jeffrey et. al.,2014; Lacroux et. al., 2017).

The research to support this claim is summarized below.

1. Transmission Studies

Goldmann and colleagues (1994) challenged groups of Cheviot sheep of various genotypes with SSBP/1 scrapie by the intra-cranial route and others by the sub cutaneous route. They also challenged other groups of Cheviot sheep with CH1641 scrapie. No sheep with genotype AA136/RR171 developed scrapie. The only sheep that were heterozygotes at 171 and were diagnosed with scrapie were sheep that were AV136/AR171 (Goldmann et. al., 1994).

A paper by Jeffrey et. al. (2014) summarized the results of six separate studies that reported results on the susceptibility of ARR/ARR sheep to classical scrapie. A total of ninety-five sheep with genotype ARR/ARR were naturally exposed (n=18) or experimentally challenged (n=77) with either natural or experimental sources of classical scrapie. Eighteen ARR/ARR sheep originating from a closed Suffolk flock with an average scrapie incidence of 84% in ARQ/ARQ (over nine years) showed no evidence of scrapie. They were allowed to survive for over 48 months until 96 months of age and were continually in contact with scrapie infected sheep. A complete necropsy was performed. Tissues including LRS, digestive tract, CNS, peripheral nerves, muscle, and other organs were collected and examined by an immunohistochemical method (IHC) (Jeffrey et. al., 2014).

ARR/ARR sheep that were experimentally challenged by the oral, intra-intestinal and subcutaneous routes showed no evidence of classical scrapie. The oral challenge is detailed below (González et. al., 2014). The study that involved intracerebral (IC) inoculations consisted of 10 Suffolks and 10 Cheviots. The Suffolks were challenged IC at 4 months of age with brain homogenate from ARQ/ARQ naturally infected Suffolk sheep. The Cheviots were challenged at the same age with brain homogenate from VRQ/VRQ Cheviot sheep. One animal from each group was found to have evidence of being infected with classical scrapie. The Suffolk was culled at 72 months post-inoculation (PI) due to other health conditions. Evidence of PrPsc was found in the brain but not in any peripheral tissues. One of the Cheviots displayed signs of a neurological disease and was culled at 75 months PI. PrPsc and histopathological lesions were found in the brain. No PrPsc was found in the LRS and other organs with the exception of the peripheral nervous system. (Jeffrey et. al., 2014)

A study by González et. al. (2014) looked to determine the effect of the Prnp genotype on the role of the lymphoreticular system (LRS) and incubation in the pathogenesis of classical scrapie. One hundred and twelve Cheviot lambs were dosed orally with a brain pool inoculum. The five groups of lambs were genotypes VRQ/ARQ; ARQ/ARQ; VRQ/ARR; ARQ/ARR and ARR/ARR. Tissues were examined by IHC and included multiple LRS, CNS and enteric nervous system (ENS) tissues. None of the ARR/ARR sheep were found to have PrPsc in any of the tissues examined indicating a zero attack rate. The attack rate for the VRQ/ARQ, ARQ/ARQ, VRQ/ARR groups was 100% when only the animals examined later than the first indication of infection in each group was considered in the calculations. The ARQ/ARR group found 9 of 14 inoculated sheep were infected for a 64% attack rate. The detection of PrPsc in the LRS was minimal and inconsistent. The first evidence of PrPsc found in 1 of 3 sheep in the ARQ/ARR group was at 1086 days post inoculation (PI) and was in the LRS and CNS but not the ENS. Clinical signs of scrapie were noted in this group at 2,252 + day PI. The authors noted that an attack rate of 64% in ARQ/ARR animals was not expected. Since natural cases in this genotype in Great Britain are rare, they attributed the high attack rate to the young age at inoculation and the high infectious dose from the brain inoculum (González et. al., 2014).

Several papers from Great Britain provide evidence to support the infrequent finding of scrapie in ARQ/ARR sheep. Only 6 out of 1763 confirmed cases of scrapie in Great Britain between 1998 and 2002 were ARQ/ARR sheep despite the genotype being approximately 30% of the population (Tongue et. al, 2006). In a different study involving 30 flocks with a total of 415 scrapie-confirmed animals, McIntyre et al. (2008) did not find a single case in ARQ/ARR sheep, despite these accounting for 26.5% of the 8,595 animals involved.

Lacroux et. al. (2017) conducted research to explore the ability of the scrapie agent to adapt to an ARR/ARR host. An inoculum of scrapie from an ARQ/ARQ infected sheep was transmitted IC to 5 ARQ/ARQ and 10 ARR/ARR sheep. All the ARQ/ARQ sheep developed signs of scrapie after 329+5 days PI. Four of the ten ARR/ARR sheep exhibited clinical signs with incubation periods that ranged from 2377 to 3011 days PI. Both groups had PrPsc in the brain but only the ARQ/ARQ group had deposits in the LRS. None of the remaining 6 ARR/ARR sheep had any evidence of scrapie by either IHC or Western blot. The second passage inoculum was made from the brain stem of the clinically affected ARR/ARR sheep and was IC inoculated into 5 ARR/ARR sheep. Two of the 5 developed scrapie between 1766 and 2053 days PI. Like the results of the first passage there was no evidence or PrPsc in any lymphoid tissue. (Lacroux et. al., 2017). As pointed out by Lacroux (Lacroux et. al., 2017) and Jeffrey (Jeffrey et. al., 2014), the IC route of transmission is the most efficient and does not reflect routes of natural exposure. Lacroux and colleagues (2017) came to the following conclusion:

“…they [data] support the view that classical scrapie has limited capacity to propagate and to adapt to ARR/ARR sheep. These results strongly reinforce our confidence in the fact that breeding for resistance is a safe and sustainable approach to control and eradicate classical scrapie in affected flocks and in general sheep population.”

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1. Epidemiological Evidence

In addition to transmission studies that provide evidence of genetic resistance associated with ARR/ARR sheep, successful scrapie control programs utilizing these genetics also demonstrate how they reduce and even eliminate the spread of scrapie. This was illustrated by several studies in Great Britain. One such study extracted the total number and genotypes of animals in holdings selected for genotype and cull options in the Compulsory Scrapie Flock Scheme (CSFS) from the National Scrapie Plan data warehouse. Models were used to examine the association between the use of breeding for specific genotypes and scrapie prevalence. An increased proportion of the ARR/ ARR genotype corresponded to a decrease in the number of scrapie cases. Regardless of the management practices, the increased susceptibility that the non-ARR alleles confer on an individual could be extrapolated at the population level. Increasing prevalence of ARR allele reduces the overall risk of scrapie at population level. (Ortiz-Pelaez et al. 2011)

A study in the Netherlands assessed the effectiveness of breeding programs in commercial flocks that used ARR/ARR rams with the intent of controlling classical scrapie at a flock level. The study showed that using these rams not only increased the numbers of resistant sheep but decreased the number of scrapie cases in susceptible sheep.  Flocks that were consistent in the application of the breeding program were able to bring the disease under control in four years or less (Nodelijk et. al. 2011). The scrapie control program in the Netherlands started in 1988 as voluntary breeding program and was made mandatory in 2004. In addition to using ARR/ARR rams, the program consists of active surveillance of sheep at slaughter and fallen stock as well as either culling entire flocks or using a combination of genetics and culling. An analysis of the program found that the level of genetic resistance has increased and the estimated scrapie prevalence level per head of susceptible genotypes is declining significantly which reflects a population effect (Hagenaars et. al., 2010)

The successful use of genetics as part of a national control program can also be demonstrated by showing an increase in resistant genotypes of sheep and a significant decrease in the prevalence of classical scrapie. As an example, the National Scrapie Eradication Program (NSEP) is a cooperative State-Federal-industry program to control and eradiate classical scrapie from the USA. The United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA, APHIS) is the lead agency for the NSEP. The program relies on finding cases of scrapie through surveillance at slaughter and on farms and responding to these detections with a detailed epidemiological investigation which includes determining genotype and affected species. In most cases a genetic based flock cleanup plan is implemented. Indemnity is provided for animals removed as part of a flock plan. The NSEP has sampled more than 731,000 sheep and goats for scrapie since April 2003. In addition, 13,197 slaughter sheep have been genotyped during 2019-2022 and found that over 70% of these sheep were not genetically susceptible, an increase of 10% since the 2002-2003 [Scrapie Ovine Slaughter Surveillance Study](https://www.aphis.usda.gov/animal_health/nahms/sheep/downloads/soss/SOSS_dr_phase2.pdf) (USDA 2003). Since APHIS adopted a genetic based approach to flock clean up, very few flocks have become reinfected. Most of the reinfections occurred when there was still a significant number of scrapie cases in the country and were the result of susceptible sheep being reintroduced to the environment.

The US program has proved to be successful in decreasing the overall prevalence of scrapie. There have been no cases of classical scrapie identified in the US since January 2021. Since this finding over 60,000 sheep and goats have been tested for scrapie with no detections. This is compared to finding 1 in 379 sheep sampled at slaughter being positive for classical scrapie in 2002-2003.

The European Union has also introduced breeding programs for sheep based on the selection of resistant genetics. The intent of the breeding program is to focus on increasing the frequency of the ARR allele in flocks of high genetic merit. Likewise male sheep carrying the VRQ allele should be slaughtered or castrated. There are different levels to the program based the genetic composition of the animals. For example, a Level I flock is composed entirely of ARR/ARR ovines (<https://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX%3A32002D1003>**).** The European Union summary report on surveillance for the presence of transmissible spongiform encephalopathies (TSE) in 2021 reports that, “when looking at the long-term trends of classical scrapie in terms of cases per 10,000 tests, the situation up to 2021 confirmed the 10-year statistically significant decrease in sheep … as estimated by modelling of the available epidemiological data”. The success of individual Member State control programs using the combination of culling and genetics, specifically breeding with ARR/ARR rams has also been documented (Hagenaars et. al., 2010)

As illustrated by the IC transmission studies summarized above, ARR/ARR sheep cannot be considered as fully resistant to classical scrapie. It is recognized that several countries have reported a case of classical scrapie in sheep of genotype ARR/ARR. Germany and France (Groschup et. al. 2007), Japan (Ikeda et. al., 1995), Spain (2019) and Romania (2020) (EFSA 2021) have reported 1 case and Brazil has reported 2 cases (de Souza Leal et. al., 2015). This is an extremely small number given that the European Union and other countries have conducted millions of tests. Further proof can be found in retrospective studies of scrapie-positive sheep showing similar resultsin other countries (Billinis et. al., 2004; Tranulis et.al., 1999).

Although arginine at codon 171 does not confer absolute resistance to classical scrapie, these animals pose a negligible risk of transmitting the disease. To date research has shown ARR/ARR sheep have experimentally been infected only by the IC route, and even when infected, the distribution of the partially protease resistant form of the prion protein has not been shown to be in LRS or other peripheral tissues that would allow for transmission.

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Genetics in Goats

**Background**

Like ovine genetics, the caprine prion protein gene has polymorphisms that have been shown to influence incubation period and susceptibility to classical scrapie. Despite the general similarities, the prion genetics of goats is much more diverse. The caprine has more than 70 polymorphisms that result in amino acid changes documented in different breeds and countries (Vaccari et. al., 2009; Goldman et. al., 2011; EFSA, 2017). However, these polymorphisms do not include the ones at codons 136 and 171 that are found with sheep.

Some of the polymorphisms are associated with scrapie resistance but the degree of such resistance is variable. Others are associated with incubation times.

* 127 – GS, SS – prolonged incubation but susceptibility to infection (Goldmann et. al, 2011; Dassanayake et. al., 2015)
* 142 – IM, MM – incomplete resistance and prolonged incubation (Goldmann et. al. 1996; Barillet et. al. 2009; EFSA, 2017)
* 143 – HR, RR – prolonged incubation and incomplete resistance (Billinis et. al., 2002)
* 145 – DD – Small Italian study showed resistance to oral experimental challenge and then natural exposure (Maestrale et. al., 2015)
* 146 – NS, ND, SS, DD, SD -confers higher resistance (see below)
* 154 – RH, HH – prolonged incubation and incomplete resistance (Barillet et al. 2009; Billinis et al. 2002; Papasavva-Stylianou et al. 2007; Vaccari et al. 2006)
* 211 – RQ, QQ – prolonged incubation and partial resistance which may be strain dependent (Barillet et al. 2009; EFSA, 2017)
* 222 – QK, KK - Confers higher resistance (see below)

[Amino acid codes: D-aspartic acid; G-glycine; H-histidine; I-isoleucine; K-lysine; M-methionine; N-asparagine; Q-glutamine; R- arginine; and S-serine]

**Research Summary for Codons 146 and 222**

A comprehensive review of the literature performed by the European Food Safety Authority Panel on Biological Hazards in 2017 concluded that the K222, D146, and S146 alleles conferred genetic resistance against classical scrapie in European goat populations (EFSA, 2017).

Codon 146

For codon 146, there are limited studies supporting the potential importance of mutations S146 and D146 to scrapie resistance. An oral challenge study demonstrated that no challenged NS146 goats developed scrapie during the experimental period, and all NN146 goats developed scrapie (White et. al., 2012; Cinar et. al., 2018). A study performed in Cyprus cited in the EFSA review(EFSA, 2017) reported results from oral and intracerebral challenge of goats with mutations at codon 146 and goats that were wild-type at codon 146 (NN146). This study found that DD146, NS146, and SS146 goats challenged intracerebrally with scrapie eventually succumbed to scrapie but the incubation period was significantly longer than that in NN146 goats. Following oral challenge, all of the NN146 succumbed to scrapie while none of the SS146, DD146, NS146, or ND146 goats have developed scrapie. Most epidemiological studies found that only NN146 goats were affected with scrapie, while mutants were not affected.(Fragkiadaki et. al., 2011; Papasavva-Stylianou et al. 2007; Ortiz-Pelaez et. al., 2014; Georgiadou et. al., 2017)

One epidemiological study found 5/288 affected goats were NS146 or ND146 while the remainder were NN146, reporting that the risk of scrapie in NN146 goats was about 13 times that of NS146 or ND146 goats (Papasavva-Stylianou et al. 2011).

Codon 222

The importance of codon 222 is supported by several experimental studies in which goat subjects were orally inoculated with scrapie; no challenged QK222 goats developed scrapie during the experimental period, and all QQ222 goats developed scrapie.(Lacroux et.al., 2014; Maestrale et. al., 2015; White et. al., 2012; Cinar et. al., 2018)

One intracerebral challenge study(Acutis et. al., 2012) found that all QK222 goats were protected and all QQ222 goats developed scrapie. However, a second intracerebral challenge study (Lacroux et. al., 2014) found that scrapie did occur in challenged QK222 and KK222 goats, although the incidence was lower than in QQ222 goats and lesions were confined to the brain rather than extending to peripheral lymphoid tissue. In many epidemiological studies, only QQ222 goats were affected with scrapie.(Maestrale et. al., 2015; Acin et. al. 2013; Acutis et. al., 2006; Bouzalas et. al., 2010; Goldmann et. al., 2011; Vaccari et. al., 2006)

Two studies documented a few cases of scrapie in QK222 goats but still found a significantly increased odds of scrapie in QQ222 goats (Barillet et. al., 2009; Corbiere et. al., 2013).

Fragkiadaki et. al., 2011investigated the genetic variability of scrapie affected and non-scrapie affected goats from numerous flocks in Greece. There were 5 QK222 goats identified in this study as scrapie affected. This was not designed as a case-control study, so it was not possible to calculate associations between scrapie resistance and genetic variability. The authors noted that the scrapie affected QK222 goats were from heavily infected flocks and the scrapie strains affecting them were biochemically different from the scrapie strains typically affecting Greek flocks. The potential importance of codon 222 is also supported by the fact that the K mutation in humans at position 219 of the human prion gene (analogous to goat codon 222) is protective against sporadic CJD (Shibuya et. al., 1998). There are little data available about the susceptibility of KK222 goats to scrapie because of the generally low prevalence of the mutation in goat populations so additional research is also needed in this area.

The 2017 EFSA Opinion on the Genetic Resistance to Transmissible Spongiform Encephalopathies (TSE) in Goats provided a ranking of alleles which is based on “weight of evidence” and “strength of resis­tance”. This is, from high to weak scrapie resistance: K222 > D146 = S146 > Q211 = H154 = M142. The As a consequence, both haplotypes 146S/D and 222 K can now be used for TSE resistance breeding and eradication programs for goats in the European Union.

The USA proposes that goats with genotypes selected for increased resistance to classical scrapie be traded between countries not recognized free of scrapie. We do not identify specific genotypes at this time due to the diversity of caprine prion genetic protection. The importing country would determine acceptable risk based on the scrapie prevalence and the affected genotypes in the goat population of the exporting country.

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**Live animal testing for scrapie proposal:**

Sheep and goats based on antemortem testing that achieves a predictive value of a negative test that meets the risk tolerance of the importing county as is done for other widespread diseases of concern. The risk of importing scrapie can be reduced to a negligible level by combining a low prevalence in the exporting country with flock/herd level and/or individual animal testing. The importing country could determine acceptable risk based on the country’s scrapie prevalence and other factors.

**Background**

Early scrapie research in sheep and goats suggested an oral route of infection with initial replication/propagation of the agent in lymphoreticular tissues including tonsil, spleen, retropharyngeal and mesenteric lymph nodes. Usually, replication/propagation continues in the lymphoreticular system (LRS) for months before evidence of infectivity can be detected in the brain. The early investigations used mouse bioassay systems to detect infectivity. (Hadlow et. al. et. al. 1974; Hadlow et. al., 1980; Hadlow et. al., 1982)

Testing by immunohistochemistry (IHC) was validated to detect the partially protease resistant form of the prion protein (PrPsc – scrapie associated prion protein). The finding of PrPsc in CNS and peripheral tissues by IHC showed there was a strong correlation with the detection of infectivity in these tissues, thus allowing for tests that could be done with greater ease and speed (van Keulen et. al. 1995; van Keulen et. al., 1996). Various studies found that PrPsc was found in lymphoreticular tissues months prior to the onset of clinical signs (Heggebo et. al., 2003; Schreuder et. al., 1996). This prompted investigations looking for accessible lymphoid tissue that could be biopsied and tested, thus serving as a preclinical test. Schreuder et. al., 1998 examined the possibility of using tonsil for a preclinical test. In the most susceptible genotypes, they found that PrPsc could be detected at between one third and one half of the expected incubation period which is more than a year before the onset of clinical signs (Schreuder et. al., 1998).

**Scientific Justification for Live Animal Tests**

The logistics involved with performing tonsil biopsies limited the ability to use these as a field application. Discovery that PrPsc was also deposited in the lymphoid tissue associated with the nictitating membrane (3rd eyelid) and rectoanal mucosa-associated lymphoid tissue (RAMALT) led to the validation of scrapie tests using these tissues (O’Rourke et. al., 1998; O’Rourke et. al., 2000; González et. al. 2005; González et. al. 2006). Studies using rectoanal mucosa-associated lymphoid tissue, and nictitating membrane lymphoid tissue as a live animal test for scrapie have demonstrated sensitivities of 85 to over 90% in sheep(Dennis et. al., 2009; González et. al., 2006) and 41.7 to 50% in goats(González et. al., 2009; Konold et. al., 2020). When serial sampling was conducted on goats during the incubation period, the percentage of detections by RAMALT biopsy rose to 77% (Mammadova et. al., 2020). A study by González et. al. (2008) in sheep showed that the sensitivity could be increased by increasing the number of follicles evaluated, with the probability of a false negative result being less than 5% for 34 follicles, less than 10% for samples with at least 10 follicles, and 28% for samples containing only two follicles.

Third eyelid tissue (89.4%) has been shown to result in comparable sensitivities to RAMALT (85.3-89.4%), as shown in a study by Dennis et. al. (2009) but was contingent upon collecting third eyelid tissues from both eyes in order to obtain enough follicles for evaluation. Third eyelid biopsies might be a viable alternative to RAMALT, with sensitivities as high as 85-90%(O’Rourke et. al., 2002) but often requires analysis of additional sections of tissue to have sufficient follicles for a valid test (Monleón et. al., 2011).

In two studies by González et. al*.* where both tonsil and RAMALT were evaluated, sensitivities for each test were not significantly different (89.2% vs 86%, respectively), and the mean number of follicles in RAMALT (35; 95% CI) was significantly higher than in the tonsil (11.4; 95% CI) (González et. al., 2006; González et. al., 2008). A Norwegian study also compared RAMALT and tonsil samples and found the proportion of positive follicles was similar (Espenes et. al 2006).

In goats, few studies have evaluated the efficiency of using any particular tissue (RAMALT, palatine tonsil, or 3rd eyelid). PrPsc was detected in samples using RAMALT and third eyelid in a study by Acinet. al. (2013) but details were not provided on test sensitivity. González et. al. (2009) reported 41.7% sensitivity for RAMALT, and 52.6% for tonsil. In that same study, the frequency of PrPsc detection rose to 84% during the preclinical disease when there was widespread lymphoreticular involvement (González et. al., 2009). In a study that included both sheep and goats, Monleón et. al. (2011) reported sensitivity results for RAMALT at 36% (and up to 48% with retesting), as well as up to 44% sensitivity in 3rd eyelid with retesting; the results for goats alone could not be determined.

Another study designed specifically to evaluate antemortem diagnostic techniques in goats naturally affected with scrapie monitored 28 goats (11 does and 17 kids) for 30 months. The goats were moved to a research facility from a scrapie infected herd. A series of 5 RAMALT biopsies were taken on each animal and tested by IHC and RT-QuIC. At the end of the 30 months, nine of the 28 animals were determined to be scrapie positive based on the detection of PrPsc in the obex, tonsil, and/or the retropharyngeal lymph node. “In the study, of the nine goats that were PrPsc positive by IHC at the end of the observation period, seven goats (77%) had at least one positive antemortem RAMALT biopsy before the onset of clinical signs. The results indicated that antemortem rectal biopsy demonstrated PrPSc in 100% of scrapie-positive offspring born to positive dams, but in only 50% of positive offspring born to negative dams possibly due to a delayed disease process resulting from a difference in the route of transmission. Three out of these seven scrapie-positive goats had positive antemortem RAMALT biopsies ~21 months before the onset of clinical signs (~58% of the incubation period). Two out of seven scrapie-positive goats had positive antemortem RAMALT biopsies at ~21 and ~23% of their incubation period”. The two positive offspring from the negative dams had positive RAMALT tests with detection only by RT-QuIC (Mammadova et. al., 2020).

 Work by Heggebo *et. al*. (2003) concluded that although abnormal prion proteins could be detected in experimentally infected sheep as early as 5 weeks post inoculation (PI), the findings were limited to Peyer’s patches, mesenteric lymph nodes, tonsil, and retropharyngeal lymph node from animals of the most susceptible genotypes (VV136/QQ171). The abnormal prion protein could not be detected in all lymphoid tissues until 11 months of age. Further, the majority of data available regarding sensitivity of biopsies for preclinical scrapie is restricted to animals aged 12-14 months or older. (O’Rourke et. al. 2002; Caplazi et. al. 2004; Monleón et.al. 2011)

The study by González et. al. (2006) indicated that a significantly higher proportion of PrPsc positive follicles could be detected in rectal mucosa in older compared with younger sheep, suggesting a minimum age for lymphoid tissue biopsy of 14 months. If flock level testing is used, the USA recommends that at least 6 follicles should be evaluated for sheep, based on two recent studies where at least 80% of the sheep tested had at least 6 follicles in the biopsy, with a resulting sensitivity of 85-90% (O’Rourke et. al., 2002; Dennis et. al., 2009). This provides an acceptable sensitivity and a reasonable level of retests required to achieve a negative result in a flock setting.

Live animal testing can be very useful and has been incorporated into some national control programs. It must be noted that there are limitations that should be considered but can be managed. An accurate test relies on the ability to get a sufficient number of follicles. In addition, genotype appears to restrict the peripheral deposition of PrPsc (Espenes et. al., 2006; González et. al., 2006; Greenlee et. al., 2014; Rechzeh et. al., 2007).

**Negative Predictive Value of a Live Animal Test**

The purpose of testing live animals intended for export is to reduce the risk of exporting an animal infected with scrapie. Example calculations for rectal biopsies used for export are performed below. Sensitivity of the rectal biopsy increases as the number of follicles evaluated per biopsy increases. Sensitivity of a rectal biopsy evaluating 6 follicles will be considered to be 87%, whereas the sensitivity of a rectal biopsy evaluating at least 34 follicles will be considered to be 95%. If a positive animal intended for export is individually tested for scrapie with a rectal biopsy evaluating at least 34 follicles, there is already a low risk of 1 - 0.95 or 5% chance that the animal will test negative without consideration of any other population factors. When live animal testing is utilized only in animals over 14 months of age with no known scrapie exposure from a low prevalence country or *zone,* this low risk is reduced even further as shown by the following illustrations. These examples are based on USDA National Agricultural Statistical Services (NASS) and scrapie sheep data from 2012 and 2013\* and are intended to demonstrate how various testing protocols can mitigate the risk of mistakenly exporting an animal infected with scrapie from a low prevalence country or *zone.*

The negative predictive value (NPV) of an individual test takes into account the prevalence of the disease in the population and provides the probability that the animal tested is negative (not infected) given a test result that is negative in the particular population being considered. This example demonstrates the calculation of the negative predictive value of the use of a test with 95% sensitivity on a live animal in a population with an estimated national scrapie prevalence of 0.02 %, such as the U.S. in 2012-2013:

$$NPV= \frac{specificity\* (1-scrapie prevalence)}{[1-\left(test sensitivity\*scrapie prevalence\right)]}$$

$$NPV= \frac{1\* (1-0.0002)}{[1-\left(0.95\*0.0002\right)]}$$

 NPV of an individual test = 99.999%

This high negative predictive value indicates that when the scrapie prevalence of the population is considered, the probability of exporting an animal that individually tested negative and was actually infected is 1 – 99.999%, or 0.001%, which is lower than one would assume if only the sensitivity of the test was considered.

The above example demonstrates how testing a single live animal from a country or zone with relatively low prevalence of scrapie can achieve a high negative predictive value by itself, meeting the threshold NPV of 99.999% stated in the suggested changes to the Scrapie Code Chapter. However, in cases where an even higher negative predictive value is desired, or where the prevalence of scrapie in the country or zone is higher than in the above example, the predictive value of the test can be even further improved by considering clustering of the disease in the population. The previous calculation of NPV assumed a random distribution of infected animals in the country. However, we know that there is typically clustering of scrapie in countries and zones in that not all flocks are infected and not all animals are infected within affected flocks. If one was to mistakenly export an animal infected with scrapie, one would first have to select an animal for export from a flock that was infected, and then select one of the animals within that flock that was infected. Then, a test of that positive animal would have to be returned negative incorrectly. To account for clustering by flock in calculations of probability, additional information is needed: the probability that a flock is infected, prevalence within infected flocks, the average size of infected flocks, the number of sheep over a year of age in the country, and the number of flocks in the country. From this you can estimate: the probability that a flock will be infected, the probability that an infected animal within the infected flock will be selected for export and testing, and the probability that an infected animal will not be detected by the test, thus calculating the overall system probability that an infected animal from an infected flock would be selected for export and tested with a false negative finding.

Consider the following illustration, again using 2012/2013 USDA data.\*

Probability of selecting a sheep for export from an infected flock

= 0.003

Probability of selecting an infected sheep in an infected flock

3 / 99 = 0.0303 = 3.03%

X

Probability of selecting an infected flock and also selecting an infected sheep within that flock

= 9.13 x 10-5

Probability of a false negative result from a rectal biopsy evaluating at least 34 follicles

= 1 - 0.95 = 0.05

X

Probability of selecting an infected flock and also selecting an infected sheep within that flock AND a false negative test = 4.57 x 10-6

Thus, when considering the export of an individual sheep from a low prevalence population not homogenously infected with scrapie, the overall probability of 1) the selected sheep being from an infected flock 2) selecting a positive animal from within that flock and then 3) not detecting it as positive with an individual test is 0.000457%.

This low risk of exporting a positive animal due to a false negative test could be even further mitigated by performing targeted testing of other animals in the flock. Assume that the animal intended for export was born and spent its entire life in a flock of 100 sheep, of which 40 were genetically susceptible to scrapie. If the flock was infected with scrapie, 3 of those 40 animals would be expected to be scrapie infected. If any of these sheep were detected as scrapie infected, then this would block the exportation of any animal from the flock. If the animal selected for export from the other 60 animals was scrapie infected, this would be very unexpected and it would be unlikely that more than 1 of these 60 animals would be infected. Therefore, selecting an infected animal from that group would represent a 1/60 chance. Assuming that the animal intended for export was still tested with a rectal biopsy evaluating 34 follicles with a sensitivity of 95%, and each of the 40 susceptible sheep was tested with the less sensitive rectal biopsy evaluating 6 follicles (sensitivity 87%; probability of false negative for each test of 13%), the following calculation would provide the probability of mistakenly exporting an infected sheep.

Probability of a false negative result from a rectal biopsy evaluating at least 34 follicles

= 1 - 0.95 = 0.05

Probability of selecting an animal in the less susceptible group for export that is infected = 1/60 = 0.017

X

Probability of not detecting an infected animal in the group of 40 susceptible if all are tested with 6 follicle rectal biopsy = 0.13 \* 0.13 \* 0.13 = 0.0022

Probability of selecting infected sheep AND false negative test = 0.00083

X

Probability of infection of flock

= 0.003

Probability of failure of flock test AND selection of infected sheep and failure of individual test = 1.83 x 10-6

X

Probability of exporting an infected sheep if flock testing and individual testing are performed = 5.5 x 10-9 or 0.00000055%

Thus, assuming that overall prevalence of scrapie is low in the country, individual testing of the animal to be exported can be combined with targeted flock testing to reduce the risk of exporting a positive animal to an extremely low level.

\*2012/2013 USDA NASS and scrapie data and derivations:

U.S. scrapie prevalence = 0.02%

# sheep > 1 year of age in U.S. = 2,967,908

# sheep flocks in U.S. = 65,690

# sheep/infected flock = 99

Scrapie prevalence in infected flock = 2.63%

# infected sheep/infected flock = 99 \* 0.0263 = 2.60 = 3 sheep

# of scrapie infected sheep in U.S. = 2,967,908 \* 0.0002 = 594

# infected flocks in U.S. = 594/3 = 198

Prevalence of infected flocks in U.S. = 198/65,690 = 0.003

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