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(alphabetical)

Polymorphisms in prion protein of Icelandic sheep and their susceptibility to classical scrapie

relying on extensive case control studies
and
Protein Misfolding Cyclic Amplification (PMCA) tests

Summary and recommendations

State of science: 6th of November 2023

0. Introduction

- scrapie since 150 years in Northern Iceland
- some farmers detected inheritability of susceptibility -> targeted breeding for resistance
- in the 90s, polymorphisms in prion protein found in European countries, connected to susceptibility/resistance to classical scrapie
- culling only removes the infected animals, but not necessarily the scrapie agent, so breeding for resistance is the best solution
- three classical codons 136, 154, 171; ARR/ARR as solution for the classical scrapie problem in the EU
- Iceland 1999: about 1000 sheep sequenced – found: wild type, V136, T137, N138, C151 and H154, but no R171[1]
- next 4 to 5 years about 3000 additional sheep sequenced, no R171 -> search stopped and only codons 136 and 154 genotyped
- since 1986 always culling of the whole flock
- only measure connected to breeding: reducing V136
- H154 seemed to be protective; single farmers started to breed for it (e.g. near Varmahlíð)
- some decreasing of scrapie cases were followed by increasing again from 2015 in Northern Iceland
- after 5 outbreaks in Skagafjörður in autumn 2020 search for new solutions began
- extensive research project with sheep farmers and scrapie experts from Germany, Italy, France, UK and Spain, besides Iceland
- first steps financed by sheep farmers association, later steps within the context of the Cofund ERA-NETs ICRAD (Classical scrapie in Iceland, a model for prion diseases worldwide, „ScIce“, 325-06.01-2823ERA31D)
- priority: finding new resistant genotypes due to lack of R171; it was then found unexpectedly but only on one farm
- new approach: not relying on R171 only but search for new resistant or protective polymorphisms in order to maintain the unique genetic diversity of Icelandic sheep breed and avoid inbreeding issues
- in Italy important researches in that direction when heterozygous T137 was detected as protective by chance in infection experiments with scrapie and BSE[2]
- then followed by case control studies in 5 flocks with over 7000 sheep and by PMCA tests[3]
- all main results of these Icelandic research which began in spring 2021, are available now and will be reported here:
    1. large scale sequencing/genotyping

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[1] Thorgeirsdottir S, Sigurdarson S, Thorisson HM, Georgsson G, Palsdottir A. PrP gene polymorphism and natural scrapie in Icelandic sheep. J Gen Virol. 1999 Sep;80 (Pt 9):2527-2534. doi: 10.1099/0022-1317-80-9-2527. PMID: 10501510

[2] Vaccari G, D'Agostino C, Nonno R, et al. Prion protein alleles showing a protective effect on the susceptibility of sheep to scrapie and bovine spongiform encephalopathy. J Virol. 2007;81(13):7306-7309. doi:10.1128/JVI.02880-06

[3] Vaccari G, Scavia G, Sala M, et al. Protective effect of the AT137RQ and ARQK176 PrP allele against classical scrapie in Sarda breed sheep. Vet Res. 2009;40(3):19. doi:10.1051/vetres/2009002;
Bucalossi C, Cosseddu G, D'Agostino C, et al. Assessment of the genetic susceptibility of sheep to scrapie by protein misfolding cyclic amplification and comparison with experimental scrapie transmission studies. J Virol. 2011;85(16):8386-8392. doi:10.1128/JVI.00241-11

2. PMCA tests with all main genotypes and 10 different Icelandic scrapie isolates
3. case control studies of 14 Icelandic scrapie flocks between 1997 and 2023 with nearly 4000 sheep

- due to congruence of these results, clear possible recommendations regarding:
  - breeding goals in Iceland
  - regulations about sheep transport within and between scrapie zones/areas
  - measures in case of scrapie outbreaks
- research is nevertheless ongoing, especially the ScIce project
- and frequency/distribution of scrapie outbreaks and affected genotypes next years -> new information
- therefore important to update goals/regulations continuously according to new knowlegde

To make reading easier, the following variants are written like that:
$V_{136}RQ$ = V136 or VRQ
$AT_{137}RQ$ = T137
$AN_{138}RQ$ = N138
$AC_{151}RQ$ = C151
$AH_{154}Q$ = H154 or AHQ
ARR_{171} = R171 or ARR

Not numbered codons are A_{136}, R_{154}, Q_{171}.

1. Current situation about polymorphisms in Iceland – sequencing/genotyping

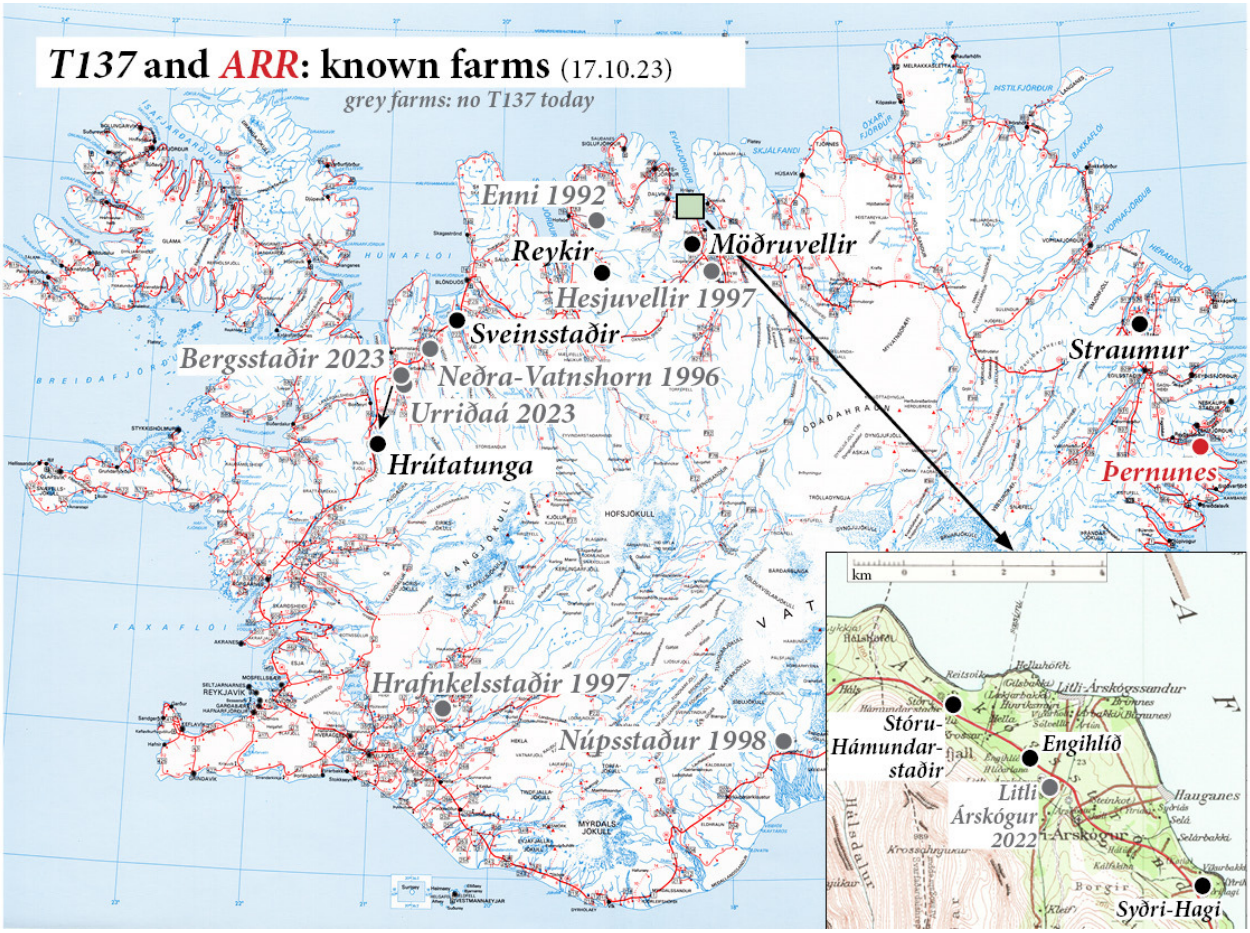
1.1 Sequencing – the basics

- first step: sequencing the current sheep population in Iceland
- aims: Are same polymorphisms found now as in 1999? Is T137 still existing? Can R171 be found with extended search?
- due to that, the results do not necessarily reflect a representative picture of the current population as a whole (this was not the main aim)
- participants (alphabetical): Eyþór Einarsson, Gesine Lühken, Karólína Elísabetardóttir, Stefanía Þorgeirsdóttir, Vilhjálmur Svansson
- flocks for sequencing selected, e.g.:
    - scrapie in the flock in the old days but disappeared
    - never scrapie at this farm in spite of scrapie all around
    - connection to (scrapie negative) sheep from scrapie farm
    - "traditionally" scrapie free area within a scrapie protection zone
    - kind of old fashioned flock, few inseminations/bought sheep
    - in comparison: much insemination/bought sheep
    - four horned sheep/"four polled" sheep
    - leadersheep
    - areas not affected by extensive culling due to other diseases (e.g. Mæði-Visna) – especially Eastern Iceland
    - complete scrapie flocks: Stóru-Akrar (2020), Vatnshóll (2021)
    - about 240 scrapie positive samples from Keldur sample bank originating from 45 different flocks/cases where classical scrapie was detected since year 2000
- farmers didn´t have to pay; they got the results for every sheep, summary/explanations
- sequenced altogether:
    - 4279 sheep
    - from 162 flocks
    - in all parts of Iceland
- Results:
    - wild type ARQ
    - V136
    - T137 (Sveinsstaðir, Straumur)
    - N138
    - C151
    - H154
    - R171 (Þernunes)
    - R231R+L237L (silent mutation)

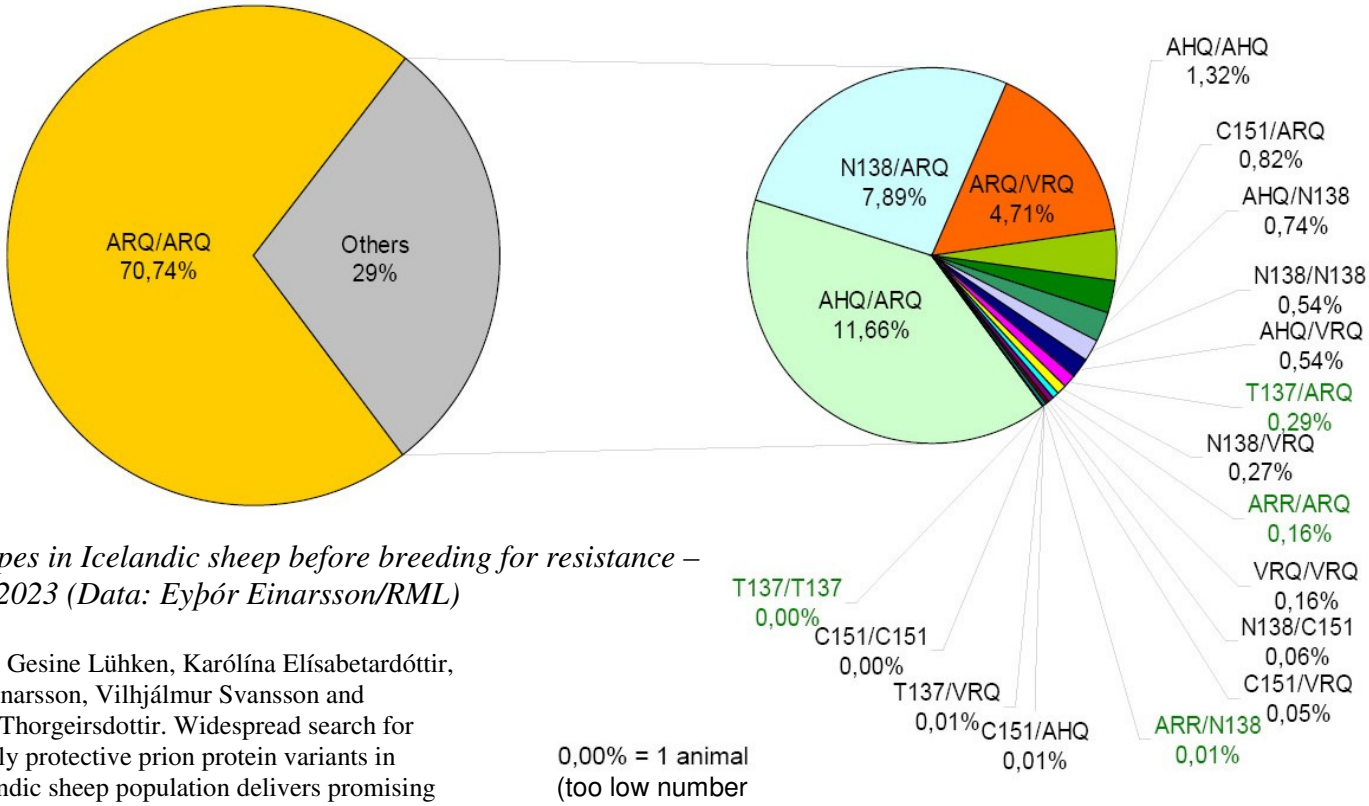
1.2 Six-codon-genotyping developed

- to save money, the German lab Agrobiogen was asked to develop 6-codon-genotyping (codons 136, 137, 138, 151, 154, 171)
- do not alter the gene product (prion protein), therefore they were not determined with this method
- extensive grants to farmers for genotyping -> very good participation
- from spring 2022 to summer 2023 about 35.000 sheep genotyped
- more unrelated farms with T137 found: Stóru-Hámundarstaðir (first ram), Syðri-Hagi, Engihlíð, Litli-Árskógur, Reykir, Möðruvellir

- with genotyping of the most recent scrapie flocks, Bergsstaðir and Syðri-Urriðaá, was T137 also found there (in summer 2023), and in connection with that in living animals



(data: Stefanía Þorgeirsdóttir, map: Karólína Elísabetardóttir, unpublished)



Genotypes in Icelandic sheep before breeding for resistance – Spring 2023 (Data: Eyþór Einarsson/RML)

See also: Gesine Lühken, Karólína Elísabetardóttir, Eyþór Einarsson, Vilhjálmur Svansson and Stefanía Thorgeirsdottir. Widespread search for potentially protective prion protein variants in the Icelandic sheep population delivers promising

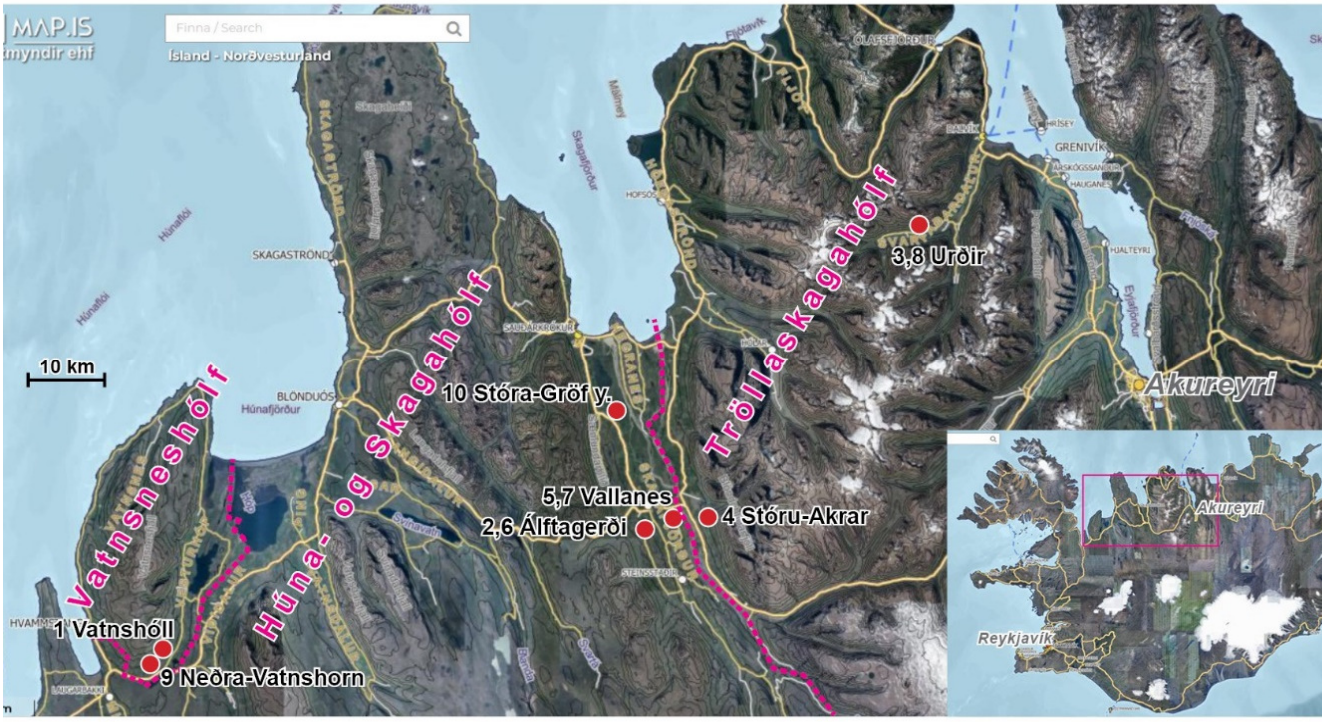
2. How susceptible/protective are the polymorphisms/genotypes in Iceland?

2.1 PMCA tests

- PMCA tests were performed at INRAE in France by Vincent Béringue, initiated by Karólína and Eyþór, with finances from the Icelandic sheep farmers association in cooperation with RML and then Cofund ERA-NETs ICRAD (Classical scrapie in Iceland, a model for prion diseases worldwide, „ScIce“, 325-06.01-2823ERA31D)
- INRAE Staff involved in the tests: Vincent Béringue, Angélique Igel, Fabienne Reine, Laetitia Herzog, Mohammed Moudjou, Pierre Sibille.
- protein misfolding cyclic amplification (PMCA) assay mimicks host PrP conversion process into prions within a short time. In essence, prion seeds (dilutions of scrapie infected brain homogenates) are mixed with healthy brain substrate containing normal PrP^C and submitted to cycles of sonication / incubation. Successive rounds with refreshening of the substrate can be performed to increase the sensitivity of the technique. The presence of prions in the PMCA amplification products is tested by western blotting.
- Several scrapie strains (5+) are circulating in scrapie infected flocks in Europe, albeit in variable proportions[4]. Co-infection can occur in the same infected animal.
- strains can prefer and adapt to certain genotypes over time
- by using isolates (“brain pieces”) from different scrapie cases, both from different farms and from same farm, but different outbreaks, it is, among others, possible to see differences between isolates/strains regarding susceptibility of certain genotypes
- (healthy) brain substrates of different genotypes gained mainly from Icelandic sheep (sampling at Keldur under strict conditions), but samples of rare genotypes in Iceland came from Swiss (Torsten Seuberlich, Bern) and Germany (Holger Thoms, Enniger)
- Positive scrapie isolates (both clinical and non clinical samples) from sample archive at Keldur; Christine Fast, Greifswald, prepared them for PMCA
- Italian isolate (used for the mentioned susceptibility researches there) came by Romolo Nonno, Rome
- Other islates and VRQ- or ARR-adapted ovine strains and working by PMCA from INRAE
- Following genotypes were tested:
    - T137/T137 (Swiss)
    - T137/ARQ (Iceland)
    - T137/H154 (Swiss)
    - T137/ARR (Swiss)
    - N138/N138 (Iceland)
    - N138/ARQ (Iceland)
    - N138/H154 (Iceland)
    - C151/C151 (Iceland)
    - C151/ARQ (Iceland)
    - C151/H154 (Iceland)
    - H154/H154 (Iceland)
    - R171/ARQ (Germany)
    - R171/H154 (Swiss)
- resistant reference sample for comparison:
    - R171/R171 (Germany)
- susceptible reference samples for comparison:

[4] Igel et al. 2022; DOI: 10.1007/s00441-022-03700-2

    - V136/V136 (Iceland)
    - ARQ/ARQ (Iceland)
- Negative control: brain from transgenic mice knock-outed for normal mouse PrP^{C} (will inform on any residual PrP^{Sc} signal in the PMCA product)
- Following isolates were used, host genotype all ARQ/ARQ if not marked different (see map below – *Karólína Elísabetardóttir, unpublished*):
    - Vatnshóll 1999 – VRQ/ARQ
    - Álftagerði 2008
    - Álftagerði 2019
    - Urðir 2017
    - Urðir 2017 –VRQ/ARQ
    - Stóru-Akrar 2020
    - Vallanes 2007
    - Vallanes 2018
    - Neðra-Vatnshorn 2015
    - Stóra-Gröf Ytri 2016
    - in progress: Bergsstaðir 2023 (latest scrapie outbreak in different zone)
    - in progress: Italy
    - in progress: $T2^{OV}$ (ARR-adapted strain) and 127S (*fast* scrapie strain)

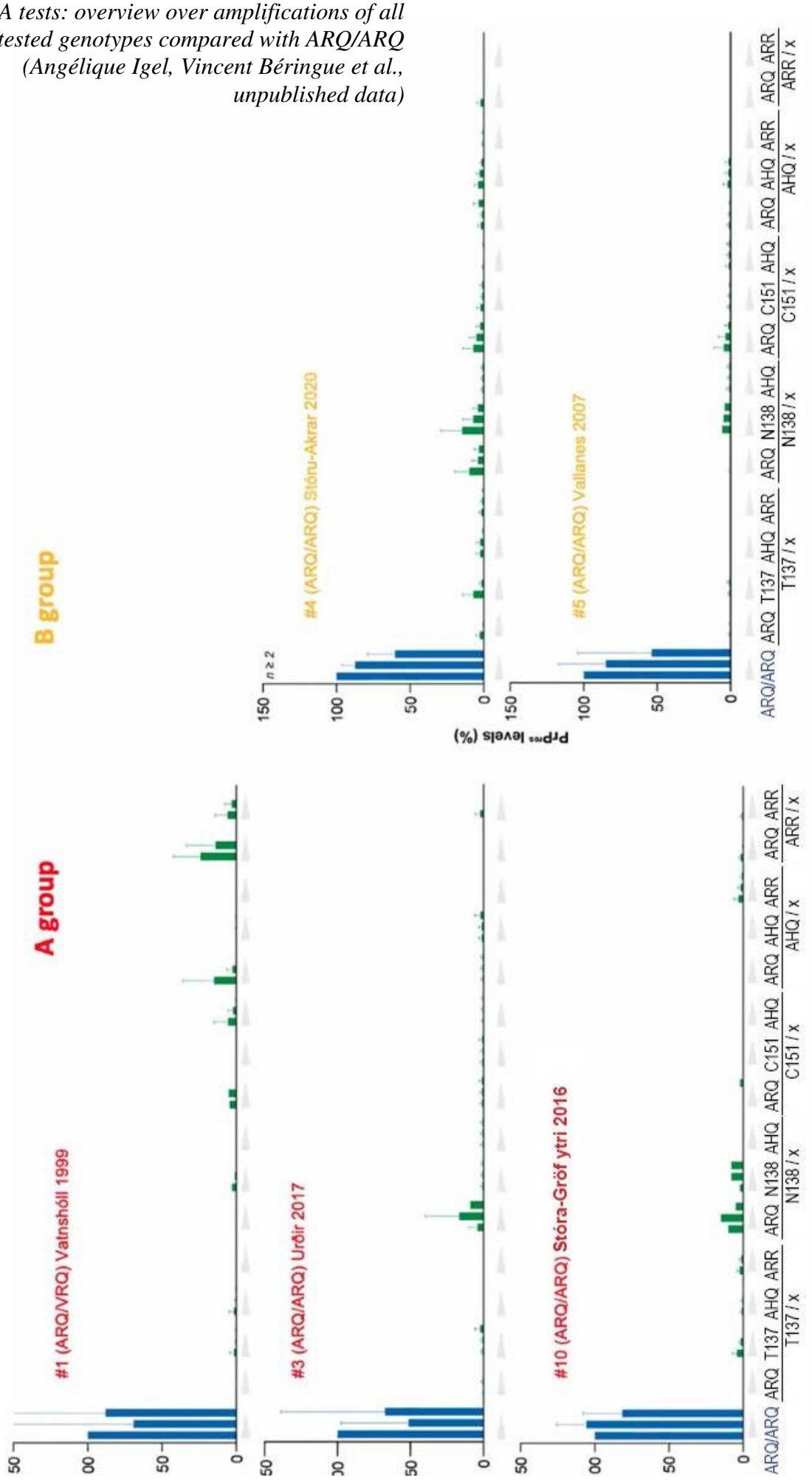


- all substrates/genotypes tested with all isolates, at least in three independent triplicates for the scrapie samples that show the highest levels of PMCA activity (runs); every run contains four rounds; this standard ensures statistically relevant results and prevents false results
- Serial 1:3 dilutions of each isolates are tested in each PMCA run to better quantify the inihibitory effects of the genotypes compared to VRQ/VRQ or ARQ/ARQ
- Western Blot after every run in order to check amplification (typical three bands) and, if so, how strong (the darker, the more)
- in background is often seen light grey band pattern ("residual signal") independent from amplification, therefore for comparison always included substrates from known

susceptible genotypes (VRQ/VRQ and ARQ/ARQ) *(positive control)*, from known resistant genotypes (ARR/ARR) and from PrP-knock-out mice *(negative control)*

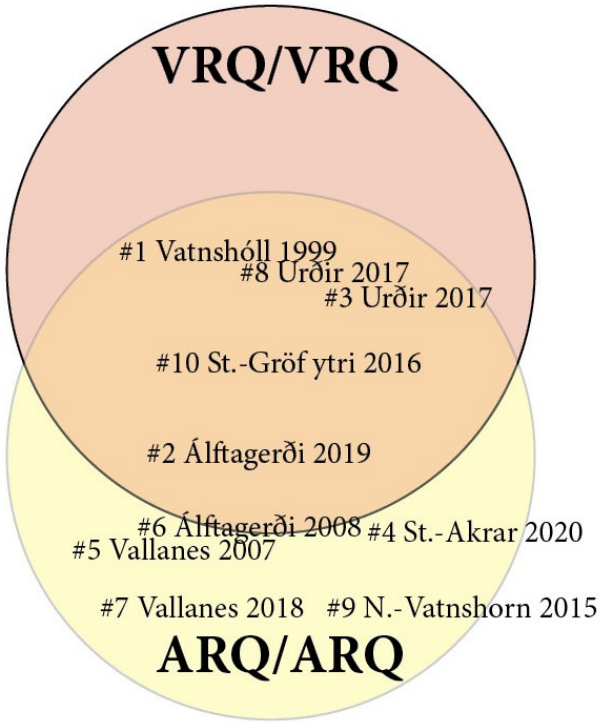
- genotypes have strong protective effect for PrP conversion at the molecular level if they
    - don´t amplify or
    - only to a very little extent and at lowest dilution
- on next page, diagrams of the five isolates which gave clearest results due to the fact that they contain the highest levels of seeding activity (e.g. from clinically sick animals). Experiments were performed in triplicates to quintuplicates (3 times to 5 times)
- columns show the extent of amplification for every single dilution of three
- important: if amplification only in one or two of three dilutions (= one or two columns visible, but not three), there is likely low/limited amplification, particularly as compared with ARQ/ARQ (blue columns on the left), which was used as baseline for quantitation (at the lowest dilution).

PMCA tests: overview over amplifications of all tested genotypes compared with ARQ/ARQ (Angélique Igel, Vincent Béringue et al., unpublished data)



- in short words ...
    - ... have all combinations with T137, N138, C151 and H154 (AHQ) clear protective effect against prion conversion which seems to be in the range of R171 (ARR)
    - this effect is already achieved in heterozygous form with ARQ
    - however, N138/ARQ and N138/N138 seem to be to a certain extent less protective than the other genotypes
- in order to see the strength of protective effect better, comparison of these in vitro results with results of case control studies from real Icelandic scrapie flocks (see next chapter 2.2)
- clear difference between isolates, some obviously amplifying indifferently VRQ/VRQ and ARQ/ARQ or preferring VRQ/VRQ, while others amplify much better ARQ, see next picture. This suggests the possibillity of two different types of strain circulating in the infected flocks

*Classification of the isolates according to PMCA reactivity with VRQ ("**A group**") or/and ARQ ("**B group**") (Vincent Béringue, unpublished)*



2.2 Comparison of genotypes in scrapie flocks: case control studies (CCS)

- protective effects of genotypes observed in vitro/at the molecular level (here: PMCA tests) need to be corroborated by epidemiological data from real scrapie flocks: genotypes of positive animals compared with genotypes of negative animals in the same scrapie flock (case control studies – here "CCS")
- important to have data from Icelandic scrapie cases, although first preliminary results of analysing strains suggest that the Icelandic strains might be similar to those in Middle and Southern Europe
- positive scrapie samples from all registered scrapie outbreaks stored at Keldur from 1957, first in paraffin blocks (not well suitable for genotyping), from 1997 frozen
- in some cases also negative samples
- very valuable because data from only 2 or 3 flocks would possibly lead to false results
- example: if only would have been looked at Stóru-Akrar 2020, Vatnshóll 2021 and Bergsstaðir 2023, result would have been that VRQ is protective – only ARQ/ARQ got infected in spite of animals with ARQ/VRQ or VRQ/VRQ existing in the flocks
- to ensure a realistic picture as much as possible, following criteria with selection of flocks:
    - flocks from different years since 1997
    - different protection zones
    - "undisturbed" development of disease – best if diagnozed because of symptoms; no "secondary" flocks with only one positive animal coming from the original outbreak farm (false results due to long incubation time)
- bigger flocks better than small (less coincidence influence)
- samples of the whole flock or at least the main part, especially if random sampling
- high prevalence (about 10% or more) or high number of positive animals (in big flocks – in spite of perhaps lower prevalence)
- 2 different outbreaks at same farm, if available
- as many different genotypes as possible (susceptibility of genotype which does not appear in flock, cannot be estimated)
- included were:
    - 14 flocks (see Annex, **table 1**)
    - from 5 protection zones, but 11 different geographical areas
    - 3989 sheep, including 327 scrapie positive animals (WB/IHC), 19 different genotypes altogether, all Icelandic variants included, except R171
- every flock was analysed separately, but mainly all flocks together in order a) to get animal numbers which allow statistical significance and b) to have as broadest base as possible (see annex, **table 3**)
- finally analysed four flocks which matched best regarding the criteria from table 1; results were very similar to them of all the 14 – strong hint that these flocks together are representative for Icelandic reality
- analysing by alleles (see annex, **table 2**) leads to these main results:
    - differences in allele frequencies between positive and negative sheep were statistically significant for V136 (VRQ), N138, C151 and H154 compared to wild type (ARQ)
    - that means: likeliness for N138, C151 and H154 to get infected is lower than for ARQ and VRQ
    - too low numbers with T137 for significancy (and no numbers for R171)
- analysing by genotypes (see annex, **table 3**) leads to these results:

- differences in genotype frequencies between positive and negative sheep were statistically significant for:
  VRQ/VRQ
  VRQ/ARQ
  N138/VRQ
  N138/ARQ
  C151/ARQ
  H154/ARQ
  compared to wild type (ARQ/ARQ)
- OR (Odd´s Ratio) allows to estimate likeliness to get infected, compared to ARQ/ARQ
  VRQ/VRQ: 22,4 times **more** likely to get infected
  VRQ/ARQ: 4,5 times **more** likely
  N138/VRQ: 3,4 times **more** likely
  C151/VRQ: 1,2 times **more** likely
  N138/ARQ: 4 times **less** likely (OR = 0,24)
  H154/ARQ: 33 times **less** likely (OR = 0,03)
- if OR is 0, no positive animal with this genotype had been found:
  all combinations with T137
  all combinations with C151 and H154 (except the above mentioned)
  all combinations with neither ARQ nor VRQ

- important: when estimating susceptibility of animals, genotypes are relevant, not alleles (every animal has 2 alleles)
- too low numbers of T137 and therefore not significant, but in Italy CCS with highly significant results (when T137 frequency in flock 10–30%), also is T137 scoring very good in PMCA (with Icelandic isolates)
- ARQ and even more VRQ as "allele combination partner" increase susceptibility of the animal, even if the other allele is protective
- this is even true when in association with the protective allele ARR, animals with ARR/ARQ and ARR/VRQ can get infected, but these animals are not positive in their lymph nodes; these semi-resistant genotypes with no replication in lymph nodes are less prone to spread the disease, even in the rare cases in which they become infected[5]
- therefore, the group of 88 animals without ARQ or VRQ allele was analysed separately: no one positive, result highly significant

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[5] *Justin Greenlee et al.: Lack of prion accumulation in lymphoid tissues of PRNP ARQ/ARR sheep intracranially inoculated with the agent of scrapie, PLOS one 2014. "Immunoreactivity for PrPSc in lymphoid tissue was consistently abundant in VRQ/VRQ, present but confined to tonsil or retropharyngeal lymph node in 4/5 VRQ/ARR, and totally absent in ARQ/ARR sheep. The results of this study demonstrate the susceptibility of sheep with the ARQ/ARR genotype to scrapie by the intracranial inoculation route with PrPSc accumulation in CNS tissues, but prolonged incubation times and lack of PrPSc in lymphoid tissue."*

Justin Greenlee et al.: Oral inoculation of neonatal Suffolk sheep with the agent of classical scrapie results in PrP(Sc) accumulation in sheep with the PRNP ARQ/ARQ but not the ARQ/ARR genotype, Research in veterinary science, 2016. "Results of this study suggest that ARQ/ARR sheep are resistant to oral infection with the scrapie isolate used even during the neonatal period."

Remark: In the UK, between 2002 and 2013 had been found (by passive surveillance) 3 sheep with ARR/ARQ with classical scrapie in spite of very high frequency of this genotype in the population, but 56 with ARR/VRQ;by fallen stock: 0 and 10; by active surveillance:1 and 39.

2.3 Lymph nodes: T137, N138

- participation of lymph nodes/periphal tissues crucial for the question whether the animal is likely of being infected when in a scrapie-infected flock and whether it could spread the disease (see Justin Greenlee et al., p. 12)
- in Italy, there had been analysed several peripheral tissues in sheep inoculated by the oral route with classical scrapie: sheep carrying T137 have been always found negative in lymphoid tissues (as well as in CNS and any other tissue analysed by Western Blotting)
- at Brúsastaðir/Snæringsstaðir (Vatnsdalur, scrapie 2002), 58% of the flock or 28 sheep carried the N138 allele (Thorgeirsdottir et al., unpublished data); only one of them had been tested positive in the brain, but 10 of them had been tested positive in the lymph nodes by immunohistochemistry (IHC) (Georgsson et al., 2008[6]), all in association with ARQ or VRQ (no one with homozygous N138). Details see Annex, **table 4**.
- T137 is a strongly protective polymorphism in Italy and the above results from PMCA and CCS strongly indicate that it has a similar effect in Iceland although so far no lymphoid tissue has been analysed from T137-animals from an Icelandic scrapie flock
- N138: as lymph nodes were detected positive in the above case, this suggests an only partially protective effect compared to the wild type and V136, at least in heterozygosis (in agreement with PMCA and CSS)
- there are no lymph nodes available from animals carrying C151 or AHQ in Icelandic scrapie flocks

[6] *Georgsson, G., Adolfsdottir, J. A., Palsdottir, A., Jorundsson, E., Sigurdarson, S., & Thorgeirsdottir, S. (2008). High incidence of subclinical infection of lymphoid tissues in scrapie-affected sheep flocks. Arch Virol, 153(4), 637-644. https://doi.org/10.1007/s00705-008-0035-8*

2.4 Summary of results of PMCA and CCS

- the results of these two studies are supporting each other and no inconsistency appeared
- this fact is a strong hint that results represent Icelandic reality -> possible to build breeding goals, regulations regarding transport/trade and measures in scrapie outbreaks on them
- they are in short words:
    - genotype without ARQ or VRQ is strongly protective – this is true for all combinations of T137, N138, C151, H154 or R171
    - protective influence appears already in combinations with ARQ
    - N138/ARQ, possibly also N138/N138 (according to PMCA tests, although no animal with N138/N138 had been diagnozed with scrapie so far) seems to be somehow less protective than the other genotypes, but nevertheless several times less likely to get infected than ARQ/ARQ

2.5 Continuous monitoring by ongoing or planned research will provide important information

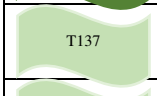
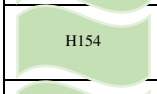
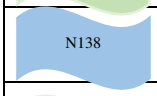
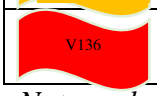
- **intensive monitoring of new scrapie flocks** – at the same time rapidly increasing frequency of T137, N138, C151 and R171 which allows continous evaluation of their protective effect even when no longer rare (statistically better quality):
    - as before: all scrapie positive animals will be 6-codon-genotyped
    - new: all animals of the flock will be 6-codon-genotyped if scrapie is confirmed, before decisions about culling is done
    - new: all dead/slaughtered animals (genotypes known) > 18 months will be scrapie tested in brain and lymph nodes for several years
- **PMCA** tests with isolates from Bergsstaðir and Italy ongoing:
    - as the Bergsstaðir cases are clinical, they might have strong seeding activity by PMCA and thus are likely to provide relevant information regarding the potential genotypes of interest; it will also inform on whether the same types of strain are circulating in Iceland over the last years
    - the Italian sheep scrapie sample is tested to provide a strain of reference and see whether the same is circulating in Iceland; if no or only slight difference to Italian isolate, it would be possible to use results from older Italian researches with PMCA and link with T137 even more
- transmission studies in the ICRAD ScIce project to **identify the strains** circulating in Iceland (to check presence of CH1641/LA19K strain or other strains which are not amplifiable in PMCA) and further **model (by transgenic mice)** the interest of **T137**
- other in vitro assay **(RT-quIC)** to comfort the PMCA results within the ICRAD ScIce project
- **infection experiments** with Icelandic isolates and Icelandic sheep of different genotypes (at least i.c., better oral) would provide additional important ~~key~~ data and would support the ongoing breeding program by a controlled experimental infection with highly infectious material

Manuscript for a scientific paper including the presented results (PCMA tests and CCS) is in preparation.

3. Recommendations for the near future

3.1 Categorizing alleles and genotypes in Iceland by coloured flags according to established and potential resistance to classical scrapie

- next step: easy colour system for farmers and veterinarians, also for use in the national sheep data and pedigree system Fjárvís
- following suggestion, based on results from PMCA, CCS and lymph node analysis above – **continously updated** when there are new/additional results:

Flagg	Haplotype	Status
	ARR	Resistant (internationally accepted)
	AT_{137}RQ	Likely resistant in Italy (published*) and Iceland (new research results)
	AHQ	Likely resistant in Iceland (new research results and published epidemiological data**)
	AC_{151}RQ	Likely resistant in Iceland (new research results)
	AN_{138}RQ	Less susceptible than ARQ (new research results)
	ARQ	Wild type – susceptible (internationally accepted)
	VRQ	Highly susceptible (internationally accepted)

Not numbered letters are codons 136, 154, 171.

** Gabriele Vaccari, Romolo Nonno et al.: Prion Protein Alleles Showing a Protective Effect on the Susceptibility of Sheep to Scrapie and BSE, Journal of Virology 2007*
Gabriele Vaccari, Romolo Nonno et al.: Protective effect of the AT137RQ and ARQK176 PrP alleles against classical scrapie in Sarda breed sheep, Veterinary Research 2009
Cecilia Bucalossi, Romolo Nonno et al: Assessment of the Genetic Susceptibility of Sheep to Scrapie by PMCA and Comparison with Experimental Scrapie Transmission Studies, Journal of Virology 2011
***Stefanía Þorgeirsdóttir et al.: Search for healthy carriers of scrapie [...], Archives of Virology 2002*

- the difference between dark green and light green is the international acceptance and wealth of data including absence of transmission to ARR animals experimentally, while protection might be the same according knowlegde nowadays
- **light green** compared to **blue**: Odd´s Ratio in association with wild type < 0,05 AND very good result in PMCA = light green;
  Odd´s Ratio in association with wild type < 0,5 AND equivalent results in PMCA = blue (given that lymph nodes are positive in infected animals, not only the brain)

3.2 Transport of *live lambs* between flocks within and between scrapie areas; keeping or culling in scrapie cases

The most important practical aspects of the categorization are:

- **transport** – especially of live lambs to begin with – **from scrapie free flocks** which are situated in scrapie zones to other flocks in scrapie zones; it is not recommended to allow transport from flocks with scrapie outbreaks during the last 7 years (in analogy to the categorization of MAST for “high risk” scrapie areas)
- **keeping instead of culling** in case of scrapie in the flock

Here, genotypes (2 alleles) are important instead of alleles only because every animal has 2 alleles and each of them has influence on the susceptibility of the animal. According to the above results and categories in 3.1, the following categorization should be applied – in analogy to the EU-regulations; "yes" means transport allowed and keeping of the animal in case of scrapie outbreak:

Genotype	**transport allowed; animal kept, not culled**
	yes
	yes
	yes
	yes
	no
	yes
	yes
	yes
	no
	no
	no
	no
	no
	no
	no

As next step the regulation regarding **adult sheep** should be revised for

- flocks which did not have new scrapie cases last 20 years
- in areas without scrapie the last 7 years –

according to EU regulations where there is no difference made regarding age (and the deadline is max 5 years, often shorter – which might be too short).

Annex

Overview: Variants

(Stefanía Þorgeirsdóttir, Vilhjálmur Svansson, unpublished data)

	1999[7]		**2023**	
allele	*number*	*%*	*number*	*%*
ARQ	742	81,18	47.182	83,43
VRQ (V136)	82	8,97	1666	2,95
T137	8	0,88	86	0,15
N138	40	4,38	2847	5,03
C151	7	0,77	267	0,47
AHQ (H154)	35	3,83	4454	7,88
ARR (R171)	0	0,00	50	0,09
	914	**100**	**56.552**	**100**

[7] *Thorgeirsdottir S, Sigurdarson S, Thorisson HM, Georgsson G, Palsdottir A. PrP gene polymorphism and natural scrapie in Icelandic sheep. J Gen Virol. 1999 Sep;80 (Pt 9):2527-2534. doi: 10.1099/0022-1317-80-9-2527. PMID: 10501510*

Table 1: CCS – overview over flocks *(unpublished data)*

	different genotypes	whole flock/rand.
S-Skörðugil 2021 551 samples *prevalence 6,7%*	7	yes
Grófargil 2020 106 samples *prevalence 3,8%*	9	yes
Álftagerði 2019 349 samples *prevalence 6,0%*	6	yes
Vallanes 2018 325 samples *prevalence 4,0%*	11	yes
Bergsstaðir 2023 669 samples *prevalence 7,8%*	13	yes
Vatnshóll 2021 747 samples *prevalence 2,3%*	10	yes
Stóru-Akrar 2020 402 samples *prevalence 11,2%*	7	yes
Hesjuvellir 1997 64 samples *prevalence 42,2%*	14	yes
Urðir 2017 108 samples *prevalence 8,3%*	11	yes
Hurðarbak 2010 129 samples *prevalence 3,9%*	5	yes
Álftagerði 2008 281 samples *prevalence 21,0%*	7	no
Syðri-Völlur 2006 57 samples *prevalence 26,3%*	6	yes
Forsæludalur 1998 105 samples *prevalence 17,1%*	5	no
Ingvarir 1998 96 samples *prevalence 5,2%*	6	yes

Table 2: CCS: Alleles of all 14 flocks; Fisher´s exact test

(Eva Hauksdóttir, Stefanía Þorgeirsdóttir, unpublished data)

Farm 1-14							Fisher´s exact test
Codon	Amino acid in codon	Pos	Neg	Allele	Pos	Neg	P
136	A/A	221	3425	A	533	7079	<0.0001
	V/A	91	229	V	121	245	
	V/V	15	8	Total:	654	7324	
137	M/M	327	3651	M	654	7313	>0.9999
	M/T	0	11	T	0	11	
	T/T	0	0	Total:	654	7324	
138	S/S	317	3288	S	644	6924	<0.0001
	S/N	10	348	N	10	400	
	N/N	0	26	Total:	654	7324	
151	R/R	326	3511	R	653	7128	<0.0001
	R/C	1	106	C	1	196	
	C/C	0	45	Total:	654	7324	
154	R/R	326	3273	R	653	6925	<0.0001
	R/H	1	379	H	1	399	
	H/H	0	10	Total:	654	7324	
171	Q/Q	327	3662	Q	654	7324	>0.9999
	Q/R	0	0	R	0	0	
	R/R	0	0	Total:	654	7324	

Table 3: CCS – Genotypes of all 14 flocks (summary); OR, Fisher´s exact test and Pearson´s Chi square test

(Gesine Lühken, Karólína Elísabetardóttir, unpublished data)

Allar hjarðir		ARQ/ARQ	ARQ/VRQ	VRQ/VRQ	T137/ARQ	T137/VRQ	T137/N138	T137/AHQ	N138/ARQ	N138/VRQ	N138/AHQ	N138/C151	N138/N138	C151/ARQ	C151/AHQ	C151/VRQ	C151/C151	AHQ/ARQ	AHQ/VRQ	AHQ/AHQ	
3662	neikv	2551	229	8	7	1	1	2	295	14	25	13	26	73	10	10	1	355	31	10	3662
327	jákv	214	86	15	0	0	0	0	6	4	0	0	0	0	0	1	0	1	0	0	327
3989	samt.	2765	315	23	7	1	1	2	301	18	25	13	26	73	10	11	1	356	31	10	3989
Allar hjarðir, %		ARQ/ARQ	ARQ/VRQ	VRQ/VRQ	T137/ARQ	T137/VRQ	T137/N138	T137/AHQ	N138/ARQ	N138/VRQ	N138/AHQ	N138/C151	N138/N138	C151/ARQ	C151/AHQ	C151/VRQ	C151/C151	AHQ/ARQ	AHQ/VRQ	AHQ/AHQ	
minna	neikv	69,7%	6,3%	0,2%	0,2%	0,0%	0,0%	0,1%	8,1%	0,4%	0,7%	0,4%	0,7%	2,0%	0,3%	0,3%	0,0%	9,7%	0,8%	0,3%	100,0%
eins/hlóg svipað	jákv	65,4%	26,3%	4,6%	0,0%	0,0%	0,0%	0,0%	1,8%	1,2%	0,0%	0,0%	0,0%	0,0%	0,0%	0,3%	0,0%	0,3%	0,0%	0,0%	100,0%
meira	samt.	69,3%	7,9%	0,6%	0,2%	0,0%	0,0%	0,1%	7,5%	0,5%	0,6%	0,3%	0,7%	1,8%	0,3%	0,3%	0,0%	8,9%	0,8%	0,3%	100,0%
% jákvæð innan arfgerða		7,7%	27,3%	65,2%	0,0%	0,0%	0,0%	0,0%	2,0%	22,2%	0,0%	0,0%	0,0%	0,0%	0,0%	9,1%	0,0%	0,3%	0,0%	0,0%	
hver arfgerð og ARQ/ARQ - Fisher's exact Pearson's Chi - "	OR	1	**4.48**	**22.35**	**0.00**	**0.00**	**0.00**	**0.00**	**0.24**	**3.41**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**1.19**	**0.00**	**0.03**	**0.00**	**0.00**	
		0,0000	*0,0000*	*0,0000*	*1,0000*	*1,0000*	*1,0000*	*1,0000*	*0,0000*	*0,0468*	*0,2545*	*0,6164*	*0,2578*	*0,0056*	*1,0000*	*0,5887*	*1,0000*	*0,0000*	*0,1668*	*1,0000*	
		<0,0001	*<0,0001*	*<0,0001*	*0,4436*	*0,7721*	*0,7721*	*0,6822*	*0,0002*	*0,0227*	*0,1478*	*0,2965*	*0,1399*	*0,0135*	*0,3599*	*0,8671*	*0,7721*	*<0,0001*	*0,1071*	*0,3599*	

reikna út Odds ratio (OR) - áhættan er OR sinnum eins mikið og viðmið = 1 (hér villigerð); ef < 1, þá er áhættan minna en viðmið

XY villigerð (ARQ/ARQ)

fjöldi jákv	a	b
fjöldi neikv	c	d

odds ratio (OR)
OR = a*d / b*c

villige XY

fjöldi neikv	d	c
fjöldi jákv	b	a

Table 4: Brúsastaðir/Snæringsstaðir, genotypes of 48 sheep with lymph node samples

(Stefanía Þorgeirsdóttir, unpublished data)

Genotype	pos	%	neg	%	sum	sum%
ARQ/ARQ	7	41	13	42	20	42
ARQ/N138	9	53	16	52	25	52
N138/N138	0	0	2	6	2	4
VRQ/N138	1	6	0	0	1	2
sum	**17**	**100**	**31**	**100**	**48**	**100**

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