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1 # The emergence of classical BSE from atypical/Nor98 scrapie

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25

Abstract

Atypical/Nor98 Scrapie (AS) is a prion disease of small ruminants. Currently there are no efficient measures to control this form of prion disease and, importantly, the zoonotic potential and the risk that AS might represent for other farmed animal species remains largely unknown. In this study, we investigated the capacity of AS to propagate in bovine PrP transgenic mice. Unexpectedly, the transmission of AS isolates originating from five different European countries to bovine PrP mice resulted in the propagation of the classical BSE (c-BSE) agent. Detection of prion seeding activity *in vitro* by protein misfolding cyclic amplification (PMCA) demonstrated that low levels of the c-BSE agent were present in the original AS isolates. C-BSE prion seeding activity was also detected in brain tissue of ovine PrP mice inoculated with limiting dilutions (end-point titration) of ovine AS isolates. These results are consistent with the emergence and replication of c-BSE prions during the *in-vivo* propagation of AS isolates in the natural host. These data also indicate that c-BSE prions, a known zoonotic in humans, can emerge as a dominant prion strain during passage of AS between different species. These findings provide an unprecedented insight into the evolution of mammalian prion strain properties triggered by intra- and inter-species passage. From a public health perspective, the presence of c-BSE in AS isolates suggest that cattle exposure to small ruminant tissues and products could lead to new occurrences of c-BSE.

(235 words)

49 **Significance Statement**

50 The origin of transmissible BSE in cattle remains unestablished. Sheep scrapie is a potential
51 source of this known zoonotic. Here we investigated the capacity of sheep scrapie to
52 propagate in bovine PrP transgenic mice. Unexpectedly, transmission of atypical but not
53 classical scrapie in bovine PrP mice resulted in propagation of classical BSE prions. Detection
54 of prion seeding activity by *in vitro* protein misfolding cyclic amplification demonstrated BSE
55 prions in the original atypical scrapie isolates. BSE prion seeding activity was also detected in
56 ovine PrP mice inoculated with limiting dilutions of atypical scrapie. Our data demonstrate
57 that classical BSE prions can emerge during intra- and inter-species passage of atypical
58 scrapie and provide an unprecedented insight into the evolution of mammalian prions.
59

60 **Introduction**

61 Transmissible spongiform encephalopathies (TSEs), or prion diseases, are fatal
62 neurodegenerative disorders that affect a large spectrum of mammalian species. These
63 conditions include scrapie in small ruminants, classical bovine spongiform encephalopathy (c-
64 BSE) in cattle and sporadic Creutzfeldt-Jakob disease (sCJD) or variant CJD (vCJD) in
65 humans.

66

67 The fundamental event in prion propagation is the conversion of the normal cellular prion
68 protein (PrP^{C}) into an abnormal disease-associated isoform (PrP^{Sc}) in tissues of infected
69 individuals. PrP^{C} is completely degraded by digestion with proteinase K (PK) whereas PrP^{Sc} is
70 N-terminally truncated resulting in a PK resistant core termed PrP^{res} (1). According to the
71 prion concept, PrP^{Sc} is the principal, if not sole component of the transmissible prion agent (2)
72 and PrP^{res} is a disease marker for prion diseases (1, 3). Particular biochemical properties of
73 PrP^{Sc}, such as detergent solubility, PK resistance and electromobility evidenced by western
74 blot can be used to distinguish between different prion agents or strains (4, 5).

75

76 Intra-species transmission of prion disease between individuals is typically quite efficient. In
77 contrast, inter-species transmission of prions can be unpredictable with apparent failure of
78 disease transmission on many occasions. In other cases, clinical prion disease may not be
79 evident but rather there is the presence of subclinical infection (6). When inter-species prion
80 transmission does occur, the propagating agent can remain identical to the original prion
81 strain, or can display different biological properties compared to the original inoculum (7, 8).
82 This complex set of outcomes for inter-species prion challenge are collectively referred to as
83 the transmission barrier phenomenon.

84

85 After identification of the gene encoding PrP it was soon discovered that differences in amino
86 acid sequence between host PrP^{C} and donor PrP^{Sc} constitutes the principal determinant of the
87 transmission barrier. For example, the resistance of wild-type mice to clinical prion disease
88 induced by hamster scrapie is abrogated by transgenic expression of hamster PrP^{C} in mice (9,
89 10). As a consequence, mice genetically engineered to express particular species forms of PrP
90 sequence, in the absence of endogenous mouse PrP, have emerged as relevant models to
91 experimentally characterize the outcome of prion strain transmission between species (11). It
92 is also now well established that strain properties have a significant impact on the ability of
93 prions to cross the species barrier. For instance, human vCJD can be transmitted readily to
94 conventional mice but it is extremely difficult for sCJD to propagate in the same mouse lines
95 (12, 13). Furthermore, the amino acid sequence of PrP^{Sc} influences the efficacy of inter-
96 species prion transmissions since studies in human PrP transgenic mouse models indicate that
97 the human species barrier is more permeable to sheep-passaged BSE compared to its cattle
98 counterpart (14).
99
100 From a public health perspective, the transmission barrier phenomenon and its capacity to
101 limit the inter-species propagation of prion disease has long been considered as an effective
102 protection of humans against animal TSEs (15). However, in 1996, the new human prion
103 disease vCJD was observed in UK individuals. Multiple lines of evidence indicated that vCJD
104 was the likely consequence of dietary exposure of humans to the agent responsible for c-BSE
105 in cattle, an epizootic prion disease that has spread in bovine hosts through the recycling of
106 prion-contaminated animal carcasses in the animal food chain (16). Since the emergence of
107 vCJD, considerable efforts have been deployed to characterize not only the zoonotic potential
108 of animal prions but also their capacity to propagate in farmed animal species.
109

110 Atypical/Nor98 scrapie (AS) probably represents the largest geographically spread known
111 animal prion disease. Since its original discovery in 1998 in Norway, AS has been identified
112 in most EU member states, in Asia and in North and South America (17). AS has also been
113 detected in Australia and New Zealand, two countries that were believed to be free of animal
114 TSEs (18, 19). Retrospective studies carried out in archived animal tissues identified an AS
115 case in a sheep that died in 1972 in the UK demonstrating that the disease has been present in
116 small ruminant populations for many decades (20).

117

118 Bioassay in ovine PrP transgenic mice provided evidence that AS comprised a single prion
119 strain (21-23). The AS prion strain was associated with a multi-band PrP^{res} signature that
120 contrasted with those normally observed in small ruminant TSE cases (24). Since there is no
121 statistical difference in the apparent prevalence of atypical scrapie between sheep flocks in
122 general and those flocks where a positive case had been identified, atypical scrapie is
123 considered by many as a non-contagious prion disease that arises sporadically in sheep and
124 goats (25). However, atypical scrapie can be experimentally transmitted via the oral route in
125 small ruminants, resulting in a similar clinico-pathological phenotype to that observed in
126 natural cases (26). Consequently, the origin of atypical scrapie (spontaneous disorder versus
127 acquired disease) remains an open question.

128

129 In this study we used mice transgenic for bovine PrP (tgBov mouse line) to characterize the
130 capacity of sheep AS isolates to cross the bovine transmission barrier. Unexpectedly, the TSE
131 agent that propagated in tgBov mice was indistinguishable from the prion strain that was
132 responsible for the c-BSE epizootic in cattle. In addition, our sensitive detection of c-BSE by
133 *in vitro* PMCA methodology indicated that this bovine prion strain was present as a minor
134 prion strain in the original sheep AS isolates, and that AS prion strain replication in an ovine

135 PrP host was accompanied by the generation of c-BSE prions. Collectively, these data provide
136 compelling evidence for the emergence and the propagation of zoonotic mammalian prions
137 during intra- and inter-host transmission of the AS prion strain.
138

139 **Results**

140 A panel of 8 atypical scrapie (AS) cases collected from sheep and goats in five different
141 European countries was obtained (Table1). All of the AS isolates displayed a multi-band
142 abnormal PrP (PrP^{res}) western blot profile that was considered to be specific for small
143 ruminant AS (SI appendix Figure S1). This panel of AS isolates was transmitted to the VRQ
144 ovine PrP transgenic mice (tg338). The transmission properties such as incubation period (SI
145 appendix Table S1), vacuolar lesion profile (Figure 1a) and PrP^{res} western blot profile in the
146 brain (SI appendix Figure S1), of the propagated AS isolates observed after two or three
147 iterative passages in tg338 were similar and were the same as that previously reported for AS
148 passage in tg338 mice (21-23).

149

150 The panel of 8 AS isolates was individually serially transmitted (2 or 3 iterative passages) in
151 bovine PrP transgenic mice (tgBov) (Table 1). On first passage, signs of clinical prion disease
152 were observed in a low proportion of inoculated tgBov mice. PrP^{res} was detected by western
153 blot in the brains of clinically affected mice and in some mice that displayed no apparent
154 clinical signs of prion disease when euthanised at the end of their life expectancy. No PrP^{res}
155 accumulation was observed in tgBov mice after inoculation with several of the AS isolates
156 (AS 2, AS 3, AS 5, AS 6 and AS 7), as was the case with classical scrapie PS42 (Table 1).

157

158 Second passage of the AS isolates in tgBov mice was performed using either first passage
159 PrP^{res}-positive brains or pooled PrP^{res}-negative brains as inoculum. During this process, 7 out
160 of the 8 AS isolates caused the occurrence of clinical prion disease in a proportion of animals
161 in each group inoculated. On third passage (available for AS 1, 2, 3, 4 and 7) 100% attack
162 rates and mean incubation periods that ranged between 235 and 286 dpi were recorded (Table
163 1).

164 At each passage stage, a three band PrP^{res} western blot profile characterised by a prominent
165 di-glycosylated PrP band was observed in the brains of the clinically-positive tgBov mice
166 (Figure 1b). Strikingly, the lesion profile (Figure 1a), the PrP^{res} western blot profile (Figure
167 1b) and the histopathological lesions (Figure 1c) in the brains of AS inoculated tgBov mice
168 were identical to those observed for transmission of c-BSE (sheep or cattle origin) to tgBov
169 mice. Importantly, the inoculation of one classical scrapie isolate (PS42) to the same mouse
170 models resulted in the occurrence of a 100% attack rate for prion disease with a short
171 incubation period in tg338 mice (SI appendix Table S1), but no clinical disease or PrP^{res}
172 accumulation in the brains of tgBov (Table 1).
173
174 In order to further characterize the nature of the TSE agent that propagated in tgBov mice
175 inoculated with the AS isolates, prions obtained after second passage (isolate AS 2) or third
176 passage (isolate AS 3) in this mouse line were transmitted (two iterative passages) to VRQ
177 (tg338) and ARQ (tgARQ) ovine PrP transgenic mice (Table 2). The incubation periods
178 (Table 2 and SI appendix Table S1), the lesion profile (Figure 2a) and the PrP^{res} western blot
179 profile in the brain (Figure 2b) of tg338 mice inoculated with tgBov-adapted AS isolates
180 clearly differed from those observed in the same mouse line inoculated with the original AS
181 isolates.
182 No PrP^{res} deposition could be detected in the spleen of tg338 mice inoculated with the original
183 AS isolates (Figure 3a). Conversely, transmission in tg338 of tgBov-adapted AS isolates was
184 associated with a PrP^{res} accumulation in the spleen as was transmission of ovine and cattle c-
185 BSE (Figure 3a). PrP^{res} WB profile in the spleen of tg338 mice that were inoculated with c-
186 BSE and AS isolates passaged in tgBov were identical (Figure 3b).
187 Transmission of tgBov-adapted AS isolates in both tg338 and tgARQ mice resulted in prion
188 incubation periods (Table 2), brain vacuolar lesion profiles (Figure 2a), PrP^{res} western blot

189 profile patterns (Figure 2b) and PrP^{res} distribution patterns in the brain (Figure 3a) that were
190 similar to c-BSE passaged in tg338 and tgARQ mice. Collectively these results demonstrate
191 beyond reasonable doubt that our transmission of AS in a bovine PrP host resulted in the
192 propagation of the c-BSE agent. Since the bioassays reported here were performed in three
193 independent institutes (located in France and Spain) that used inoculum prepared by five
194 distinct laboratories, we exclude the possibility of a cross contamination of the original AS
195 isolates by the c-BSE agent.

196

197 Two hypotheses could explain the emergence of the c-BSE agent in tgBov mice after their
198 inoculation with AS isolates. Firstly, c-BSE prions could be present at a low level in the
199 original AS isolates. The high sensitivity of tgBov mice for detection of the c-BSE agent
200 could allow this potentially low level of bovine prions to be identified during passage of the
201 original AS isolates in the bovine PrP host. Alternatively, the occurrence of c-BSE in AS-
202 inoculated tgBov mice could result from a mutation of AS strain properties triggered by
203 passage across the bovine transmission barrier for this particular ovine prion strain.

204

205 In order to explore the origin of the c-BSE agent observed in tgBov mice inoculated with AS,
206 we employed *in vitro* protein misfolding cyclic amplification (PMCA), a methodology that
207 mimics prion replication *in vitro*, but in an accelerated form, allowing amplification of minute
208 amounts of PrP^{Sc} and prion infectivity (27). In PMCA, a PrP^{C}-containing substrate is
209 combined with a seed that contains PrP^{Sc}. Following repeated cycles of incubation and
210 sonication, the amount of PrP^{Sc} increases.

211

212 PMCA has been previously reported to amplify the c-BSE agent with a great efficacy using
213 either tgARQ or tgBov mouse brain homogenate as substrate (28). Using this protocol two

214 (tgARQ substrate) or three (tgBov substrate) amplification rounds were sufficient to reach the
215 detection limit for c-BSE prion seeding activity (SI appendix Figure S2). The level of prion
216 infectivity and prion seeding activity of a reference sheep-passaged c-BSE isolate were end-
217 point titrated by both bioassay in tgBov mice and PMCA, respectively (SI appendix Table
218 S2). The infectious prion titer of the sheep-passaged c-BSE isolate was $\approx 10^{7.2}$ LD_{50}/g IC in
219 tgBov mice. The prion seeding titer (SA_{50}) was estimated to be $\approx 10^{11.1}$ SA_{50}/g using tgARQ
220 mouse tissue as substrate and $10^{11.05}$ SA_{50}/g using tgBov mouse tissue as substrate.
221 Considering the fact that mice were inoculated using 20µL of sample and the PMCA reactions
222 were seeded using 5µL of the same sample, the PMCA can be considered to be about 1500
223 fold more sensitive than the bioassay in tgBov. This also means that 1 c-BSE LD_{50} in tgBov
224 mice corresponds to ≈1500 SA_{50} assessed by PMCA.

225

226 In addition to its high sensitivity, *in vitro* PMCA can reproduce, at least partly, the
227 transmission barrier phenomenon observed during the *in vivo* prion bioassay (29). Therefore,
228 amplification of prion seeding activity in AS isolates by PMCA using tgBov mouse tissue as
229 substrate offered an opportunity to characterize the potential impact of the bovine
230 transmission barrier on AS strain properties.

231

232 The AS isolates that were originally transmitted to tgBov mice (except AS8) and 18 additional
233 AS isolates (originating from Norway, France, and Portugal) were subjected to PMCA (Table
234 4). Each AS isolate was used to seed reactions containing either bovine PrP or ovine ARQ PrP
235 substrate (10 to 18 replicates per substrate). After amplification, PrP^{res} was detected by
236 western blot in a low proportion of the reactions seeded with 19 out of the 25 AS isolates for
237 tgBov and tgARQ combined (Table 3). In most instances, a similar proportion of PrP^{res}-
238 positive PMCA reactions were observed when either bovine PrP or ovine ARQ PrP was used

239 as substrate. However, in some cases (n=3), a low number of PrP^{res}-positive reactions were
240 observed when bovine PrP was used as substrate (in the case of AS 10) or when ovine ARQ
241 PrP was used as substrate (in the case of AS 9, and AS 25). Whatever combination of AS
242 isolate and substrate PrP used, the PrP^{res} western blot profile in PMCA-positive reaction
243 products and its reactivity with 12B2 antibody were indistinguishable from those observed for
244 PMCA reaction products seeded with authentic ovine c-BSE prions (Figure 4). No PrP^{res} was
245 observed in PMCA reactions that were unseeded (n=120) or in those reactions seeded (n=60)
246 with prion-free sheep brain homogenate (representative samples shown in Figure 4). It should
247 be noted that the PrP amino sequence was 100% homologous between certain AS isolates (AS
248 5, AS 26) and the ovine PrP substrate (tgARQ) used in PMCA reactions. Therefore, *in vitro*
249 amplification of c-BSE prions in PMCA reactions seeded with these AS isolates using ovine
250 ARQ PrP as substrate cannot be a consequence of mutation of prion strain properties triggered
251 by a transmission barrier.
252
253 Taken together, the tgBov mouse bioassay and PMCA results strongly support the view that a
254 low level of c-BSE prions was initially present in at least 21 out of the 26 AS isolates tested.
255
256 To further clarify the origin of the c-BSE agent detected in AS isolates, two of these isolates
257 (AS 25 and AS 26) were end-point titrated in tg338 mice (1/10 dilution series, 6 to 7 tg338
258 mice inoculated per dilution). For both isolates, the last positive transmissions were observed
259 in mice that received a 10^{-6} $\log_{10}$ dilution of the original 10% w/vol brain material (SI
260 appendix Table S3). The brains of these end-point titration tg338 mice were subsequently
261 subjected to PMCA. Irrespective of the substrate used for PMCA, either bovine or ovine ARQ
262 PrP, PrP^{res} was observed in a similar proportion of the PMCA reactions seeded with either the
263 original AS isolates, or AS isolates passaged in tg338 mice (Table 4). The PrP^{res} western blot

264 profile observed in all the PMCA-positive reactions was identical to that seen in reactions
265 seeded with authentic c-BSE prions (Figure 5). PMCA reactions seeded with brain
266 homogenate prepared from age matched non-inoculated tg338 mice remained PrP^{res} negative
267 (Table 4 and Figure 5).
268
269 Considering the level of c-BSE seeding activity originally present in isolates AS 25 and AS
270 26 (less than 100 SA_{50}/mL, Table 5), there is an extremely low level of probability that one of
271 the six tg338 mice inoculated with 20µL of a 10^{-6} diluted AS isolate (<2 10^{-6} SA_{50} per dose of
272 inoculum) could be exposed to 1 infectious dose of c-BSE agent (1 c-BSE LD_{50} is ≈1500
273 SA_{50}). Consequently, the presence of c-BSE prion seeding activity in the brains of tg338 mice
274 inoculated with a 10^{-6} log_{10} dilution of original AS isolate implies that a low titer of c-BSE
275 prions was generated during the propagation of ovine AS prions in a host that expressed ovine
276 PrP, namely tg338 mice.
277
278

279 **Discussion**

280 The mechanism(s) that lead to an alteration in the phenotype of prion strains as these
281 transmissible entities undergo transmission between different host species remain uncertain.
282 This is despite the identification that differences in amino acid sequence between host PrP^{C}
283 and donor PrP^{Sc}, together with prion strain identity are principal determinants of the
284 transmission barrier (9, 10). Based on the concept that conformation of PrP^{Sc}
285 molecules/aggregates encode prion strain information (2, 4, 5, 30, 31), at least two non-
286 exclusive hypotheses, 'deformed templating' (32, 33) and the 'conformational selection
287 model' (32-35) have been proposed to explain the mutation of prion strains.

288

289 The 'deformed templating' hypothesis postulates that a prion strain replicates as a clone of
290 PrP^{Sc} molecules/aggregates. When confronted by a transmission barrier that does not allow
291 clonal prion replication, the propagation process is modified so that 'altered' PrP^{Sc} structural
292 variants are generated in an attempt to convert the new host PrP^{C}. While the majority of these
293 presumably fail to replicate efficiently in the new host, variants eventually emerge that are
294 successful and adapt to the new PrP environment through multiple trial-and-error replication
295 events. In this 'deformed templating' model, confrontation of the transmission barrier serves
296 as the triggering event that initiates the generation of new prion variant(s) and as a filter for
297 their selection (35).

298

299 The 'conformational selection model' proposes that a prion strain naturally propagates in its
300 host as an ensemble of PrP^{Sc} conformers dominated by a stable energetically favourable
301 conformation responsible for the observed prion strain phenotype. Furthermore, this model
302 predicts that the number of stable PrP^{Sc} conformers is limited for each PrP amino acid
303 sequence, which would explain the existence of a finite number of stable prion strains that can

304 propagate in a given species. It is further proposed that during transmission of a prion strain to
305 a new host, one of the less dominant PrP^{Sc} conformers of those present in the ensemble is
306 selected with a resultant change, or mutation, in the properties of the newly propagating prion
307 strain. In the 'conformational selection model', the transmission barrier acts simply as a
308 selective filter for new prion variants, and ease of permeation of the barrier results from the
309 extent of overlap of PrP^{Sc} conformers that exist between the interacting species (32, 33).
310
311 Our data reported here showed that c-BSE prions are present as a minor variant in natural
312 isolates of ovine AS. In addition, transmission of ovine AS to bovine PrP mice demonstrated
313 that c-BSE can emerge during these transmissions as the dominant prion strain. These results
314 provide a cogent argument in favour of the 'conformational selection model' as the
315 mechanism for prion strain mutation during inter-species prion transmission. This would be
316 expected to occur by selection of a pre-existing PrP^{Sc} variant in AS isolates, one best suited to
317 the new replicative environment. Within this conceptual framework, the occurrence of prion
318 strain mutation is dependent upon the particular repertoire of PrP^{Sc} variants associated with
319 distinct prion strains. This notion is supported by our observation that c-BSE prions emerged
320 during serial transmission of ovine AS in tgBov mice but not from serial passage of classical
321 scrapie in the same mouse line (Table 1 and Cassard et al (15)).
322
323 The diversity of prion strains that exist in small ruminants remains undefined although it is
324 established that at least 5 different natural ovine prion strains exist including AS (6, 36-39).
325 According to the 'conformational selection model', each of these different ovine prion strains
326 is associated with a unique and stable PrP^{Sc} conformer and a distinct set of minor variants.
327 The tgBov mouse line has previously been reported to support the propagation of a variety of
328 natural ovine prions, of which several displayed significantly shorter incubation periods than

329 c-BSE (15). Strikingly, in our experiments the diversity of prion variants in the AS isolates
330 (seven different cases) revealed by the serial passage in tgBov was restricted to the c-BSE
331 agent (Table 1). This consistent emergence of a single prion strain argues against the view that
332 AS prion replication in sheep can randomly generate all the existing stable PrP^{Sc} variants
333 associated with a particular ovine PrP^{C} amino acid sequence. Instead, our data support the
334 view that individual prion strains are associated with a restricted repertoire of stable PrP^{Sc}
335 variants in a given host. Whether AS is unique in its ability to generate c-BSE prion particles
336 during its replication process remains to be established.
337
338 Classical BSE was first recognized in 1984-85 as a novel prion disease affecting cattle in the
339 UK (40). Epidemiological data clearly established that the number of cases of c-BSE was
340 amplified by the recycling of infected animal carcasses into cattle feed in the form of meat
341 and bone meal (MBM) (41). Since bovine prion disease had not been recognized in cattle
342 prior to the c-BSE epizootic and the disease is apparently non-contagious between cattle,
343 several hypotheses were proposed to explain its emergence. These range from the spontaneous
344 occurrence of c-BSE in cattle to the passage and adaptation of a prion originating from
345 another species (42, 43). Our studies here that show the presence of c-BSE prions in AS
346 isolates combined with the demonstrated presence of AS in the UK long before the
347 appearance of the c-BSE epizootic in cattle suggests that the recycling of AS cases in MBM
348 might be a source of bovine prion disease (20). In addition to its potential role in the initial
349 emergence of c-BSE in cattle, the presence of c-BSE prions in natural cases of AS has current
350 and direct implications for both the continued risk of this ovine prion disease to other farmed
351 animals and for human exposure risks. The distribution of AS cases are widespread across the
352 world (17-19). A recent retrospective analysis of surveillance data collected over a period
353 exceeding 10 years in the European Union (EU) concluded that the prevalence of detected AS

354 cases has remained relatively stable in the different member states with between 2-6 positive
355 cases per 10,000 tested animals per year. This implies that a substantial number of AS-
356 infected animals could enter either the animal or human food chain each year (44, 45), and
357 each case represents a potential source of exposure to the c-BSE agent for farmed animals
358 (MBM derived from rendered small ruminants) and human consumers (consumption of
359 healthy slaughtered animals), respectively. The epidemiological features of AS within the EU
360 is likely to reflect the situation of the disease in other countries that breed and maintain small
361 ruminants.
362
363 In Europe, the c-BSE crisis and the emergence of vCJD resulted in the implementation of a
364 strong and coherent policy (EU regulation 999/2001) aimed at control and eradication of this
365 animal prion disease. The total feed ban on the use of MBM in animal feed and the systematic
366 retrieval from the food chain of ruminant tissues that have the potential to contain high levels
367 of prion infectivity, so called Specified Risk Material (SRM) measures, were instrumental for
368 control of c-BSE in cattle and preventing dietary human exposure to these bovine prions (46,
369 47). As a side effect, these measures also strongly limited the exposure of farmed animals and
370 human consumers to the other TSE agents circulating in farmed animal species, including AS.
371
372 With the decline of the c-BSE epizootic in cattle and the combined increase in pressure from
373 industry, EU authorities have begun to consider discontinuing certain TSE control measures.
374 The abrogation of the SRM measures for small ruminants and the partial re-authorization of
375 the use of processed animal protein, formerly known as MBM in animal feed are part of the
376 EU authorities' agenda. Our observation of the presence of the c-BSE agent in AS-infected
377 small ruminants suggest that modification of the TSE control measures could result in an
378 increased risk of exposure to c-BSE prions for both animals and humans. Whether or not this

379 exposure will result in further c-BSE transmission in cattle and/or humans remains an open
380 and important question.
381
382

383 **Methods**

384 ***Ethics Statement***

385 All animal experiments were performed in compliance with institutional and French national
386 guidelines and in accordance with the European Directives 86/609/EEC and 2010/63/EU. In
387 France, the animal experiments that are part of this study (national registration 01734.01)
388 were approved by the local ENVT ethics committee. Experiments developed in CISA-INIA
389 (Madrid, Spain) were approved by the Committee on the Ethics of Animal Experiments of the
390 Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria and the General
391 Directorate of the Madrid Community Government (permit numbers: CEEA 2009/004 and
392 PROEX 228-16). Mouse inoculations were performed under anaesthesia (isofluorane).
393 Experiments developed in IRTA-CReSA (Barcelona, Catalonia) involving animals were
394 approved by the animal experimentation ethics committee of the Autonomous University of
395 Barcelona (Reference number: 585-3487) in agreement with Article 28, sections a), b), c) and
396 d) of the "Real Decreto 214/1997 de 30 de Julio" and the European Directive 86/609/CEE and
397 the European council Guidelines included in the European Convention for the Protection of
398 Vertebrate Animals used for Experimental and Other Scientific Purposes.
399 Mice that displayed clinical signs were anesthetized with isofluorane before sacrifice using
400 CO_2 inhalation.

401

402 ***Atypical/Nor98 scrapie cases and control sheep***

403 Natural atypical scrapie (AS) cases identified through active or passive surveillance programs
404 were selected according to their geographical origin (France, Spain, Italy, Norway and
405 Portugal) and *PRNP* genotypes (SI appendix Table S1 and Table 1). These cases have been
406 originally classified as AS by TSE national reference laboratories in each country. All the
407 cases corresponded to sheep except the AS 7 case (goat).

408

409 In all cases, PrP genotype was checked by sequencing the Exon 3 of the *PRNP* gene as
410 previously described (48). The polymorphisms at codons 136 (A/V), 154 (H/R) and 171
411 (R/Q/H), which have been demonstrated to strongly influence the susceptibility to TSE in
412 sheep are indicated (49). Additionally, the presence of a phenylalanine at codon 141 (F/L),
413 which has been shown to impact on the susceptibility to atypical/Nor98 scrapie, are also
414 indicated (Table 1 and 4) (23, 48). Brain material collected in TSE-free Poll-Dorset sheep
415 (APHA, Weybridge, UK) was used as control (50).

416

417 ***c-BSE isolates***

418 Cattle and ovine classical BSE (c-BSE) isolates were used as control. The cattle c-BSE isolate
419 was a natural case originating from France. This isolate was used in previous studies aimed at
420 the characterization of c-BSE strain properties though transmission to mice over-expressing
421 the PrP sequence of various host species (51). The ovine c-BSE isolate was obtained by the
422 intracerebral inoculation of the same cattle c-BSE isolate in ARQ/ARQ TSE free sheep (first
423 passage) as described in Andreoletti et al 2004 (50).

424

425 **Mouse bioassays**

426 Bioassays were carried out using mice expressing bovine PrP (tgBov /tg110) (52, 53) and/or
427 mice expressing ovine ARQ (tgARQ) (54) or VRQ (tg338) PrP (55).

428

429 Groups of six- to ten-week-old female mice (n ≥6) were anesthetized and inoculated with
430 20µL of a 10% tissue homogenate in the right parietal lobe using a 25-gauge disposable
431 hypodermic needle. Mice were observed daily and their neurological status was assessed
432 weekly. When clinically progressive TSE disease was evident, the animals were euthanized

433 and their brains harvested. Half of the brain was fixed by immersion in 10% formol saline and
434 the other half was frozen at -20ºC. Tissues from animals found dead were frozen (no formalin
435 fixation). In animals where no clinical signs were observed, mice were killed at the end of
436 their natural life-span (650 to 750 days). In those cases, incubation periods reported in the
437 table as >650 dpi, corresponded to the survival time observed in at least three out of the six
438 mice.

439

440 **PMCA Amplification**

441 Brains from tgBov, tgARQ and tg338 were used to prepare the PMCA substrates. PMCA was
442 performed as previously described (28, 56). Briefly PMCA reactions (50µL final volume)
443 were seeded with 5µL of sample to be tested. PMCA reactions were then subjected to 3
444 amplification rounds each comprising 96 cycles (10s sonication-14 minutes and 50 seconds
445 incubation at 39.5°C) in a Qsonica700. After each round, reaction products (1 volume) were
446 mixed with fresh substrate (9 volumes) to seed the following round. The PMCA reaction
447 products were analysed by western blot for the presence of PK-resistant PrP. Each WB line
448 was loaded with the equivalent of 20 µL of PK digested PMCA product. Each PMCA run
449 included a reference ovine BSE sample (10% brain homogenate) as a control for the
450 amplification efficiency. Unseeded controls (2 unseeded controls for 8 seeded reactions) were
451 also included in each run.

452

453 **Western blot detection of abnormal PrP**

454 PK-resistant abnormal PrP (PrP^{res}) extraction and western blot were performed as previously
455 described (57). Immunodetection was performed using two different PrP-specific monoclonal
456 antibodies: Sha31 (1 µg/ml) (58), and 12B2 (4 µg/ml) (59), which recognize the amino acid
457 sequences YEDRYYRE (145-152), and WGQGG (89-93) respectively (60).

458

459

460 **Paraffin embedded tissue blot**

461 Paraffin embedded brain tissue from inoculated mice was analysed as previously described

462 (61-63).

463

464 **Lesion profiling and abnormal PrP immunohistochemistry.**

465 Vacuolar brain lesion profiles were established following the method described by Fraser et al

466 (64). *In situ* PrP^{Sc} immune-labelling was performed as previously described (63), using 6H4

467 anti PrP antibody (epitope: $_{147}DYEDRYYRE_{155}$ of the bovine PrP- concentyrtation 3µg/mL).

468

469 **Infectious and seeding activity titer estimates**

470 A series of 1/10 dilutions of a reference 10% w/vol brain stem from an ovine-BSE

471 (ARQ/ARQ) isolate and two AS isolates (AS 25 and AS 26) were prepared. Successive 1/10

472 dilutions of brain homogenate were inoculated intra-cerebrally (20µl) into tgBov or tg338

473 mice (n=6 per inoculum). Dilutions of the same c-BSE isolate were used to seed PMCA

474 reactions that used brain tissue from either bovine PrP (tgBov mice) or ovine ARQ PrP

475 (tgARQ mice) as substrate. Twelve individual replicates of each sample dilution were tested.

476 Reactions were then subjected to three amplification rounds. PMCA reaction products (third

477 amplification round) were analysed by western blot for the presence of PrP^{res}. The titer of

478 prion seeding activity was estimated by the Spearman-Kärber's method (65).

479

480 For AS and AS passaged in tg338 isolates (10% brain homogenate), 1/50 diluted material was

481 used to seed twelve individual reactions (tgBov or tgARQ substrates). After three

482 amplification rounds the number of PrP^{res} western blot positive reactions / total number of

483 reactions was established. These ratios were used to estimate seeding activity titers (SA/μL of
484 10% brain homogenate) by the limiting dilution titration method (application of Poisson's
485 probabilistic model) described by Brown et al (66) or by the Spearman-Kärber's method.
486 According to Fisher et al (67) and as previously used for prion infectivity comparisons (68)
487 one SA_{50} was considered to be equivalent to 0.693 SA.

488

489 **Data availability**

490

491 All data is available in the manuscript and SI.

492

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494

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502

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658
659

660 **Legends of Figures**
661
662 **Figure 1: Brain lesion profile and PrP^{res} western blot profiles in tgBov and tg338 mice**
663 **inoculated with atypical/Nor98 scrapie (AS) or c-BSE**
664
665 Groups of mice (n≥6) that express either ovine VRQ PrP (tg338 mice) or bovine PrP (tgBov
666 mice) were intra-cerebrally challenged with atypical scrapie isolates (AS) or an ovine classical
667 BSE isolate (c-BSE).
668 (**a**) After two or three iterative passages in each mouse line a standard lesion profile was
669 established by scoring the vacuolar changes observed in pre-defined brain areas. In c-BSE
670 graphs ●: ovine c-BSE, ○: cattle c-BSE. In AS graphs: ○ : AS 1, △: AS 2, ▽: AS 3).
671 (**b**) The accumulation of PK-resistant PrP (PrP^{res}) in the brain of tgBov mice was established
672 by western blot using anti PrP monoclonal antibodies Sha31 (epitope 145-YEDRYYRE-152)
673 and/or 12B2 (epitope 89-WGQGG-93). The same western blot PrP^{res} control (classical scrapie
674 isolate) was used on all the gels labelled as WB control. Cattle c-BSE and an ovine c-BSE
675 (original isolate and isolate passaged in tgBov) were included as controls.
676 (**c**) Vacuolar lesions (thalamus level, conventional histology; hematoxylin-eosin, bar: 25 µm)
677 and abnormal PrP deposition (Mesencephalon: tegmentum, immunohistochemistry using 6H4
678 anti PrP antibody, epitope 147-DYEDRYYRE-155, bar: 50µm) in tgBov mice inoculated with
679 AS3 and cattle BSE.
680
681
682 **Figure 2: Brain lesion profile and PrP^{res} Western blot in the brain of tgARQ and tg338**
683 **mice inoculated with AS scrapie adapted in tgBov**
684
685 Groups of mice (n≥6) that express ovine VRQ PrP (tg338 mice) or ovine ARQ PrP (tgARQ
686 mice) were intra-cerebrally challenged with atypical scrapie isolates (AS) or an ovine c-BSE
687 isolate that had previously been adapted (2 iterative passages) in tgBov mice.
688 (**a**) After two iterative passages in each mouse line a standard lesion profile was established
689 by scoring the vacuolar changes observed in pre-defined brain areas. In AS graphs △: AS 2,
690 ▽: AS 3.
691 (**b**) The western blot profile of PK resistant PrP (PrP^{res}) in the original AS isolates and in the
692 brain of inoculated mice was established by western blot using anti PrP monoclonal antibody
693 Sha31 (epitope 145-YEDRYYRE-152). The same western blot PrP^{res} control (classical
694 scrapie isolate) was used on all the gels labelled as WB control.
695
696 **Figure 3: Brain and spleen PrP^{res} accumulation in tg338 mice inoculated with AS scrapie**
697 **adapted in tgBov**
698
699 Groups of mice (n≥6) that express ovine VRQ PrP (tg338 mice) were intra-cerebrally
700 challenged with atypical scrapie isolates (AS) and AS that had previously been adapted (2
701 iterative passages) in tgBov mice. In parallel, cattle c-BSE isolate and ovine BSE isolate
702 (adapted in tg Bov) were transmitted (2 iterative passages) in tg338 mice.
703 (**a**) In tg338 mice (2nd passage) the PrP^{res} distribution pattern in the brain (thalamic coronal
704 section: bar: 160 µm) and in the spleen (bar: 100 µm) was established by paraffin embedded
705 tissue blot using anti PrP monoclonal antibody Sha31 (epitope 145-YEDRYYRE-152).
706 (**b**) The western blot profile of PK resistant PrP (PrP^{res}) in the spleen of tg338 mice (2nd
707 passage) inoculated with AS isolates, AS isolates passaged in tgBov and c-BSE (cattle and
708 ovine origin) was established using anti PrP monoclonal antibody Sha31.
709

710
711 **Figure 4: PrP^{res} detection in PMCA reactions seeded with atypical/Nor98 scrapie**
712 **isolates.**
713
714 Protein misfolding cyclic amplification (PMCA) reactions were seeded with Atypical/Nor98
715 scrapie (AS) isolates (1/50 diluted 10% brain homogenate) that had been identified in five
716 European countries (see Table 3). PMCA reactions seeded with brain homogenate from a
717 TSE-free sheep (originating from New Zealand) and unseeded PMCA reactions were included
718 as specificity controls. PMCA substrate consisted of brain homogenate from either bovine PrP
719 (tgBov) or ovine PrP (tgARQ) mice. PMCA reactions were subjected to three (tgARQ
720 substrate) or four (tgBov substrate) amplification rounds each comprising 96 cycles (10s
721 sonication-14 minutes and 50 seconds incubation at 39.5°C) in a Qsonica700. The PMCA
722 reactions were analysed by western blot for the presence of abnormal PK-resistant PrP (PrP^{res})
723 using anti PrP monoclonal antibodies Sha31 (epitope 145-YEDRYYRE-152) and/or 12B2
724 (epitope 89-WGQGG-93). Each western blot included a classical scrapie isolate (labelled as
725 WB control) and an ovine c-BSE isolate as controls.
726
727 **Figure 5: PMCA seeding activity detection in two bioassay end-point titrated**
728 **Atypical/Nor98 isolates**
729
730 Two atypical/Nor98 isolates AS 25 and AS 26 (Table 3) were end-point titrated in tg338 mice
731 (1/10 dilution series, 6 tg338 mice per dilution). For both isolates, the last positive
732 transmissions were observed in mice that received a 10^{-6} dilution of the original 10% w/vol
733 brain material (SI appendix Table S3). The original AS isolates and the brains of clinically
734 affected mice inoculated with neat and 10^{-6} diluted isolates were used to seed PMCA reactions
735 that either used tgARQ or tgBov as substrate. PMCA reactions seeded with age matched
736 inoculated tg338 mice and unseeded reactions were included as specificity controls. Reactions
737 were subjected to three (tgARQ substrate) or four (tgBov substrate) amplification rounds each
738 comprising 96 cycles (10s sonication-14 minutes and 50 seconds incubation at 39.5°C) in a
739 Qsonica700. The PMCA reactions were analysed by western blot for the presence of
740 abnormal PK resistant PrP (PrP^{res}) using the Sha31 (epitope 145-YEDRYYRE-152) and/or
741 the 12B2 (89-WGQGG-93) anti PrP antibodies. Each western blot included a classical scrapie
742 isolate (labelled as WB control) and an ovine c-BSE isolate as controls.
743
744

Table 1: Inoculation of atypical scrapie isolates in bovine PrP (tgBov) expressing mice

Isolates			TgBov					
			1st passage		2nd passage		3rd passage	
Identifiant	**Origin**	**Genotype**	**Positive mice**	**Incubation (mean±SD)**	**Positive mice**	**Incubation (mean±SD)**	**Positive mice**	**Incubation (mean±SD)**
AS 1	Fr	ARQ*/ARQ	1/6	533	3/5	317±63	7/7	235±16
AS 2	Sp	ARR/ARQ	0/6	>650	7/9	354±26	5/5	273±5
AS 3	Sp	ARQ/ARH	0/6	>650	2/11	504, 525‡	12/12	269±13
AS 4	Nor	ARQ*/ARQ*	3/4	395± 44	6/6	230±17	6/6	271±18
AS 5	Sp	ARQ/ARQ	0/6	>650	0/4	>650	NA	
AS 6	Sp	ARQ/ARH	0/6	>650	1/4	>650‡	NA	
AS 7†	It	ARQ/AHQ	0/6	>650	1/7	424	6/6	286±14
AS 8	Po	ARQ/ARQ	1/5	439	5/5	297±14	6/6	250±4
PS42	Fr	VRQ/VRQ	0/6	>650	0/6	>650	0/6	>650
Ovine c-BSE	Fr	ARQ/ARQ	6/6	254±19	6/6	234±12	6/6	232±6
Cattle c-BSE	Fr	-	6/6	295±12	6/6	265±35	6/6	243±7

Transgenic mice that express the bovine PrP (tgBov) were inoculated intra-cerebrally (6 to 12 mice, 20µL per mouse) with 8 sheep or goat (†) atypical scrapie (AS) isolates originating from five different countries; France (Fr), Spain (Sp), Norway (Nor), Italy (It) or Portugal (Po). The AS affected animals displayed a different *PRNP* genotype at codons 136, 154 and 171. Some also displayed a F/L dimorphism at codon 141 (*). Cattle classical BSE (c-BSE), ovine c-BSE (first passage of cattle c-BSE in an ARQ/ARQ sheep by the intracerebral route) and classical scrapie (PS42) isolates were inoculated in the same mouse model. After first and second passage, clinically affected or asymptomatic mice that had lived for more than 500 days post inoculation were pooled and used for subsequent passage in the same line. Mice were considered positive when abnormal PrP deposition was detected in the brain. (‡) indicate abnormal PrP positive and found dead animals (without symptoms). Incubation periods (in days) are shown as mean±SD except when less than 50% of the mice were found to be positive. In that case the incubation periods of the positive mice are individually presented. NA: not available. Cattle c-BSE transmission in tgBov data were already reported in Torres et al 2014 (51)

Table 2: Inoculation of atypical scrapie adapted in tgBov and c-BSE isolates in ovine PrP expressing mouse models (tg338 and tgARQ)

Isolates		Tg338				TgARQ			
		1st passage		2nd passage		1st passage		2nd passage	
Identifiant	Origin	Positive mice	Incubation (mean±SD)	Positive mice	Incubation (mean±SD)	Positive mice	Incubation (mean±SD)	Positive mice	Incubation (mean±SD)
AS 2	2nd pass in TgBov	6/6‡	>650	6/6	617±75	6/6	350±9	6/6	260±3
AS 3	3rd pass in TgBov	6/6‡	>650	6/6	672±83	6/6	354±21	6/6	257±2
Ovine c-BSE	2nd pass in TgBov	6/6‡	>750	6/6	653±32	6/6	270±12	6/6	259±4
Cattle c-BSE	cattle	6/6‡	>700	6/6	682±52	6/6	321±16	6/6	263±7

Transgenic mice that express the VRQ (tg338) or ARQ (tgARQ) variants of ovine PrP were inoculated intra-cerebrally (6 mice, 20µL per mouse) with atypical scrapie isolates or ovine c-BSE isolate that had previously been adapted in tgBov (2 iterative passages). Cattle BSE was also included as control. After first passage, clinically affected or asymptomatic mice that had lived for more than 500 days post inoculation were pooled and used for second passage in the same line. Mice were considered positive when abnormal PrP deposition was detected in the brain. (‡) indicate abnormal PrP positive and found dead animals (without symptoms). Incubation periods (in days) are shown as mean±SD.

Table 3: Protein Misfolding Cyclic Amplification seeding activity in atypical scrapie isolates

Isolates			PMCA positive reactions	
Identifiant	**Origin**	**Genotype**	**TgBov substrate**	**TgARQ substrate**
AS 1	Fr	ARQ*/ARQ	3/12	5/12
AS 2	Sp	ARR/ARQ	2/12	3/12
AS 3	Sp	ARQ/ARH	3/12	4/18
AS 4	No	ARQ*/AFRQ	12/12	9/12
AS 5	Sp	ARQ/ARQ	4/12	3/12
AS 6	Sp	ARQ/ARH	5/12	1/12
AS 7	It	ARQ/AHQ	1/12	2/12
AS 8	Po	ARQ/ARQ	ND	ND
AS 9	Nor	ARR/ARQ	0/12	2/10
AS 10		ARQ*/AHQ	1/12	0/10
AS 11		AHQ/ARQ	3/12	7/12
AS 12		ARR/ARQ	0/12	0/12
AS 13		ARR/AHQ	0/12	0/12
AS 14		ARQ/AHQ	1/12	1/12
AS 15		ARR/ARR	3/12	1/12
AS 16		ARR/AHQ	1/12	3/10
AS 17		ARQ*/AHQ	1/12	1/10
AS 18		ARQ*/AHQ	0/12	0/10
AS 19	Po	ARR/ARR	0/12	0/12
AS 20		ARR/AHQ	3/12	1/12
AS 21		ARR/ARR	0/12	0/10
AS 22		ARQ*/AHQ	0/12	0/12
AS 23		ARQ*/ARQ	1/12	1/12
AS 24		ARQ*/ARQ	2/12	4/12
AS 25	Fr	AHQ/AHQ	0/12	1/12
AS 26		ARQ/ARQ	1/12	1/12
TSE free sheep		ARQ/ARQ	0/12	0/12
Unseeded		-	0/120	0/120

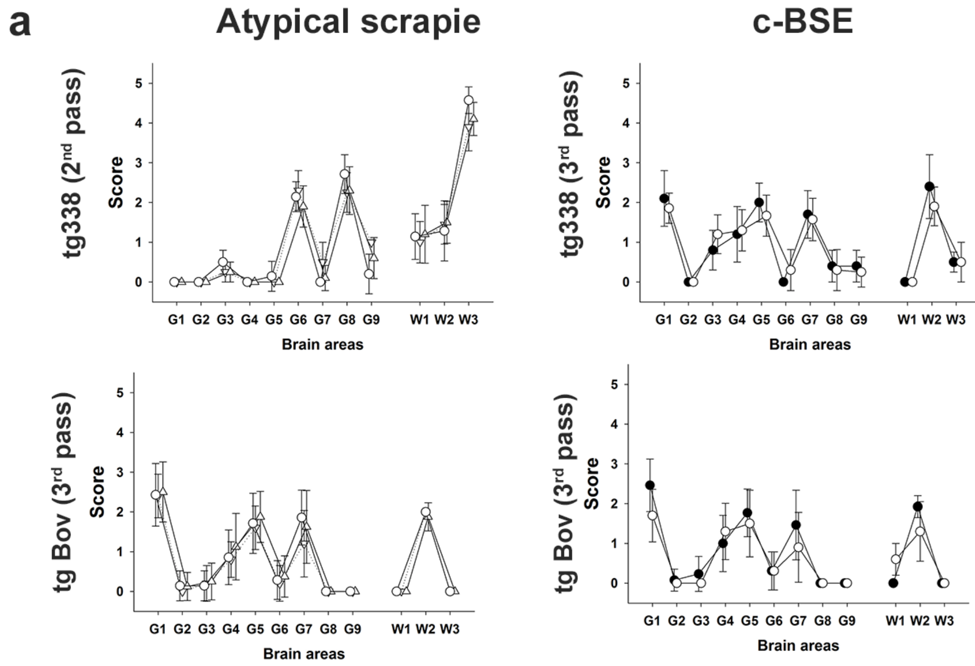
Twenty-six AS scrapie cases (1/50 diluted 10% brain homogenates) originating from five different countries (France (Fr), Spain (Sp), Italy (It), Portugal (Po) and Norway (Nor)) were used to seed PMCA reactions (5 µl of seed per reaction). The AS affected animals displayed different *Prnp* genotypes at codons 136, 154 and 171. Some also displayed a F/L dimorphism at codon 141 (*).

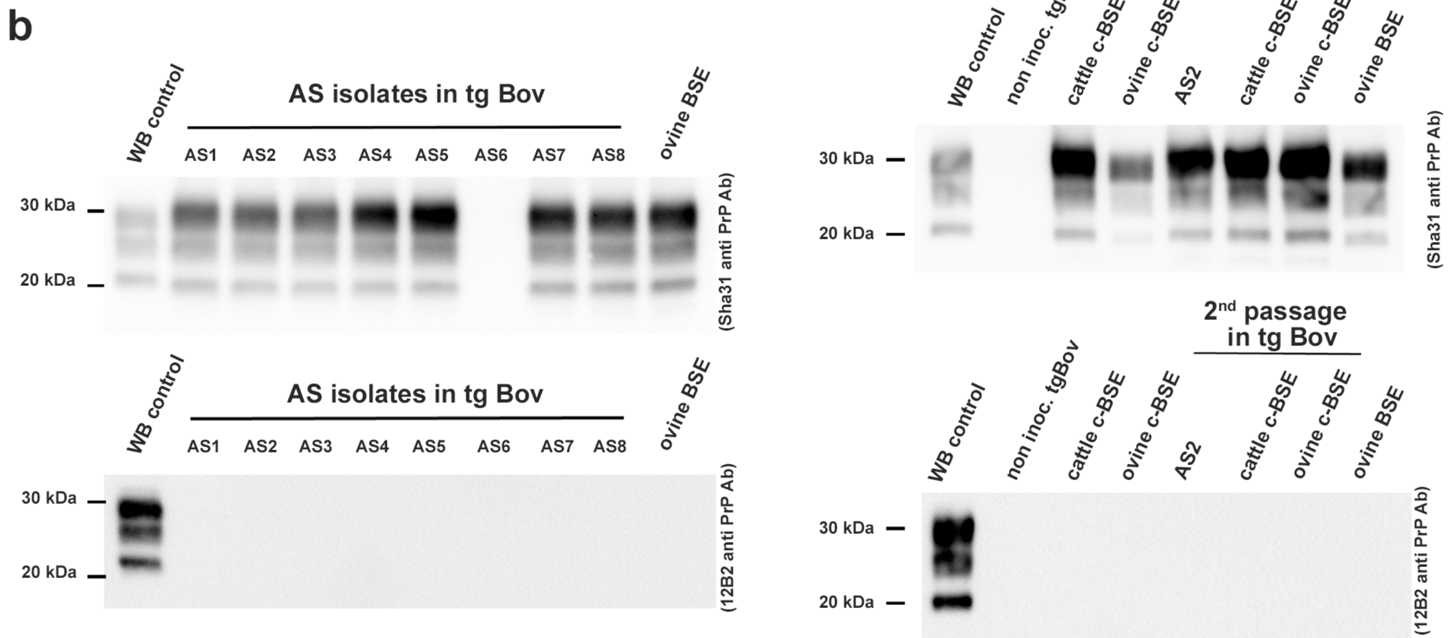
Two different PMCA substrates were used. The first one was prepared using brains from transgenic mice over-expressing the ARQ variant of the sheep prion protein (tgARQ). The second was prepared using brains from transgenic mice over-expressing the bovine prion protein (tgBov). For each isolate and substrate ten to eighteen individual replicates were tested. Reactions were subjected to 3 amplification rounds. After each round reaction products (1 volume) were mixed with fresh substrate (9 volumes) to seed the following round. PMCA reaction products (third amplification round) were analysed by western blot for the presence of PrP^{res}. The number of PrP^{res} western blot positive reactions / total number of reactions are reported. Unseeded reactions and reactions seeded with brain homogenate prepared from a TSE free sheep were included as specificity controls. ND: not done.

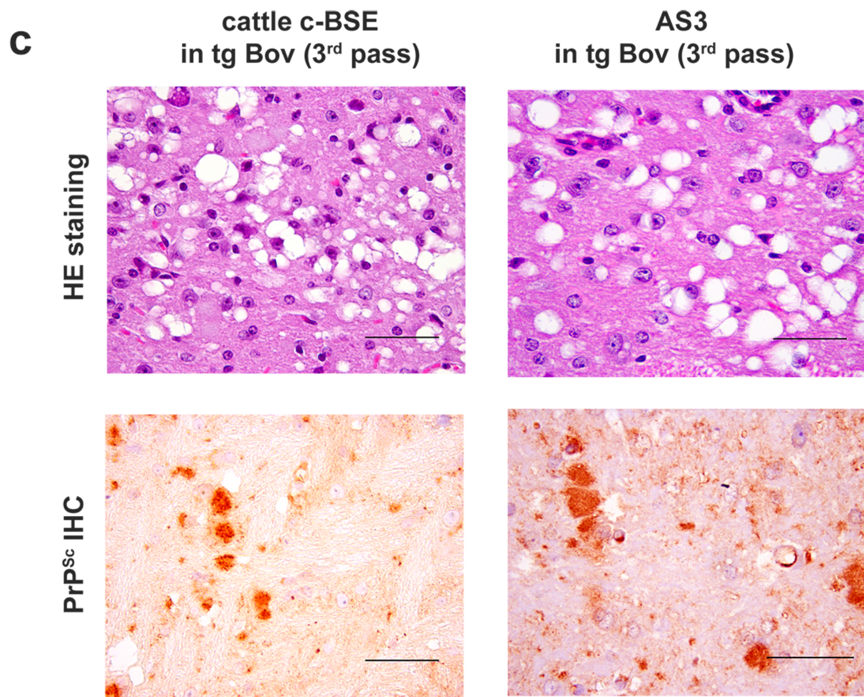
Table 4: PMCA seeding activity in atypical scrapie passaged in tg338

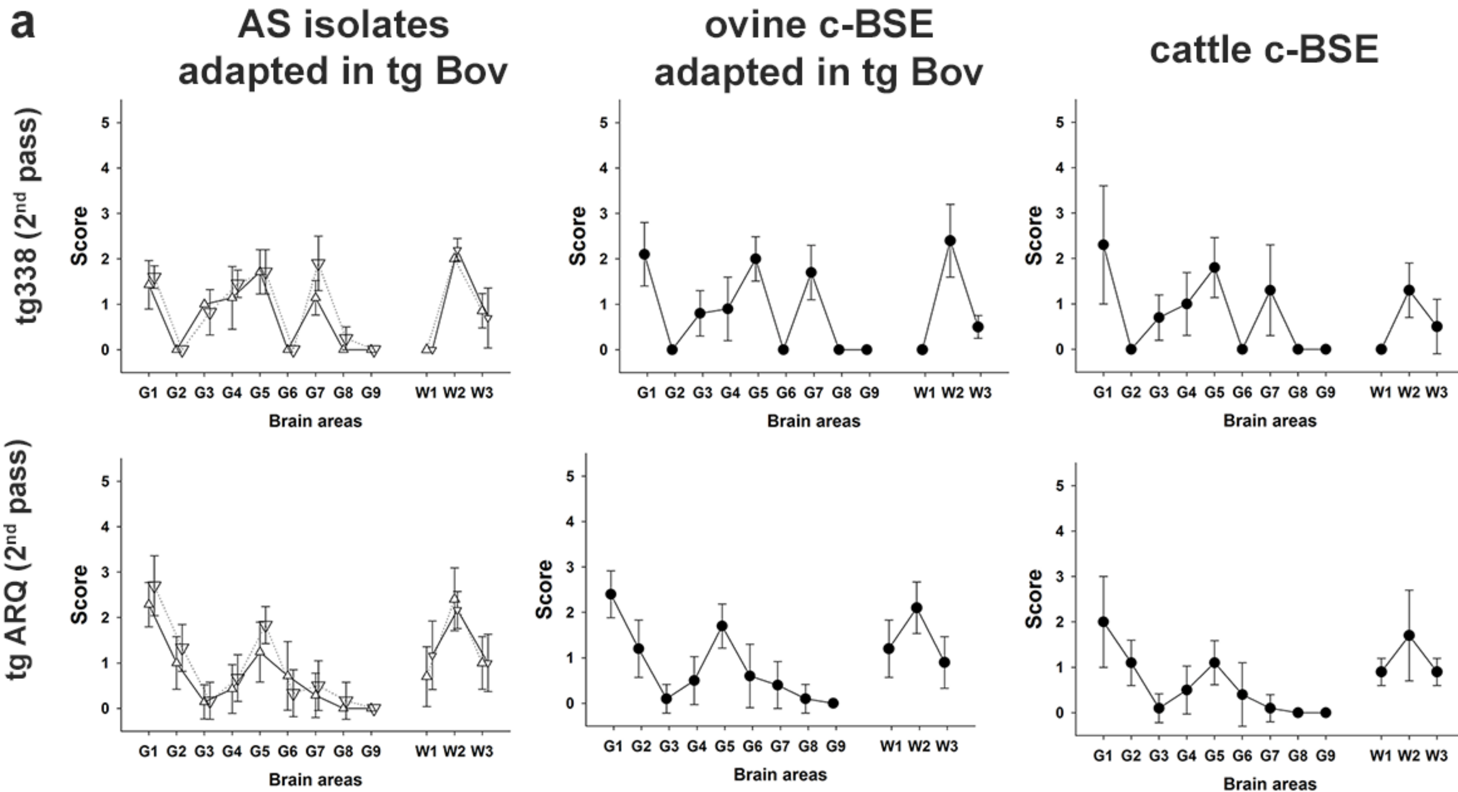
PMCA seeds			PrP^{res} positive PMCA reactions		Seeding activity (SA_{50}/mL)	
Case	**Origin**		**TgBov substrate**	**TgARQ substrate**	**TgBov substrate**	**TgARQ substrate**
AS 25	**Sheep**		0/12	1/12	0 (0-$10^{1.86}$)*	$10^{1.40}$
	1st passage in tg338 (neat)	**Mouse 1**	2/12	1/12	$10^{1.72}$	$10^{1.40}$
	2nd passage in tg338 (neat)	**Mouse 1**	2/12	1/12	$10^{1.72}$	$10^{1.40}$
	End-point titration in tg338 (10^{-6} dilution)	**Mouse 1**	2/12	3/12	$10^{1.72}$	$10^{1.92}$
		Mouse 2	1/12	3/12	$10^{1.40}$	$10^{1.92}$
		Mouse 3	2/12	2/12	$10^{1.72}$	$10^{1.72}$
AS 26	**Sheep**		1/12	1/12	$10^{1.40}$	$10^{1.40}$
	1st passage in tg338 (neat)	**Mouse 1**	2/12	0/12	$10^{1.72}$	0 (0-$10^{1.86}$) *
	2nd passage in tg338 (neat)	**Mouse 1**	2/12	0/12	$10^{1.72}$	0 (0-$10^{1.86}$) *
	End-point titration in tg338 (10^{-6} dilution)	**Mouse 1**	2/12	1/12	$10^{1.72}$	$10^{1.40}$
		Mouse 2	1/12	1/12	$10^{1.40}$	$10^{1.40}$
	Non inoculated tg338	**Mouse 1**	0/12	0/12	-	-
		Mouse 2	0/12	0/12	-	-
		Mouse 3	0/12	0/12	-	-

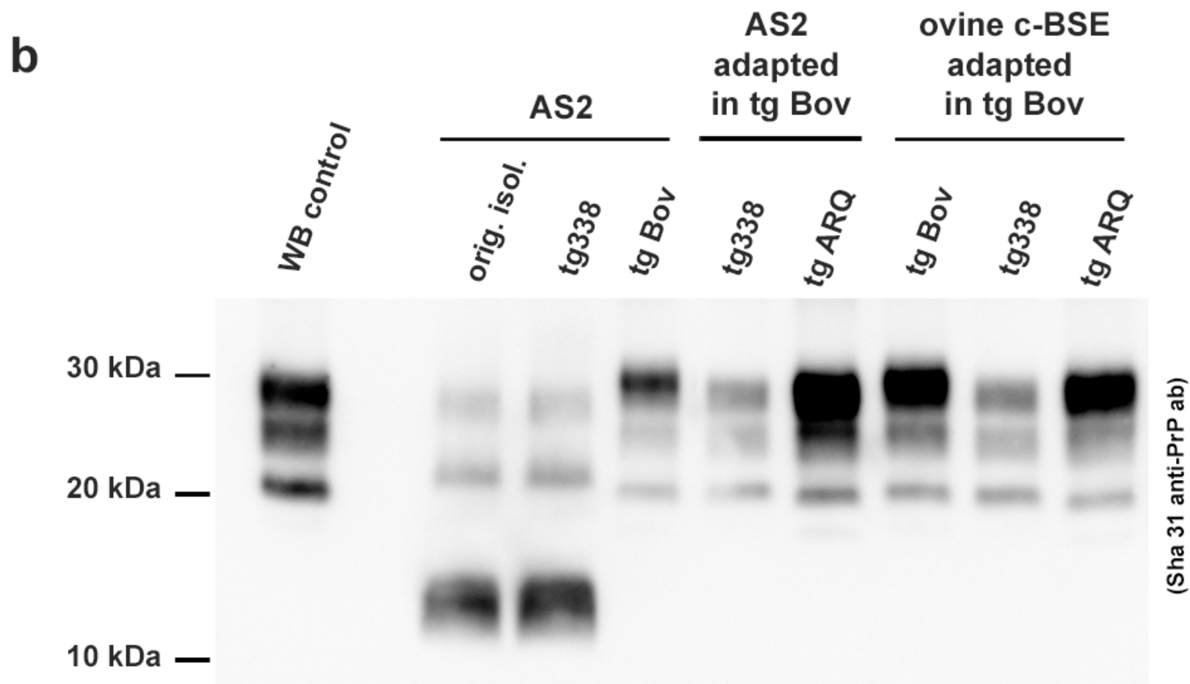
Two sheep atypical scrapie (AS) isolates were selected. The 10% w/vol brain homogenates were inoculated into tg338 mice (2 iterative passages). Groups of 6 tg338 mice were inoculated intra-cerebrally with 20µL of serial ten-fold dilutions of the same homogenates. Transmission was observed in 3 (AS 25) and 2 (AS 26) mice inoculated with 10^{-6} brain homogenate. No transmission was observed at lower dilutions. PMCA reactions (12 replicates) were seeded with 1/50 diluted brain homogenate (10% w/vol) from (i) the original sheep, (ii) the second passage tg338 mice (pool of brains) and (iii) individual brain from positive tg338 in the end-point titration experiment. Brain homogenates (10% w/vol) from age matched, non-inoculated tg338 mice were also used as seeds (1/50 diluted). Two different PMCA substrates were used. The first one was prepared using brains from transgenic mice over-expressing the ARQ variant of the sheep prion protein. The second was prepared using brains from transgenic mice over-expressing the bovine prion protein (tgBov). Reactions were subjected to up to 4 amplification rounds. After each round reaction products (1 volume) were mixed with fresh substrate (9 volumes) to seed the following round. PMCA reaction products were analysed by western blot for the presence of PrP^{res}. The number of PrP^{res} western blot positive reactions / total number of reactions are reported. Seeding activity titers were estimated using the Spearman Karber's limiting dilution titration method (most likely value) or when no positive reaction was observed, by the Poisson's probabilistic model (*: most likely value and IC 95%) as described by Brown et al (66). Titers are given as the number of PMCA SA_{50} per mL of 10% brain homogenate.


a
Atypical scrapie
c-BSE
tg338 (2nd pass)
tg338 (3rd pass)
tg Bov (3rd pass)
Score
Brain areas
G1 G2 G3 G4 G5 G6 G7 G8 G9 W1 W2 W3


b
WB control
AS isolates in tg Bov
AS1 AS2 AS3 AS4 AS5 AS6 AS7 AS8
ovine BSE
30 kDa
20 kDa
(Sha31 anti PrP Ab)
(12B2 anti PrP Ab)
2nd passage in tg Bov
non inoc. tgBov
cattle c-BSE
ovine c-BSE
AS2


c
cattle c-BSE in tg Bov (3rd pass)
AS3 in tg Bov (3rd pass)
HE staining
PrPSc IHC


a
AS isolates
adapted in tg Bov
ovine c-BSE
adapted in tg Bov
cattle c-BSE
tg338 (2nd pass)
tg ARQ (2nd pass)
Score
Brain areas
G1 G2 G3 G4 G5 G6 G7 G8 G9 W1 W2 W3

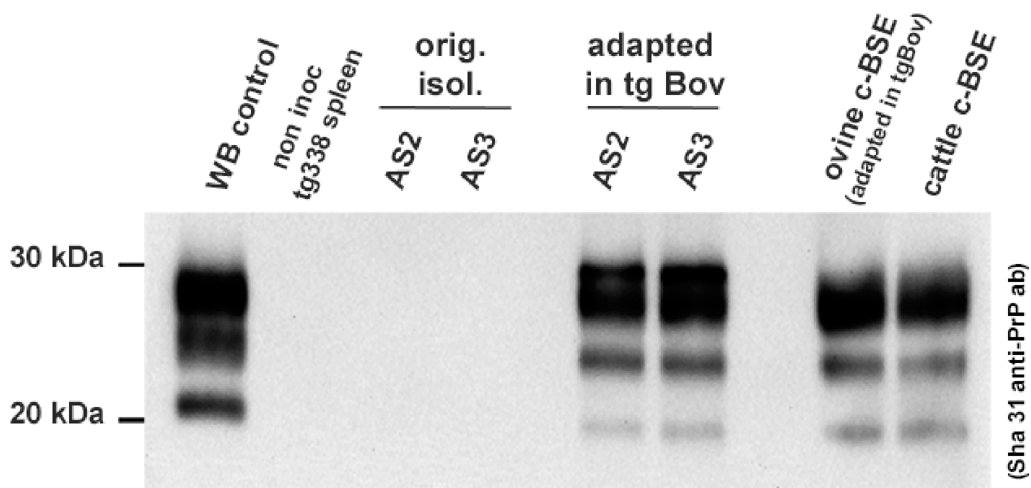

b
AS2
AS2
adapted
in tg Bov
ovine c-BSE
adapted
in tg Bov
WB control
orig. isol.
tg338
tg Bov
tg338
tg ARQ
tg Bov
tg338
tg ARQ
30 kDa
20 kDa
10 kDa
(Sha 31 anti-PrP ab)

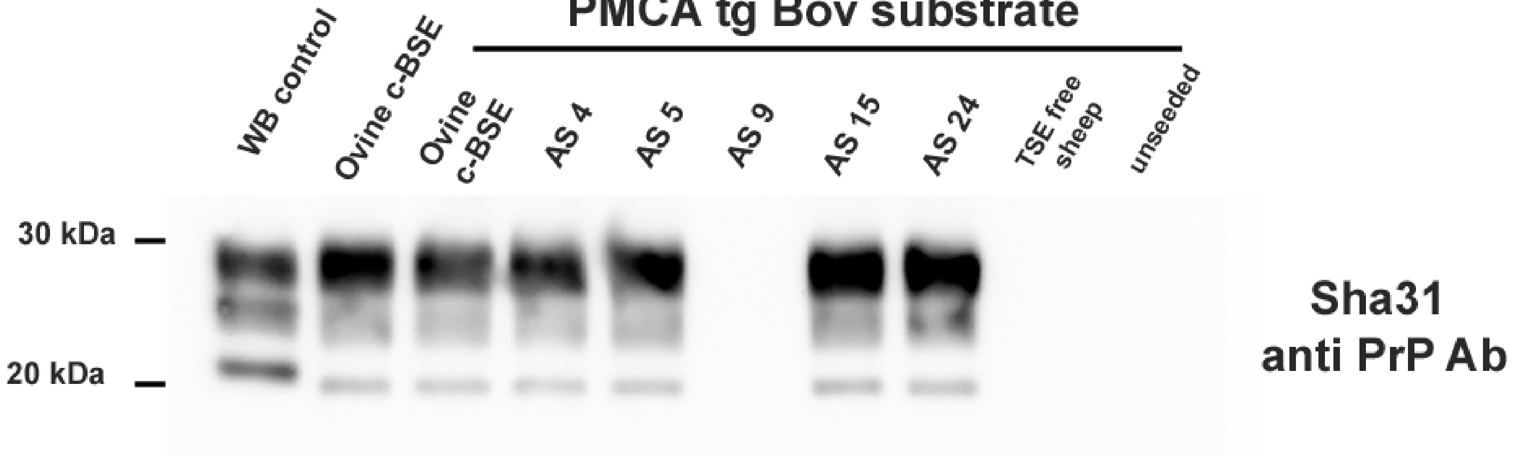
a

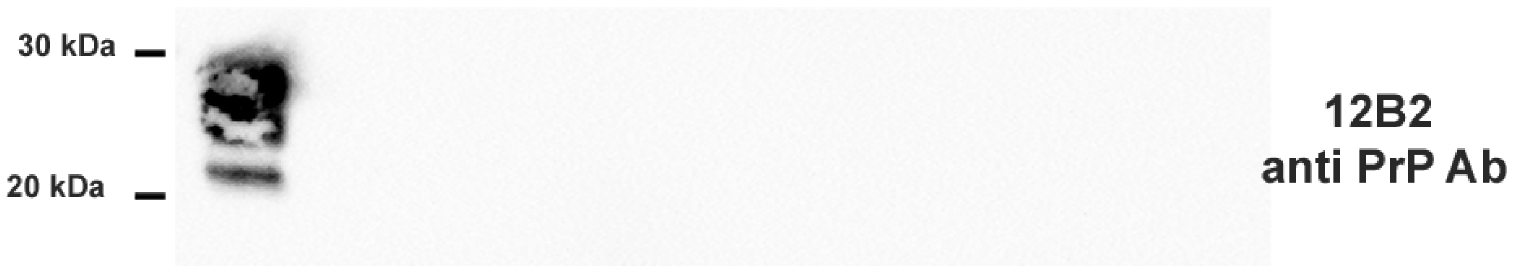
2nd passage in tg338

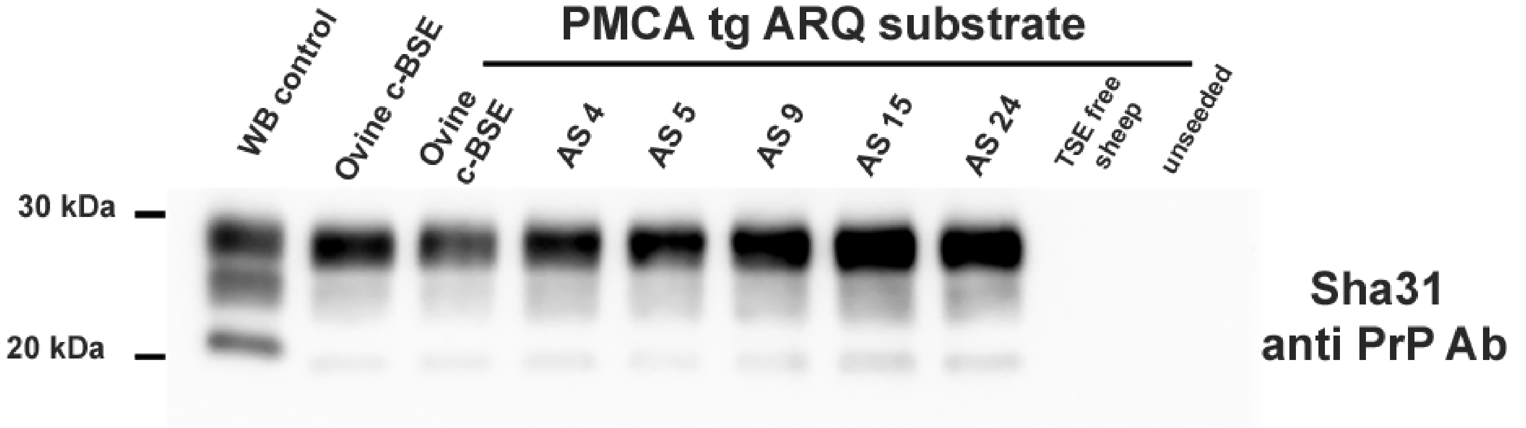
	AS2 original isolate	AS2 adapted in tg Bov	Ovine c-BSE adpated in tg Bov
Brain			
Spleen			

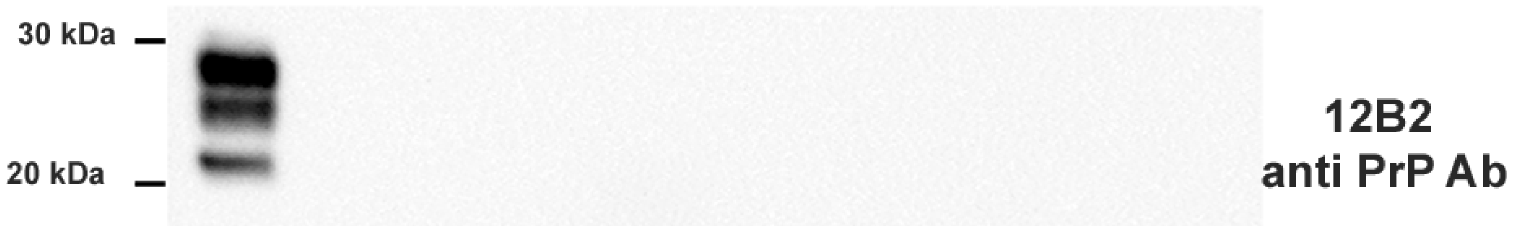
b

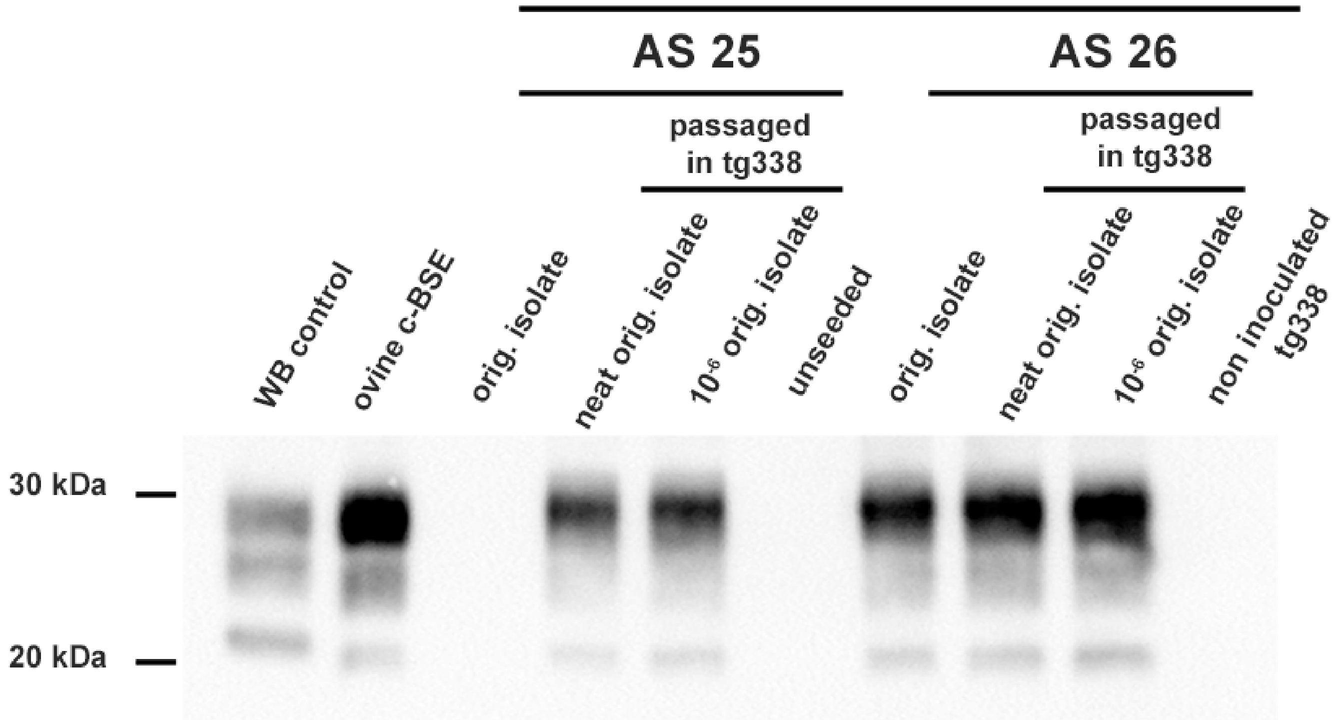


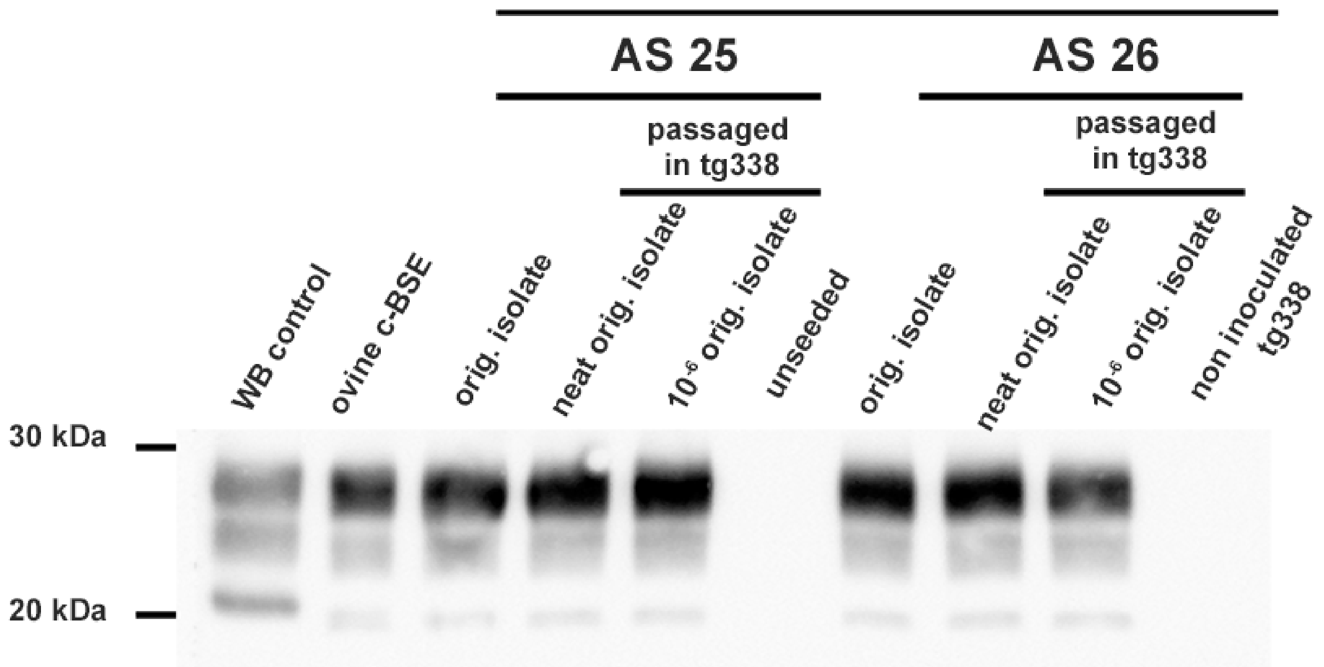

PMCA tg Bov substrate
WB control
Ovine c-BSE
Ovine c-BSE
AS 4
AS 5
AS 9
AS 15
AS 24
TSE free sheep
unseeded
30 kDa
20 kDa
Sha31 anti PrP Ab


30 kDa
20 kDa
12B2 anti PrP Ab


PMCA tg ARQ substrate
WB control
Ovine c-BSE
Ovine c-BSE
AS 4
AS 5
AS 9
AS 15
AS 24
TSE free sheep
unseeded
30 kDa
20 kDa
Sha31 anti PrP Ab


30 kDa
20 kDa
12B2 anti PrP Ab


PMCA amplification tg Bov substrate
AS 25
AS 26
passaged in tg338
passaged in tg338
WB control
ovine c-BSE
orig. isolate
neat orig. isolate
10^{-6} orig. isolate
unseeded
orig. isolate
neat orig. isolate
10^{-6} orig. isolate
non inoculated tg338
30 kDa
20 kDa


PMCA amplification tg ARQ substrate
AS 25
AS 26
passaged in tg338
passaged in tg338
WB control
ovine c-BSE
orig. isolate
neat orig. isolate
10^{-6} orig. isolate
unseeded
orig. isolate
neat orig. isolate
10^{-6} orig. isolate
non inoculated tg338
30 kDa
20 kDa

1 # The emergence of classical BSE from atypical/Nor98 scrapie

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25

26 **Abstract**

27 Atypical/Nor98 Scrapie (AS) is a prion disease of small ruminants. Currently there are no
28 efficient measures to control this form of prion disease and, importantly, the zoonotic
29 potential and the risk that AS might represent for other farmed animal species remains largely
30 unknown. In this study, we investigated the capacity of AS to propagate in bovine PrP
31 transgenic mice. Unexpectedly, the transmission of AS isolates originating from five different
32 European countries to bovine PrP mice resulted in the propagation of the classical BSE (c-
33 BSE) agent. Detection of prion seeding activity *in vitro* by protein misfolding cyclic
34 amplification (PMCA) demonstrated that low levels of the c-BSE agent were present in the
35 original AS isolates. C-BSE prion seeding activity was also detected in brain tissue of ovine
36 PrP mice inoculated with limiting dilutions (end-point titration) of ovine AS isolates. These
37 results are consistent with the emergence and replication of c-BSE prions during the *in-vivo*
38 propagation of AS isolates in the natural host. These data also indicate that c-BSE prions, a
39 known zoonotic in humans, can emerge as a dominant prion strain during passage of AS
40 between different species. These findings provide an unprecedented insight into the evolution
41 of mammalian prion strain properties triggered by intra- and inter-species passage. From a
42 public health perspective, the presence of c-BSE in AS isolates suggest that cattle exposure to
43 small ruminant tissues and products could lead to new occurrences of c-BSE.
44 **(235 words)**

45
46
47
48

Significance Statement

The origin of transmissible BSE in cattle remains unestablished. Sheep scrapie is a potential source of this known zoonotic. Here we investigated the capacity of sheep scrapie to propagate in bovine PrP transgenic mice. Unexpectedly, transmission of atypical but not classical scrapie in bovine PrP mice resulted in propagation of classical BSE prions. Detection of prion seeding activity by *in vitro* protein misfolding cyclic amplification demonstrated BSE prions in the original atypical scrapie isolates. BSE prion seeding activity was also detected in ovine PrP mice inoculated with limiting dilutions of atypical scrapie. Our data demonstrate that classical BSE prions can emerge during intra- and inter-species passage of atypical scrapie and provide an unprecedented insight into the evolution of mammalian prions.

Introduction

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are fatal neurodegenerative disorders that affect a large spectrum of mammalian species. These conditions include scrapie in small ruminants, classical bovine spongiform encephalopathy (c-BSE) in cattle and sporadic Creutzfeldt-Jakob disease (sCJD) or variant CJD (vCJD) in humans.

The fundamental event in prion propagation is the conversion of the normal cellular prion protein (PrP^{C}) into an abnormal disease-associated isoform (PrP^{Sc}) in tissues of infected individuals. PrP^{C} is completely degraded by digestion with proteinase K (PK) whereas PrP^{Sc} is N-terminally truncated resulting in a PK resistant core termed PrP^{res} (1). According to the prion concept, PrP^{Sc} is the principal, if not sole component of the transmissible prion agent (2) and PrP^{res} is a disease marker for prion diseases (1, 3). Particular biochemical properties of PrP^{Sc}, such as detergent solubility, PK resistance and electromobility evidenced by western blot can be used to distinguish between different prion agents or strains (4, 5).

Intra-species transmission of prion disease between individuals is typically quite efficient. In contrast, inter-species transmission of prions can be unpredictable with apparent failure of disease transmission on many occasions. In other cases, clinical prion disease may not be evident but rather there is the presence of subclinical infection (6). When inter-species prion transmission does occur, the propagating agent can remain identical to the original prion strain, or can display different biological properties compared to the original inoculum (7, 8). This complex set of outcomes for inter-species prion challenge are collectively referred to as the transmission barrier phenomenon.

85 After identification of the gene encoding PrP it was soon discovered that differences in amino
86 acid sequence between host PrP^C and donor PrP^{Sc} constitutes the principal determinant of the
87 transmission barrier. For example, the resistance of wild-type mice to clinical prion disease
88 induced by hamster scrapie is abrogated by transgenic expression of hamster PrP^C in mice (9,
89 10). As a consequence, mice genetically engineered to express particular species forms of PrP
90 sequence, in the absence of endogenous mouse PrP, have emerged as relevant models to
91 experimentally characterize the outcome of prion strain transmission between species (11). It
92 is also now well established that strain properties have a significant impact on the ability of
93 prions to cross the species barrier. For instance, human vCJD can be transmitted readily to
94 conventional mice but it is extremely difficult for sCJD to propagate in the same mouse lines
95 (12, 13). Furthermore, the amino acid sequence of PrP^{Sc} influences the efficacy of inter-
96 species prion transmissions since studies in human PrP transgenic mouse models indicate that
97 the human species barrier is more permeable to sheep-passaged BSE compared to its cattle
98 counterpart (14).
99
100 From a public health perspective, the transmission barrier phenomenon and its capacity to
101 limit the inter-species propagation of prion disease has long been considered as an effective
102 protection of humans against animal TSEs (15). However, in 1996, the new human prion
103 disease vCJD was observed in UK individuals. Multiple lines of evidence indicated that vCJD
104 was the likely consequence of dietary exposure of humans to the agent responsible for c-BSE
105 in cattle, an epizootic prion disease that has spread in bovine hosts through the recycling of
106 prion-contaminated animal carcasses in the animal food chain (16). Since the emergence of
107 vCJD, considerable efforts have been deployed to characterize not only the zoonotic potential
108 of animal prions but also their capacity to propagate in farmed animal species.
109

110 Atypical/Nor98 scrapie (AS) probably represents the largest geographically spread known
111 animal prion disease. Since its original discovery in 1998 in Norway, AS has been identified
112 in most EU member states, in Asia and in North and South America (17). AS has also been
113 detected in Australia and New Zealand, two countries that were believed to be free of animal
114 TSEs (18, 19). Retrospective studies carried out in archived animal tissues identified an AS
115 case in a sheep that died in 1972 in the UK demonstrating that the disease has been present in
116 small ruminant populations for many decades (20).

117

118 Bioassay in ovine PrP transgenic mice provided evidence that AS comprised a single prion
119 strain (21-23). The AS prion strain was associated with a multi-band PrP^{res} signature that
120 contrasted with those normally observed in small ruminant TSE cases (24). Since there is no
121 statistical difference in the apparent prevalence of atypical scrapie between sheep flocks in
122 general and those flocks where a positive case had been identified, atypical scrapie is
123 considered by many as a non-contagious prion disease that arises sporadically in sheep and
124 goats (25). However, atypical scrapie can be experimentally transmitted via the oral route in
125 small ruminants, resulting in a similar clinico-pathological phenotype to that observed in
126 natural cases (26). Consequently, the origin of atypical scrapie (spontaneous disorder versus
127 acquired disease) remains an open question.

128

129 In this study we used mice transgenic for bovine PrP (tgBov mouse line) to characterize the
130 capacity of sheep AS isolates to cross the bovine transmission barrier. Unexpectedly, the TSE
131 agent that propagated in tgBov mice was indistinguishable from the prion strain that was
132 responsible for the c-BSE epizootic in cattle. In addition, our sensitive detection of c-BSE by
133 *in vitro* PMCA methodology indicated that this bovine prion strain was present as a minor
134 prion strain in the original sheep AS isolates, and that AS prion strain replication in an ovine

135 PrP host was accompanied by the generation of c-BSE prions. Collectively, these data provide
136 compelling evidence for the emergence and the propagation of zoonotic mammalian prions
137 during intra- and inter-host transmission of the AS prion strain.
138

139 **Results**

140 A panel of 8 atypical scrapie (AS) cases collected from sheep and goats in five different
141 European countries was obtained (Table1). All of the AS isolates displayed a multi-band
142 abnormal PrP (PrP^{res}) western blot profile that was considered to be specific for small
143 ruminant AS (SI appendix Figure S1). This panel of AS isolates was transmitted to the VRQ
144 ovine PrP transgenic mice (tg338). The transmission properties such as incubation period (SI
145 appendix Table S1), vacuolar lesion profile (Figure 1a) and PrP^{res} western blot profile in the
146 brain (SI appendix Figure S1), of the propagated AS isolates observed after two or three
147 iterative passages in tg338 were similar and were the same as that previously reported for AS
148 passage in tg338 mice (21-23).
149

150 The panel of 8 AS isolates was individually serially transmitted (2 or 3 iterative passages) in
151 bovine PrP transgenic mice (tgBov) (Table 1). On first passage, signs of clinical prion disease
152 were observed in a low proportion of inoculated tgBov mice. PrP^{res} was detected by western
153 blot in the brains of clinically affected mice and in some mice that displayed no apparent
154 clinical signs of prion disease when euthanised at the end of their life expectancy. No PrP^{res}
155 accumulation was observed in tgBov mice after inoculation with several of the AS isolates
156 (AS 2, AS 3, AS 5, AS 6 and AS 7), as was the case with classical scrapie PS42 (Table 1).
157

158 Second passage of the AS isolates in tgBov mice was performed using either first passage
159 PrP^{res}-positive brains or pooled PrP^{res}-negative brains as inoculum. During this process, 7 out
160 of the 8 AS isolates caused the occurrence of clinical prion disease in a proportion of animals
161 in each group inoculated. On third passage (available for AS 1, 2, 3, 4 and 7) 100% attack
162 rates and mean incubation periods that ranged between 235 and 286 dpi were recorded (Table
163 1).

164 At each passage stage, a three band PrP^{res} western blot profile characterised by a prominent
165 di-glycosylated PrP band was observed in the brains of the clinically-positive tgBov mice
166 (Figure 1b). Strikingly, the lesion profile (Figure 1a), the PrP^{res} western blot profile (Figure
167 1b) and the histopathological lesions (Figure 1c) in the brains of AS inoculated tgBov mice
168 were identical to those observed for transmission of c-BSE (sheep or cattle origin) to tgBov
169 mice. Importantly, the inoculation of one classical scrapie isolate (PS42) to the same mouse
170 models resulted in the occurrence of a 100% attack rate for prion disease with a short
171 incubation period in tg338 mice (SI appendix Table S1), but no clinical disease or PrP^{res}
172 accumulation in the brains of tgBov (Table 1).
173
174 In order to further characterize the nature of the TSE agent that propagated in tgBov mice
175 inoculated with the AS isolates, prions obtained after second passage (isolate AS 2) or third
176 passage (isolate AS 3) in this mouse line were transmitted (two iterative passages) to VRQ
177 (tg338) and ARQ (tgARQ) ovine PrP transgenic mice (Table 2). The incubation periods
178 (Table 2 and SI appendix Table S1), the lesion profile (Figure 2a) and the PrP^{res} western blot
179 profile in the brain (Figure 2b) of tg338 mice inoculated with tgBov-adapted AS isolates
180 clearly differed from those observed in the same mouse line inoculated with the original AS
181 isolates.
182 No PrP^{res} deposition could be detected in the spleen of tg338 mice inoculated with the original
183 AS isolates (Figure 3a). Conversely, transmission in tg338 of tgBov-adapted AS isolates was
184 associated with a PrP^{res} accumulation in the spleen as was transmission of ovine and cattle c-
185 BSE (Figure 3a). PrP^{res} WB profile in the spleen of tg338 mice that were inoculated with c-
186 BSE and AS isolates passaged in tgBov were identical (Figure 3b).
187 Transmission of tgBov-adapted AS isolates in both tg338 and tgARQ mice resulted in prion
188 incubation periods (Table 2), brain vacuolar lesion profiles (Figure 2a), PrP^{res} western blot

189 profile patterns (Figure 2b) and PrP^{res} distribution patterns in the brain (Figure 3a) that were
190 similar to c-BSE passaged in tg338 and tgARQ mice. Collectively these results demonstrate
191 beyond reasonable doubt that our transmission of AS in a bovine PrP host resulted in the
192 propagation of the c-BSE agent. Since the bioassays reported here were performed in three
193 independent institutes (located in France and Spain) that used inoculum prepared by five
194 distinct laboratories, we exclude the possibility of a cross contamination of the original AS
195 isolates by the c-BSE agent.
196
197 Two hypotheses could explain the emergence of the c-BSE agent in tgBov mice after their
198 inoculation with AS isolates. Firstly, c-BSE prions could be present at a low level in the
199 original AS isolates. The high sensitivity of tgBov mice for detection of the c-BSE agent
200 could allow this potentially low level of bovine prions to be identified during passage of the
201 original AS isolates in the bovine PrP host. Alternatively, the occurrence of c-BSE in AS-
202 inoculated tgBov mice could result from a mutation of AS strain properties triggered by
203 passage across the bovine transmission barrier for this particular ovine prion strain.
204
205 In order to explore the origin of the c-BSE agent observed in tgBov mice inoculated with AS,
206 we employed *in vitro* protein misfolding cyclic amplification (PMCA), a methodology that
207 mimics prion replication *in vitro*, but in an accelerated form, allowing amplification of minute
208 amounts of PrP^{Sc} and prion infectivity (27). In PMCA, a PrP^{C}-containing substrate is
209 combined with a seed that contains PrP^{Sc}. Following repeated cycles of incubation and
210 sonication, the amount of PrP^{Sc} increases.
211
212 PMCA has been previously reported to amplify the c-BSE agent with a great efficacy using
213 either tgARQ or tgBov mouse brain homogenate as substrate (28). Using this protocol two

214 (tgARQ substrate) or three (tgBov substrate) amplification rounds were sufficient to reach the
215 detection limit for c-BSE prion seeding activity (SI appendix Figure S2). The level of prion
216 infectivity and prion seeding activity of a reference sheep-passaged c-BSE isolate were end-
217 point titrated by both bioassay in tgBov mice and PMCA, respectively (SI appendix Table
218 S2). The infectious prion titer of the sheep-passaged c-BSE isolate was $\approx 10^{7.2}$ LD_{50}/g IC in
219 tgBov mice. The prion seeding titer (SA_{50}) was estimated to be $\approx 10^{11.1}$ SA_{50}/g using tgARQ
220 mouse tissue as substrate and $10^{11.05}$ SA_{50}/g using tgBov mouse tissue as substrate.
221 Considering the fact that mice were inoculated using 20µL of sample and the PMCA reactions
222 were seeded using 5µL of the same sample, the PMCA can be considered to be about 1500
223 fold more sensitive than the bioassay in tgBov. This also means that 1 c-BSE LD_{50} in tgBov
224 mice corresponds to $\approx$1500 SA_{50} assessed by PMCA.

225

226 In addition to its high sensitivity, *in vitro* PMCA can reproduce, at least partly, the
227 transmission barrier phenomenon observed during the *in vivo* prion bioassay (29). Therefore,
228 amplification of prion seeding activity in AS isolates by PMCA using tgBov mouse tissue as
229 substrate offered an opportunity to characterize the potential impact of the bovine
230 transmission barrier on AS strain properties.

231

232 The AS isolates that were originally transmitted to tgBov mice (except AS8) and 18 additional
233 AS isolates (originating from Norway, France, and Portugal) were subjected to PMCA (Table
234 4). Each AS isolate was used to seed reactions containing either bovine PrP or ovine ARQ PrP
235 substrate (10 to 18 replicates per substrate). After amplification, PrP^{res} was detected by
236 western blot in a low proportion of the reactions seeded with 19 out of the 25 AS isolates for
237 tgBov and tgARQ combined (Table 3). In most instances, a similar proportion of PrP^{res}-
238 positive PMCA reactions were observed when either bovine PrP or ovine ARQ PrP was used

239 as substrate. However, in some cases (n=3), a low number of PrP^{res}-positive reactions were
240 observed when bovine PrP was used as substrate (in the case of AS 10) or when ovine ARQ
241 PrP was used as substrate (in the case of AS 9, and AS 25). Whatever combination of AS
242 isolate and substrate PrP used, the PrP^{res} western blot profile in PMCA-positive reaction
243 products and its reactivity with 12B2 antibody were indistinguishable from those observed for
244 PMCA reaction products seeded with authentic ovine c-BSE prions (Figure 4). No PrP^{res} was
245 observed in PMCA reactions that were unseeded (n=120) or in those reactions seeded (n=60)
246 with prion-free sheep brain homogenate (representative samples shown in Figure 4). It should
247 be noted that the PrP amino sequence was 100% homologous between certain AS isolates (AS
248 5, AS 26) and the ovine PrP substrate (tgARQ) used in PMCA reactions. Therefore, *in vitro*
249 amplification of c-BSE prions in PMCA reactions seeded with these AS isolates using ovine
250 ARQ PrP as substrate cannot be a consequence of mutation of prion strain properties triggered
251 by a transmission barrier.
252
253 Taken together, the tgBov mouse bioassay and PMCA results strongly support the view that a
254 low level of c-BSE prions was initially present in at least 21 out of the 26 AS isolates tested.
255
256 To further clarify the origin of the c-BSE agent detected in AS isolates, two of these isolates
257 (AS 25 and AS 26) were end-point titrated in tg338 mice (1/10 dilution series, 6 to 7 tg338
258 mice inoculated per dilution). For both isolates, the last positive transmissions were observed
259 in mice that received a 10^{-6} $\log_{10}$ dilution of the original 10% w/vol brain material (SI
260 appendix Table S3). The brains of these end-point titration tg338 mice were subsequently
261 subjected to PMCA. Irrespective of the substrate used for PMCA, either bovine or ovine ARQ
262 PrP, PrP^{res} was observed in a similar proportion of the PMCA reactions seeded with either the
263 original AS isolates, or AS isolates passaged in tg338 mice (Table 4). The PrP^{res} western blot

264 profile observed in all the PMCA-positive reactions was identical to that seen in reactions
265 seeded with authentic c-BSE prions (Figure 5). PMCA reactions seeded with brain
266 homogenate prepared from age matched non-inoculated tg338 mice remained PrP^{res} negative
267 (Table 4 and Figure 5).
268
269 Considering the level of c-BSE seeding activity originally present in isolates AS 25 and AS
270 26 (less than 100 SA_{50}/mL, Table 5), there is an extremely low level of probability that one of
271 the six tg338 mice inoculated with 20µL of a 10^{-6} diluted AS isolate (<2 10^{-6} SA_{50} per dose of
272 inoculum) could be exposed to 1 infectious dose of c-BSE agent (1 c-BSE LD_{50} is ≈1500
273 SA_{50}). Consequently, the presence of c-BSE prion seeding activity in the brains of tg338 mice
274 inoculated with a 10^{-6} log_{10} dilution of original AS isolate implies that a low titer of c-BSE
275 prions was generated during the propagation of ovine AS prions in a host that expressed ovine
276 PrP, namely tg338 mice.
277
278

279 **Discussion**

280 The mechanism(s) that lead to an alteration in the phenotype of prion strains as these
281 transmissible entities undergo transmission between different host species remain uncertain.
282 This is despite the identification that differences in amino acid sequence between host PrP^{C}
283 and donor PrP^{Sc}, together with prion strain identity are principal determinants of the
284 transmission barrier (9, 10). Based on the concept that conformation of PrP^{Sc}
285 molecules/aggregates encode prion strain information (2, 4, 5, 30, 31), at least two non-
286 exclusive hypotheses, 'deformed templating' (32, 33) and the 'conformational selection
287 model' (32-35) have been proposed to explain the mutation of prion strains.
288

289 The 'deformed templating' hypothesis postulates that a prion strain replicates as a clone of
290 PrP^{Sc} molecules/aggregates. When confronted by a transmission barrier that does not allow
291 clonal prion replication, the propagation process is modified so that 'altered' PrP^{Sc} structural
292 variants are generated in an attempt to convert the new host PrP^{C}. While the majority of these
293 presumably fail to replicate efficiently in the new host, variants eventually emerge that are
294 successful and adapt to the new PrP environment through multiple trial-and-error replication
295 events. In this 'deformed templating' model, confrontation of the transmission barrier serves
296 as the triggering event that initiates the generation of new prion variant(s) and as a filter for
297 their selection (35).
298

299 The 'conformational selection model' proposes that a prion strain naturally propagates in its
300 host as an ensemble of PrP^{Sc} conformers dominated by a stable energetically favourable
301 conformation responsible for the observed prion strain phenotype. Furthermore, this model
302 predicts that the number of stable PrP^{Sc} conformers is limited for each PrP amino acid
303 sequence, which would explain the existence of a finite number of stable prion strains that can

304 propagate in a given species. It is further proposed that during transmission of a prion strain to
305 a new host, one of the less dominant PrP^{Sc} conformers of those present in the ensemble is
306 selected with a resultant change, or mutation, in the properties of the newly propagating prion
307 strain. In the ‘conformational selection model’, the transmission barrier acts simply as a
308 selective filter for new prion variants, and ease of permeation of the barrier results from the
309 extent of overlap of PrP^{Sc} conformers that exist between the interacting species (32, 33).
310

311 Our data reported here showed that c-BSE prions are present as a minor variant in natural
312 isolates of ovine AS. In addition, transmission of ovine AS to bovine PrP mice demonstrated
313 that c-BSE can emerge during these transmissions as the dominant prion strain. These results
314 provide a cogent argument in favour of the ‘conformational selection model’ as the
315 mechanism for prion strain mutation during inter-species prion transmission. This would be
316 expected to occur by selection of a pre-existing PrP^{Sc} variant in AS isolates, one best suited to
317 the new replicative environment. Within this conceptual framework, the occurrence of prion
318 strain mutation is dependent upon the particular repertoire of PrP^{Sc} variants associated with
319 distinct prion strains. This notion is supported by our observation that c-BSE prions emerged
320 during serial transmission of ovine AS in tgBov mice but not from serial passage of classical
321 scrapie in the same mouse line (Table 1 and Cassard et al (15)).
322

323 The diversity of prion strains that exist in small ruminants remains undefined although it is
324 established that at least 5 different natural ovine prion strains exist including AS (6, 36-39).
325 According to the ‘conformational selection model’, each of these different ovine prion strains
326 is associated with a unique and stable PrP^{Sc} conformer and a distinct set of minor variants.
327 The tgBov mouse line has previously been reported to support the propagation of a variety of
328 natural ovine prions, of which several displayed significantly shorter incubation periods than

329 c-BSE (15). Strikingly, in our experiments the diversity of prion variants in the AS isolates
330 (seven different cases) revealed by the serial passage in tgBov was restricted to the c-BSE
331 agent (Table 1). This consistent emergence of a single prion strain argues against the view that
332 AS prion replication in sheep can randomly generate all the existing stable PrP^{Sc} variants
333 associated with a particular ovine PrP^{C} amino acid sequence. Instead, our data support the
334 view that individual prion strains are associated with a restricted repertoire of stable PrP^{Sc}
335 variants in a given host. Whether AS is unique in its ability to generate c-BSE prion particles
336 during its replication process remains to be established.
337
338 Classical BSE was first recognized in 1984-85 as a novel prion disease affecting cattle in the
339 UK (40). Epidemiological data clearly established that the number of cases of c-BSE was
340 amplified by the recycling of infected animal carcasses into cattle feed in the form of meat
341 and bone meal (MBM) (41). Since bovine prion disease had not been recognized in cattle
342 prior to the c-BSE epizootic and the disease is apparently non-contagious between cattle,
343 several hypotheses were proposed to explain its emergence. These range from the spontaneous
344 occurrence of c-BSE in cattle to the passage and adaptation of a prion originating from
345 another species (42, 43). Our studies here that show the presence of c-BSE prions in AS
346 isolates combined with the demonstrated presence of AS in the UK long before the
347 appearance of the c-BSE epizootic in cattle suggests that the recycling of AS cases in MBM
348 might be a source of bovine prion disease (20). In addition to its potential role in the initial
349 emergence of c-BSE in cattle, the presence of c-BSE prions in natural cases of AS has current
350 and direct implications for both the continued risk of this ovine prion disease to other farmed
351 animals and for human exposure risks. The distribution of AS cases are widespread across the
352 world (17-19). A recent retrospective analysis of surveillance data collected over a period
353 exceeding 10 years in the European Union (EU) concluded that the prevalence of detected AS

354 cases has remained relatively stable in the different member states with between 2-6 positive
355 cases per 10,000 tested animals per year. This implies that a substantial number of AS-
356 infected animals could enter either the animal or human food chain each year (44, 45), and
357 each case represents a potential source of exposure to the c-BSE agent for farmed animals
358 (MBM derived from rendered small ruminants) and human consumers (consumption of
359 healthy slaughtered animals), respectively. The epidemiological features of AS within the EU
360 is likely to reflect the situation of the disease in other countries that breed and maintain small
361 ruminants.
362
363 In Europe, the c-BSE crisis and the emergence of vCJD resulted in the implementation of a
364 strong and coherent policy (EU regulation 999/2001) aimed at control and eradication of this
365 animal prion disease. The total feed ban on the use of MBM in animal feed and the systematic
366 retrieval from the food chain of ruminant tissues that have the potential to contain high levels
367 of prion infectivity, so called Specified Risk Material (SRM) measures, were instrumental for
368 control of c-BSE in cattle and preventing dietary human exposure to these bovine prions (46,
369 47). As a side effect, these measures also strongly limited the exposure of farmed animals and
370 human consumers to the other TSE agents circulating in farmed animal species, including AS.
371
372 With the decline of the c-BSE epizootic in cattle and the combined increase in pressure from
373 industry, EU authorities have begun to consider discontinuing certain TSE control measures.
374 The abrogation of the SRM measures for small ruminants and the partial re-authorization of
375 the use of processed animal protein, formerly known as MBM in animal feed are part of the
376 EU authorities' agenda. Our observation of the presence of the c-BSE agent in AS-infected
377 small ruminants suggest that modification of the TSE control measures could result in an
378 increased risk of exposure to c-BSE prions for both animals and humans. Whether or not this

379 exposure will result in further c-BSE transmission in cattle and/or humans remains an open
380 and important question.
381
382

383 **Methods**

384 ***Ethics Statement***

385 All animal experiments were performed in compliance with institutional and French national
386 guidelines and in accordance with the European Directives 86/609/EEC and 2010/63/EU. In
387 France, the animal experiments that are part of this study (national registration 01734.01)
388 were approved by the local ENVT ethics committee. Experiments developed in CISA-INIA
389 (Madrid, Spain) were approved by the Committee on the Ethics of Animal Experiments of the
390 Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria and the General
391 Directorate of the Madrid Community Government (permit numbers: CEEA 2009/004 and
392 PROEX 228-16). Mouse inoculations were performed under anaesthesia (isofluorane).
393 Experiments developed in IRTA-CReSA (Barcelona, Catalonia) involving animals were
394 approved by the animal experimentation ethics committee of the Autonomous University of
395 Barcelona (Reference number: 585-3487) in agreement with Article 28, sections a), b), c) and
396 d) of the "Real Decreto 214/1997 de 30 de Julio" and the European Directive 86/609/CEE and
397 the European council Guidelines included in the European Convention for the Protection of
398 Vertebrate Animals used for Experimental and Other Scientific Purposes.
399 Mice that displayed clinical signs were anesthetized with isofluorane before sacrifice using
400 CO_2 inhalation.
401

402 ***Atypical/Nor98 scrapie cases and control sheep***

403 Natural atypical scrapie (AS) cases identified through active or passive surveillance programs
404 were selected according to their geographical origin (France, Spain, Italy, Norway and
405 Portugal) and *PRNP* genotypes (SI appendix Table S1 and Table 1). These cases have been
406 originally classified as AS by TSE national reference laboratories in each country. All the
407 cases corresponded to sheep except the AS 7 case (goat).

408

409 In all cases, PrP genotype was checked by sequencing the Exon 3 of the *PRNP* gene as
410 previously described (48). The polymorphisms at codons 136 (A/V), 154 (H/R) and 171
411 (R/Q/H), which have been demonstrated to strongly influence the susceptibility to TSE in
412 sheep are indicated (49). Additionally, the presence of a phenylalanine at codon 141 (F/L),
413 which has been shown to impact on the susceptibility to atypical/Nor98 scrapie, are also
414 indicated (Table 1 and 4) (23, 48). Brain material collected in TSE-free Poll-Dorset sheep
415 (APHA, Weybridge, UK) was used as control (50).

416

417 ***c-BSE isolates***

418 Cattle and ovine classical BSE (c-BSE) isolates were used as control. The cattle c-BSE isolate
419 was a natural case originating from France. This isolate was used in previous studies aimed at
420 the characterization of c-BSE strain properties though transmission to mice over-expressing
421 the PrP sequence of various host species (51). The ovine c-BSE isolate was obtained by the
422 intracerebral inoculation of the same cattle c-BSE isolate in ARQ/ARQ TSE free sheep (first
423 passage) as described in Andreoletti et al 2004 (50).

424

425 **Mouse bioassays**

426 Bioassays were carried out using mice expressing bovine PrP (tgBov /tg110) (52, 53) and/or
427 mice expressing ovine ARQ (tgARQ) (54) or VRQ (tg338) PrP (55).

428

429 Groups of six- to ten-week-old female mice (n ≥6) were anesthetized and inoculated with
430 20µL of a 10% tissue homogenate in the right parietal lobe using a 25-gauge disposable
431 hypodermic needle. Mice were observed daily and their neurological status was assessed
432 weekly. When clinically progressive TSE disease was evident, the animals were euthanized

433 and their brains harvested. Half of the brain was fixed by immersion in 10% formol saline and
434 the other half was frozen at -20ºC. Tissues from animals found dead were frozen (no formalin
435 fixation). In animals where no clinical signs were observed, mice were killed at the end of
436 their natural life-span (650 to 750 days). In those cases, incubation periods reported in the
437 table as >650 dpi, corresponded to the survival time observed in at least three out of the six
438 mice.
439

440 **PMCA Amplification**

441 Brains from tgBov, tgARQ and tg338 were used to prepare the PMCA substrates. PMCA was
442 performed as previously described (28, 56). Briefly PMCA reactions (50µL final volume)
443 were seeded with 5µL of sample to be tested. PMCA reactions were then subjected to 3
444 amplification rounds each comprising 96 cycles (10s sonication-14 minutes and 50 seconds
445 incubation at 39.5°C) in a Qsonica700. After each round, reaction products (1 volume) were
446 mixed with fresh substrate (9 volumes) to seed the following round. The PMCA reaction
447 products were analysed by western blot for the presence of PK-resistant PrP. Each WB line
448 was loaded with the equivalent of 20 µL of PK digested PMCA product. Each PMCA run
449 included a reference ovine BSE sample (10% brain homogenate) as a control for the
450 amplification efficiency. Unseeded controls (2 unseeded controls for 8 seeded reactions) were
451 also included in each run.
452

453 **Western blot detection of abnormal PrP**

454 PK-resistant abnormal PrP (PrP^{res}) extraction and western blot were performed as previously
455 described (57). Immunodetection was performed using two different PrP-specific monoclonal
456 antibodies: Sha31 (1 µg/ml) (58), and 12B2 (4 µg/ml) (59), which recognize the amino acid
457 sequences YEDRYYRE (145-152), and WGQGG (89-93) respectively (60).

458

459

460 **Paraffin embedded tissue blot**

461 Paraffin embedded brain tissue from inoculated mice was analysed as previously described

462 (61-63).

463

464 **Lesion profiling and abnormal PrP immunohistochemistry.**

465 Vacuolar brain lesion profiles were established following the method described by Fraser et al

466 (64). *In situ* PrP^{Sc} immune-labelling was performed as previously described (63), using 6H4

467 anti PrP antibody (epitope: $_{147}DYEDRYYRE_{155}$ of the bovine PrP- concentyrtation 3µg/mL).

468

469 **Infectious and seeding activity titer estimates**

470 A series of 1/10 dilutions of a reference 10% w/vol brain stem from an ovine-BSE

471 (ARQ/ARQ) isolate and two AS isolates (AS 25 and AS 26) were prepared. Successive 1/10

472 dilutions of brain homogenate were inoculated intra-cerebrally (20µl) into tgBov or tg338

473 mice (n=6 per inoculum). Dilutions of the same c-BSE isolate were used to seed PMCA

474 reactions that used brain tissue from either bovine PrP (tgBov mice) or ovine ARQ PrP

475 (tgARQ mice) as substrate. Twelve individual replicates of each sample dilution were tested.

476 Reactions were then subjected to three amplification rounds. PMCA reaction products (third

477 amplification round) were analysed by western blot for the presence of PrP^{res}. The titer of

478 prion seeding activity was estimated by the Spearman-Kärber's method (65).

479

480 For AS and AS passaged in tg338 isolates (10% brain homogenate), 1/50 diluted material was

481 used to seed twelve individual reactions (tgBov or tgARQ substrates). After three

482 amplification rounds the number of PrP^{res} western blot positive reactions / total number of

483 reactions was established. These ratios were used to estimate seeding activity titers (SA/μL of 484 10% brain homogenate) by the limiting dilution titration method (application of Poisson's 485 probabilistic model) described by Brown et al (66) or by the Spearman-Kärber's method. 486 According to Fisher et al (67) and as previously used for prion infectivity comparisons (68) 487 one SA_{50} was considered to be equivalent to 0.693 SA.

488

489 **Data availability**

490

491 All data is available in the manuscript and SI.

492

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494

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Legends of Figures

Figure 1: Brain lesion profile and PrP^{res} western blot profiles in tgBov and tg338 mice inoculated with atypical/Nor98 scrapie (AS) or c-BSE

Groups of mice (n≥6) that express either ovine VRQ PrP (tg338 mice) or bovine PrP (tgBov mice) were intra-cerebrally challenged with atypical scrapie isolates (AS) or an ovine classical BSE isolate (c-BSE).

(**a**) After two or three iterative passages in each mouse line a standard lesion profile was established by scoring the vacuolar changes observed in pre-defined brain areas. In c-BSE graphs ●: ovine c-BSE, ○: cattle c-BSE. In AS graphs: ○ : AS 1, △: AS 2, ▽: AS 3).

(**b**) The accumulation of PK-resistant PrP (PrP^{res}) in the brain of tgBov mice was established by western blot using anti PrP monoclonal antibodies Sha31 (epitope 145-YEDRYYRE-152) and/or 12B2 (epitope 89-WGQGG-93). The same western blot PrP^{res} control (classical scrapie isolate) was used on all the gels labelled as WB control. Cattle c-BSE and an ovine c-BSE (original isolate and isolate passaged in tgBov) were included as controls.

(**c**) Vacuolar lesions (thalamus level, conventional histology; hematoxylin-eosin, bar: 25 µm) and abnormal PrP deposition (Mesencephalon: tegmentum, immunohistochemistry using 6H4 anti PrP antibody, epitope 147-DYEDRYYRE-155, bar: 50µm) in tgBov mice inoculated with AS3 and cattle BSE.

Figure 2: Brain lesion profile and PrP^{res} Western blot in the brain of tgARQ and tg338 mice inoculated with AS scrapie adapted in tgBov

Groups of mice (n≥6) that express ovine VRQ PrP (tg338 mice) or ovine ARQ PrP (tgARQ mice) were intra-cerebrally challenged with atypical scrapie isolates (AS) or an ovine c-BSE isolate that had previously been adapted (2 iterative passages) in tgBov mice.

(**a**) After two iterative passages in each mouse line a standard lesion profile was established by scoring the vacuolar changes observed in pre-defined brain areas. In AS graphs △: AS 2, ▽: AS 3.

(**b**) The western blot profile of PK resistant PrP (PrP^{res}) in the original AS isolates and in the brain of inoculated mice was established by western blot using anti PrP monoclonal antibody Sha31 (epitope 145-YEDRYYRE-152). The same western blot PrP^{res} control (classical scrapie isolate) was used on all the gels labelled as WB control.

Figure 3: Brain and spleen PrP^{res} accumulation in tg338 mice inoculated with AS scrapie adapted in tgBov

Groups of mice (n≥6) that express ovine VRQ PrP (tg338 mice) were intra-cerebrally challenged with atypical scrapie isolates (AS) and AS that had previously been adapted (2 iterative passages) in tgBov mice. In parallel, cattle c-BSE isolate and ovine BSE isolate (adapted in tg Bov) were transmitted (2 iterative passages) in tg338 mice.

(**a**) In tg338 mice (2nd passage) the PrP^{res} distribution pattern in the brain (thalamic coronal section: bar: 160 µm) and in the spleen (bar: 100 µm) was established by paraffin embedded tissue blot using anti PrP monoclonal antibody Sha31 (epitope 145-YEDRYYRE-152).

(**b**) The western blot profile of PK resistant PrP (PrP^{res}) in the spleen of tg338 mice (2nd passage) inoculated with AS isolates, AS isolates passaged in tgBov and c-BSE (cattle and ovine origin) was established using anti PrP monoclonal antibody Sha31.

710
711 **Figure 4: PrP^{res} detection in PMCA reactions seeded with atypical/Nor98 scrapie**
712 **isolates.**
713
714 Protein misfolding cyclic amplification (PMCA) reactions were seeded with Atypical/Nor98
715 scrapie (AS) isolates (1/50 diluted 10% brain homogenate) that had been identified in five
716 European countries (see Table 3). PMCA reactions seeded with brain homogenate from a
717 TSE-free sheep (originating from New Zealand) and unseeded PMCA reactions were included
718 as specificity controls. PMCA substrate consisted of brain homogenate from either bovine PrP
719 (tgBov) or ovine PrP (tgARQ) mice. PMCA reactions were subjected to three (tgARQ
720 substrate) or four (tgBov substrate) amplification rounds each comprising 96 cycles (10s
721 sonication-14 minutes and 50 seconds incubation at 39.5°C) in a Qsonica700. The PMCA
722 reactions were analysed by western blot for the presence of abnormal PK-resistant PrP (PrP^{res})
723 using anti PrP monoclonal antibodies Sha31 (epitope 145-YEDRYYRE-152) and/or 12B2
724 (epitope 89-WGQGG-93). Each western blot included a classical scrapie isolate (labelled as
725 WB control) and an ovine c-BSE isolate as controls.
726
727 **Figure 5: PMCA seeding activity detection in two bioassay end-point titrated**
728 **Atypical/Nor98 isolates**
729
730 Two atypical/Nor98 isolates AS 25 and AS 26 (Table 3) were end-point titrated in tg338 mice
731 (1/10 dilution series, 6 tg338 mice per dilution). For both isolates, the last positive
732 transmissions were observed in mice that received a 10^{-6} dilution of the original 10% w/vol
733 brain material (SI appendix Table S3). The original AS isolates and the brains of clinically
734 affected mice inoculated with neat and 10^{-6} diluted isolates were used to seed PMCA reactions
735 that either used tgARQ or tgBov as substrate. PMCA reactions seeded with age matched
736 inoculated tg338 mice and unseeded reactions were included as specificity controls. Reactions
737 were subjected to three (tgARQ substrate) or four (tgBov substrate) amplification rounds each
738 comprising 96 cycles (10s sonication-14 minutes and 50 seconds incubation at 39.5°C) in a
739 Qsonica700. The PMCA reactions were analysed by western blot for the presence of
740 abnormal PK resistant PrP (PrP^{res}) using the Sha31 (epitope 145-YEDRYYRE-152) and/or
741 the 12B2 (89-WGQGG-93) anti PrP antibodies. Each western blot included a classical scrapie
742 isolate (labelled as WB control) and an ovine c-BSE isolate as controls.
743
744

Table 1: Inoculation of atypical scrapie isolates in bovine PrP (tgBov) expressing mice

Isolates			TgBov					
			1st passage		2nd passage		3rd passage	
Identifiant	Origin	Genotype	Positive mice	Incubation (mean±SD)	Positive mice	Incubation (mean±SD)	Positive mice	Incubation (mean±SD)
AS 1	Fr	ARQ*/ARQ	1/6	533	3/5	317±63	7/7	235±16
AS 2	Sp	ARR/ARQ	0/6	>650	7/9	354±26	5/5	273±5
AS 3	Sp	ARQ/ARH	0/6	>650	2/11	504, 525‡	12/12	269±13
AS 4	Nor	ARQ*/ARQ*	3/4	395± 44	6/6	230±17	6/6	271±18
AS 5	Sp	ARQ/ARQ	0/6	>650	0/4	>650	NA	
AS 6	Sp	ARQ/ARH	0/6	>650	1/4	>650‡	NA	
AS 7†	It	ARQ/AHQ	0/6	>650	1/7	424	6/6	286±14
AS 8	Po	ARQ/ARQ	1/5	439	5/5	297±14	6/6	250±4
PS42	Fr	VRQ/VRQ	0/6	>650	0/6	>650	0/6	>650
Ovine c-BSE	Fr	ARQ/ARQ	6/6	254±19	6/6	234±12	6/6	232±6
Cattle c-BSE	Fr	-	6/6	295±12	6/6	265±35	6/6	243±7

Transgenic mice that express the bovine PrP (tgBov) were inoculated intra-cerebrally (6 to 12 mice, 20µL per mouse) with 8 sheep or goat (†) atypical scrapie (AS) isolates originating from five different countries; France (Fr), Spain (Sp), Norway (Nor), Italy (It) or Portugal (Po). The AS affected animals displayed a different *PRNP* genotype at codons 136, 154 and 171. Some also displayed a F/L dimorphism at codon 141 (*). Cattle classical BSE (c-BSE), ovine c-BSE (first passage of cattle c-BSE in an ARQ/ARQ sheep by the intracerebral route) and classical scrapie (PS42) isolates were inoculated in the same mouse model. After first and second passage, clinically affected or asymptomatic mice that had lived for more than 500 days post inoculation were pooled and used for subsequent passage in the same line. Mice were considered positive when abnormal PrP deposition was detected in the brain. (‡) indicate abnormal PrP positive and found dead animals (without symptoms). Incubation periods (in days) are shown as mean±SD except when less than 50% of the mice were found to be positive. In that case the incubation periods of the positive mice are individually presented. NA: not available. Cattle c-BSE transmission in tgBov data were already reported in Torres et al 2014 (51)

Table 2: Inoculation of atypical scrapie adapted in tgBov and c-BSE isolates in ovine PrP expressing mouse models (tg338 and tgARQ)

Isolates		Tg338				TgARQ			
		1st passage		2nd passage		1st passage		2nd passage	
Identifiant	Origin	Positive mice	Incubation (mean±SD)	Positive mice	Incubation (mean±SD)	Positive mice	Incubation (mean±SD)	Positive mice	Incubation (mean±SD)
AS 2	2nd pass in TgBov	6/6‡	>650	6/6	617±75	6/6	350±9	6/6	260±3
AS 3	3rd pass in TgBov	6/6‡	>650	6/6	672±83	6/6	354±21	6/6	257±2
Ovine c-BSE	2nd pass in TgBov	6/6‡	>750	6/6	653±32	6/6	270±12	6/6	259±4
Cattle c-BSE	cattle	6/6‡	>700	6/6	682±52	6/6	321±16	6/6	263±7

Transgenic mice that express the VRQ (tg338) or ARQ (tgARQ) variants of ovine PrP were inoculated intra-cerebrally (6 mice, 20µL per mouse) with atypical scrapie isolates or ovine c-BSE isolate that had previously been adapted in tgBov (2 iterative passages). Cattle BSE was also included as control. After first passage, clinically affected or asymptomatic mice that had lived for more than 500 days post inoculation were pooled and used for second passage in the same line. Mice were considered positive when abnormal PrP deposition was detected in the brain. (‡) indicate abnormal PrP positive and found dead animals (without symptoms). Incubation periods (in days) are shown as mean±SD.

Table 3: Protein Misfolding Cyclic Amplification seeding activity in atypical scrapie isolates

Isolates			PMCA positive reactions	
Identifiant	**Origin**	**Genotype**	**TgBov substrate**	**TgARQ substrate**
AS 1	Fr	ARQ*/ARQ	3/12	5/12
AS 2	Sp	ARR/ARQ	2/12	3/12
AS 3	Sp	ARQ/ARH	3/12	4/18
AS 4	No	ARQ*/AFRQ	12/12	9/12
AS 5	Sp	ARQ/ARQ	4/12	3/12
AS 6	Sp	ARQ/ARH	5/12	1/12
AS 7	It	ARQ/AHQ	1/12	2/12
AS 8	Po	ARQ/ARQ	ND	ND
AS 9	Nor	ARR/ARQ	0/12	2/10
AS 10		ARQ*/AHQ	1/12	0/10
AS 11		AHQ/ARQ	3/12	7/12
AS 12		ARR/ARQ	0/12	0/12
AS 13		ARR/AHQ	0/12	0/12
AS 14		ARQ/AHQ	1/12	1/12
AS 15		ARR/ARR	3/12	1/12
AS 16		ARR/AHQ	1/12	3/10
AS 17		ARQ*/AHQ	1/12	1/10
AS 18		ARQ*/AHQ	0/12	0/10
AS 19	Po	ARR/ARR	0/12	0/12
AS 20		ARR/AHQ	3/12	1/12
AS 21		ARR/ARR	0/12	0/10
AS 22		ARQ*/AHQ	0/12	0/12
AS 23		ARQ*/ARQ	1/12	1/12
AS 24		ARQ*/ARQ	2/12	4/12
AS 25	Fr	AHQ/AHQ	0/12	1/12
AS 26		ARQ/ARQ	1/12	1/12
TSE free sheep		ARQ/ARQ	0/12	0/12
Unseeded		-	0/120	0/120

Twenty-six AS scrapie cases (1/50 diluted 10% brain homogenates) originating from five different countries (France (Fr), Spain (Sp), Italy (It), Portugal (Po) and Norway (Nor)) were used to seed PMCA reactions (5 µl of seed per reaction). The AS affected animals displayed different *Prnp* genotypes at codons 136, 154 and 171. Some also displayed a F/L dimorphism at codon 141 (*).

Two different PMCA substrates were used. The first one was prepared using brains from transgenic mice over-expressing the ARQ variant of the sheep prion protein (tgARQ). The second was prepared using brains from transgenic mice over-expressing the bovine prion protein (tgBov). For each isolate and substrate ten to eighteen individual replicates were tested. Reactions were subjected to 3 amplification rounds. After each round reaction products (1 volume) were mixed with fresh substrate (9 volumes) to seed the following round. PMCA reaction products (third amplification round) were analysed by western blot for the presence of PrP^{res}. The number of PrP^{res} western blot positive reactions / total number of reactions are reported. Unseeded reactions and reactions seeded with brain homogenate prepared from a TSE free sheep were included as specificity controls. ND: not done.

Table 4: PMCA seeding activity in atypical scrapie passaged in tg338

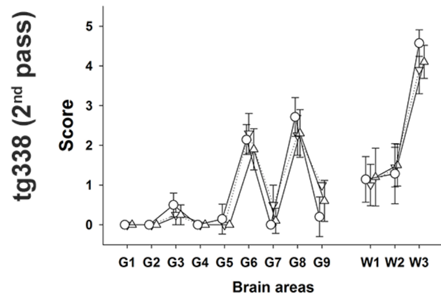
PMCA seeds			PrP^{res} positive PMCA reactions		Seeding activity (SA_{50}/mL)	
Case	**Origin**		**TgBov substrate**	**TgARQ substrate**	**TgBov substrate**	**TgARQ substrate**
AS 25	**Sheep**		0/12	1/12	0 (0-$10^{1.86}$)*	$10^{1.40}$
	1st passage in tg338 (neat)	**Mouse 1**	2/12	1/12	$10^{1.72}$	$10^{1.40}$
	2nd passage in tg338 (neat)	**Mouse 1**	2/12	1/12	$10^{1.72}$	$10^{1.40}$
	End-point titration in tg338 (10^{-6} dilution)	**Mouse 1**	2/12	3/12	$10^{1.72}$	$10^{1.92}$
		Mouse 2	1/12	3/12	$10^{1.40}$	$10^{1.92}$
		Mouse 3	2/12	2/12	$10^{1.72}$	$10^{1.72}$
AS 26	**Sheep**		1/12	1/12	$10^{1.40}$	$10^{1.40}$
	1st passage in tg338 (neat)	**Mouse 1**	2/12	0/12	$10^{1.72}$	0 (0-$10^{1.86}$) *
	2nd passage in tg338 (neat)	**Mouse 1**	2/12	0/12	$10^{1.72}$	0 (0-$10^{1.86}$) *
	End-point titration in tg338 (10^{-6} dilution)	**Mouse 1**	2/12	1/12	$10^{1.72}$	$10^{1.40}$
		Mouse 2	1/12	1/12	$10^{1.40}$	$10^{1.40}$
	Non inoculated tg338	**Mouse 1**	0/12	0/12	-	-
		Mouse 2	0/12	0/12	-	-
		Mouse 3	0/12	0/12	-	-

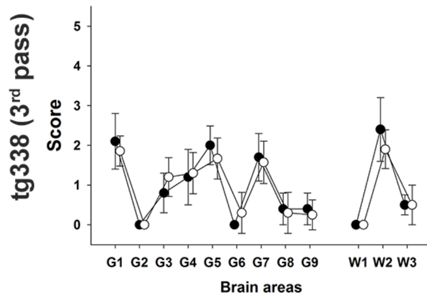
Two sheep atypical scrapie (AS) isolates were selected. The 10% w/vol brain homogenates were inoculated into tg338 mice (2 iterative passages). Groups of 6 tg338 mice were inoculated intra-cerebrally with 20µL of serial ten-fold dilutions of the same homogenates. Transmission was observed in 3 (AS 25) and 2 (AS 26) mice inoculated with 10^{-6} brain homogenate. No transmission was observed at lower dilutions. PMCA reactions (12 replicates) were seeded with 1/50 diluted brain homogenate (10% w/vol) from (i) the original sheep, (ii) the second passage tg338 mice (pool of brains) and (iii) individual brain from positive tg338 in the end-point titration experiment. Brain homogenates (10% w/vol) from age matched, non-inoculated tg338 mice were also used as seeds (1/50 diluted). Two different PMCA substrates were used. The first one was prepared using brains from transgenic mice over-expressing the ARQ variant of the sheep prion protein. The second was prepared using brains from transgenic mice over-expressing the bovine prion protein (tgBov). Reactions were subjected to up to 4 amplification rounds. After each round reaction products (1 volume) were mixed with fresh substrate (9 volumes) to seed the following round. PMCA reaction products were analysed by western blot for the presence of PrP^{res}. The number of PrP^{res} western blot positive reactions / total number of reactions are reported. Seeding activity titers were estimated using the Spearman Karber's limiting dilution titration method (most likely value) or when no positive reaction was observed, by the Poisson's probabilistic model (*: most likely value and IC 95%) as described by Brown et al (66). Titers are given as the number of PMCA SA_{50} per mL of 10% brain homogenate.

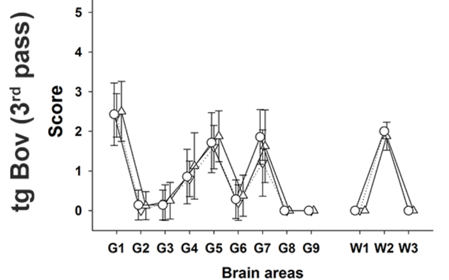
a

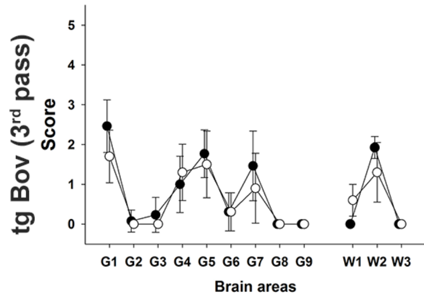
Atypical scrapie

c-BSE









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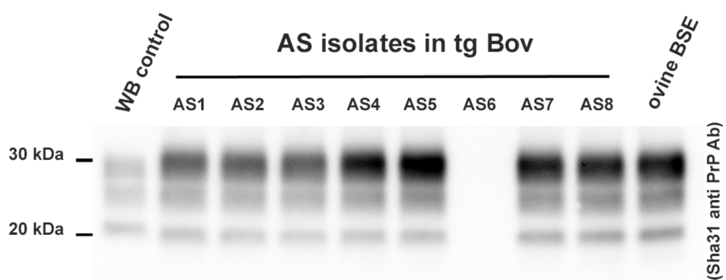
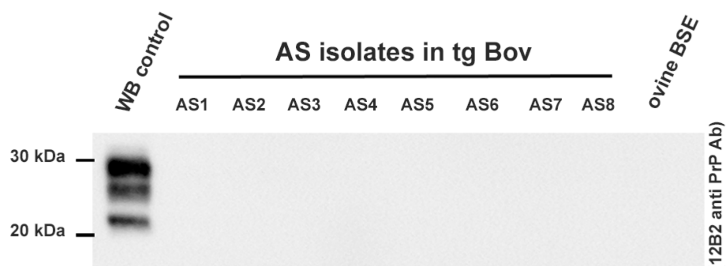
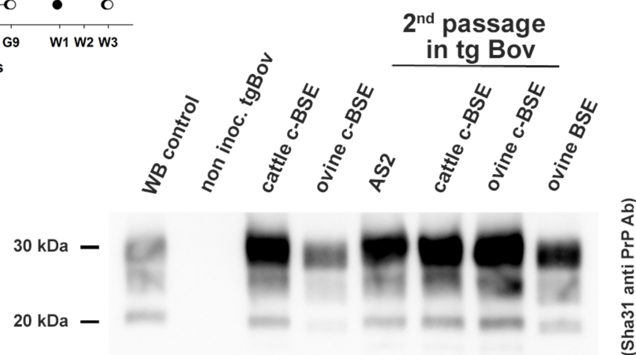
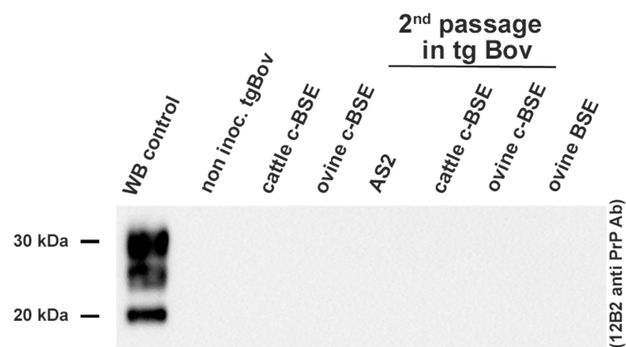






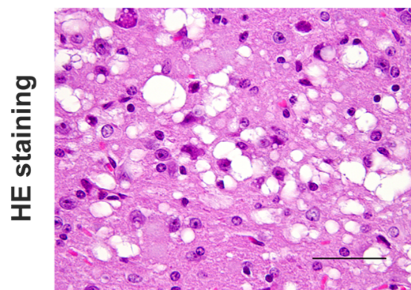


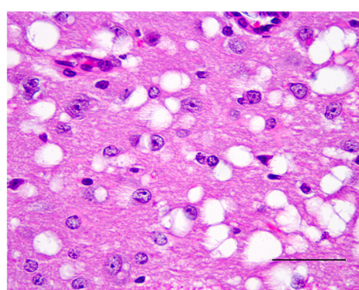
c

cattle c-BSE in tg Bov (3rd pass)

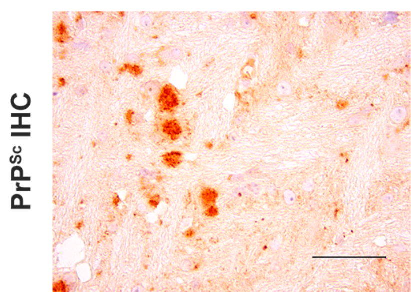
AS3 in tg Bov (3rd pass)

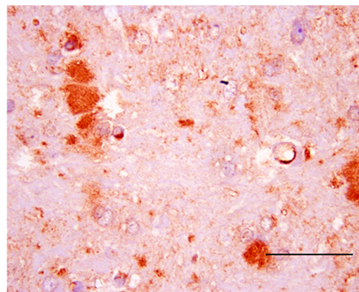
HE staining

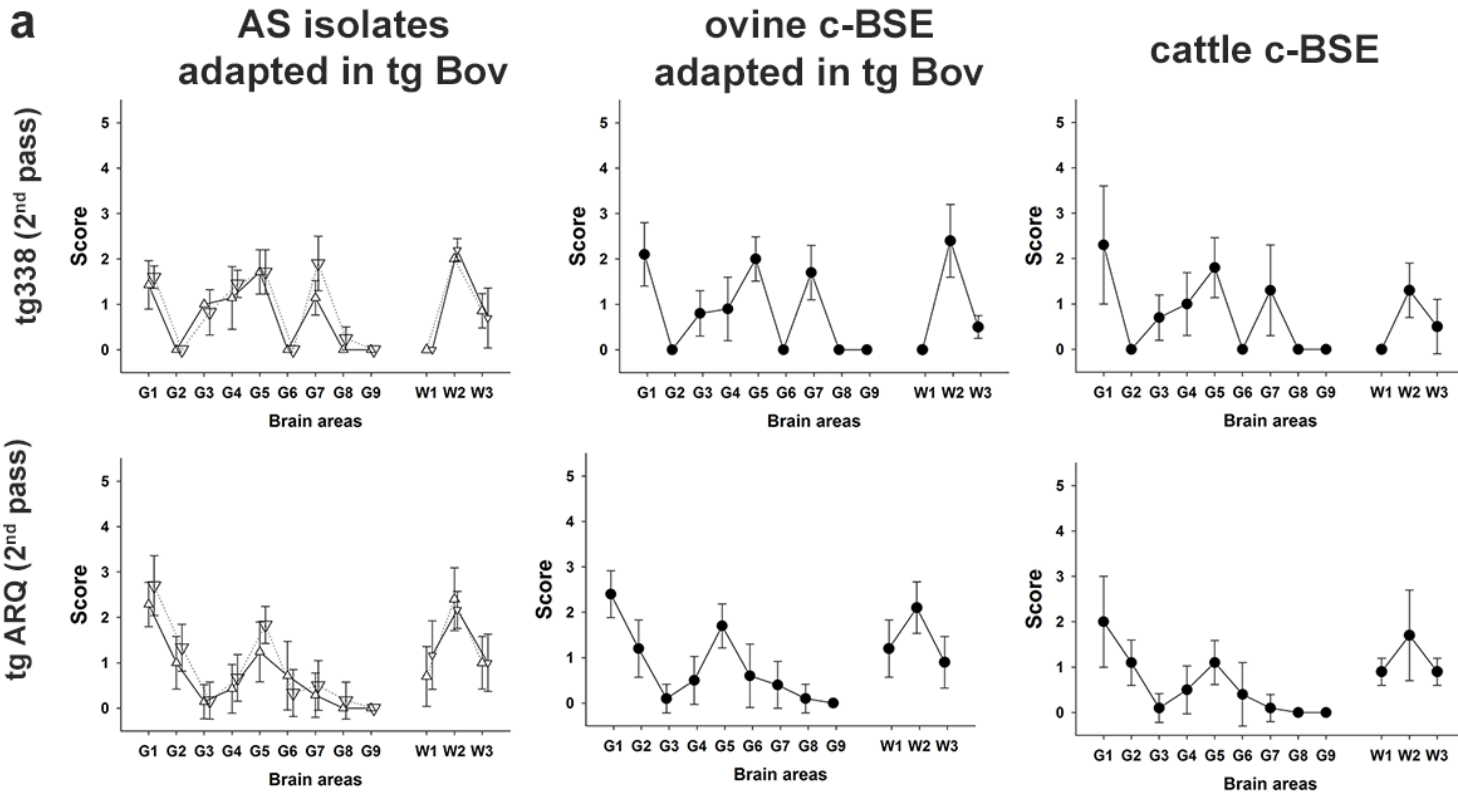


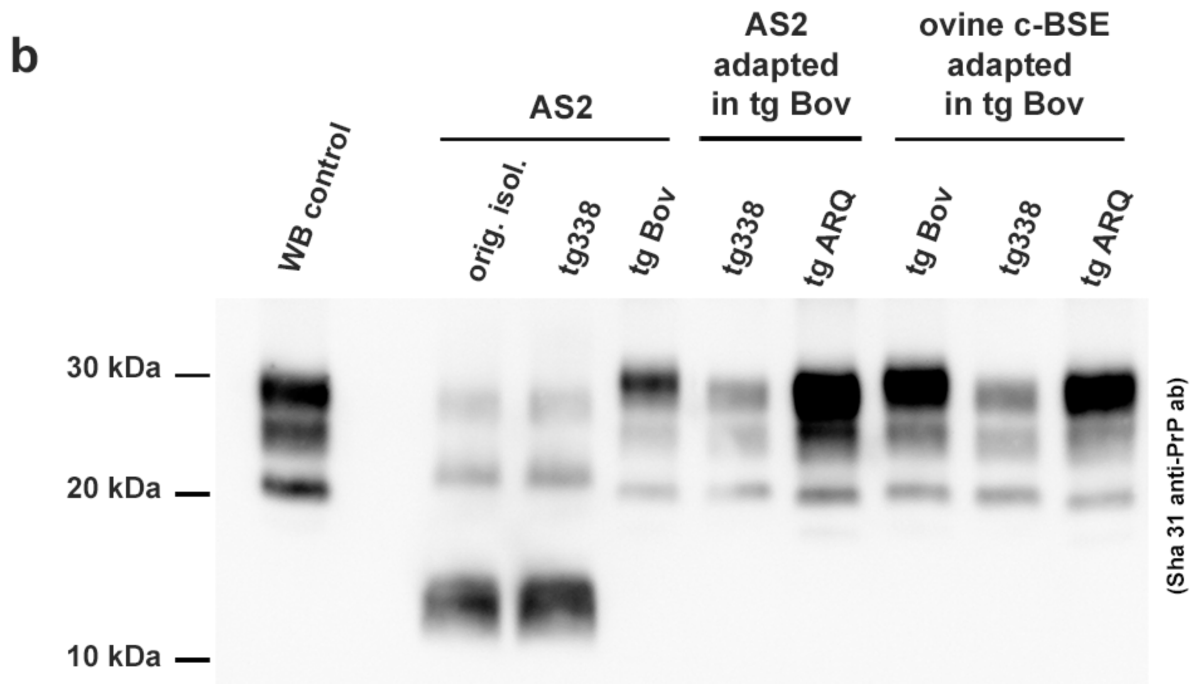


PrP^{Sc} IHC






a
AS isolates adapted in tg Bov
ovine c-BSE adapted in tg Bov
cattle c-BSE
tg338 (2nd pass)
tg ARQ (2nd pass)
Score
Brain areas
G1 G2 G3 G4 G5 G6 G7 G8 G9 W1 W2 W3


b
AS2
AS2 adapted in tg Bov
ovine c-BSE adapted in tg Bov
WB control
orig. isol.
tg338
tg Bov
tg338
tg ARQ
tg Bov
tg338
tg ARQ
30 kDa
20 kDa
10 kDa
(Sha 31 anti-PrP ab)

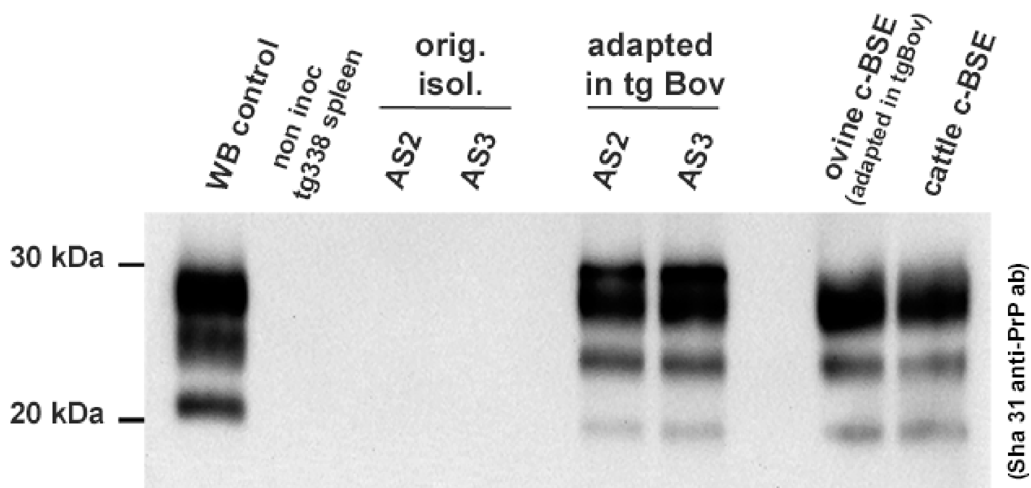
a

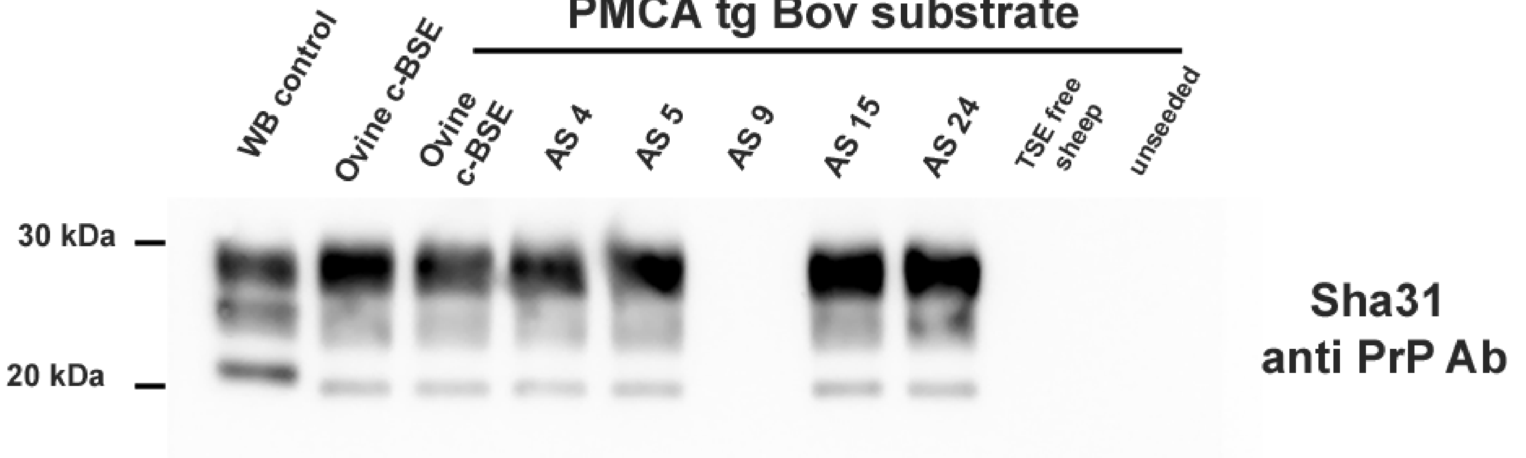
2nd passage in tg338

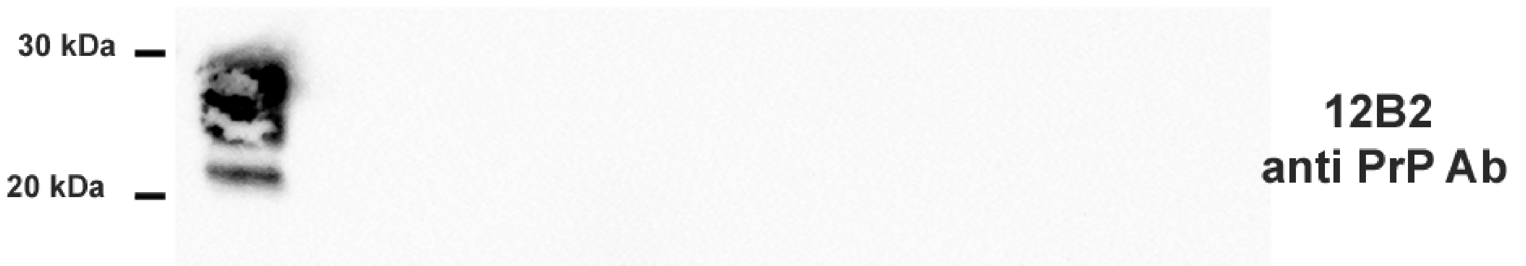
	AS2 original isolate	AS2 adapted in tg Bov	Ovine c-BSE adpated in tg Bov
Brain			
Spleen			

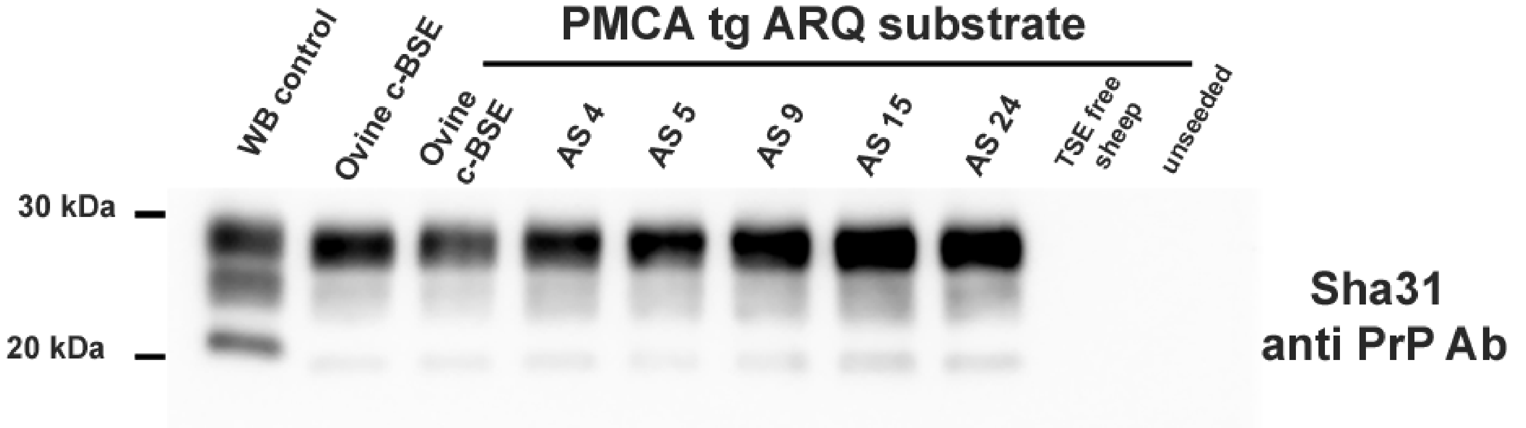
b

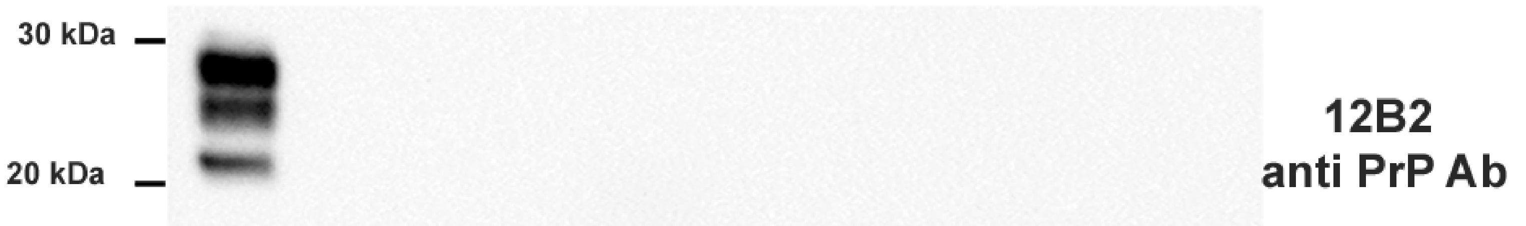
Spleen from tg338 inoculated with

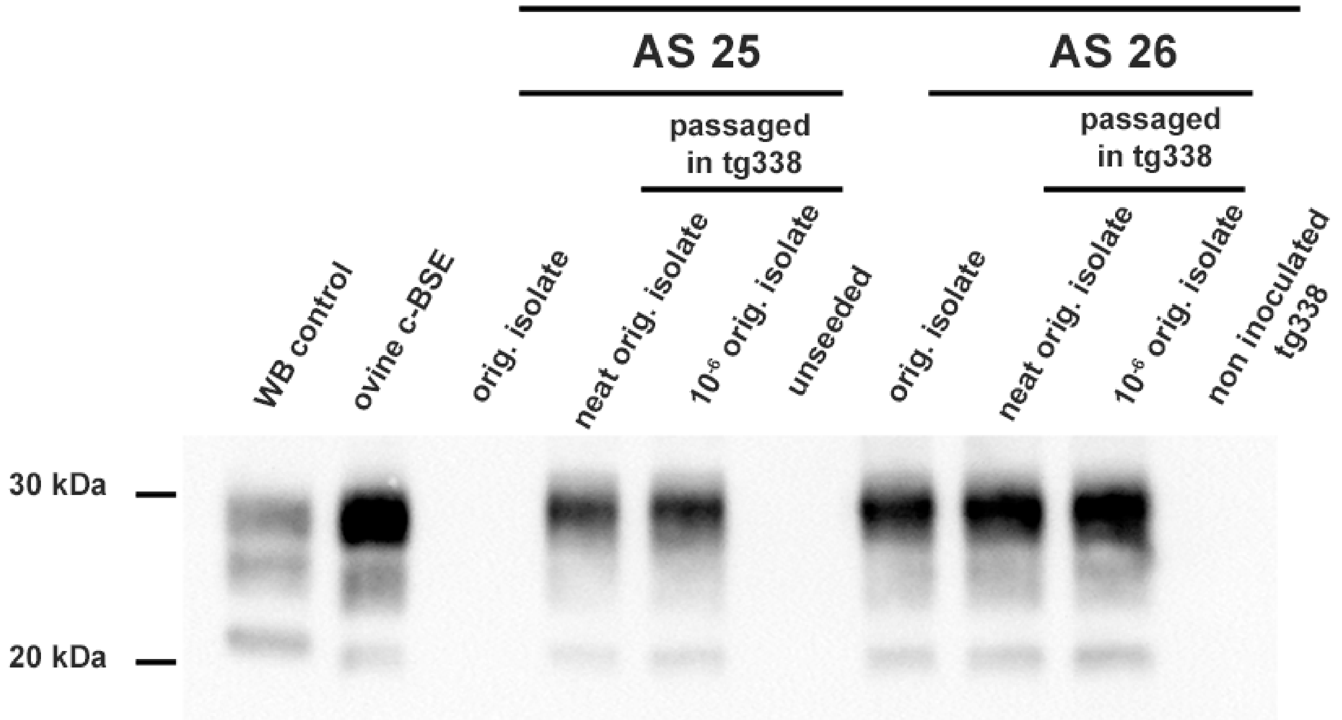


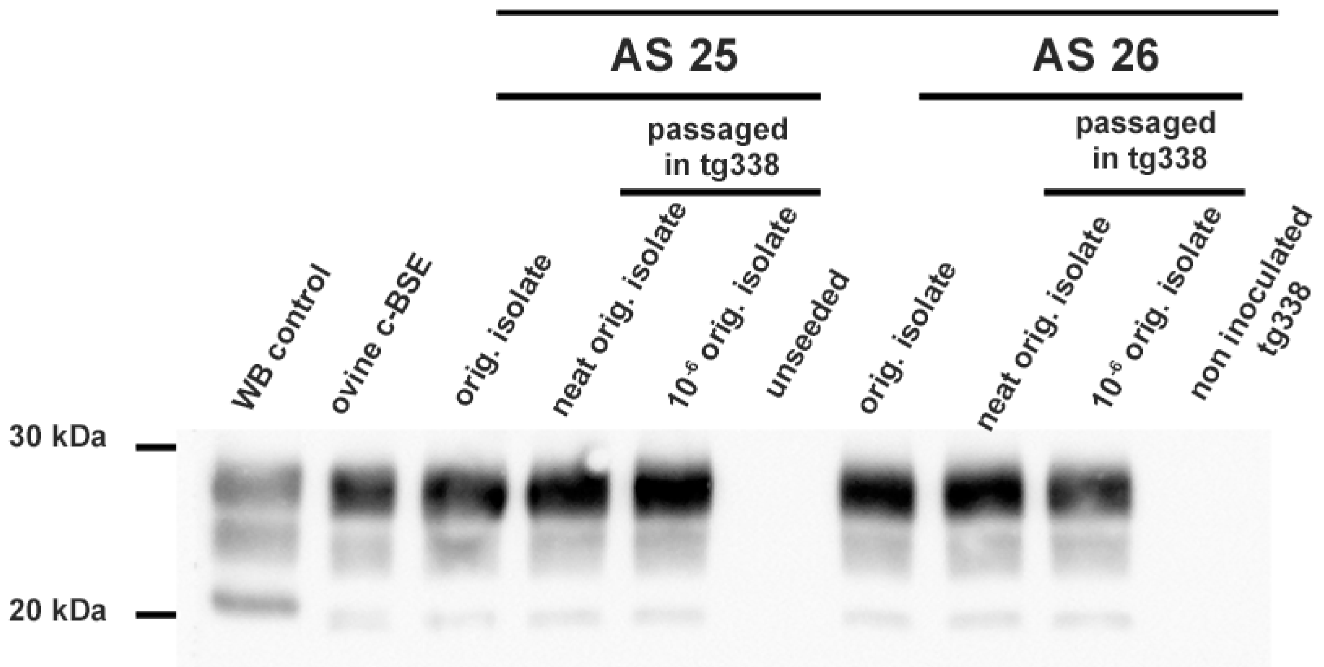

PMCA tg Bov substrate
WB control
Ovine c-BSE
Ovine c-BSE
AS 4
AS 5
AS 9
AS 15
AS 24
TSE free sheep
unseeded
30 kDa
20 kDa
Sha31
anti PrP Ab


30 kDa
20 kDa
12B2
anti PrP Ab


PMCA tg ARQ substrate
WB control
Ovine c-BSE
Ovine c-BSE
AS 4
AS 5
AS 9
AS 15
AS 24
TSE free sheep
unseeded
30 kDa
20 kDa
Sha31
anti PrP Ab


30 kDa
20 kDa
12B2
anti PrP Ab


PMCA amplification tg Bov substrate
AS 25
AS 26
passaged in tg338
passaged in tg338
WB control
ovine c-BSE
orig. isolate
neat orig. isolate
10^{-6} orig. isolate
unseeded
orig. isolate
neat orig. isolate
10^{-6} orig. isolate
non inoculated tg338
30 kDa
20 kDa


PMCA amplification tg ARQ substrate
AS 25
AS 26
passaged in tg338
passaged in tg338
WB control
ovine c-BSE
orig. isolate
neat orig. isolate
10^{-6} orig. isolate
unseeded
orig. isolate
neat orig. isolate
10^{-6} orig. isolate
non inoculated tg338
30 kDa
20 kDa