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Abstract

Ovine scrapie is a worldwide spread prion disease that is transmitted horizontally under field conditions. Placenta from scrapie-infected ewes is an important source of infection, since this tissue can accumulate high amounts of PrPSc depending on the foetal genotype. Therefore, placentas carrying susceptible foetuses can accumulate PrPSc but there is not PrPSc accumulation in presence of foetuses with at least one ARR haplotype. In scrapie eradication programs, ARR/ARR males are used for breeding to increase the resistant progeny and reduce the horizontal transmission of the disease through the placenta. The development of highly sensitive techniques, that allow the detection of minimal amounts of PrPSc, has caused many secretions/excretions and tissues that had previously been deemed negative to be relabeled as positive for PrPSc. This has raised concerns about the possible presence of minimal amounts of PrPSc in placentas from ARR foetuses that conventional techniques had indicated were negative. In the present study we examined 30 placentas from a total of 23 gestations; 15 gestations resulted from naturally ARQ/ARQ scrapie-infected ewes mated with ARR/ARR rams. The absence of PrPSc in placentas carrying the foetal ARR haplotype (n=19) was determined by IDEXX HerdChek scrapie/BSE Antigen EIA Test, Prionics®-Check WESTERN and corroborated by the highly sensitive Protein Misfolding Cyclic Amplification technique (saPMCA). By immunohistochemistry, several unspecific stainings that might mislead a diagnosis were observed. The results of the present study support that using ARR/ARR males in scrapie eradication programs efficiently decreases the spreading of the agent in the environment via shed placentas.

Keywords	Scrapie; prion; saPMCA; placenta.
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Protein misfolding cyclic amplification ~~confirms~~ corroborates the absence of PrP^{Sc} accumulation in placenta from foetuses with the ARR/ARQ genotype in natural scrapie.

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Abstract

Ovine scrapie is a worldwide spread prion disease that is transmitted horizontally under field conditions. Placenta from scrapie-infected ewes is an important source of infection, since this tissue can accumulate high amounts of PrP^{Sc} depending on the foetal genotype. Therefore, placentas carrying susceptible foetuses can accumulate PrP^{Sc} but there is not PrP^{Sc} accumulation in presence of foetuses with at least one ARR haplotype. In scrapie eradication programs, ARR/ARR males are used for breeding to increase the resistant progeny and reduce the horizontal transmission of the disease through the placenta. The development of highly sensitive techniques, that allow the detection of minimal amounts of PrP^{Sc}, has caused many secretions/excretions and tissues that had previously been deemed negative to be relabeled as positive for PrP^{Sc}. This has raised concerns about the possible presence of minimal amounts of PrP^{Sc} in placentas from ARR foetuses that conventional techniques had indicated were negative. In the present study we examined 30

placentas from a total of 23 gestations; 15 gestations resulted from naturally ARQ/ARQ scrapie-infected ewes mated with ARR/ARR rams. The absence of PrP^{Sc} in placentas carrying the foetal ARR haplotype (n=19) was determined by IDEXX HerdChek scrapie/BSE Antigen EIA Test, Prionics®-Check WESTERN and corroborated by the highly sensitive Protein Misfolding Cyclic Amplification technique (saPMCA). By immunohistochemistry, several unspecific stainings that might mislead a diagnosis were observed. The results of the present study support ~~We can confirm~~ that using ARR/ARR males in scrapie eradication programs efficiently decreases the spreading of the agent in the environment via shed placentas.

Keywords: scrapie; prion; saPMCA; placenta.

Introduction

Ovine scrapie is a worldwide spread disease caused by the infectious isoform of the host encoded prion protein (PrP^{Sc}). The bovine spongiform encephalopathy (BSE) outbreak in Great Britain provoked strong efforts by European governments to eradicate both prion diseases: BSE and scrapie. A dramatic decrease in prevalence has been achieved for BSE, whereas the control of scrapie remains complicated, mostly due to the difficulty of reducing its horizontal transmission. Scrapie is known to be both horizontally transmitted by direct contact between animals and indirectly transmitted through the contaminated environment, where prions can persist for several years (Hoinville, 1996). In particular, the delivery period and the placenta play key roles in transmission, which is sustained by high levels of PrP^{Sc} and infectivity located in the placentas (Race et al., 1998).

Susceptibility to ovine scrapie is controlled by polymorphisms in the PrP gene (*PRNP*), which are mainly found at codons 136 (A or V), 154 (R or H) and 171 (R, Q or H). The ancestral *PRNP* gene encodes for A136, R154 and Q171 (haplotype ARQ) and is associated with an average resistance to scrapie. Indeed, the substitution of Q by R at the codon 171 (haplotype ARR) is clearly associated with resistance (reviewed by Goldmann, 2008). The foetal *PRNP* gene also controls PrP^{Sc}

deposition in placenta because no PrP^{Sc} has been detected in this tissue when foetuses have at least one ARR haplotype (Andréoletti et al., 2002; Lacroux et al., 2007; Tuo et al., 2002). Therefore, the use of ARR/ARR breeding ~~males~~ rams ~~appears to effectively~~ reduces the dissemination of scrapie infection ~~the transmission of the disease~~ through the shed placenta and effectively controls the transmission of the disease ~~(~~(Nodelijk et al., 2011). However, the absence of PrP^{Sc} in placentas with ARR foetuses has been determined only by using conventional techniques such as immunohistochemistry (IHC), western blotting (WB) and ELISA. The aim of the present study was to use the highly sensitive serial automated Protein Misfolding Cyclic Amplification technique (saPMCA; Saá et al., 2006), to assess the presence of PrP^{Sc} in placentas from foetuses carrying an ARR haplotype and consequently, the effectiveness of a part of the genetic programme for scrapie control and eradication (Dawson et al., 2008).

Material and methods

Animals and sample collection

Seventeen naturally scrapie-infected ewes were selected from different outbreaks after a PrP^{Sc} positive biopsy of rectal mucosal-associated lymphoid tissue (Monleón et al., 2011). The animals were brought to the University of Zaragoza facilities and genotyped for the *PRNP* gene, with each presenting an ARQ/ARQ genotype. All animals under investigation were examined for pregnancy by ultrasound and were regularly checked by clinical examination. Non-pregnant dams (n=8) were subjected to natural mating with negative scrapie rams of the ARR/ARR genotype. After parturition, 5 dams were in good condition ~~and were~~to be mated again with an ARR/ARR ram, obtaining 2 gestations from 4 dams and 3 gestations from 1 dam. All lambs/foetuses were genotyped for the *PRNP* gene.

Thirty placentas from foetuses of ARQ/ARQ (n=11) and ARR/ARQ (n=19) genotypes were collected. Seventeen of those placentas were collected after parturition (shed- placentas), and the remaining 13 were collected at different time points of gestations because it became necessary to

humanely euthanize the dams. Cotyledons/placentomes were taken as samples and were divided into halves. One half was fixed in 10% formalin for IHC, and the other half was stored at -20°C for IDEXX HerdChek scrapie/BSE Antigen EIA Test ("IDEXX EIA", Idexx, US), Prionics®-Check WESTERN (Prionics AG, Switzerland) and saPMCA. ~~Data for the ewes and placentas are detailed in Table 1.~~In addition, 8 placental samples (foetal genotypes ARQ/ARQ n=4 and~~,~~ ARR/ARQ n=4) from ARQ/ARQ non ~~-~~scrapie-infected ~~sheep~~ ewes were used as negative controls. Data for the ewes and placentas are detailed in Table 1.

All ewes included in the present study were humanly euthanized by intravenous injections of sodium pentobarbital, and the scrapie status was confirmed *post-mortem* for PrP^{Sc} detection in the brainstem (obex) and retropharyngeal lymph node by IHC.

This study was performed in strict accordance with the recommendations for the care and use of experimental animals of the University of Zaragoza (R.D.169 1201/2005). The protocol was approved by the associated Committee on the Ethics of Animal Experiments (Permit Number: PI02/08).

~~*Laboratory examinations*~~ *Immunohistochemistry*

Formalin-fixed samples were trimmed and processed according to standard histopathological procedures. PrP^{Sc} IHC was performed as previously described (Monleón et al., 2011) using the L42 antibody (R Biopharm Ltd., Germany; 1:500). Briefly, the protocol included formic acid, proteinase K digestion (4 µg/ml; F. Hoffmann La Roche Ltd, 211 Switzerland) and hydrated autoclaving as pretreatments. EnVision was used as a visualization system, and the two chromogens aminoethilcarbazole and diaminobenzidine were used to distinguish actual PrP^{Sc} deposits from commonly found brown pigments in placenta (all products from Dako Denmark A/S, Denmark). PrP^{Sc} signal was subjectively scored (from + to ++++) based on the extent of immunostaining. In some selected placentas, the F89/160.1.5 (0.5 mg/ml; NC-Neopharma (Abcam Inc, Cambridge, MA, USA)) and R145 (1:3000 Genómica, Spain) antibodies were used to confirm the results obtained by the L42 antibody. No digestion with proteinase K was required as a pretreatment for the

R145 antibody (Sisó et al., 2008). In addition, BAR224 (1:20, Spi-Bio, France), which presents great affinity for the ovine PrP (Féraudet et al., 2005), was used to detect ~~PrP~~^{e}-PrP^{C} in placentas with only hydrated autoclaved in citrate buffer for 20 minutes as pretreatment (Lacroux et al., 2007).

ELISA IDEXX HerdCheck BSE-Scrapie Antigen Test Kit, EIA

All frozen placental samples were tested in duplicate by "IDEXX EIA" according to the manufacturer's protocol for nervous tissue with a previous homogenization step. A medial cross section for each cotyledon/placentome was homogenized (20% w/v) in 0.01 M Tris HCl (pH 7.5) and then subjected to 4 cycles in the TeSeE Precess 48 Homogenizer (Bio-Rad, USA) with ceramic beads. Each cycle consisted of 2 agitation phases of 45 seconds at 6,500 rpm, with 60 seconds between phases. The samples were allowed to cool for 5 minutes between cycles. A sample was considered positive if the optical density values of both replicates were greater than the manufacturer's cut-off criterion.

Prionics-Check Western Small Ruminant test

Frozen placental samples were analysed by WB using the *Prionics-Check Western Small Ruminant test*. The procedure was modified by using the placental homogenate at 20% w/v diluted with 0.01 M Tris HCl (pH 7.5) to a final concentration of 10% (w/v). Next, the homogenates were analysed according to the manufacturer´s protocol, except for the primary antibody, which was substituted for P4 antibody (R-Biopharm Ltd., Germany; 1:5,000).

Serial automated Protein Misfolding Cyclic Amplification (saPMCA)

A total of 13 ARR/ARQ placental samples from scrapie-infected dams were selected for saPMCA analysis, as described previously (Garza et al., 2011). Briefly, brains from tg338 mice over-expressing the VRQ allele of ovine PrP (Vilotte et al., 2001) were used as the substrate for *in vitro* prion conversion and as unseeded-negative controls. Mouse brain homogenates (10% w/v) were prepared in a conversion buffer (PBS containing 150 mM NaCl and 1% Triton X-100 with the addition of Complete Protease Inhibitors; Roche Pharmaceuticals, Indianapolis, IN). Placental

tissue samples were disrupted in a Dounce homogenizer at 10% (w/v) in PBS (Gibco calcium and magnesium free) with Complete Protease Inhibitors. Four aliquots per placenta were analysed by adding 5 µl of placental sample to 50 µl of substrate with 5 mM EDTA final concentration. Tubes were placed on an adaptor on the plate holder of a microsonicator (Misonix, USA, model S3000MP sonicator). The amplified product was digested with proteinase K (85 µg/ml) for 60 min at 42°C with shaking, and PrP^{Sc} was detected by WB using P4 antibody. In addition, 4 placental samples were used as controls: 2 ARQ/ARQ that had been deemed positive by IHC and "IDEXX EIA" [P9 (1st G), P12.2] and 2 negative controls (ARQ/ARQ and ARQ/ARR).

To evaluate the relative sensitivity of saPMCA on placental tissue compared to WB, limiting dilution experiments were conducted in four placentas: 2 negative (P10 (2^{nd} G), P18.1) and 2 positive (P8 (1^{st} G), P9 (1^{st} G)). The placental homogenates (10% w/v) were serially diluted (10^{-1} to 10^{-6}) in the substrate and assased by WB before and after amplification.

To evaluate a potential inhibitory effect of foetal ARR-PrP^{C} and blood on the detection of misfolding activity by saPMCA, the following experiments were performed. To test the effect of ARR-PrP^{C}, 5 µl of negative foetal ARR/ARQ placenta (P10 (2^{nd} G)) were added to the substrate along with 5 µl of positive foetal ARQ/ARQ placenta seed (P9 (1^{st} G). To test the inhibitory effect of the blood on placental and nervous tissue, 5 µl of blood from an ARQ/ARQ non scrapie-infected sheep were added to the substrate in the presence of 2 different seeds: 5 µl of positive foetal ARQ/ARQ placenta (P9 (1^{st} G) and 5 µl of positive nervous tissue. Serial dilutions (10^{-1} to 10^{-6}) from all samples described were assessed by PMCA and detecetd by WB.

Results

None of the 19 ARR/ARQ placentas from naturally scrapie-infected sheep, collected at several times throughout gestation, showed PrP^{Sc} deposits by IHC, "IDEXX EIA" or WB (hereafter referred to as conventional techniques). Thirteen of those negative placentas were analysed by saPMCA and no seeding activity was detected ~~showed a total absence of PrP^{Sc} replication even~~ after the 4^{th} round of saPMCA. In placentas from foetuses presenting an ARQ/ARQ genotype, PrP^{Sc}

deposits were detected in all samples; 3 were collected during the 5th month of gestation, and 8 were shed placentas (Fig. 1 ~~B~~C, ~~E~~D). The results of the PrP^{Sc} detection techniques are detailed in Table 1. Two ARQ/ARQ placentas that had been deemed positive by conventional techniques were used as positive control for saPMCA [P9 (1stG), P12.2; Fig. 2] and showed PrP^{Sc} after the 3rd round in all tested aliquots. Remarkably, the positive P12.2 placenta belonged to a multiple gestation shared with an ARR/ARQ placenta (P12.1) that remained negative to all techniques, including saPMCA (Fig. 2). This case underpinned the absence of cross-contamination and the clear inhibitory effect on PrP replication caused by the foetal ARR haplotype. No PrP^{Sc} was detected by any technique in the 8 placentas from the non scrapie-infected ewes that were used as negative controls (Fig. 1 A,B). By saPMCA, no seeding activity was detected in ARR/ARQ placentas, even after the 7th round neither from non scrapie nor scrapie-infected sheep (Fig. 3 B).

Since there are not previous reports testing placental tissue by saPMCA, the relative sensitivity of the assay to a conventional technique (WB) was determined by limiting dilution experiments (Fig 3 A-C). We detected scrapie-associated seeding activity present on placental tissue from the 3th round (Fig. 2), reaching the detection limit of 10^{-5} to 10^{-6} by the 7th round that corresponded to an increase of at least 4 logs in sensitivity compared to WB (~~FIG~~Fig. 3 A,B). An inhibitory effect on the scrapie-associated seeding activity caused by foetal ARR-PrP^{eC} and blood was observed in placenta. Presence of ARR-PrP^{eC} reduced 3 logs the detection limit of the assay in placentas, and the presence of blood reduced 2 logs either in placental or nervous tissues compared to those obtained under standard saPMCA conditions (Fig.3 B, C). Despite sensitivity of saPMCA assay on placental tissue was slightly lower in presence of foetal ARR-PrP^{eC} or blood, this assay achieved higher sensitivity (around 2 logs) than the conventional technique (WB; ~~FIG~~Fig. 3 B,C).

~~Despite the negative results obtained using other techniques, some~~ Unspecific immunostainings were observed by IHC in ~~both~~ some ~~the~~ negative control placentas and ~~the~~ ARR/ARQ placentas from scrapie-infected ewes (Table 1). These unspecific immunostainings were classified into 3 different types according to the ~~type of~~ stained cells, antibody used and location in the placentome

(Fig. 1 ~~C, F, G, H~~E-J). Type 1 was the most frequently observed and primarily affected the foeto-maternal syncytia at the top of the foetal villus tree surrounding the extravasated maternal blood (arcade area; Fig. 1 ~~C~~E, G~~, F~~). It was characterized as diffused staining that mainly delineated the cell shape, although in some cases, it resembled fine/gross particulate. The foeto-maternal syncytia at the arcade area presented an increase in ~~PrP^{e}~~ PrP^{C} staining compared with the remaining cells (trophoblast and endometrial cells), as revealed by the BAR224 antibody (Fig. 1 ~~I~~H). Type 2 was an intracellular immunostaining that was exclusively related to the binucleate trophoblasts located at the base of the endometrial crypts. This type of staining was only found in placentas at earlier stages of gestation (3rd-4th month; Fig. 1 ~~G~~I). Finally, type 3 was a granular staining of binucleate trophoblast cells and, in turn, syncytia (formed as result of the fusion of those binucleate with the endometrial cells) appearing all across the placentome (Fig. 1 ~~H~~J). The latter type of immunostaining was detected only with the R145 antibody, whereas types 1 and 2 were detected with all of the antibodies included in the study. In control sections with the primary antibody omitted and with an isotype-matched primary antibody (Universal Negative Control Mouse, Dako Denmark A/S, Denmark; Fig. 1 F)), no staining was observed.

Discussion

Scrapie eradication programs are largely based on genetic selection targeting the elimination of susceptible animals and the maintenance of rams carrying the ARR/ARR genotype for breeding (Dawson et al., 2008). The purpose of these programs is to increase the frequency of the ARR haplotype, thus conferring greater resistance to sheep population, and to reduce the dissemination of scrapie infection through placentas with PrP^{Sc} deposition (Andréoletti et al., 2002). Over the last several years, the development of highly sensitive methodologies, such as transgenic rodent bioassay or the saPMCA technique (Saá et al., 2006), have allowed the detection of minimal amounts of PrP^{Sc} in different secretions/excretions that are otherwise undetectable by conventional techniques (Gough and Maddison, 2010). To date, the absence of PrP^{Sc} in placentas from foetuses

with ARR haplotypes has been determined only by using conventional techniques (Alverson et al., 2006; Andréoletti et al., 2002; Lacroux et al., 2007; Santucciu et al., 2010; Tuo et al., 2002). Our results corroborate this previous finding since no misfolding activity was detected by saPMCA in ~~Using saPMCA, our results confirm that~~ placentas carrying foetal ARR ~~haplotypes do not accumulate any PrP^{Sc}~~. In agreement with previously published studies (Alverson et al., 2006; Lacroux et al., 2007), the case of P12.1 emphasizes this lack of PrP^{Sc} accumulation, even when sharing the uterus of other susceptible foetuses with a positive placenta. However, one weakly positive placenta of an ARR/ARQ foetus that shared the same uterine horn with an ARQ/ARQ foetus has been described in the literature, likely caused by blood sharing between foetuses (Alverson et al., 2006).

The ARQ/ARQ sheep in an advanced clinical stage of the scrapie disease show wide dissemination of PrP^{Sc} in many organs other than the central nervous (CNS) and lympho-reticular systems (Garza et al., 2014). However, we did not detect PrP^{Sc} in any of the ARR/ARQ placenta, even from the 13 ARQ/ARQ sheep that were at a terminal clinical stage. Moreover, 4 of the 19 negative ARR/ARQ placentas came from sheep having previous gestations with positive ARQ/ARQ placentas. It is worth noting that the Dam 9 had up to 3 gestations. To our knowledge, this case is the first description of an animal having up to 3 pregnancies during the progression of scrapie disease. Only the first gestation, as a consequence of being mated with a susceptible ram, presented PrP^{Sc} accumulation in placenta. The other 2 gestations, in which the dam was in a more advanced clinical stage and presumably had a higher dissemination of PrP in the organism, did not lead to a threat of scrapie dissemination through placentas. In conclusion, our data highlight the efficiency of using ARR-carrying males for breeding to reduce the dissemination of scrapie during lambing seasons. Furthermore, the gestation periods do not seem to exacerbate or accelerate the progression of disease, as observed for the 5 animals that each had at least 2 gestations during the progression of the disease.

ARR/ARQ sheep, which are widely viewed as highly resistant to classical scrapie, have been shown to be infected by oral inoculation, although with a much longer incubation period than ARQ/ARQ sheep (Jeffrey et al., 2014). Sheep placenta is a unique tissue that only lasts 5 months, and its short "life-~~spam~~span" together with even a minimal interference in the conversion process caused by the ARR haplotype (Bossers et al., 2000) might explain the total absence of PrP^{Sc} in placenta in presence of foetal ARR.

It is still not clear how the prions access the placenta, but in general terms, prions disseminate throughout an organism via two pathways: by using peripheral nerves as physical conduits (neural neuroinvasion) or via the blood (haematogenous neuroinvasion; Sisó et al., 2009). The restricted innervation of the uterus and placenta during pregnancy, with very few fibres in the internal myometrium (Marzioni et al., 2004), and the existence of maternal blood pools ("haemophagous areas") in the ovine placenta (Sammin et al., 2009) suggest that prion access occurs via blood rather than the peripheral nerves. Haematogenous dissemination has also been proposed for prion access to some peripheral organs/secretions, such as the kidney (Sisó et al., 2008) or milk (Lacroux et al., 2008). The detection of infectivity in the blood by bioassay studies (Houston et al., 2008) and the presence of PrP^{Sc} by using PMCA (Thorne and Terry, 2008) –has been demonstrated in scrapie cases. Recently, Andréoletti *et al*. (2012) concluded that the intravenous administration of a few hundred microliters of blood is sufficient to infect an ovine transfusion recipient. Therefore, the flow of maternal blood through the ovine placenta and the maternal blood pools ~~should~~ might be sufficient to infect the tissue. However, the PMCA did not amplify any PrP^{Sc} in placentas carrying foetal ARR haplotype (not even from circulating blood), which indicates that PrP^{Sc} can only be detected when replicates in the placentomes. We also exclude the possibility that factors like blood or the ARR-PrP^{eC} are limiting the sensitivity of detection by this technique hence we show that the co-presence of these factors in the seed represented a maximum reduction of 3 logs still leading the saPMCA to a 1000-fold increase in sensitivity over the standard detection method, WB. This clearly substantial gain in sensitivity of saPMCA compared with WB reassures that such factors are not

responsible for the absence of seeding activity determined by saPMCA in ARR placentas. We can also speculate that the contact required for PrP^{C} conversion to PrP^{Sc} in placenta initially occurs on the trophoblastic cells, since only the foetal side of the placenta expresses the ARR haplotype.

Finally, we present in this study a detailed description of the non-specific staining/artefacts resembling PrP in placentas that might ~~complicate~~ confuse ~~a~~ the diagnosis of scrapie when only IHC is applied. We confirmed that these were non-specific stainings because they were observed in both the negative control placentas and the saPMCA-negative ARR/ARQ placentas from scrapie-infected ewes. The unspecific staining that was most frequently found, type 1, could have been due to an excess staining of PrP^{C}~~PrP^{c}~~, as demonstrated for the antibody BAR224 detecting PrP^{C}~~PrP^{c}~~. Its co-existence with the actual PrP^{Sc} deposits in positive placentas and its presence in negative placentas from infected animals highlight the importance of its clear recognition and the use of other techniques such as WB or ELISA to prevent any misleading diagnoses.

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~~**Figure 1. Specific and unspecific immunostaining obtained by PrP antibodies in ARQ/ARQ and ARR/ARQ placentas.**~~

~~PrP^{Sc} detection by IHC in ARR/ARQ (A, C, D, F, G, H, I) and ARQ/ARQ (B, E) placentas. No immunostaining was observed in an ARR/ARQ placenta collected at 5th month of gestation (A) neither an ARR/ARQ shed-placentas from non-scrapie-infected sheep (D). Specific PrP^{Sc} immunostaining was observed in an ARQ/ARQ placenta collected at 5th month of gestation (B) and an ARQ/ARQ shed-placentas from scrapie-infected sheep (E). The PrP^{Sc} deposits observed in these ARQ/ARQ placentas were in some extend similar to the unspecific stainings observed in ARR/ARQ placentas negative by the rest of the techniques, named type 1 (C with antibody L42, F with antibody F89), type 2 (G) and type 3 (H). The unspecific staining type 1 resembles to an excess of PrP^{c} present in syncitia, as detected with antibody BAR224 (I).~~

Figure 1. Immnohistochemical detection of PrP^{Sc} in placentas.

A) ARR/ARQ placenta collected at 5th month of gestation from a scrapie-infected ewe. No PrP^{Sc} immunostaining is present (mAb L42; x20). B) ARR/ARQ shed placenta from a non scrapie-infected ewe. No PrP^{Sc} immunostaining is present (mAb L42; x20). C) ARQ/ARQ placenta collected at 5th month of gestation from a scrapie-infected ewe. Intense PrP^{Sc} immunostaining located in trophoblast and foeto-maternal interface (mAb L42; x20). D) ARQ/ARQ shed placenta from a scrapie-infected sheep. PrP^{Sc} immunostaining located in decidual tissue (mAb L42; x20). E) ARR/ARQ placenta collected at 5th month of gestation from a non scrapie-infected ewe. Unspecific immunostaining type 1 mostly located in foetal side delineating sincitial cells (mAb L42; x10). A detailed image of the unspecific immunostaining is shown in the inset picture. F) A subsequent

histological section from E. No PrP^{Sc} immunostaining is present when replacing the L42 antibody for an isotype matched antibody (Negative control mouse antibody; x10). G) ARR/ARQ placenta collected at 5th month of gestation from a scrapie-infected ewe. F89 antibody shows unspecific immunostaining type 1 (x20). H) ARR/ARQ placenta collected at 5th month of gestation from a scrapie-infected ewe. mAb BAR224 reveals an increased PrP^{C} immunostaining at foeto-maternal syncytia (x20). I) ARR/ARQ placenta collected at 3rd month of gestation from a scrapie-infected ewe. Unspecific immunostaining type 2 featured by an intracellular immunostaining related to the binucleate trophoblasts located at the base of the endometrial crypts. (mAb L42. x20). A detailed image of the unspecific immunostaining is shown in the inset picture. J) ARR/ARQ placenta collected at 4th month of gestation from a scrapie-infected ewe. Unspecific immunostaining type 3 detected by mAb R145: was characterized by an extensive inmunostainig related to binucleate trophoblast cells and syncytia (x20).

Figure 2. WB detection of PrP^{Sc} in placentas after the 4th round of saPMCA

The two placentas fromof the ~~unique~~ twin gestation ~~from~~ of the ewe No. 12 presented ~~the~~ ~~opposed~~opposite results: the four aliquots analyzed from the ARQ/ARQ placenta (P12.2) showed PrP^{Sc} amplification but no amplification was observed in any of the aliquots from the ARR/ARQ placenta (P12.1). In the case of 3 placentas from ewe No. 9 coming from 3 different gestations, PrP^{Sc} amplification was observed only in the first gestation with an ARQ/ARQ ~~fetus~~ placenta [P9 (1st G)]; ARR/ARQ placentas from the other two gestations showed not PrP^{Sc} amplification [P9 (2nd G), P9 (3rd G)].

Figure 3.Relative sensitivity of PMCA on placental tissue compared with WB. A) PrP^{Sc} detection by WB of the pre-amplified placental and obex samples (undiluted and diluted) from a non scrapie-infected ewe (ARQ/ARQ P18.1) and 4 scrapie-infected ewes (ARR/ARQ P10 (2ndG), ARQ/ARQ P8 (1st G), ARQ/ARQ P9 (1st G), ARR/ARQ P14 and ARQ/ARQ obex). B) PrP^{Sc}

detection by WB of amplified products of the previous placental samples (diluted from 10^{-1} to 10^{-6}) after the 7th round of saPMCA. The amplified samples resulted in an increase of sensitivity of 4-5 logs compared to WB. No amplification was detected neither in the placenta from negative ewe (P18.1) nor the ARR placenta (P10 (2^{nd}G)). C) PrP^{Sc} detection by WB of amplified products of a positive placenta (P9 (1^{st}G)) in presence of ARR-PrP^{C} (P10 (2^{nd} G)) and blood to test any potential inhibitory effect of these factors. The effect of blood was also evaluated on nervous tissue. In placentas the presence of ARR-PrP^{C} reduced the sensitivity by 3 logs whereas the blood by 2 logs.

Table 1. Details of the naturally scrapie-affected ewes and placentas under study. From each ewe the number and time of gestation, clinical stage, placenta identification, foetal genotype and results of PrP^{Sc} detection in placenta by IHC, IDEXX EIA, WB and PMCA are included. ~~The score of immunostaining extension is indicated (+ to ++++)~~.

Protein misfolding cyclic amplification corroborates the absence of PrP^{Sc} accumulation in placenta from foetuses with the ARR/ARQ genotype in natural scrapie.

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Abstract

Ovine scrapie is a worldwide spread prion disease that is transmitted horizontally under field conditions. Placenta from scrapie-infected ewes is an important source of infection, since this tissue can accumulate high amounts of PrP^{Sc} depending on the foetal genotype. Therefore, placentas carrying susceptible foetuses can accumulate PrP^{Sc} but there is not PrP^{Sc} accumulation in presence of foetuses with at least one ARR haplotype. In scrapie eradication programs, ARR/ARR males are used for breeding to increase the resistant progeny and reduce the horizontal transmission of the disease through the placenta. The development of highly sensitive techniques, that allow the detection of minimal amounts of PrP^{Sc}, has caused many secretions/excretions and tissues that had previously been deemed negative to be relabeled as positive for PrP^{Sc}. This has raised concerns about the possible presence of minimal amounts of PrP^{Sc} in placentas from ARR foetuses that conventional techniques had indicated were negative. In the present study we examined 30

placentas from a total of 23 gestations; 15 gestations resulted from naturally ARQ/ARQ scrapie-infected ewes mated with ARR/ARR rams. The absence of PrP^{Sc} in placentas carrying the foetal ARR haplotype (n=19) was determined by IDEXX HerdChek scrapie/BSE Antigen EIA Test, Prionics®-Check WESTERN and corroborated by the highly sensitive Protein Misfolding Cyclic Amplification technique (saPMCA). By immunohistochemistry, several unspecific stainings that might mislead a diagnosis were observed. The results of the present study support that using ARR/ARR males in scrapie eradication programs efficiently decreases the spreading of the agent in the environment via shed placentas.

Keywords: scrapie; prion; saPMCA; placenta.

Introduction

Ovine scrapie is a worldwide spread disease caused by the infectious isoform of the host encoded prion protein (PrP^{Sc}). The bovine spongiform encephalopathy (BSE) outbreak in Great Britain provoked strong efforts by European governments to eradicate both prion diseases: BSE and scrapie. A dramatic decrease in prevalence has been achieved for BSE, whereas the control of scrapie remains complicated, mostly due to the difficulty of reducing its horizontal transmission. Scrapie is known to be both horizontally transmitted by direct contact between animals and indirectly transmitted through the contaminated environment, where prions can persist for several years (Hoinville, 1996). In particular, the delivery period and the placenta play key roles in transmission, which is sustained by high levels of PrP^{Sc} and infectivity located in the placentas (Race et al., 1998).

Susceptibility to ovine scrapie is controlled by polymorphisms in the PrP gene (*PRNP*), which are mainly found at codons 136 (A or V), 154 (R or H) and 171 (R, Q or H). The ancestral *PRNP* gene encodes for A136, R154 and Q171 (haplotype ARQ) and is associated with an average resistance to scrapie. Indeed, the substitution of Q by R at the codon 171 (haplotype ARR) is clearly associated with resistance (reviewed by Goldmann, 2008). The foetal *PRNP* gene also controls PrP^{Sc}

53 deposition in placenta because no PrP^{Sc} has been detected in this tissue when foetuses have at least
54 one ARR haplotype (Andréoletti et al., 2002; Lacroux et al., 2007; Tuo et al., 2002). Therefore, the
55 use of ARR/ARR breeding rams reduces the dissemination of scrapie infection through the shed
56 placenta and effectively controls the transmission of the disease(Nodelijk et al., 2011). However,
57 the absence of PrP^{Sc} in placentas with ARR foetuses has been determined only by using
58 conventional techniques such as immunohistochemistry (IHC), western blotting (WB) and ELISA.
59 The aim of the present study was to use the highly sensitive serial automated Protein Misfolding
60 Cyclic Amplification technique (saPMCA; Saá et al., 2006), to assess the presence of PrP^{Sc} in
61 placentas from foetuses carrying an ARR haplotype and consequently, the effectiveness of a part of
62 the genetic programme for scrapie control and eradication (Dawson et al., 2008).
63
64 **Material and methods**
65 *Animals and sample collection*
66 Seventeen naturally scrapie-infected ewes were selected from different outbreaks after a PrP^{Sc}
67 positive biopsy of rectal mucosal-associated lymphoid tissue (Monleón et al., 2011). The animals
68 were brought to the University of Zaragoza facilities and genotyped for the *PRNP* gene, with each
69 presenting an ARQ/ARQ genotype. All animals under investigation were examined for pregnancy
70 by ultrasound and were regularly checked by clinical examination. Non-pregnant dams (n=8) were
71 subjected to natural mating with negative scrapie rams of the ARR/ARR genotype. After
72 parturition, 5 dams were in good condition to be mated again with an ARR/ARR ram, obtaining 2
73 gestations from 4 dams and 3 gestations from 1 dam. All lambs/foetuses were genotyped for the
74 *PRNP* gene.
75 Thirty placentas from foetuses of ARQ/ARQ (n=11) and ARR/ARQ (n=19) genotypes were
76 collected. Seventeen of those placentas were collected after parturition (shed placentas), and the
77 remaining 13 were collected at different time points of gestations because it became necessary to
78 humanely euthanize the dams. Cotyledons/placentomes were taken as samples and were divided

79 into halves. One half was fixed in 10% formalin for IHC, and the other half was stored at -20°C for
80 IDEXX HerdChek scrapie/BSE Antigen EIA Test ("IDEXX EIA", Idexx, US), Prionics®-Check
81 WESTERN (Prionics AG, Switzerland) and saPMCA. In addition, 8 placental samples (foetal
82 genotypes ARQ/ARQ n=4 and ARR/ARQ n=4) from ARQ/ARQ non scrapie-infected ewes were
83 used as negative controls. Data for the ewes and placentas are detailed in Table 1.
84 All ewes included in the present study were humanly euthanized by intravenous injections of
85 sodium pentobarbital, and the scrapie status was confirmed *post-mortem* for PrP^{Sc} detection in the
86 brainstem (obex) and retropharyngeal lymph node by IHC.
87 This study was performed in strict accordance with the recommendations for the care and use of
88 experimental animals of the University of Zaragoza (R.D.169 1201/2005). The protocol was
89 approved by the associated Committee on the Ethics of Animal Experiments (Permit Number:
90 PI02/08).
91 *Immunohistochemistry*
92 Formalin-fixed samples were trimmed and processed according to standard histopathological
93 procedures. PrP^{Sc} IHC was performed as previously described (Monleón et al., 2011) using the L42
94 antibody (R Biopharm Ltd., Germany; 1:500). Briefly, the protocol included formic acid, proteinase
95 K digestion (4 μg/ml; F. Hoffmann La Roche Ltd, 211 Switzerland) and hydrated autoclaving as
96 pretreatments. EnVision was used as a visualization system, and the two chromogens
97 aminoethilcarbazole and diaminobenzidine were used to distinguish actual PrP^{Sc} deposits from
98 commonly found brown pigments in placenta (all products from Dako Denmark A/S, Denmark).
99 PrP^{Sc} signal was subjectively scored (from + to ++++) based on the extent of immunostaining. In
100 some selected placentas, the F89/160.1.5 (0.5 mg/ml; NC-Neopharma (Abcam Inc, Cambridge,
101 MA, USA)) and R145 (1:3000 Genómica, Spain) antibodies were used to confirm the results
102 obtained by the L42 antibody. No digestion with proteinase K was required as a pretreatment for the
103 R145 antibody (Sisó et al., 2008). In addition, BAR224 (1:20, Spi-Bio, France), which presents

great affinity for the ovine PrP (Féraudet et al., 2005), was used to detect PrP^C in placentas with only hydrated autoclaved in citrate buffer for 20 minutes as pretreatment (Lacroux et al., 2007).

ELISA IDEXX HerdCheck BSE-Scrapie Antigen Test Kit, EIA

All frozen placental samples were tested in duplicate by "IDEXX EIA" according to the manufacturer's protocol for nervous tissue with a previous homogenization step. A medial cross section for each cotyledon/placentome was homogenized (20% w/v) in 0.01 M Tris HCl (pH 7.5) and then subjected to 4 cycles in the TeSeE Precess 48 Homogenizer (Bio-Rad, USA) with ceramic beads. Each cycle consisted of 2 agitation phases of 45 seconds at 6,500 rpm, with 60 seconds between phases. The samples were allowed to cool for 5 minutes between cycles. A sample was considered positive if the optical density values of both replicates were greater than the manufacturer's cut-off criterion.

Prionics-Check Western Small Ruminant test

Frozen placental samples were analysed by WB using the *Prionics-Check Western Small Ruminant test*. The procedure was modified by using the placental homogenate at 20% w/v diluted with 0.01 M Tris HCl (pH 7.5) to a final concentration of 10% (w/v). Next, the homogenates were analysed according to the manufacturer´s protocol, except for the primary antibody, which was substituted for P4 antibody (R-Biopharm Ltd., Germany; 1:5,000).

Serial automated Protein Misfolding Cyclic Amplification (saPMCA)

A total of 13 ARR/ARQ placental samples from scrapie-infected dams were selected for saPMCA analysis, as described previously (Garza et al., 2011). Briefly, brains from tg338 mice over-expressing the VRQ allele of ovine PrP (Vilotte et al., 2001) were used as the substrate for *in vitro* prion conversion and as unseeded-negative controls. Mouse brain homogenates (10% w/v) were prepared in a conversion buffer (PBS containing 150 mM NaCl and 1% Triton X-100 with the addition of Complete Protease Inhibitors; Roche Pharmaceuticals, Indianapolis, IN). Placental tissue samples were disrupted in a Dounce homogenizer at 10% (w/v) in PBS (Gibco calcium and magnesium free) with Complete Protease Inhibitors. Four aliquots per placenta were analysed by

adding 5 µl of placental sample to 50 µl of substrate with 5 mM EDTA final concentration. Tubes were placed on an adaptor on the plate holder of a microsonicator (Misonix, USA, model S3000MP sonicator). The amplified product was digested with proteinase K (85 µg/ml) for 60 min at 42°C with shaking, and PrP^{Sc} was detected by WB using P4 antibody. In addition, 4 placental samples were used as controls: 2 ARQ/ARQ that had been deemed positive by IHC and "IDEXX EIA" [P9 (1st G), P12.2] and 2 negative controls (ARQ/ARQ and ARQ/ARR).

To evaluate the relative sensitivity of saPMCA on placental tissue compared to WB, limiting dilution experiments were conducted in four placentas: 2 negative (P10 (2^{nd} G), P18.1) and 2 positive (P8 (1^{st} G), P9 (1^{st} G)). The placental homogenates (10% w/v) were serially diluted (10^{-1} to 10^{-6}) in the substrate and assased by WB before and after amplification.

To evaluate a potential inhibitory effect of foetal ARR-PrP^{C} and blood on the detection of misfolding activity by saPMCA, the following experiments were performed. To test the effect of ARR-PrP^{C}, 5 µl of negative foetal ARR/ARQ placenta (P10 (2^{nd} G)) were added to the substrate along with 5 µl of positive foetal ARQ/ARQ placenta seed (P9 (1^{st} G). To test the inhibitory effect of the blood on placental and nervous tissue, 5 µl of blood from an ARQ/ARQ non scrapie-infected sheep were added to the substrate in the presence of 2 different seeds: 5 µl of positive foetal ARQ/ARQ placenta (P9 (1^{st} G) and 5 µl of positive nervous tissue. Serial dilutions (10^{-1} to 10^{-6}) from all samples described were assessed by PMCA and detecetd by WB.

Results

None of the 19 ARR/ARQ placentas from naturally scrapie-infected sheep, collected at several times throughout gestation, showed PrP^{Sc} deposits by IHC, "IDEXX EIA" or WB (hereafter referred to as conventional techniques). Thirteen of those negative placentas were analysed by saPMCA and no seeding activity was detected after the 4^{th} round of saPMCA. In placentas from foetuses presenting an ARQ/ARQ genotype, PrP^{Sc} deposits were detected in all samples; 3 were collected during the 5^{th} month of gestation, and 8 were shed placentas (Fig. 1 C, D). The results of the PrP^{Sc} detection techniques are detailed in Table 1. Two ARQ/ARQ placentas that had been

deemed positive by conventional techniques were used as positive control for saPMCA [P9 (1stG), P12.2; Fig. 2] and showed PrP^{Sc} after the 3rd round in all tested aliquots. Remarkably, the positive P12.2 placenta belonged to a multiple gestation shared with an ARR/ARQ placenta (P12.1) that remained negative to all techniques, including saPMCA (Fig. 2). This case underpinned the absence of cross-contamination and the clear inhibitory effect on PrP replication caused by the foetal ARR haplotype. No PrP^{Sc} was detected by any technique in the 8 placentas from the non scrapie-infected ewes that were used as negative controls (Fig. 1 A,B). By saPMCA, no seeding activity was detected in ARR/ARQ placentas, even after the 7th round neither from non scrapie nor scrapie-infected sheep (Fig. 3 B).

Since there are not previous reports testing placental tissue by saPMCA, the relative sensitivity of the assay to a conventional technique (WB) was determined by limiting dilution experiments (Fig 3 A-C). We detected scrapie-associated seeding activity present on placental tissue from the 3th round (Fig. 2), reaching the detection limit of 10^{-5} to 10^{-6} by the 7th round that corresponded to an increase of at least 4 logs in sensitivity compared to WB (Fig. 3 A,B). An inhibitory effect on the scrapie-associated seeding activity caused by foetal ARR-PrP^{C} and blood was observed in placenta. Presence of ARR-PrP^{C} reduced 3 logs the detection limit of the assay in placentas, and the presence of blood reduced 2 logs either in placental or nervous tissues compared to those obtained under standard saPMCA conditions (Fig.3 B, C). Despite sensitivity of saPMCA assay on placental tissue was slightly lower in presence of foetal ARR-PrP^{C} or blood, this assay achieved higher sensitivity (around 2 logs) than the conventional technique (WB; Fig. 3 B,C).

Unspecific immunostainings were observed by IHC in some negative control placentas and ARR/ARQ placentas from scrapie-infected ewes (Table 1). These unspecific immunostainings were classified into 3 different types according to the stained cells, antibody used and location in the placentome (Fig. 1 E-J). Type 1 was the most frequently observed and primarily affected the foeto-maternal syncytia at the top of the foetal villus tree surrounding the extravasated maternal blood (arcade area; Fig. 1 E, G). It was characterized as diffused staining that mainly delineated the cell

shape, although in some cases, it resembled fine/gross particulate. The foeto-maternal syncytia at the arcade area presented an increase in PrP^{C} staining compared with the remaining cells (trophoblast and endometrial cells), as revealed by the BAR224 antibody (Fig. 1 H). Type 2 was an intracellular immunostaining that was exclusively related to the binucleate trophoblasts located at the base of the endometrial crypts. This type of staining was only found in placentas at earlier stages of gestation (3^{rd}-4^{th} month; Fig. 1 I). Finally, type 3 was a granular staining of binucleate trophoblast cells and, in turn, syncytia (formed as result of the fusion of those binucleate with the endometrial cells) appearing all across the placentome (Fig. 1 J). The latter type of immunostaining was detected only with the R145 antibody, whereas types 1 and 2 were detected with all of the antibodies included in the study. In control sections with the primary antibody omitted and with an isotype-matched primary antibody (Universal Negative Control Mouse, Dako Denmark A/S, Denmark; Fig. 1 F)), no staining was observed.

Discussion

Scrapie eradication programs are largely based on genetic selection targeting the elimination of susceptible animals and the maintenance of rams carrying the ARR/ARR genotype for breeding (Dawson et al., 2008). The purpose of these programs is to increase the frequency of the ARR haplotype, thus conferring greater resistance to sheep population, and to reduce the dissemination of scrapie infection through placentas with PrP^{Sc} deposition (Andréoletti et al., 2002). Over the last several years, the development of highly sensitive methodologies, such as transgenic rodent bioassay or the saPMCA technique (Saá et al., 2006), have allowed the detection of minimal amounts of PrP^{Sc} in different secretions/excretions that are otherwise undetectable by conventional techniques (Gough and Maddison, 2010). To date, the absence of PrP^{Sc} in placentas from foetuses with ARR haplotypes has been determined only by using conventional techniques (Alverson et al., 2006; Andréoletti et al., 2002; Lacroux et al., 2007; Santucciu et al., 2010; Tuo et al., 2002). Our results corroborate this previous finding since no misfolding activity was detected by saPMCA in

placentas carrying foetal ARR. In agreement with previously published studies (Alverson et al., 2006; Lacroux et al., 2007), the case of P12.1 emphasizes this lack of PrP^{Sc} accumulation, even when sharing the uterus of other susceptible foetuses with a positive placenta. However, one weakly positive placenta of an ARR/ARQ foetus that shared the same uterine horn with an ARQ/ARQ foetus has been described in the literature, likely caused by blood sharing between foetuses (Alverson et al., 2006).

The ARQ/ARQ sheep in an advanced clinical stage of the scrapie disease show wide dissemination of PrP^{Sc} in many organs other than the central nervous (CNS) and lympho-reticular systems (Garza et al., 2014). However, we did not detect PrP^{Sc} in any of the ARR/ARQ placenta, even from the 13 ARQ/ARQ sheep that were at a terminal clinical stage. Moreover, 4 of the 19 negative ARR/ARQ placentas came from sheep having previous gestations with positive ARQ/ARQ placentas. It is worth noting that the Dam 9 had up to 3 gestations. To our knowledge, this case is the first description of an animal having up to 3 pregnancies during the progression of scrapie disease. Only the first gestation, as a consequence of being mated with a susceptible ram, presented PrP^{Sc} accumulation in placenta. The other 2 gestations, in which the dam was in a more advanced clinical stage and presumably had a higher dissemination of PrP in the organism, did not lead to a threat of scrapie dissemination through placentas. In conclusion, our data highlight the efficiency of using ARR-carrying males for breeding to reduce the dissemination of scrapie during lambing seasons. Furthermore, the gestation periods do not seem to exacerbate or accelerate the progression of disease, as observed for the 5 animals that each had at least 2 gestations during the progression of the disease.

ARR/ARQ sheep, which are widely viewed as highly resistant to classical scrapie, have been shown to be infected by oral inoculation, although with a much longer incubation period than ARQ/ARQ sheep (Jeffrey et al., 2014). Sheep placenta is a unique tissue that only lasts 5 months, and its short "life-span" together with even a minimal interference in the conversion process caused by the ARR

haplotype (Bossers et al., 2000) might explain the total absence of PrP^{Sc} in placenta in presence of foetal ARR.

It is still not clear how the prions access the placenta, but in general terms, prions disseminate throughout an organism via two pathways: by using peripheral nerves as physical conduits (neural neuroinvasion) or via the blood (haematogenous neuroinvasion; Sisó et al., 2009). The restricted innervation of the uterus and placenta during pregnancy, with very few fibres in the internal myometrium (Marzioni et al., 2004), and the existence of maternal blood pools ("haemophagous areas") in the ovine placenta (Sammin et al., 2009) suggest that prion access occurs via blood rather than the peripheral nerves. Haematogenous dissemination has also been proposed for prion access to some peripheral organs/secretions, such as the kidney (Sisó et al., 2008) or milk (Lacroux et al., 2008). The detection of infectivity in the blood by bioassay studies (Houston et al., 2008) and the presence of PrP^{Sc} by using PMCA (Thorne and Terry, 2008) has been demonstrated in scrapie cases. Recently, Andréoletti *et al*. (2012) concluded that the intravenous administration of a few hundred microliters of blood is sufficient to infect an ovine transfusion recipient. Therefore, the flow of maternal blood through the ovine placenta and the maternal blood pools might be sufficient to infect the tissue. However, the PMCA did not amplify any PrP^{Sc} in placentas carrying foetal ARR haplotype (not even from circulating blood), which indicates that PrP^{Sc} can only be detected when replicates in the placentomes. We also exclude the possibility that factors like blood or the ARR-PrP^{C} are limiting the sensitivity of detection by this technique hence we show that the co-presence of these factors in the seed represented a maximum reduction of 3 logs still leading the saPMCA to a 1000-fold increase in sensitivity over the standard detection method, WB. This clearly substantial gain in sensitivity of saPMCA compared with WB reassures that such factors are not responsible for the absence of seeding activity determined by saPMCA in ARR placentas. We can also speculate that the contact required for PrP^{C} conversion to PrP^{Sc} in placenta initially occurs on the trophoblastic cells, since only the foetal side of the placenta expresses the ARR haplotype.

Finally, we present in this study a detailed description of the non-specific staining/artefacts resembling PrP in placentas that might confuse the diagnosis of scrapie when only IHC is applied. We confirmed that these were non-specific stainings because they were observed in both the negative control placentas and the saPMCA-negative ARR/ARQ placentas from scrapie-infected ewes. The unspecific staining that was most frequently found, type 1, could have been due to an excess staining of PrP^{C}, as demonstrated for the antibody BAR224 detecting PrP^{C}. Its co-existence with the actual PrP^{Sc} deposits in positive placentas and its presence in negative placentas from infected animals highlight the importance of its clear recognition and the use of other techniques such as WB or ELISA to prevent any misleading diagnoses.

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Figure 1. Immnohistochemical detection of PrP^{Sc} in placentas.

A) ARR/ARQ placenta collected at 5th month of gestation from a scrapie-infected ewe. No PrP^{Sc} immunostaining is present (mAb L42; x20). B) ARR/ARQ shed placenta from a non scrapie-infected ewe. No PrP^{Sc} immunostaining is present (mAb L42; x20). C) ARQ/ARQ placenta collected at 5th month of gestation from a scrapie-infected ewe. Intense PrP^{Sc} immunostaining located in trophoblast and foeto-maternal interface (mAb L42; x20). D) ARQ/ARQ shed placenta from a scrapie-infected sheep. PrP^{Sc} immunostaining located in decidual tissue (mAb L42; x20). E) ARR/ARQ placenta collected at 5th month of gestation from a non scrapie-infected ewe. Unspecific immunostaining type 1 mostly located in foetal side delineating sincitial cells (mAb L42; x10). A detailed image of the unspecific immunostaining is shown in the inset picture. F) A subsequent histological section from E. No PrP^{Sc} immunostaining is present when replacing the L42 antibody for an isotype matched antibody (Negative control mouse antibody; x10). G) ARR/ARQ placenta collected at 5th month of gestation from a scrapie-infected ewe. F89 antibody shows unspecific immunostaining type 1 (x20). H) ARR/ARQ placenta collected at 5th month of gestation from a scrapie-infected ewe. mAb BAR224 reveals an increased PrP^{C} immunostaining at foeto-maternal syncytia (x20). I) ARR/ARQ placenta collected at 3rd month of gestation from a scrapie-infected ewe. Unspecific immunostaining type 2 featured by an intracellular immunostaining related to the binucleate trophoblasts located at the base of the endometrial crypts. (mAb L42. x20). A detailed image of the unspecific immunostaining is shown in the inset picture. J) ARR/ARQ placenta collected at 4th month of gestation from a scrapie-infected ewe. Unspecific immunostaining type 3 detected by mAb R145: was characterized by an extensive inmunostainig related to binucleate trophoblast cells and syncytia (x20).

Figure 2. WB detection of PrP^{Sc} in placentas after the 4th round of saPMCA

388 The two placentas from the twin gestation of the ewe No. 12 presented opposite results: the four
389 aliquots analyzed from the ARQ/ARQ placenta (P12.2) showed PrP^{Sc} amplification but no
390 amplification was observed in any of the aliquots from the ARR/ARQ placenta (P12.1). In the case
391 of 3 placentas from ewe No. 9 coming from 3 different gestations, PrP^{Sc} amplification was observed
392 only in the first gestation with an ARQ/ARQ placenta [P9 (1^{st} G)]; ARR/ARQ placentas from the
393 other two gestations showed not PrP^{Sc} amplification [P9 (2^{nd} G), P9 (3^{rd} G)].
394
395 **Figure 3.Relative sensitivity of PMCA on placental tissue compared with WB.** A) PrP^{Sc}
396 detection by WB of the pre-amplified placental and obex samples (undiluted and diluted) from a
397 non scrapie-infected ewe (ARQ/ARQ P18.1) and 4 scrapie-infected ewes (ARR/ARQ P10 (2^{nd}G),
398 ARQ/ARQ P8 (1^{st} G), ARQ/ARQ P9 (1^{st} G), ARR/ARQ P14 and ARQ/ARQ obex). B) PrP^{Sc}
399 detection by WB of amplified products of the previous placental samples (diluted from 10^{-1} to 10^{-6})
400 after the 7^{th} round of saPMCA. The amplified samples resulted in an increase of sensitivity of 4-5
401 logs compared to WB. No amplification was detected neither in the placenta from negative ewe
402 (P18.1) nor the ARR placenta (P10 (2^{nd}G)). C) PrP^{Sc} detection by WB of amplified products of a
403 positive placenta (P9 (1^{st}G)) in presence of ARR-PrP^{C} (P10 (2^{nd} G)) and blood to test any potential
404 inhibitory effect of these factors. The effect of blood was also evaluated on nervous tissue. In
405 placentas the presence of ARR-PrP^{C} reduced the sensitivity by 3 logs whereas the blood by 2 logs.
406
407 **Table 1. Details of the naturally scrapie-affected ewes and placentas under study.** From each
408 ewe the number and time of gestation, clinical stage, placenta identification, foetal genotype and
409 results of PrP^{Sc} detection in placenta by IHC, IDEXX EIA, WB and PMCA are included..

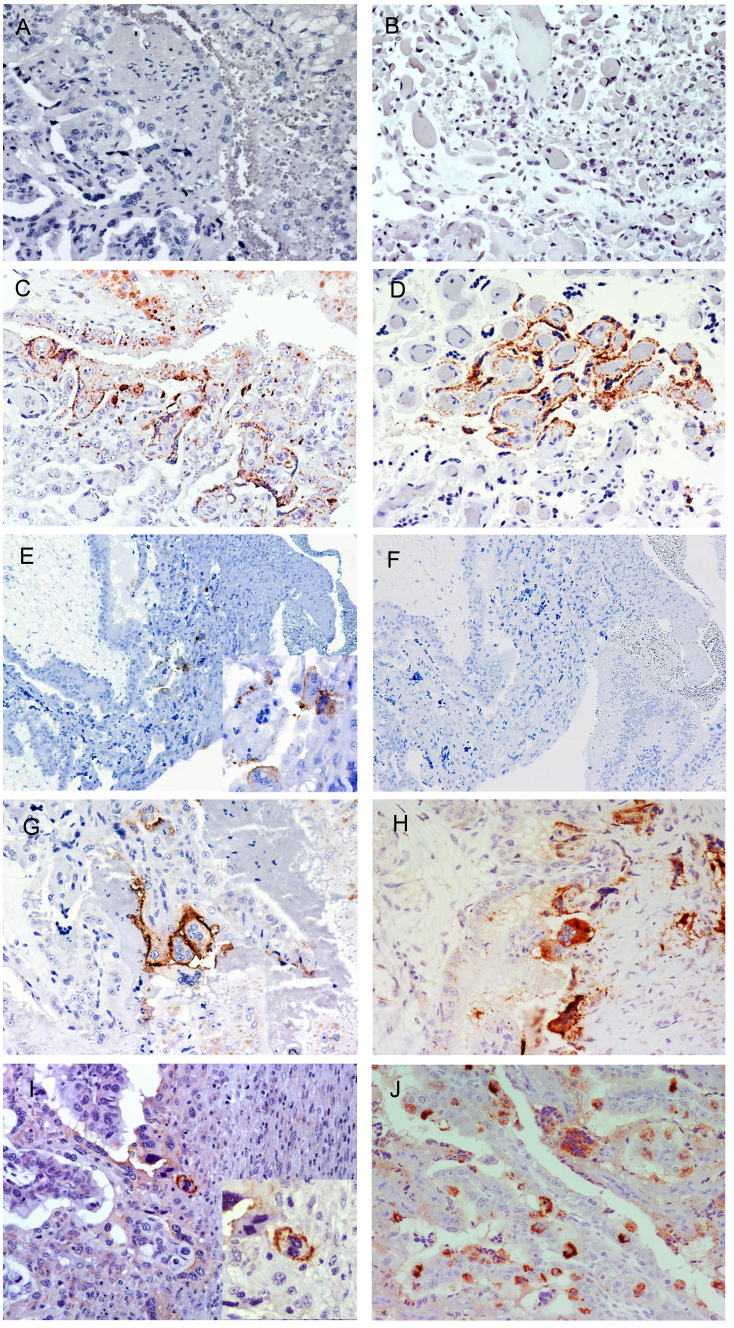
A
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Figure 2. WB detection of PrP^{Sc} in placentas after the 4^{th} round of saPMCA.

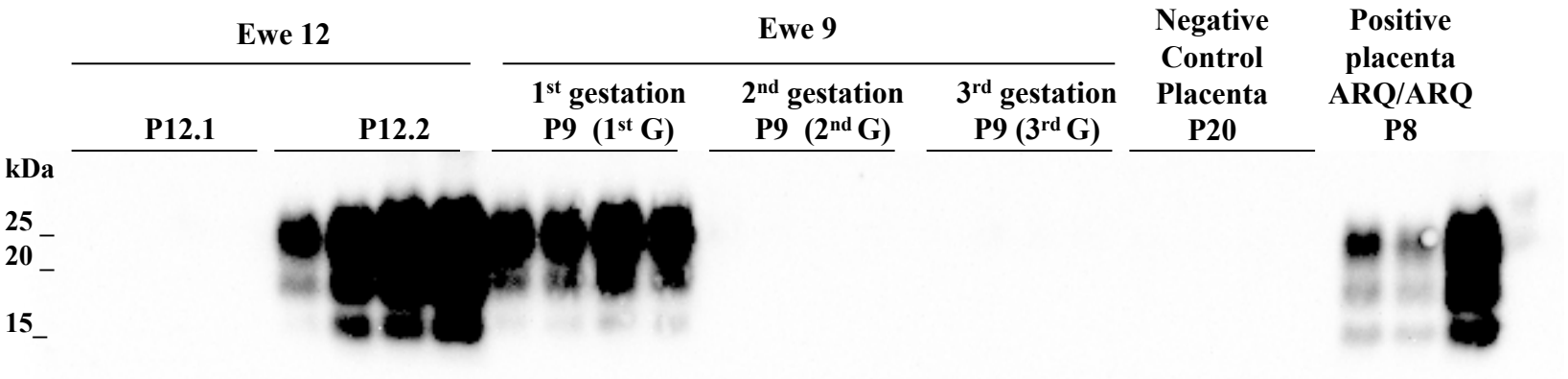

Figure 3. Relative sensitivity of saPMCA on placental tissue compared with WB.

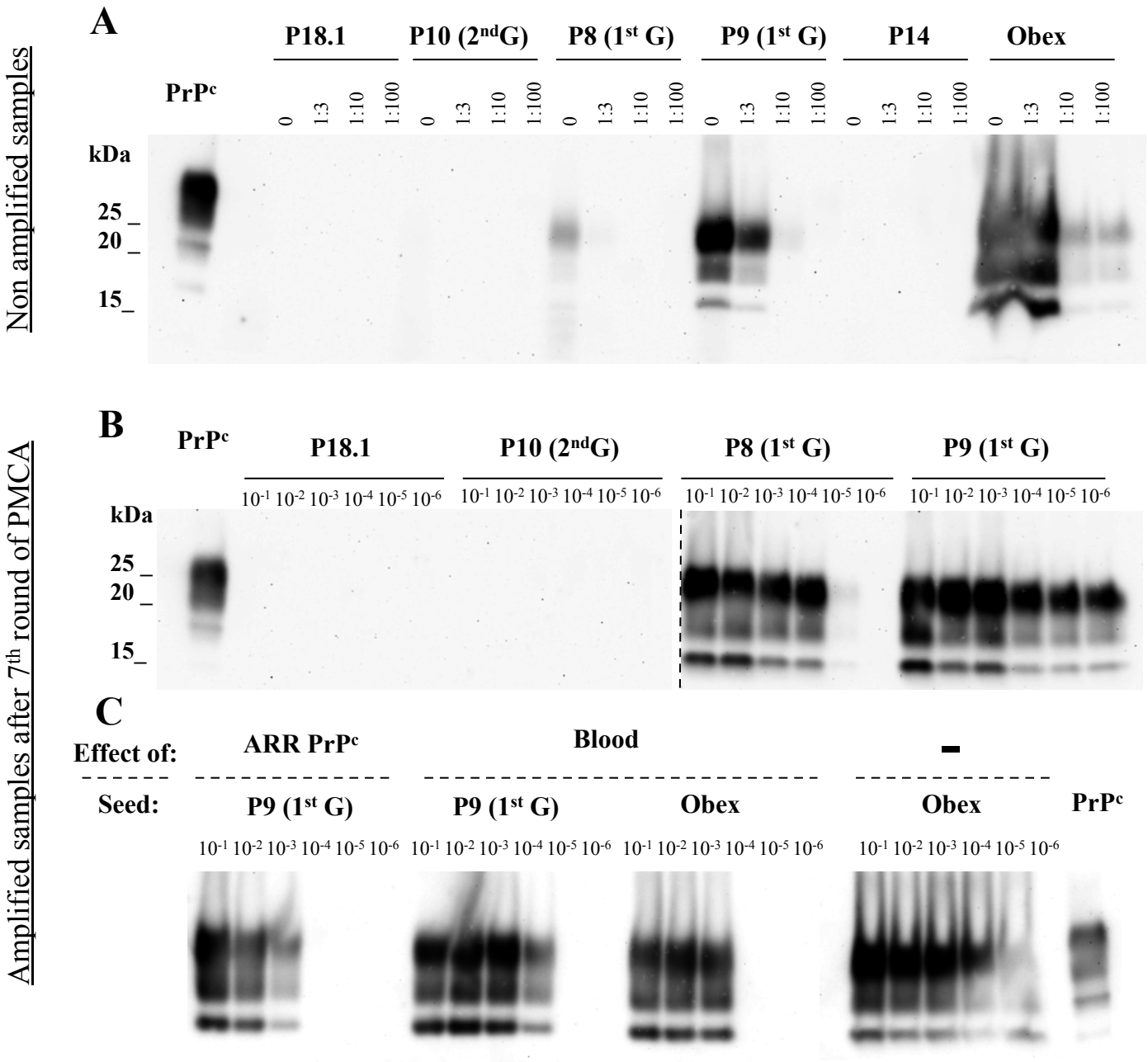

Table 1.

Ewe no.	Gestation no.	Time of gestation	Clinical status of ewes	Placenta no.	Foetus genotype	PrP^{Sc} detection in placenta			
						IHC*	EIA	WB	PMCA
1	1	Full-term	Asymptomatic	P1	ARQ/ARQ	++	+	+	NT
2	1	Full-term	Asymptomatic	P2	ARQ/ARQ	+	+	+	NT
3	1	Full-term	Symptomatic	P3.1	ARQ/ARQ	+++	+	+	NT
				P3.2	ARQ/ARQ	++++	+	+	NT
4	1	Full-term	Asymptomatic	P4 (1st G)	ARQ/ARQ	+	+	+	NT
	2	Full-term	Symptomatic	P4 (2nd G)	ARR/ARQ	-	-	-	NT
5	1	Full-term	Asymptomatic	P5	ARR/ARQ	-	-	-	-
6	1	Full-term	Asymptomatic	P6 (1st G)	ARR/ARQ	-	-	-	-
	2	Full-term	Asymptomatic	P6.1 (2nd G)	ARR/ARQ	-	-	-	-
				P6.2 (2nd G)	ARR/ARQ	-	-	-	-
7	1	Full-term	Symptomatic	P7.1	ARR/ARQ	-	-	-	NT
				P7.2	ARR/ARQ	-	-	-	NT
8	1	Full-term	Asymptomatic	P8 (1st G)	ARQ/ARQ	++	+	+	NT
	2	4th month	Symptomatic	P8 (2nd G)	ARR/ARQ	-[1, 3]	-	-	-
9	1	Full-term	Asymptomatic	P9 (1st G)	ARQ/ARQ	++++	+	+	+
	2	Full-term	Asymptomatic	P9 (2nd G)	ARR/ARQ	-	-	-	-
	3	3th month	Symptomatic	P9 (3rd G)	ARR/ARQ	-[1,2]	-	-	-
10	1	Full-term	Asymptomatic	P10 (1st G)	ARQ/ARQ	+	+	+	NT
	2	5th month	Symptomatic	P10 (2nd G)	ARR/ARQ	-[1, 3]	-	-	-
11	1	Full-term	Asymptomatic	P11	ARR/ARQ	-	-	-	NT
12	1	5th month	Symptomatic	P12.1	ARR/ARQ	-[1]	-	-	-
				P12.2	ARQ/ARQ	++++	+	+	+
13	1	5th month	Symptomatic	P13.1	ARQ/ARQ	++++	+	+	NT
				P13.2	ARQ/ARQ	++++	+	+	NT
14	1	4th month	Symptomatic	P14	ARR/ARQ	-[1]	-	-	NT
15	1	4th month	Symptomatic	P15.1	ARR/ARQ	-[1]	-	-	-
				P15.2	ARR/ARQ	-[1, 3]	-	-	-
16	1	3rd month	Symptomatic	P16.1	ARR/ARQ	-	-	-	-
				P16.2	ARR/ARQ	-[1, 3]	-	-	-
17	1	3rd month	Asymptomatic	P17	ARR/ARQ	-[1,2]	-	-	NT
18	1	Full-term	Control**	P18.1	ARQ/ARQ	-	-	-	-
			Control	P18.2	ARQ/ARQ	-	-	-	NT
19	1	Full-term	Control	P19.1	ARQ/ARQ	-	-	-	NT
			Control	P19.2	ARQ/ARQ	-	-	-	NT
20	1	Full-term	Control	P20	ARR/ARQ	-	-	-	-
21	1	5th month	Control	P21.1	ARR/ARQ	-[1]	-	-	NT
				P21.2	ARR/ARQ	-[1]	-	-	NT
22	1	4th month	Control	P22	ARR/ARQ	-[1]	-	-	NT

1st G: first gestation; 2nd G: second gestation; 3rd G: third gestation; NT: no tested.

*IHC: score of immunostaining extension (+ to ++++) and type of non-specific immunostaining pattern detected (1, 2 or/and 3)

**Control: ARQ/ARQ non-scrapie-infected ewes used as negative controls

PMCA does not amplify any PrPSc from blood in placentas carrying foetal ARR haplotype~~PMCA confirms the absence of PrP^{Sc} accumulation in placenta from foetuses with ARR/ARQ genotype~~.

PMCA does not amplify any PrP^{Sc} from circulating blood in placentas carrying foetal ARR haplotype.

By IHC, several unspecific stainings might mislead a scrapie diagnosis in placenta.

PMCA results support the use of ARR/ARR breeding rams to reduce scrapie dissemination~~Using ARR/ARR males for breeding efficiently reduces dissemination of~~ .