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

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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

140 **Results**

141 A panel of 8 atypical scrapie (AS) cases collected from sheep and goats in five different
142 European countries was obtained (Table 1). All of the AS isolates displayed a multi-band
143 abnormal PrP (PrP^{res}) western blot profile that was considered to be specific for small
144 ruminant AS (Figure 1c). This panel of AS isolates was transmitted to the VRQ ovine PrP
145 transgenic mice (tg338). The transmission properties such as incubation period (Table 1),
146 vacuolar lesion profile (Figure 1a and / or Figure 2a), and PrP^{res} distribution pattern in the
147 brain (Figure 2c), of the propagated AS isolates observed after two or three iterative passages
148 in tg338 were similar (Figure 1) and were the same as that previously reported for AS passage
149 in tg338 mice (21-23).

150

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

158

159 Second passage of the AS isolates in tgBov mice was performed using either first passage
160 PrP^{res}-positive brains or pooled PrP^{res}-negative brains as inoculum. During this process, 7 out
161 of the 8 AS isolates caused the occurrence of clinical prion disease in a proportion of animals
162 in each group inoculated. On third passage (available for AS 1, 2, 3, 4 and 7) 100% attack
163 rates and mean incubation periods that ranged between 235 and 286 dpi were recorded (Table
164 1).

165 At each passage stage, a three band PrP^{res} western blot profile characterised by a prominent
166 di-glycosylated PrP band was observed in the brains of the clinically-positive tgBov mice.
167 Strikingly, the PrP^{res} western blot profile, the lesion profile and the histopathological lesions
168 in the brains of AS inoculated tgBov mice were identical to those observed for transmission of
169 c-BSE (sheep or cattle origin) to tgBov mice (Figure 1). Importantly, the inoculation of one
170 classical scrapie isolate (PS42) to the same mouse models resulted in the occurrence of a
171 100% attack rate for prion disease with a short incubation period in tg338 mice, but no
172 clinical disease or PrP^{res} 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), the PrP^{res} western blot profile and the lesion profile (Figure 2) of tg338 mice
179 inoculated with tgBov-adapted AS isolates clearly differed from those observed in the same
180 mouse line inoculated with the original AS isolates (Table 1 and Figure 1). No PrP^{res}
181 deposition could be detected in the spleen of tg338 mice inoculated with the original AS
182 isolates. Conversely, transmission in tg338 of tgBov-adapted AS isolates was associated with
183 a PrP^{res} accumulation in the spleen as was transmission of ovine and cattle c-BSE (Figure 2c).
184 PrP^{res} WB profile in the spleen of tg338 mice that were inoculated with c-BSE and AS isolates
185 passaged in tgBov were identical (Figure 2d).
186
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} distribution and
189 PrP^{res} western blot profile patterns (Figure 2b) that were similar to c-BSE passaged in tg338

190 and tgARQ mice. Collectively these results demonstrate beyond reasonable doubt that our
191 transmission of AS in a bovine PrP host resulted in the propagation of the c-BSE agent. Since
192 the bioassays reported here were performed in three independent institutes (located in France
193 and Spain) that used inoculum prepared by five distinct laboratories, we exclude the
194 possibility of a cross contamination of the original AS isolates by the c-BSE agent.
195
196 Two hypotheses could explain the emergence of the c-BSE agent in tgBov mice after their
197 inoculation with AS isolates. Firstly, c-BSE prions could be present at a low level in the
198 original AS isolates. The high sensitivity of tgBov mice for detection of the c-BSE agent
199 could allow this potentially low level of bovine prions to be identified during passage of the
200 original AS isolates in the bovine PrP host. Alternatively, the occurrence of c-BSE in AS-
201 inoculated tgBov mice could result from a mutation of AS strain properties triggered by
202 passage across the bovine transmission barrier for this particular ovine prion strain.
203
204 In order to explore the origin of the c-BSE agent observed in tgBov mice inoculated with AS,
205 we employed *in vitro* protein misfolding cyclic amplification (PMCA), a methodology that
206 mimics prion replication *in vitro*, but in an accelerated form, allowing amplification of minute
207 amounts of PrP^{Sc} and prion infectivity (27). In PMCA, a PrP^{C}-containing substrate is
208 combined with a seed that contains PrP^{Sc}. Following repeated cycles of incubation and
209 sonication, the amount of PrP^{Sc} increases.
210
211 PMCA has been previously reported to amplify the c-BSE agent with a great efficacy using
212 either tgARQ or tgBov mouse brain homogenate as substrate (28). Using this protocol two
213 (tgARQ substrate) or three (tgBov substrate) amplification rounds were sufficient to reach the
214 detection limit for c-BSE prion seeding activity (Supplementary Figure 1). The level of prion

215 infectivity and prion seeding activity of a reference sheep-passaged c-BSE isolate were end-
216 point titrated by both bioassay in tgBov mice and PMCA, respectively (Table 3). The
217 infectious prion titer of the sheep-passaged c-BSE isolate was ≈$10^{7.2}$ LD_{50}/g IC in tgBov mice.
218 The prion seeding titer (SA_{50}) was estimated to be ≈$10^{11.1}$ SA_{50}/g using tgARQ mouse tissue
219 as substrate and $10^{11.05}$ SA_{50}/g using tgBov mouse tissue as substrate. Considering the fact that
220 mice were inoculated using 20µL of sample and the PMCA reactions were seeded using 5µL
221 of the same sample, the PMCA can be considered to be about 1500 fold more sensitive than
222 the bioassay in tgBov. This also means that 1 c-BSE LD_{50} in tgBov mice corresponds to
223 ≈1,500 SA_{50} assessed by PMCA.
224
225 In addition to its high sensitivity, *in vitro* PMCA can reproduce, at least partly, the
226 transmission barrier phenomenon observed during the *in vivo* prion bioassay (29). Therefore,
227 amplification of prion seeding activity in AS isolates by PMCA using tgBov mouse tissue as
228 substrate offered an opportunity to characterize the potential impact of the bovine
229 transmission barrier on AS strain properties.
230
231 The AS isolates that were originally transmitted to tgBov mice (except AS8) and 18 additional
232 AS isolates (originating from Norway, France, and Portugal) were subjected to PMCA (Table
233 4). Each AS isolate was used to seed reactions containing either bovine PrP or ovine ARQ PrP
234 substrate (10 to 18 replicates per substrate). After amplification, PrP^{res} was detected by
235 western blot in a low proportion of the reactions seeded with 19 out of the 25 AS isolates for
236 tgBov and tgARQ combined (Table 4). In most instances, a similar proportion of PrP^{res}-
237 positive PMCA reactions were observed when either bovine PrP or ovine ARQ PrP was used
238 as substrate. However, in some cases (n=3), a low number of PrP^{res}-positive reactions were
239 observed when bovine PrP was used as substrate (in the case of AS 10) or when ovine ARQ

240 PrP was used as substrate (in the case of AS 9, and AS 25). Whatever combination of AS
241 isolate and substrate PrP used, the PrP^{res} western blot profile in PMCA-positive reaction
242 products and its reactivity with 12B2 antibody were indistinguishable from those observed for
243 PMCA reaction products seeded with authentic ovine c-BSE prions (Figure 3). No PrP^{res} was
244 observed in PMCA reactions that were unseeded (n=120) or in those reactions seeded (n=60)
245 with prion-free sheep brain homogenate (representative samples shown in Figure 3**).** It should
246 be noted that the PrP amino sequence was 100% homologous between certain AS isolates (AS
247 5, AS 26) and the ovine PrP substrate (tgARQ) used in PMCA reactions. Therefore, *in vitro*
248 amplification of c-BSE prions in PMCA reactions seeded with these AS isolates using ovine
249 ARQ PrP as substrate cannot be a consequence of mutation of prion strain properties triggered
250 by a transmission barrier.

251

252 Taken together, the tgBov mouse bioassay and PMCA results strongly support the view that a
253 low level of c-BSE prions was initially present in at least 21 out of the 26 AS isolates tested.

254

255 To further clarify the origin of the c-BSE agent detected in AS isolates, two of these isolates
256 (AS 25 and AS 26) were end-point titrated in tg338 mice (1/10 dilution series, 6 tg338 mice
257 inoculated per dilution). For both isolates, the last positive transmissions were observed in
258 mice that received a 10^{-6} $\log_{10}$ dilution of the original 10% w/vol brain material
259 (Supplementary Table 1). The brains of these end-point titration tg338 mice were
260 subsequently subjected to PMCA. Irrespective of the substrate used for PMCA, either bovine
261 or ovine ARQ PrP, PrP^{res} was observed in a similar proportion of the PMCA reactions seeded
262 with either the original AS isolates, or AS isolates passaged in tg338 mice (Table 5). The
263 PrP^{res} western blot profile observed in all the PMCA-positive reactions was identical to that
264 seen in reactions seeded with authentic c-BSE prions. PMCA reactions seeded with brain

265 homogenate prepared from age matched non-inoculated tg338 mice remained PrP^{res} negative
266 (Table 5 and Figure 4).
267
268 Considering the level of c-BSE seeding activity originally present in isolates AS 25 and AS
269 26 (less than 100 SA_{50}/mL, Table 5), there is an extremely low level of probability that one of
270 the six tg338 mice inoculated with 20µL of a 10^{-6} diluted AS isolate (<2 10^{-6} SA_{50} per dose of
271 inoculum) could be exposed to 1 infectious dose of c-BSE agent (1 c-BSE LD_{50} is ≈1,500
272 SA_{50}). Consequently, the presence of c-BSE prion seeding activity in the brains of tg338 mice
273 inoculated with a 10^{-6} log_{10} dilution of original AS isolate implies that a low titer of c-BSE
274 prions was generated during the propagation of ovine AS prions in a host that expressed ovine
275 PrP, namely tg338 mice.
276
277

278 **Discussion**

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

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

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

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

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

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

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

382 **Methods**

383 ***Ethics Statement***

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

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

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

407

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

415

416 ***c-BSE isolates***

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

423

424 **Mouse bioassays**

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

427

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

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

438

439 **PMCA Amplification**

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

450

451 **Western blot detection of abnormal PrP**

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

456

457

458 **Paraffin embedded tissue blot**

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

460 (61-63).

461

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

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

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

465 PrP antibody (epitope: $_{147}$DYEDRYYRE$_{155}$ of the bovine PrP) (63).

466

467 **Infectious and seeding activity titer estimates**

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

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

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

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

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

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

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

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

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

477

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

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

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

481 reactions was established. These ratios were used to estimate seeding activity titers (SA/µL of

482 10% brain homogenate) by the limiting dilution titration method (application of Poisson's
483 probabilistic model) described by Brown et al (66) or by the Spearman-Kärber's method.
484 According to Fisher et al (67) and as previously used for prion infectivity comparisons (68)
485 one SA_{50} was considered to be equivalent to 0.693 SA.

486
487
488
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490

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498

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655
656

657 **Legends of Figures**
658
659 **Figure 1: Brain lesion profile and PrP^{res} western blot profiles in tgBov and tg338 mice**
660 **inoculated with atypical/Nor98 scrapie (AS) or ovine c-BSE**
661
662 Groups of mice (n≥6) that express either ovine VRQ PrP (tg338 mice) or bovine PrP (tgBov
663 mice) were intra-cerebrally challenged with atypical scrapie isolates (AS) or an ovine classical
664 BSE isolate (c-BSE).
665 (**a**) After two or three iterative passages in each mouse line a standard lesion profile was
666 established by scoring the vacuolar changes observed in pre-defined brain areas. In c-BSE
667 graphs ●: ovine c-BSE, ○: cattle c-BSE. In AS graphs: ○: AS 1, △: AS 2, ▽: AS 3).
668 (**b**) Vacuolar lesions (thalamus level, conventional histology; hematoxylin-eosin, Bar: 25
669 µm) and abnormal PrP deposition (Mesencephalon: tegmentum, immunohistochemistry using
670 6H4 anti PrP antibody, bar: 50µm) in tgBov mice inoculated with AS3 and cattle BSE.
671 (**c**) The accumulation of PK-resistant PrP (PrP^{res}) in the original AS isolates and in the brain
672 of inoculated mice was established by western blot using anti PrP monoclonal antibodies
673 Sha31 (epitope 145-YEDRYYRE-152) and/or 12B2 (epitope 89-WGQGG-93). The same
674 western blot PrP^{res} control (classical scrapie isolate) was used on all the gels labelled as WB
675 control. PrP signal in PK digested / undigested in AS isolates and negative control sheep brain
676 shows the specificity of Western blot banding pattern observed in AS isolates.
677
678
679 **Figure 2: Brain lesion profile and PrP^{res} accumulation in tgARQ and tg338 mice**
680 **inoculated with AS scrapie adapted in tgBov**
681
682 Groups of mice (n≥6) that express ovine VRQ PrP (tg338 mice) or ovine ARQ PrP (tgARQ
683 mice) were intra-cerebrally challenged with atypical scrapie isolates (AS) or an ovine c-BSE
684 isolate that had previously been adapted (2 iterative passages) in tgBov mice.
685 (**a**) After two iterative passages in each mouse line a standard lesion profile was established
686 by scoring the vacuolar changes observed in pre-defined brain areas. In AS graphs △: AS 2,
687 ▽: AS 3.
688 (**b**) The western blot profile of PK resistant PrP (PrP^{res}) in the original AS isolates and in the
689 brain of inoculated mice was established by western blot using anti PrP monoclonal antibody
690 Sha31 (epitope 145-YEDRYYRE-152). The same western blot PrP^{res} control (classical
691 scrapie isolate) was used on all the gels labelled as WB control.
692 (**c**) In tg338 mice (2nd passage) the PrP^{res} distribution pattern in the brain (thalamic coronal
693 section: bar: 50 µm) and in the spleen (bar: 200 µm) was established by paraffin embedded
694 tissue blot using anti PrP monoclonal antibody Sha31 (epitope 145-YEDRYYRE-152).
695 (**d**) The western blot profile of PK resistant PrP (PrP^{res}) in the spleen of tg338 mice (2nd
696 passage) inoculated with AS isolates, AS isolates passaged in tgBov and c-BSE (cattle and
697 ovine origin) was established using anti PrP monoclonal antibody Sha31.
698
699
700 **Figure 3: PrP^{res} detection in PMCA reactions seeded with atypical/Nor98 scrapie**
701 **isolates.**
702
703 Protein misfolding cyclic amplification (PMCA) reactions were seeded with Atypical/Nor98
704 scrapie (AS) isolates (1/50 diluted 10% brain homogenate) that had been identified in five
705 European countries (see Table 4). PMCA reactions seeded with brain homogenate from a
706 TSE-free sheep (originating from New Zealand) and unseeded PMCA reactions were included

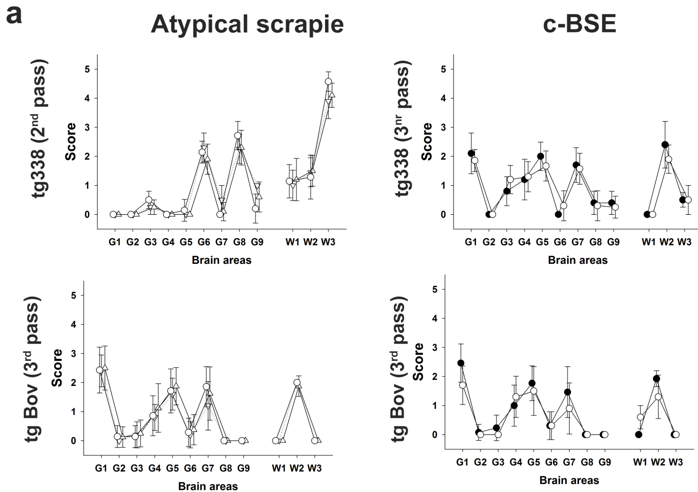
707 as specificity controls. PMCA substrate consisted of brain homogenate from either bovine PrP
708 (tgBov) or ovine PrP (tgARQ) mice. PMCA reactions were subjected to three (tgARQ
709 substrate) or four (tgBov substrate) amplification rounds each comprising 96 cycles (10s
710 sonication-14 minutes and 50 seconds incubation at 39.5°C) in a Qsonica700. The PMCA
711 reactions were analysed by western blot for the presence of abnormal PK-resistant PrP (PrP^{res})
712 using anti PrP monoclonal antibodies Sha31 (epitope 145-YEDRYYRE-152) and/or 12B2
713 (epitope 89-WGQGG-93). Each western blot included a classical scrapie isolate (labelled as
714 WB control) and an ovine c-BSE isolate as controls.
715

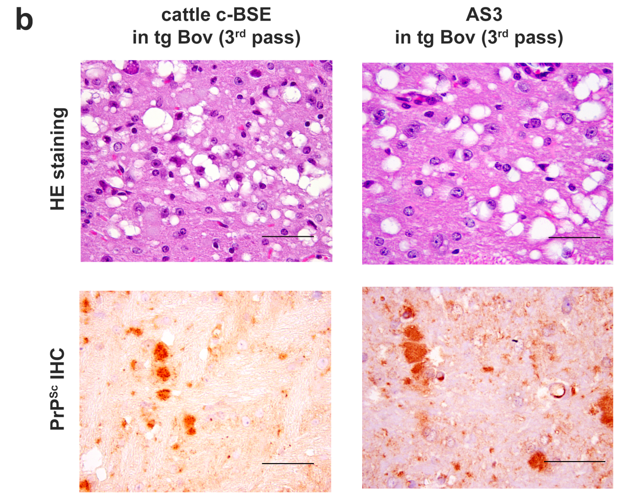
716 **Figure 4: PMCA seeding activity detection in two bioassay end-point titrated**
717 **Atypical/Nor98 isolates**
718

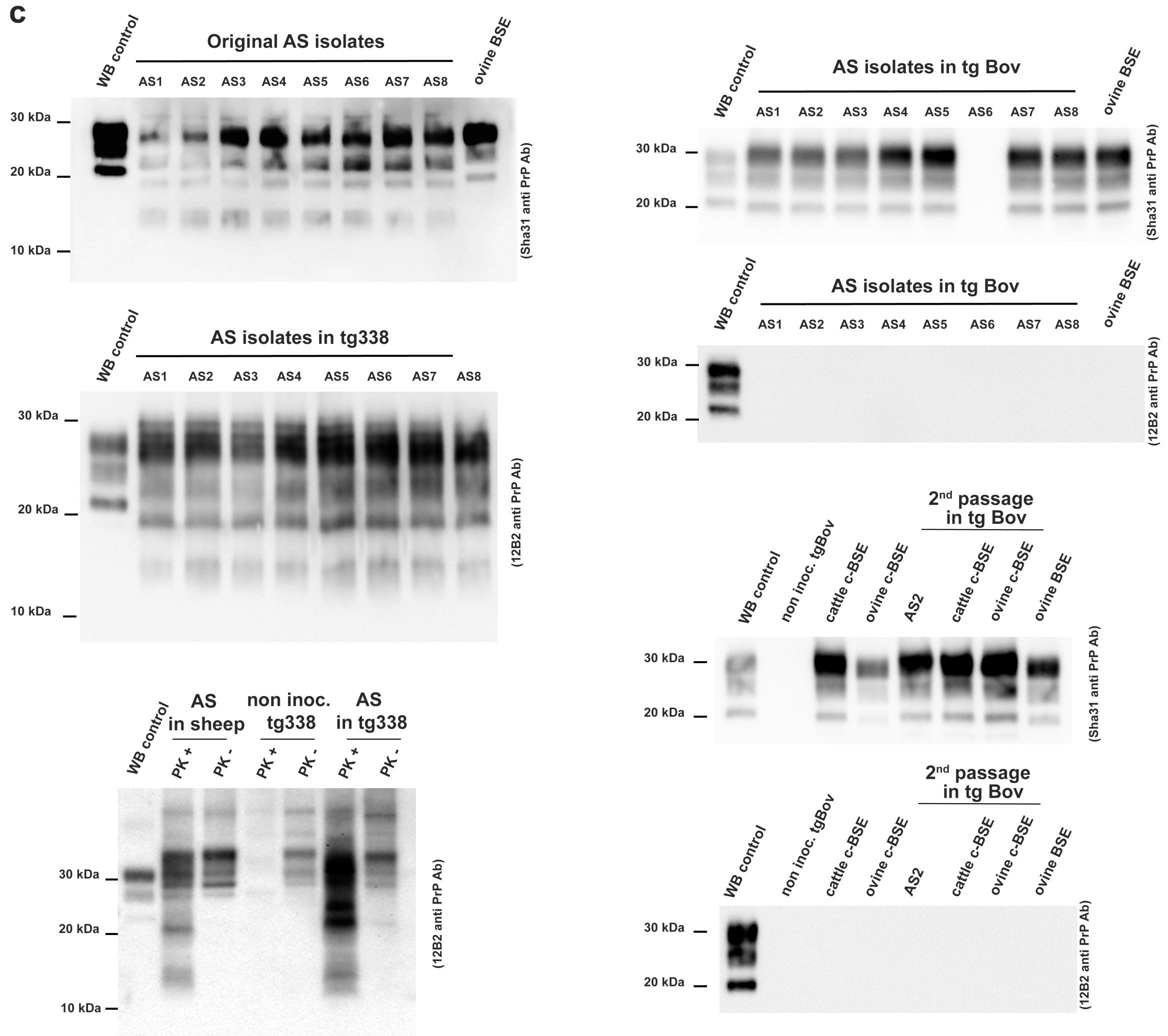
719 Two atypical/Nor98 isolates AS 25 and AS 26 (Table 4) were end-point titrated in tg338 mice
720 (1/10 dilution series, 6 tg338 mice per dilution). For both isolates, the last positive
721 transmissions were observed in mice that received a 10^{-6} dilution of the original 10% w/vol
722 brain material (Supplementary Table 1). The original AS isolates and the brains of clinically
723 affected mice inoculated with neat and 10^{-6} diluted isolates were used to seed PMCA reactions
724 that either used tgARQ or tgBov as substrate. PMCA reactions seeded with age matched
725 inoculated tg338 mice and unseeded reactions were included as specificity controls. Reactions
726 were subjected to three (tgARQ substrate) or four (tgBov substrate) amplification rounds each
727 comprising 96 cycles (10s sonication-14 minutes and 50 seconds incubation at 39.5°C) in a
728 Qsonica700. The PMCA reactions were analysed by western blot for the presence of
729 abnormal PK resistant PrP (PrP^{res}) using the Sha31 (epitope 145-YEDRYYRE-152) and/or
730 the 12B2 (89-WGQGG-93) anti PrP antibodies. Each western blot included a classical scrapie
731 isolate (labelled as WB control) and an ovine c-BSE isolate as controls.
732
733

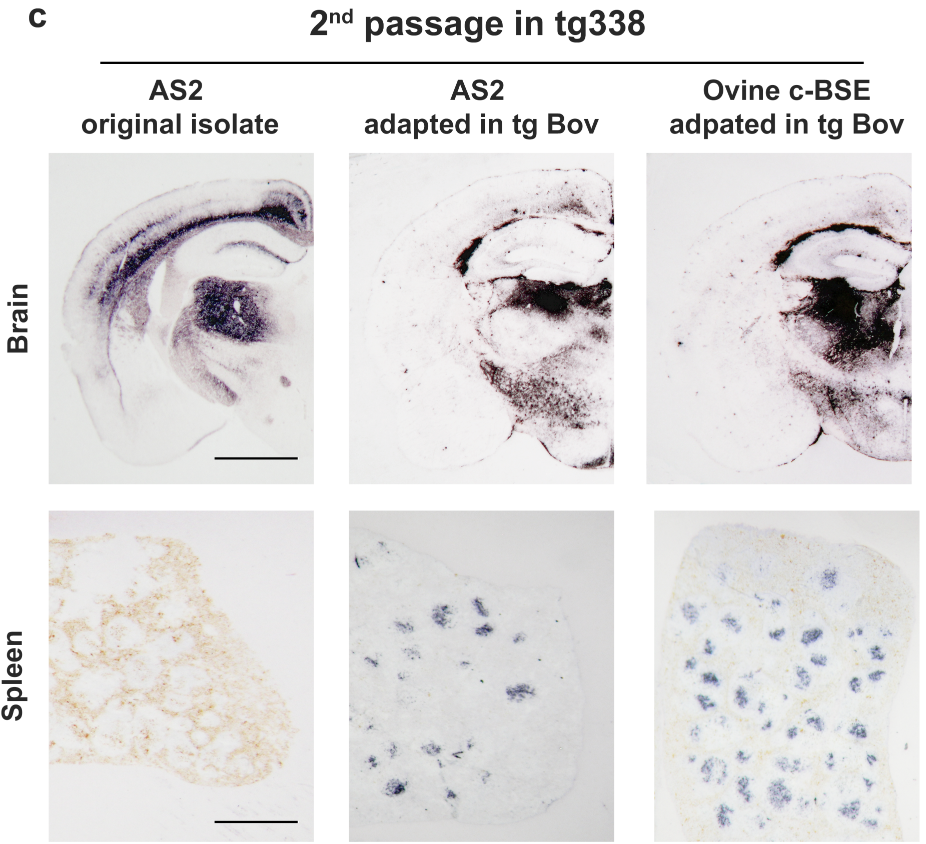
734 **Supplementary Figure 1: PMCA amplification of ovine BSE agent**

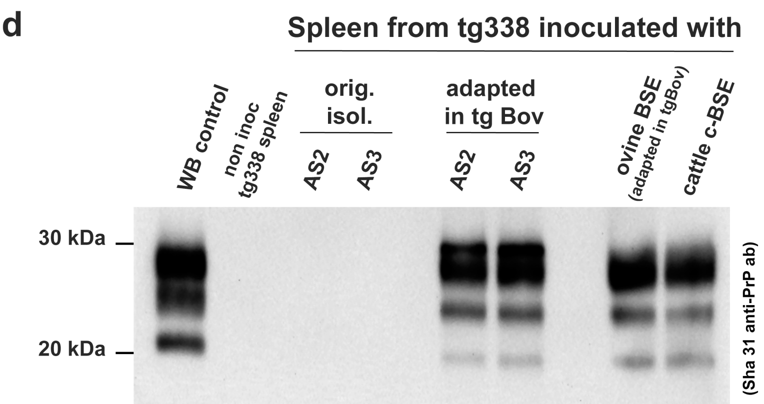
735 PMCA reactions were seeded with a 1/10 dilution series of a reference ovine BSE brain
736 homogenate (10% weight / volume -10^{-2} to 10^{-10} dilution). This homogenate has been
737 endpoint titrated by bioassay in bovine PrP expressing mice (tgBov, intracerebral route – $10^{7.2}$
738 LD_{50}/g).
739 PMCA substrate was prepared using brains from transgenic mice over-expressing the bovine
740 prion protein (tgBov) or the ARQ variant of the sheep prion protein (tgARQ). Unseeded
741 reactions were included as specificity controls. PMCA reactions were then submitted to three
742 to four amplification rounds each comprising 96 cycles (10s sonication-14 minutes and 50
743 seconds incubation at 39.5°C) in a Qsonica700. After each round, (i) reaction products (1
744 volume) were mixed with fresh substrate (9 volumes) to seed the following round while (ii) a
745 part of the same product was analysed by Western Blot (WB) for the presence of abnormal
746 PK resistant PrP (PrP^{res} -antibody Sha31 epitope YEDRYYRE).
747 On each gel a scrapie in sheep isolate was used as control (WB control).
748
749

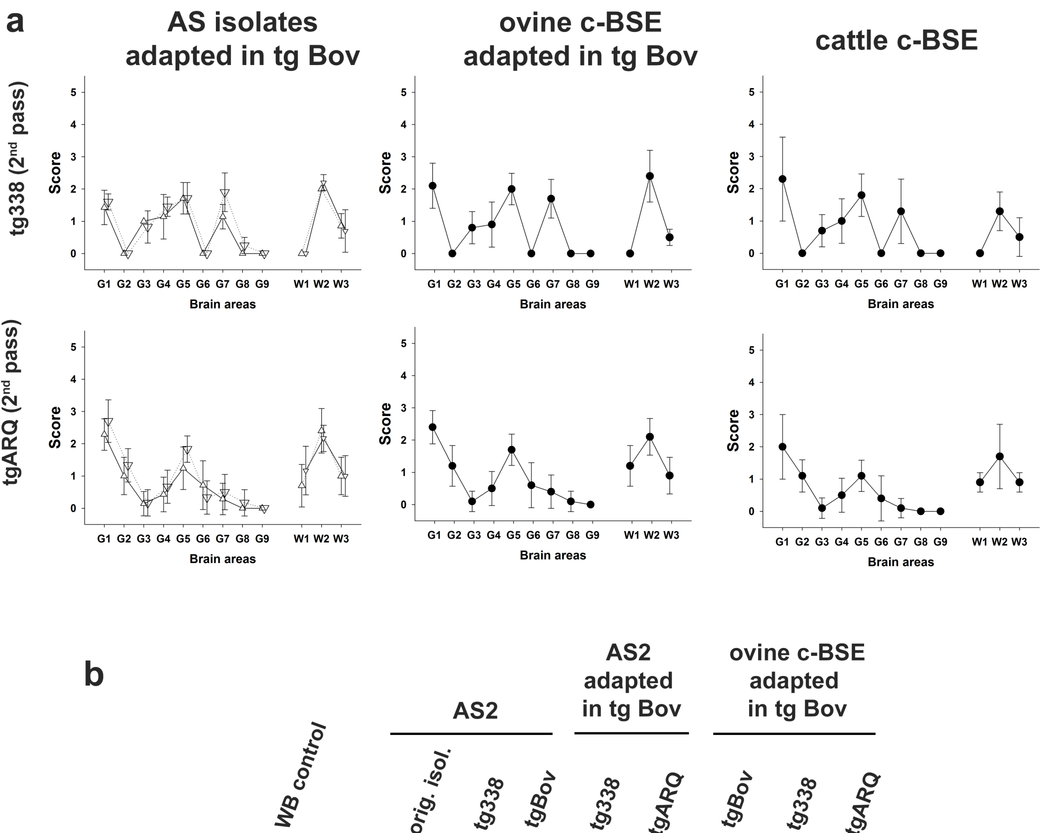

a
Atypical scrapie
c-BSE
tg338 (2nd pass)
tg338 (3rd pass)
tg Bov (3rd pass)
Score
Brain areas
G1 G2 G3 G4 G5 G6 G7 G8 G9 W1 W2 W3


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


c
WB control
Original AS isolates
AS1 AS2 AS3 AS4 AS5 AS6 AS7 AS8
ovine BSE
30 kDa
20 kDa
10 kDa
(Sha31 anti PrP Ab)
AS isolates in tg338
(12B2 anti PrP Ab)
AS in sheep
non inoc. tg338
AS in tg338
PK +
PK -
AS isolates in tg Bov
2nd passage in tg Bov
non inoc. tgBov
cattle c-BSE
ovine c-BSE
AS2
ovine BSE







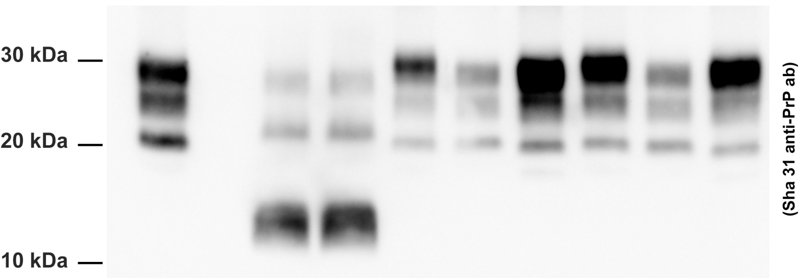
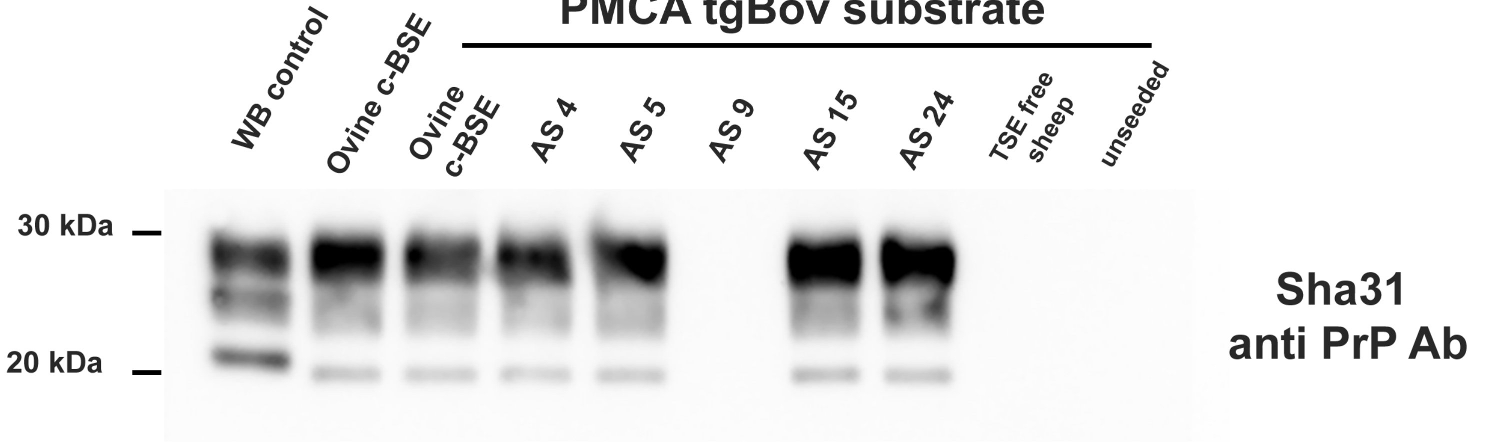


Figure 2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Ovine c-BSE
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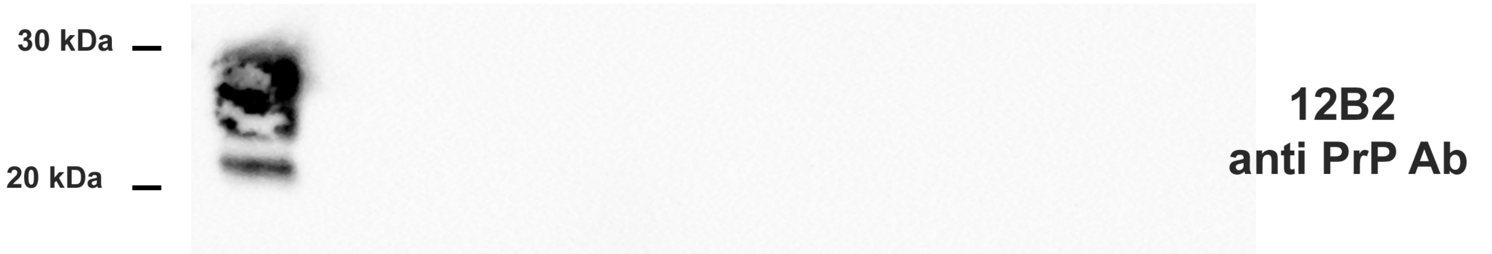
AS 5
AS 9
AS 15
AS 24
TSE free sheep
unseeded
30 kDa
20 kDa
Sha31
anti PrP Ab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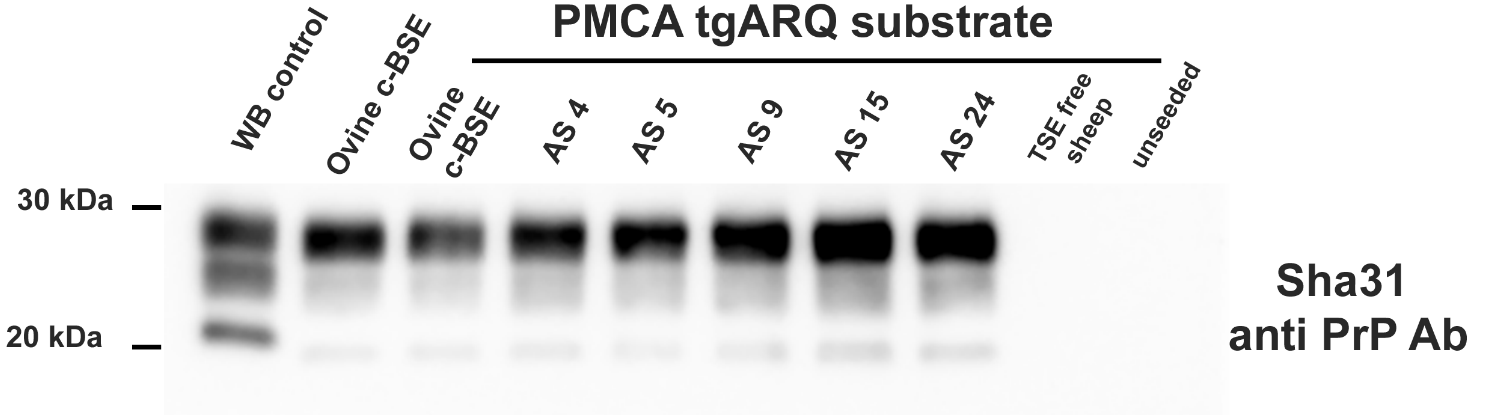
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Ovine c-BSE
Ovine c-BSE
AS 4
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TSE free sheep
unseeded
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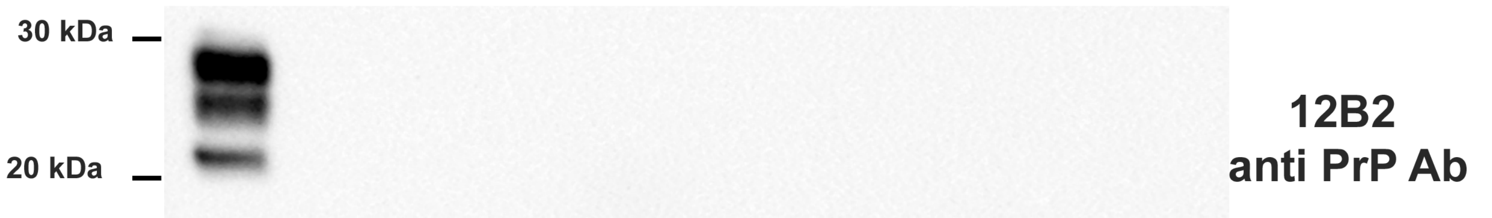
Sha31
anti PrP Ab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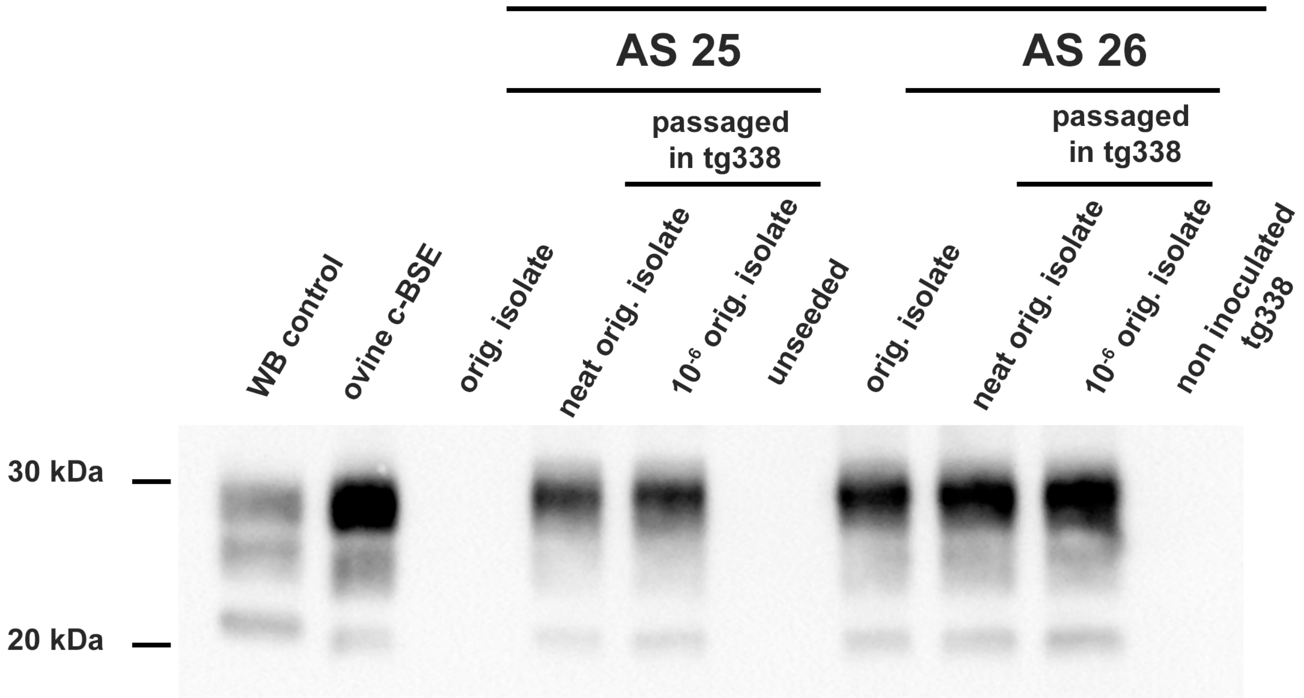
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passaged in tg338
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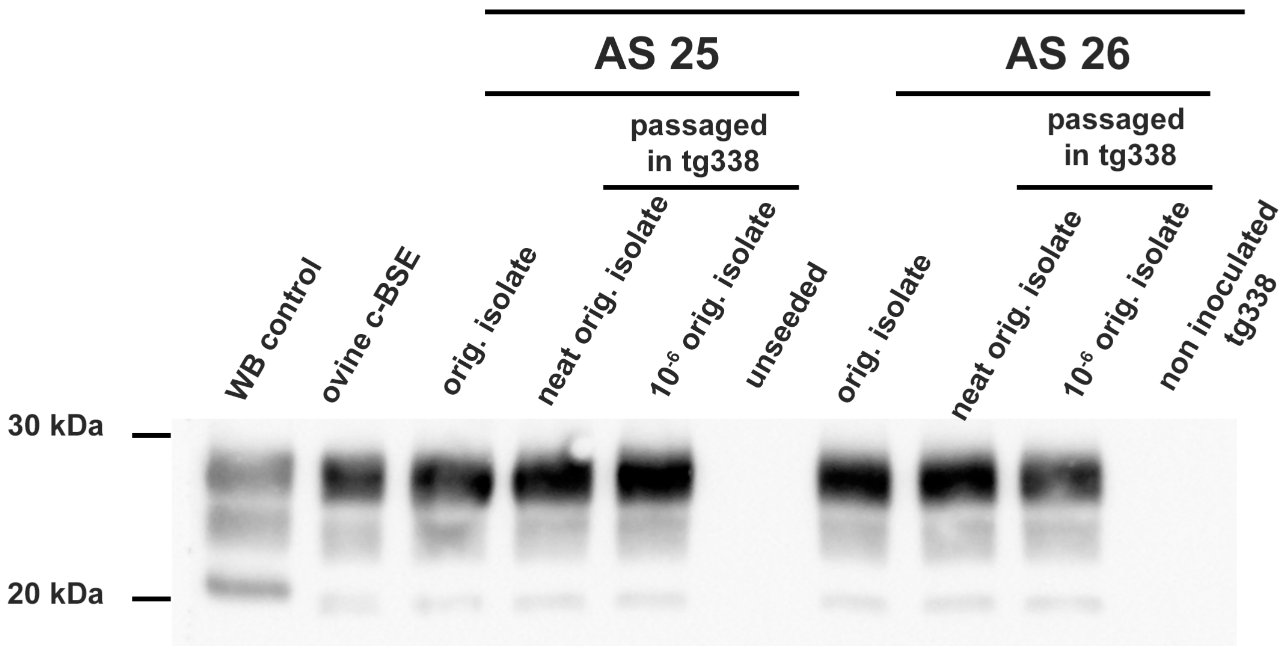
ovine c-BSE
orig. isolate
neat orig. isolate
10-6 orig. isolate
unseeded
orig. isolate
neat orig. isolate
10-6 orig. isolate
non inoculated tg338
30 kDa
20 kDa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neat orig. isolate
10-6 orig. isolate
unseeded
orig. isolate
neat orig. isolate
10-6 orig. isolate
non inoculated tg338
30 kDa
20 kDa

Table 1: Inoculation of atypical scrapie isolates in ovine PrP (tg338) and bovine PrP (tgBov) expressing mice

Isolates			Tg338						TgBov					
			1st passage		2nd passage		3rd passage		1st passage		2nd passage		3rd passage	
Identifiant	**Origin**	**Genotype**	**Positive mice**	**Incubation (mean±SD)**	**Positive mice**	**Incubation (mean±SD)**	**Positive mice**	**Incubation (mean±SD)**	**Positive mice**	**Incubation (mean±SD)**	**Positive mice**	**Incubation (mean±SD)**	**Positive mice**	**Incubation (mean±SD)**
AS 1	Fr	ARQ*/ARQ	6/6	250±18	6/6	232±13	6/6	212±9	1/6	533	3/5	317±63	7/7	235±16
AS 2	Sp	ARR/ARQ	12/12	243±15	6/6	217±15	ND		0/6	>650	7/9	354±26	5/5	273±5
AS 3	Sp	ARQ/ARH	12/12	239±15	12/12	229±12	ND		0/6	>650	2/11	504, 525‡	12/12	269±13
AS 4	Nor	ARQ*/ARQ*	5/5	235±12	ND				3/4	395± 44	6/6	230±17	6/6	271±18
AS 5	Sp	ARQ/ARQ	5/5	186±11	5/5	250±16	6/6	217±14	0/6	>650	0/4	>650	NA	
AS 6	Sp	ARQ/ARH	4/4	226±10	ND				0/6	>650	1/4	>650‡	NA	
AS 7†	It	ARQ/AHQ	6/6	228±11	ND				0/6	>650	1/7	424	6/6	286±14
AS 8	Po	ARQ/ARQ	6/6	207±11	ND				1/5	439	5/5	297±14	6/6	250±4
PS42	Fr	VRQ/VRQ	6/6	71±2	6/6	62±1	6/6	61±1	0/6	>650	0/6	>650	0/6	>650
Ovine c-BSE	Fr	ARQ/ARQ	6/6	663±94	6/6	224±36	6/6	134±2	6/6	254±19	6/6	234±12	6/6	232±6
Cattle c-BSE	Fr	-	6/6‡	>700	6/6	682±52	6/6	136±5	6/6	295±12	6/6	265±35	6/6	243±7

Transgenic mice that express the ovine PrP VRQ variant (tg338) or 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 into both mouse models. 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. ND: not done. Cattle c-BSE transmission in tgBov data were already reported in Torres et al 2014 (50)

Table 2: Inoculation of atypical scrapie and ovine BSE 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 controls. 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. ND: not done.

Table 3: End point titration of BSE in sheep reference isolate by bioassay in bovine PrP expressing mice (tgBov) and Protein Misfolding Cyclic Amplification

Sheep passaged c-BSE isolate	Bioassay tgBov		PMCA positive reactions	
	Positive mice	Incubation period (days ± SD)	TgBov substrate	TgARQ Substrate
Neat	6/6	223±4	ND	ND
10^{-1}	6/6	250±9	ND	ND
10^{-2}	6/6	290±12	12/12	12/12
10^{-3}	6/6	338±18	12/12	12/12
10^{-4}	6/6	386±38	12/12	12/12
10^{-5}	5/6	486±96	12/12	12/12
10^{-6}	1/6	402*	12/12	12/12
10^{-7}	0/6	>700	9/12	10/12
10^{-8}	0/6	>700	6/12	5/12
10^{-9}	ND		0/12	1/12
10^{-10}	ND		0/12	0/12
10^{-11}	ND		0/12	0/12

A 10% w/vol homogenate was prepared using brain stem from a clinically affected sheep (ARQ/ARQ genotype) inoculated with BSE. Groups of 6 tgBov mice were inoculated intra-cerebrally with 20µL of serial ten-fold dilutions of this homogenate. Mice were considered positive when PK resistant PrP (PrP^{res}) deposition was detected in the brain (western blot). Incubation periods (in days) are presented as mean±SD, except for those marked (*) indicating dilutions in which less than half of the mice were scored as positive. The same dilution series was used to seed PMCA reactions (5µL per reaction). Twelve individual replicates of each sample dilution were tested. 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). Reactions were then 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.

Table 4: 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 5: PMCA seeding activity in atypical scrapie passaged in tg338

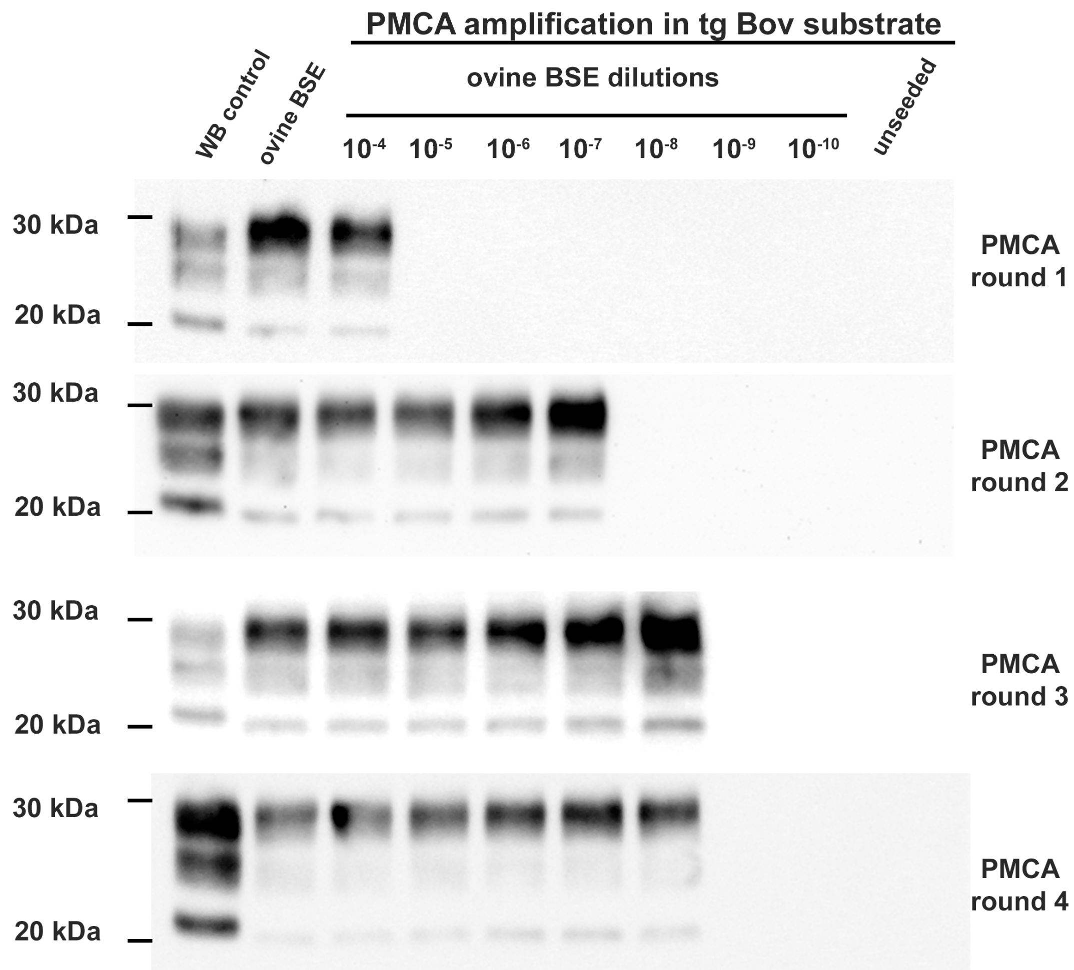
PMCA seeds			PrP^{res} positive PMCA reactions		Seeding activity (SA_{50}/mL)	
Case	**Origin**		**TgBov substrate**	**TgARQ substrate**	**TgBov substrate**	**TgARQ substrate**
AS 25	**Sheep**		0/12	1/12	$0\ (0\text{-}10^{1.86})$*	$10^{1.40}$
	1st passage in tg338 (neat)	**Mouse 1**	2/12	1/12	$10^{1.72}$	$10^{1.40}$
	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	$10^{1.72}$	$0\ (0\text{-}10^{1.86})$ *
	2nd passage in tg338 (neat)	**Mouse 1**	2/12	0/12	$10^{1.72}$	$0\ (0\text{-}10^{1.86})$ *
	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 (65). Titers are given as the number of PMCA SA_{50} per mL of 10% brain homogenate.

Supp Table 1: Atypical scrapie cases end-point titration in tg338 mice

	AS 25		AS 26	
Dilution	**Positive mice**	**Incubation period (mean±SD)**	**Positive mice**	**Incubation period (mean±SD)**
Neat	7/7	224±10	6/6	219±4
10^{-1}	ND		ND	
10^{-2}	ND		ND	
10^{-3}	ND		ND	
10^{-4}	6/6	294±41	6/6	272±23
10^{-5}	6/6	329±34	6/6	315±51
10^{-6}	3/6	360, 392, 412*	2/6	368, 451*
10^{-7}	0/6	>650	0/6	>650
10^{-8}	0/6	>650	0/6	>650
Infectious titer (ID_{50} IC tg338/g)	$10^{8.7}$		$10^{8.5}$	

A 10% w/vol homogenate was prepared using brains from two AS affected sheep. Groups of 6 or 7 tg338 mice were inoculated intra-cerebrally with 20µL of serial ten-fold dilutions of these homogenates. Mice were considered positive when PK resistant PrP (PrP^{res}) deposition was detected in the brain by western blot. Incubation periods (in days) are presented as mean±SD, except for those marked (*) indicating dilutions in which less than half of the mice were scored as positive. Infectious titers (ID_{50} / gram of brain tissue) were estimated by the Spearman-Karber's method.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ovine BSE dilutions
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10-5
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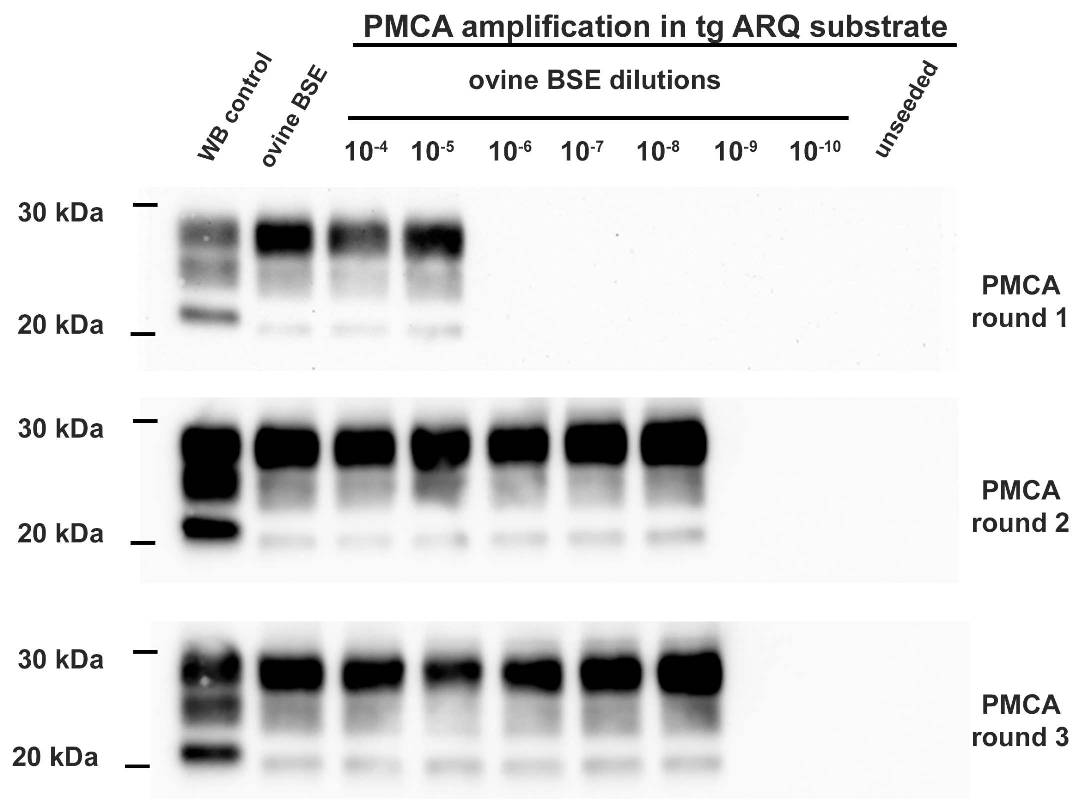
PMCA round 1
PMCA round 2
PMCA round 3
PMCA round 4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WB control
ovine BSE
ovine BSE dilutions
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10-5
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10-8
10-9
10-10
unseeded
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PMCA round 1
PMCA round 2
PMCA round 3