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Title: Rapid testing leads to the underestimation of the scrapie prevalence in an affected sheep and goat flock

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1 **Rapid testing leads to the underestimation of the scrapie prevalence in an affected sheep**
2 **and goat flock**

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22 **Abstract**

23 To obtain a more detailed understanding of the prevalence of classical scrapie
24 infections in a heavily affected German sheep flock (composed of 603 sheep and 6 goats), we
25 analysed 169 sheep and 6 goats that carried the genotypes susceptible to the disease and that
26 were therefore culled following discovery of the index case. The initial tests were performed
27 using the Biorad TeSeE ELISA and reactive results were verified by official confirmatory
28 methods (OIE-immunoblot and/or immunohistochemistry (IHC)) to demonstrate the
29 deposition of scrapie-associated PrP^{Sc} in the brain stem (obex). This approach led to the
30 discovery of 40 additional subclinically scrapie-infected sheep. Furthermore, peripheral
31 lymphatic tissue samples of the 129 sheep and six goats with a negative CNS result were
32 examined by IHC in order to identify any preclinical infections which had not already spread
33 to the central nervous system (CNS). Using this approach we found 13 additional sheep with
34 PrP^{Sc} depositions in the gut-associated lymph nodes (GALT) as well as in the enteric nervous
35 system. Moreover, in most of these cases PrP^{Sc} was also deposited in the spleen and in the
36 retropharyngeal and superficial cervical lymph nodes. Taken together, these results show a
37 30.3% infection prevalence in this scrapie-affected flock. Almost 7.4% of the infected animals
38 harboured PrP^{Sc} exclusively in the peripheral lymphatic tissue and were therefore missed by
39 the currently used testing strategy.

40

41 *Keywords:* preclinical scrapie, immunohistochemistry, lymphoid tissue

42

43 **1. Introduction**

44 Scrapie belongs to the transmissible spongiform encephalopathies (TSE) of sheep and
45 goats and is characterised by the accumulation of an abnormally folded isoform (PrP^{Sc}) of the
46 cellular prion protein (PrP^{C}). Incubation times vary between a few months and several years
47 depending on the infectious dose and route, the particular scrapie strain and the genotype and

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48 age of the recipient animal (summarised in Detwiler and Baylis, 2003) According to the prion
49 hypothesis (Prusiner et al., 1982), abnormally folded PrP^{Sc} represents the causative infectious
50 agent itself, the 'prion'. However, the existence of different strains remains as yet
51 unexplained. PrP^{Sc} is partially protease resistant, insoluble in detergents (as a consequence of
52 an increased β-sheet content which increases its hydrophobicity) and forms scrapie-associated
53 fibrils (Diringer et al., 1983; Hope et al., 1986; Oesch et al., 1985). These criteria form the
54 basis for the demonstration of PrP^{Sc} as a diagnostic marker.

55 Amino acid polymorphisms at positions 136, 154 and 171 of the prion protein
56 determine the susceptibility of sheep to classical scrapie. Alleles on the PrP gene encoding
57 alanine/arginine/arginine (PrP^{ARR}) are associated with the lowest level of susceptibility,
58 alleles encoding valine/arginine/glutamine (PrP^{VRQ}) with the highest susceptibility,
59 particularly if present in homozygous animals (Goldmann et al., 1990; Hunter, 1996; Hunter
60 et al., 1997). The PrP genotype of sheep has a strong effect on the scrapie pathogenesis in
61 sheep. While no, or only minor, traces of PrP^{Sc} depositions can be found in the peripheral
62 lymphatic tissues (e.g., spleen, tonsils, retropharyngeal lymph nodes, gut-associated lymph
63 nodes) of sheep carrying at least one PrP^{ARR} allele, $PrP^{VRQ/VRQ}$ and $PrP^{ARQ/ARQ}$ carriers can
64 show PrP^{Sc} accumulations in these lymphatic tissues as early as a few months after the
65 infection (van Keulen et al., 1996 & 2002; Andreoletti et al., 2000; Jeffrey et al., 2002; Ersdal
66 et al., 2005). Preclinical accumulation of PrP^{Sc} in lymphoreticular tissues of sheep therefore
67 provides an opportunity for an *in-vivo* diagnosis based on biopsy samples of the tonsils
68 (Schreuder et al., 1998), the third eyelid (O'Rourke et al., 2002) or the rectum (Gonzalez et
69 al., 2006; Espenes et al., 2006).

70 The gastrointestinal tract, in particular the gut-associated lymphoid tissues (GALT), is
71 regarded as the major route of entry for natural scrapie (Hadlow et al., 1982; Heggebo et al.,
72 2000). The earliest accumulation of PrP^{Sc} can be found in the ileal Peyer's patches (IPP) and
73 in the draining mesenteric lymph nodes (Andreoletti et al., 2000). From there, prions may

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74 spread to other lymphatic tissues, which are not part of the GALT, resulting in a widespread
75 distribution of disease-specific PrP deposition in the lymphoreticular system at relatively
76 early stages of infection (Andreoletti et al., 2000; Heggebo et al., 2002; van Keulen et al.,
77 2002). Neuroinvasion appears to start at the enteric nervous system (ENS) followed by a
78 centripetal and retrograde spread via sympathetic and parasympathetic efferent fibres of the
79 autonomic nervous system to the spinal cord and to the medulla oblongata (van Keulen et al.,
80 2000).

81 The aim of the study was to analyse the prevalence of preclinical scrapie in a cohort of
82 susceptible sheep that were compulsorily culled after the detection of scrapie in the flock. For
83 this purpose LRS and the peripheral nervous system samples were examined with particular
84 attention paid to animals which were tested negative in the initial rapid test on the brain stem.
85

86 **2. Materials and Methods**

87 *2.1. Sheep and goats*

88 All 169 German Blackheaded Mutton-Merino crossbreed sheep and 6 fawn goats
89 belonged to a single flock (n=603) in Saxony-Anhalt in which an outbreak of classical scrapie
90 occurred in December 2003.
91

92 *2.2. TSE rapid testing*

93 Brain stem samples from the obex region were examined using the Biorad TeSeE
94 ELISA rapid test (Biorad, Munich, Germany) following the manufacturer's instructions.
95

96 *2.3. Genotyping*

97 Sheep were genotyped as described previously (Lühken et al., 2004).
98

99 *2.4. Histopathology and immunohistochemistry*

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100 With some modifications, tissue samples were processed as described previously
101 (Hardt et al., 2000). In short, all tissue samples were fixed in 4% buffered formalin, treated
102 for one hour with 98% formic acid, rinsed for 40 minutes in tap water, embedded in paraffin,
103 sectioned at 3-4 µm and stained with haematoxylin and eosin.

104 The avidin-biotin-complex (ABC) method was used for PrP-IHC. This involved the
105 paraffin wax tissue sections being mounted on superfrost plus slides (Menzel-Gläser,
106 Braunschweig, Germany) and rehydrated. The subsequent pretreatment included incubation
107 of the slides in 98% formic acid for 15 minutes, a 5-minute rinse in tap water, inhibition of the
108 endogenous peroxidase activity with 3% H_2O_2 (Merck, Darmstadt, Germany) in methanol for
109 30 minutes, followed by 15 minutes digestion with proteinase K (4µg/ml, Boehringer,
110 Mannheim, Germany) at 37 °C. The primary monoclonal antibody (mab) L 42, which binds to
111 an epitope of amino acid 145-163 of ovine PrP, was applied at a dilution of 1:250 (Harmeyer
112 et al., 1998) in Tris-buffered saline (TBS) containing 10 % goat serum and incubated
113 overnight at 4°C. Negative control sections were treated with a monoclonal antibody against
114 GP_5 of the Porcine Respiratory and Reproductive Syndrome virus (Weiland et al., 1999). As a
115 secondary (link) antibody, biotinylated goat anti-mouse antiserum (Vector Laboratories,
116 Burlingame, CA, USA) was incubated on the sections in a 1:200 dilution for 30 minutes at
117 room temperature. Immunodetection was amplified using Vector ABC-elite avidin-
118 horseradish peroxidase/biotin complex (Vector laboratories; Peterborough, UK). The slides
119 were finally developed in DAB (diaminobenzidintetrahydrochloride) (Fluka Feinchemikalien,
120 Neu Ulm, Germany) and counterstained with Mayer`s haematoxylin. All sections were
121 examined using light microscopy.

122 All positive results were verified using mab 2G11 binding to the epitope 146-182 of
123 ovine PrP (Institut Pourquier, Montpellier, France) diluted 1:250 in TBS with 10 % goat
124 serum.

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126 **3. Results**

127 All 175 animals examined in this study came from a flock of 603 German Blackhead
128 Mutton-Merino crossbreed sheep and six fawn goats which was kept in Saxony-Anhalt in
129 Germany. The outbreak of classical scrapie was first recognised through TSE rapid testing in
130 two fallen stock sheep. After the flock was quarantined and monitored, 10 more clinical cases
131 of scrapie were identified. According to the EU 999/2001 regulation, all animals in the flock
132 were genotyped with regard to codons 136, 154 and 171 of the sheep PrP gene and all
133 genetically highly TSE-susceptible animals (169 sheep and six goats) were killed. The
134 necropsy was carried out immediately after the death of the animals and a wide range of
135 tissues of the central and peripheral nervous system as well as of the lymphoreticular system
136 (LRS) was taken. Obex samples from these animals were subsequently TSE rapid tested using
137 the Biorad TeSeE assay. Reactive results were obtained in another 40 of the 169 sheep and
138 the TSE diagnosis in these cases was confirmed by using the OIE- approved methods, SAF-
139 immunoblot and/or IHC. Table 1 shows an overview of relevant data of all sheep investigated.

140 All 135 animals with a negative rapid test result on the obex region were more closely
141 examined by IHC to detect PrP^{Sc} accumulations in the peripheral nervous and lymphoreticular
142 system using a hierarchical approach, as illustrated in Figure 1. In a first step the five most
143 likely sites of entry for the agent were analysed. In cases where there was a total absence of
144 PrP^{Sc} depositions in the samples, the animals were considered to be completely negative and
145 no further studies were undertaken. However, if there was a positive result in one of the five
146 tissues, the investigation was extended to several parts of the peripheral nervous system as
147 well as to the lymphoreticular system.

148 PrP^{Sc} was not found to be present in any of the initially investigated tissues of the six
149 goats. However, PrP^{Sc} was found to be present in the lymphoreticular system of 13 of the 129
150 investigated sheep. Table 2 summarises the results for all animals with a clear PrP^{Sc}-
151 immunolabelling.

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152 PrP^{Sc} was discovered in the lymphoreticular system in most animals and in particular
153 in the head-associated lymph nodes (i.e., tonsils, retropharyngeal or mandibular lymph
154 nodes). Interestingly, two sheep showed a strong staining reaction in these non-GALT
155 lymphoid tissues (Figure 2C), but not in the GALT. Additionally, a PrP^{Sc} accumulation in the
156 lymphoid follicles of the third eyelid was only apparent in the two sheep with the most
157 widespread PrP^{Sc} distribution in the lymphoreticular system. As the rectum samples examined
158 did not totally comply with the proposed diagnostic requirements (i.e. too low a number of
159 follicles), the absence of PrP^{Sc} staining in any of the preclinically affected sheep at this site
160 should be interpreted cautiously.

161 Only a sparse immunolabelling was found in the peripheral nervous system. While 11
162 sheep showed a strong PrP^{Sc} immunolabelling in the enteric nervous system of the distal
163 ileum (Fig. 2A), only six of these sheep showed a detectable amount of PrP^{Sc} in the coeliac
164 and mesenteric ganglion complex (Fig. 2B). Three of these sheep showed an additional PrP-
165 immunostaining in the ENS of the rectum. However, no PrP^{Sc} accumulation was
166 demonstrated in the Ganglion cervicale craniale, Ganglion stellatum and the Vagus nerve.

167 The preclinical scrapie-infected sheep carried either the $PrP^{ARQ/ARQ}$ (9 cases) or the
168 $PrP^{ARQ/AHQ}$ (4 cases) genotype. There was no obvious correlation between the number of
169 positive tissues and the age of the animal or between the quantity of accumulated PrP^{Sc} and
170 the genotype of the sheep. Although a widespread distribution of PrP^{Sc} was detected in most
171 animals, none of the 13 sheep was positive in every single sample.

172

173 **4. Discussion**

174 In this study 169 sheep carrying the susceptible genotypes and six goats out of a flock,
175 which underwent a selective culling to eradicate scrapie, were analysed. This investigation
176 revealed that 30% of these animals were preclinically scrapie-infected and that 7.4% of them
177 were PrP^{Sc} -positive only at the peripheral sites but not in the brain stem, which is routinely

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178 tested. This prevalence is within the range of 5% to 30.3% previously reported in similar
179 studies (Jeffrey et al., 2002; Thorgeirsdottir et al. 2002; Ersdal et al. 2003; Vascellari et al.
180 2005; Gonzalez et al., 2006). The classical scrapie prevalence in a flock depends on different
181 factors: a) length of time period after the introduction of the infection into the flock; b)
182 husbandry measures (e.g. postpartal removal of the placentas as the most infectious tissues,
183 separation of lambing ewes, frequency of pasture changes, general hygiene measures); c)
184 genetic status (in terms of scrapie susceptibility) of the animals in the flock; d) differences in
185 the scrapie strains (Andreoletti et al., 2001; Jeffrey et al., 2001).

186 The origin of the scrapie infection in this flock, in which the oldest scrapie-infected
187 sheep was born in 1998, is unknown. However, the large number of scrapie-infected sheep
188 may reflect the fact that this flock has had a long scrapie history which was possibly started
189 by the introduction of an infected animal from the outside. In this regard it is of particular
190 interest that most of the sheep with a positive rapid test result are two years or younger, which
191 corresponds to the reported changes in the age-genotype profile in flocks which have been
192 infected for several years (Baylis et al., 2000). The last acquisition of ewes took place in
193 1994, while rams were restocked from other flocks for many years. Interestingly, scrapie was
194 diagnosed in a neighbourhood flock in 1979/1980, but the eradication measures taken at this
195 time were not fully recorded. Little is known about the husbandry measures which were
196 applied in this particular scrapie-affected flock.

197 In our study, all affected animals were either $PrP^{ARQ/ARQ}$ or $PrP^{AHQ/ARQ}$ carriers and no
198 differences in the distribution of PrP^{Sc} were found between these groups. These results are in
199 contrast to previous studies carried out in Scotland (Jeffrey et al., 2002) and Iceland
200 (Thorgeirsdottir et al., 2002) in which these genotypes seemed to be more resistant to a
201 scrapie infection. However, the results reported here are in accordance with the results from
202 an outbreak in Italy (Vascellari et al., 2005). The most probable explanation for this difference
203 is that scrapie strains with a bias towards different genotypes had been going round the flocks

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204 in question. All seven PrP^{ARR} heterozygous sheep were negative in our study. Although most
205 of the sheep were at least four years or older (5/6), the low number of animals with this semi-
206 resistant genotype does not allow us to exclude that the scrapie agent targets genotypes with
207 an age-dependent lower prevalence as shown in a previous study (Gonzalez et al., 2006).

208 All preclinically scrapie-infected sheep showed a marked and widespread distribution
209 of PrP^{Sc} throughout the lymphoreticular system and in some parts of the peripheral nervous
210 system. However, none of the animals was positive in every tissue sampled. In accordance
211 with several studies most animals (n=11) showed an accumulation of PrP^{Sc} in the Peyer's
212 patches of the distal ileum and/or the draining lymph nodes, suggesting the intestine as the
213 port of entry for the agent (Heggebo et al., 2000; van Keulen et al., 2002). Additionally, six of
214 the sheep showed a clear staining reaction in the enteric nervous system as well as in the
215 CMGC, indicating the subsequent spread to the CNS (van Keulen et al., 2000). In contrast, in
216 two sheep (5, 11) no detectable amount of PrP^{Sc} was found in the intestine and the mesenteric
217 lymph nodes. One possible explanation could be a direct neuroinvasion (Jeffrey et al., 2002).
218 However, none of the neuronal tissues examined contained PrP^{Sc} depositions, whereas a
219 strong immunostaining was seen in the tonsil and in the Ln. retropharyngealis lateralis.
220 Therefore the oronasal cavity may have served as a port of entry in these two sheep.

221 The current EU strategy for the active and passive surveillance of sheep TSEs is based
222 on the testing of CNS samples. The results of the present study reveal that a better estimation
223 of the "real" prevalence of scrapie-infected sheep can be obtained when LRS samples are
224 examined as well.

225 However, it must be noted that a diagnostic examination of the LRS alone (as
226 suggested by Gonzalez et al., 2006) would lead to studies missing the large number of
227 atypical scrapie cases which lack a PrP^{Sc} dissemination in the LRS (Gavier-Widen et al.,
228 2005). The same applies for sheep carrying the PrP^{ARR} haplotype. Therefore a combination of

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229 CNS and LRS testing is necessary to detect all clinical and preclinically scrapie-infected
230 animals in affected flocks.
231

232 **5. Conclusion**

233 In conclusion, this study shows that a negative result of the rapid test on brain stem
234 samples does not exclude the presence of PrP^{Sc} in peripheral tissues. In consequence, the
235 actual prevalence of preclinical scrapie is significantly underestimated when it is only based
236 on studies using brain stem samples.
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Table 1: Numbers, genotypes and age of all culled sheep and goats

	Total number	Number of sheep with genotypes						Age (in years)*				
		ARQ/ARR	**ARQ/ARQ**	**ARQ/AHQ**	**AHQ/ARQ**	**VRQ/ARQ**	**VRQ/ARR**	**0-1**	**1-2**	**2-3**	**3-4**	**> 4**
Obex positive	40	--	38	--	2	--	--	13	16	2	3	0
Obex negative												
sheep	129	4	105	13	--	4	3	23	24	39	11	9
goats	6	--	--	--	--	--	--	unknown				

* 29 sheep of unknown age

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Table 2: Immunohistochemical results relative to the distribution of PrP^{Sc} in the different tissues of 13 sheep

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Sheep	IPP	Lnn. ileo-colici	RPLN	Tonsil	3rd eyelid	Spleen	Ln. cerv. superf.	ENS Ileum	ENS Rectum	CMGC	Ggl. cerv. cran.	Ggl. stella-tum	N. vagus	Tongue	PrP genotype	Age (in years)
1	+	-	+'*	+	-	+	+	-	-	-	-	-	-	-	ARQ/ARQ	>4
2	-	+	+'*	+	-	+	+	-	-	-	-	-	-	-	ARQ/ARQ	>4
3	+	+	+	+	+	+	+	+	-	+	-	-	-	-	ARQ/ARQ	2-3
4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	ARQ/ARQ	2-3
5	-	-	+	+	-	+	+	-	-	-	-	-	-	-	ARQ/ARQ	2-3
6	+	+	-	-	-	+	-	+	+	+	-	-	-	-	ARQ/ARQ	1-2
7	+	+	+	+	+	+	+	+	+	+	-	-	-	-	ARQ/ARQ	1-2
8	-	+	+	+	-	-	+	-	-	-	-	-	-	-	ARQ/ARQ	0-1
9	-	+	+	+	-	+	+	-	-	-	-	-	-	-	ARQ/ARQ	0-1
10	+	+	+	+	-	+	+	+	-	+	-	-	-	-	AHQ/ARQ	1-2
11	-	-	+	+	-	-	-	-	-	-	-	-	-	-	AHQ/ARQ	1-2

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12	+	-	-	-	-	-	-	+	+	+	-	-	-	-	AHQ/ARQ	0-1
13	+	-	-	-	-	-	-	+	-	+	-	-	-	-	AHQ/ARQ	
Total	8	7	9	9	2	8	8	6	3	6	0	0	0	0		

* Lnn. mandibulares

IPP Peyer's patches of the distal ileum

RPLN Retropharyngeal lymph node

ENS Enteric nervous system CMGC Coeliac and mesenteric ganglion complex

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Figure legends

Figure 1:

Hierarchical approach in the sample selection from culled sheep and goats that underwent a further immunohistochemical examination

Figure 2:

Distinct PrP^{Sc} immunolabelling in different tissues of sheep

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