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Does the presence of scrapie affect the ability of current statutory discriminatory tests to detect the presence of BSE?

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Running title: Detection of BSE in the presence of scrapie

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ABSTRACT

The present EC surveillance regulations require discriminatory testing of all transmissible spongiform encephalopathy (TSE) positive small ruminant samples, in order to classify them as BSE or non-BSE. This requires a range of tests including, characterisation by bioassay in mouse models. Since 2005 naturally-occurring BSE has been identified in two goats. It has also been demonstrated that more than one distinct TSE strain can co-infect a single animal in natural field situations.

This study assesses the ability of the statutory methods as listed in the regulation to identify BSE in a blinded series of brain samples in which ovine BSE and distinct isolates of scrapie are mixed in varying ratios from 99% to 1%. Additionally, these current statutory tests were compared with that of a new in vitro discriminatory method which uses serial protein misfolding cyclic amplification (sPMCA).

Western blotting consistently detected 50% BSE within a mixture, but at higher dilutions it had variable success. The ELISA method consistently detected BSE only when it was present as 99% of the mixture, with variable success at higher dilutions. Bioassay and sPMCA reported BSE in all samples where it was present, down to 1%. sPMCA could also consistently detect the presence of BSE in mixtures at 0.1%.

While bioassay is the only validated method that allows comprehensive phenotypic characterisation of an unknown TSE isolate, sPMCA assay appears to offer a fast and cost-effective alternative for the screening of unknown isolates when the purpose of the investigation is solely to determine the presence or absence of BSE.

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INTRODUCTION

The transmissible spongiform encephalopathies (TSE's) are fatal neurodegenerative diseases of animals, of which scrapie in small ruminants is the archetype, and has been recognised as a disease in sheep and goats for almost 300 years although many aspects of the disease are still poorly understood. Despite the relative uniformity of the clinical signs, scrapie can be caused by 'strains' with differing biological and biochemical features [1,2]. Polymorphisms in the host *PRNP* gene, which encodes for the cellular protein PrP^{C}, also influence scrapie susceptibility, strain selection and the ultimate disease phenotype displayed by the host [3,4]. Historically, the diversity of the scrapie agent has been demonstrated by the serial passaging of natural isolates to a panel of inbred mouse lines, but this does not provide a comprehensive and reliable picture of the diversity of the TSE agents in small ruminants. However, transgenic mice are proving to be susceptible to a wider range of TSEs, enabling more comprehensive characterisation of strains [2]. In 1998, the definition of ovine TSEs was extended by the discovery, in Norway, of an experimentally transmissible, neurological disease of sheep that was clearly distinguishable by all phenotypic parameters from the 'classical' cases that had been reported so far. It was therefore considered to be an 'atypical' form of scrapie [4].

Despite similar diseases occurring in man (e.g. [5]) the animal TSEs were not regarded as zoonotic until the emergence in 1996 of variant Creutzfeldt-Jakob Disease (vCJD), linked to bovine spongiform encephalopathy (BSE) [6] which was first described in cattle in the 1980s [7].

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Experimental studies in food animal species showed the potential transmissibility of BSE to a range of alternative hosts [8] and it became clear that the small ruminant population had been potentially exposed to infection by dissemination through concentrate feed which may have contained contaminated meat and bone meal, implicated as the origin of the BSE epidemic in cattle [9].

Experimental studies in sheep demonstrated that disease can result from oral challenge with cattle BSE [10] and, once established, it can transmit naturally [11]. In addition, the biological properties of the resulting ovine BSE in laboratory models indicate a potentially enhanced virulence for other species including man [12,13].

Although ovine BSE has not yet been identified in the field, two naturally-occurring cases of caprine BSE have been reported [14,15,16]. As a consequence of this potential risk of BSE in the small ruminant (SR) population, the current EC regulations (999/2001 as amended 36/2005) require the discriminatory testing of all TSE positive SR surveillance samples to enable the discrimination of BSE from classical scrapie in these samples.

The phenotype of experimental ovine BSE [17] bears a clear resemblance to naturally-occurring scrapie, which is endemic in many sheep populations. However, there are some subtle differences in the biochemical signatures of these diseases. The disease-specific isoform (PrP^{Sc}), of the normal host protein (PrP^{C}) is the target for all current TSE biochemical diagnostic tests. Depending on the TSE isolate, e.g. BSE or one of the various forms of scrapie, there are differences in the molecular location of protease K (pK) cleavage sites, and/or relative pK sensitivity. These differences, as visualised

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by comparing the relative binding of antibodies against various epitopes around these PK cleavage sites, form the basis of the discriminatory tests currently listed in the regulations, which use either immunohistochemistry (IHC) [18] Western blot (WB) [19,20] or ELISA [21] formats.

In the absence of identified, naturally-occurring ovine BSE, the development and evaluation of the discriminatory tests which form the basis of the current EU statutory requirements was based on panels of samples comprising naturally-occurring classical scrapie, experimentally induced ovine BSE and bovine BSE all of which were demonstrated to be readily distinguishable by these tests [22].

The only experimental study that has been undertaken [23], suggests that the picture with co-infection in sheep is complicated; the WB and IHC data from central nervous system (CNS) tissues resemble classical scrapie, while in the lymphoreticular system (LRS) they may resemble either classical scrapie *or* BSE. Subsequent bioassay using two transgenic models (tg110, a line which overexpresses bovine PrP on a null murine PrP background [24] and tg338, a line that overexpresses a VRQ allele of the ovine PrP on a null murine PrP background [25]) has shown that both BSE and scrapie can be identified by bioassay from the brains of these sheep despite only a classical scrapie signal being detectable on initial screening with WB and IHC [23]. These two transgenic mouse lines, used in combination, are widely accepted to be a robust approach to biological discrimination, because the ovinised line would preferentially propagate scrapie isolates, and the bovinised line would preferentially propagate BSE. They are endorsed by the EU Reference Laboratory Strain Typing Group in the guidance document for discriminatory

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testing in the context of EU regulation 36/2005 (http://www.tse-lab-net.eu/documents/tse-oie-rl-handbook.pdf).

Although not a widely reported occurrence, there is evidence that animals can be naturally co-infected with atypical and classical scrapie [26] and experimentally with BSE and scrapie [23,27]. There are concerns that current *in vitro* tests would not be able to provide reliable discrimination in situations where a sheep or goat was co-infected with BSE and scrapie. This uncertainty applies to a lesser extent to bioassay models using wild-type mice [27,28] although transgenic models offer greater potential through differing strain susceptibility [29,30,31].

Any *in vitro* mixing study cannot replicate a natural host co-infection situation, and results must not be extrapolated in that way. However, a preliminary estimate of how tests *might* perform can be sought through the testing of scrapie samples 'spiked' with BSE, and *vice versa*. Even this *in vitro* approach cannot cover all the potential variables relating to the scrapie isolates from donors, and issues such as the timing and route of infection (e.g. temporally separated, or not; same route, or not; compatibility of donor and recipient genotypes; age at challenge etc.) without becoming unworkably complicated, so the study reported here must be viewed in this context.

Recently, a new *in vitro* method has been developed [32] that exploits the differential amplification of PrP^{Sc} from BSE and classical scrapie sources in different substrates using serial protein misfolding cyclic amplification (sPMCA), with retention of strain-specific biochemical characteristics. Although presently sPMCA is not a statutory test it was included retrospectively in this blinded assessment to enable a preliminary direct

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comparison of this new discriminatory approach with the current statutory discriminatory methods, including the bioassay 'gold standard'.

MATERIALS AND METHODS

Materials

Experimentally generated ovine classical BSE [17] and four different naturally occurring scrapie isolates which had been characterised pathologically, biochemically and biologically, were sourced (see Table 1 for details) and prepared as a 10% (w/v) homogenate in 0.85% sterile saline [33,34]. For each scrapie source selected, the ovine BSE was mixed with it at 1%, 10%, 25%, 50%, 75%, 90% and 99% based on volume. These mixtures, together with the neat ovine BSE and scrapie samples were subdivided into aliquots, blinded and stored frozen as test panels comprising 33 samples (Panel 1).

Estimations of the PrP^{res} (the proteinase K (PK) resistant moiety of PrP^{Sc} which is usually detected by biochemical tests such as Western blot and ELISA) present in each 'neat' sample were obtained so that estimates of the relative proportions of PrP^{res} contributed by the two components of the mixture could be calculated retrospectively, but this did not affect the choice of material, since known biological phenotype criteria (i.e. distinct bioassay characteristics) were considered more relevant for this study.

The sample panels were provided 'blinded' to teams in the APHA (for discriminatory Western blotting) and CEA laboratories (for discriminatory ELISA), and both laboratories were asked to provide an initial interpretation of 'BSE-like' or 'not BSE-like' before samples were de-coded; samples were also

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provided to ADAS/University of Nottingham for sPMCA analysis, and were similarly interpreted before decoding.

All the neat sources used to prepare the panel were selected for bioassay in transgenic mice, together with the mixture with the highest undetectable percentage of BSE and the mixture containing 1% BSE from each scrapie/BSE combination. For animal bioassays, a bovinised (tg110) and an ovinised (tg338) mouse line were used. A total of 24 assays were performed.

Discriminatory Western blot

The samples were subjected to discriminatory Western immunoblotting using the APHA BioRad-Hybrid Western blot method as described in detail in the EU discriminatory testing handbook (http://www.tse-lab-net.eu/documents/tse-oie-rl-handbook.pdf). TSE strains can be characterised as 'classical' or 'atypical' based on the profile of the protein bands detected by different antibodies following Western immunoblotting. Within this study, the PrP forms in the sample panel tested originated from either classical scrapie , classical ovine BSE or atypical scrapie.

Classical forms of TSE typically present with a 3 band profile consisting of a diglycosylated (top), monoglycosylated (middle) and diglycosylated (bottom) pattern and a high molecular mass migration of the unglycosylated PrP band with the core antibody (SHA31) and a similar or stronger intensity of overall signal with the N-terminal antibody (P4) is observed for classical scrapie. A low molecular mass migration of the unglycosylated PrP band with the core antibody (SHA31) and a much reduced, or lack of, intensity of overall signal with the N-terminal antibody (P4) is observed for classical ovine BSE. Atypical

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scrapie typically presents with a four band profile where all bands give a distinctive downward shift with the lowest band at 15kD or lower.

The resulting band profile for each sample was visually assessed and categorised based on the above criteria. (Figure 1) The initial WB results were reported as BSE, scrapie or a mixture in which the BSE and each of the scrapie sources were provisionally classified

Discriminatory ELISA

The samples were tested in duplicate, using the discriminatory ELISA method that has been described in detail elsewhere [21]. This method treats each sample with one of two different PK digestion protocols (mild and stringent), and expresses the subsequent differences in antibody binding as a ratio. This ratio is further normalised against the BSE control sample in each assay run. Three internal controls were included: one classical scrapie sample (highly PK resistant, normalised ratio inferior to 0.3), an unusual scrapie sample previously reported [21] to give an 'intermediate' result (PK resistant, normalised ratio comprised between 0.3 and 0.7) and an experimental BSE sample (PK sensitive, normalised ratio comprised between 0.7 and 1.3). According to these values, the blinded samples were categorised as 'scrapie', 'intermediate scrapie' or 'BSE'. Samples with a normalised ratio above 1.3 were classified as 'atypical scrapie'.

PrP^{res} estimation

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Aliquots of the un-mixed 10% brain homogenates (in normal saline) of ovine BSE and scrapie were pelleted out by high-speed centrifugation and re-homogenised in Bio-Rad TeEsE ELISA kit proprietary buffer to give 20% w/v as specified in the kit instructions. A dilution series of each isolate was prepared, and samples were analysed in accordance with the manufacturer's instructions. The end point dilution assays were used to generate PrP^{res} protein estimations for each homogenate (Table 1), and these were normalised for the ovine BSE sample.

Animal bioassays

All intracerebral inoculations were carried out under general anaesthesia, and in accordance with the United Kingdom (UK) Animal (Scientific Procedures) Act 1986, under Licence from the UK Government Home Office (Project licence number 70/7159). Such licences are only granted following approval by the internal APHA ethical review process as mandated by the Home Office. Each inoculum (10% w/v brain homogenate in normal saline) was used to challenge 10 tg110 and 10 tg338 mice intracerebrally (20µl per mouse). The mice were allowed to develop TSE disease and were euthanized when they reached terminal disease stage, or due to other welfare reasons. At post mortem each brain was sectioned parasagitally; 2/3 was fixed and subsequently processed for histology and immunohistochemistry whilst the remaining 1/3 was kept frozen.

All samples were examined for the presence of TSE specific vacuolation in H&E slides and for PrP^{Sc} detection using immunohistochemistry with the polyclonal antibody Rb486 according to standard methodology as previously described [34].

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Discriminatory sPMCA

Following completion of this ring trial and bioassay, a potentially discriminatory sPMCA method became available. This method, which is described in detail elsewhere [32], uses five sPMCA rounds with AHQ/AHQ and VRQ/VRQ sheep brain homogenates as the substrates being used in alternate rounds, followed by PK digestion and visualisation in WB using the monoclonal antibody (mAb) SHa31. This method selects for the amplification of BSE, but not scrapie.

Amplified products that are detected in WB are then additionally probed separately by both P4 and SHa31 antibodies in order to confirm BSE status.

A panel of original aliquots from this comparative study was supplied, blinded, for testing (Panel 1), then, following initial results Panel 2, generated from the original sources, and extending the dilution range of the ovine BSE to 1:10,000, was also tested 'blind'.

RESULTS

Estimated PrP^{res} concentration in the brains of the sources that contributed to the mixtures

The PrP^{res} concentration in the brains of the sources that contributed to the mixtures is shown in Table 1. There was less than 1 log difference between the concentration of PrP^{res} in the ovine BSE source and each of the scrapie sources. Assuming that PrP^{res} is a reliable indicator for infectivity, the ratios of BSE relative to scrapie in the series of mixtures used in this study is accurate within 0.27-0.63 logs depending on the classical scrapie source. With the exception of the VRQ/VRQ classical scrapie source, the PrP^{res} concentration in the BSE source was lower compared to the other scrapie sources.

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Therefore, with the exception mentioned above, in the mixtures of scrapie sources with BSE the concentration of PrP^{res} attributed to scrapie was higher than that indicated by the percentage ratio of the scrapie source in the mixture.

Discriminatory Western blot

The WB results for each sample were recorded using the following criteria; high or low molecular migration with the core antibody (SHa31); strong, medium, weak or negative with the N-terminal antibody (P4) and a description of either classic 3 band or atypical profile for each antibody. Using the combination of results, each sample was assigned a concluding result of BSE, scrapie, atypical scrapie or a description of combined TSE types. Representative descriptions for 14 blinded samples are presented in Table 2, with corresponding Western blots shown in Fig 1.

Western blotting could consistently detect BSE present within a mixture with scrapie when it was present as 50% of the mixture (Table 3).

Discriminatory ELISA

The ELISA method could consistently detect BSE when mixed with scrapie when it was present as 99% of the mixture (Table 3). However the results vary depending on the scrapie strains. BSE was detected when it was present as 75% of the mixture (for classical scrapie VRQ/VRQ and ARQ/ARQ), 90% (classical scrapie 1-4-7 ARQ/ARQ) or 99% (atypical scrapie). When the data are unblinded, and the ELISA results grouped by scrapie type (Figure 2) it can be seen that this apparent inability to detect BSE is partly due to the

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311 restrictions of having a numerical result and cut-offs. There is no qualitative
312 data to aid interpretation of 'intermediate' cases.
313
314 A summary of the ability of the biochemical tests to discriminate BSE in the
315 presence of scrapie, at a ratio of 1% to 99% (Panel 1) when the samples are
316 blinded, is presented in Table 3. Different isolates resulted in different
317 discriminatory thresholds, which were different between the tests. For
318 example, BSE could be detected at very low levels against a background of
319 atypical scrapie in the WB, but the BSE signal was masked by small amounts
320 of atypical scrapie when present as a mixture in the ELISA.
321
322 *Bioassay*
323
324 Mixtures of BSE with VRQ/VRQ or ARQ/ARQ classical scrapie
325 Both tests, WB and ELISA, failed to identify the presence of BSE in the
326 dilution series when its ratio in the mixture with VRQ/VRQ classical scrapie
327 dropped below 50% (Table 3). Therefore the inoculum just below the cut-off
328 point (25% BSE ratio relative to scrapie) and the inoculum with the lowest
329 BSE ratio relative to scrapie (1% BSE) were subjected to bioassays. WB and
330 ELISA also failed to identify the presence of BSE when the ratio of BSE to
331 ARQ/ARQ classical scrapie was below 25% (Table 3). Therefore the inocula
332 with 10% and 1% BSE concentration relative to scrapie were subjected to
333 bioassays.
334 In tg338 mice the scrapie agents isolated from both classical scrapie sources
335 were indistinguishable, with very short incubation periods - 69.5 and 75.5
336 mean dpi for VRQ/VRQ and ARQ/ARQ scrapie isolates respectively (Figures

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3A and 4A), similar lesion profiles (Figures 3B and 4B) and similar brain distribution of PrP^{Sc} types as assessed by IHC (data not shown).

All tg338 mice that were challenged with BSE succumbed to TSE 624 dpi or later (Figure 3A). In contrast, inoculation of tg338 mice with BSE mixed with either the VRQ/VRQ or the ARQ/ARQ classical scrapie sources produced incubation periods less than 90 dpi that were compatible with those produced by the respective scrapie sources alone (Figures 3A and 4A). These data indicate that the component isolated in the mice from the BSE mixtures with VRQ/VRQ or ARQ/ARQ classical scrapie only had classical scrapie properties. The vacuolation lesion profiles alone were not conclusive because the VRQ/VRQ and the ARQ/ARQ classical scrapie, and BSE profiles were not dissimilar enough to allow unequivocal interpretation, although the mixtures did align more closely with the classical scrapie profiles produced by the 100% scrapie sources (Figures 3B and 4B).

All tg110 mice challenged with the VRQ/VRQ source were TSE negative or, in the case of the ARQ/ARQ source, showed low attack rates with the first positive animal identified 581 dpi (Figures 3A and 4A). In contrast, mixtures of BSE with either VRQ/VRQ or ARQ/ARQ classical scrapie produced clinical stage TSE with incubation periods of 236-326 dpi (Figures 3A and 4A). These incubation periods are comparable with those generated by the original BSE source (221-267 dpi) albeit slightly longer, probably as a result of the slightly reduced titre of BSE in the mixtures. These data suggest that in this mouse line only the BSE component was isolated from the mixtures. The lesion profiles from the tg110 mice (Figures 3C and 4C) further support the conclusion that the isolated agent had only BSE properties although it was not

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possible to construct lesion profiles from either scrapie source due to the lack of sufficient clinically positive mice diagnosed with TSE.

1-4-7 ARQ/ARQ classical scrapie and ovine BSE

Both tests failed to identify the presence of BSE in the dilution series when its concentration in the mixture dropped below 50% (Table 3). Therefore the inoculum just below the cut off point (25% BSE ratio relative to scrapie) and the inoculum with 1% BSE ratio relative to scrapie were subjected to bioassay.

Figure 4A shows that tg338 mice challenged with either of these mixtures succumbed to disease with incubation periods that were compatible with the incubation periods produced by the scrapie source alone, indicating that the agent isolated from the 25% and 1% BSE mixtures was the scrapie component. The lesion profiles are not conclusive because the ARQ/ARQ scrapie and BSE profiles are indistinguishable (Figure 5B).

The incubation periods of tg110 mice challenged with the 1-4-7 classical scrapie isolate were relatively shorter than the incubation periods caused by BSE in this mouse line (Figure 5A). The incubation periods produced by the 25% and 1% BSE mixture in this mouse line were aligned with the incubation periods produced by the 1-4-7 ARQ/ARQ classical scrapie source (Figure 5A). Lesion profiles concur with this interpretation as the lesion profiles produced by the mixtures align with the lesion profile of the 1-4-7 classical scrapie whilst BSE produces a distinct separate profile (Figure 5C).

Tg110 mice challenged with either the scrapie or the BSE source succumbed to disease with relatively short incubation periods, therefore the mice

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inoculated with the mixtures were further compared to those challenged with the original sources using immunohistochemistry (IHC) (Figure 6).

Tg110 mice challenged with the 1-4-7 classical scrapie source showed a pattern characterised by intraneuronal and fine punctate PrP^{Sc} deposits in the neuropil; when aggregates were present they were distinct, well demarcated and ovoid (Figure 6A). BSE challenged tg110 mice also showed intraneuronal PrP^{Sc} but the neuropil was populated with diffuse granular deposits, coalescing aggregates and plaque-like formations (Figure 6B). In addition to the IHC attributes associated with the 1-4-7 classical scrapie pattern, tg110 mice challenged with either mixture additionally showed features that were associated with the BSE-induced pattern (Figures 5C and 5B). This BSE associated pattern also appeared to be more extensive in the mice that were challenged with the 25% BSE mixture compared with the mice that received the 1% BSE mixture.

Although the incubation periods and the lesion profiles, particularly those generated by mice that were inoculated with 25% BSE mixture, suggest that the BSE agent did not propagate selectively in the tg110 mice, it was still possible to identify the BSE component reliably in the Tg110 mice using IHC.

Atypical scrapie and ovine BSE

Both WB and ELISA failed to identify the presence of BSE in the dilution series when its concentration in the mixture dropped below 10% (Table 3). Therefore the inoculum with 1% BSE concentration relative to scrapie was subjected to bioassays.

The incubation period data in tg338 indicate that the agent isolated from these mixtures was compatible with the agent isolated from the atypical scrapie

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source; in tg110 mice the incubation period data indicate that agent isolated from the mixtures was compatible with the agent isolated from the BSE source (Figure 7A). The lesion profiles from tg338 (Figure 7B) and tg110 mice (Figure 7C) provide further support to the incubation period data although it was not possible to construct lesion profiles from the scrapie source in tg110 as all of the mice challenged with this source were TSE negative.

Discriminatory sPMCA

The original panel of 33 samples (Panel 1) were all correctly reported as 'BSE present', with the correct exception of the un-mixed scrapie samples (Table 3). The analysis of Panel 2 gave identical results for the 1% to 99% mixtures. sPMCA also correctly reported the presence of BSE in all samples in which BSE was diluted to 0.1%. BSE was also successfully detected in one sample where it was diluted to 0.01% with atypical scrapie (Figure 8).

Discussion

Co-infection studies in animals using mixtures of known infectious titre remain the hypothetical ideal for this type of study, but for several reasons such studies are not necessarily as appropriate as they might appear on initial consideration. The observed titre of a TSE isolate is not an absolute measure of the infectivity of that isolate, but is also affected by the susceptibility of the host, which may differ for different isolates. For example, some scrapie strains that readily infect sheep and transgenic mouse models do not cause disease in conventional inbred mouse lines [2,33]. Therefore it is unwise to assume that two isolates with similar observed levels of infectivity in any one model will necessarily have the same infectivity potential in other species, or indeed

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444 in animals of different genotypes. Equally, PrP^{res} concentration cannot be
445 considered to be a consistent proxy for the level of infectivity in an isolate [37].
446 The interaction of strains either *in vitro* or within a single host is also very
447 poorly understood. If strain properties are conferred by tertiary molecular
448 structure, then mixing isolates together might affect the ability of a strain to
449 infect a host either in an inhibitory or potentiating way. This may also affect
450 tests applied to a sample with both isolates represented. However, the data
451 from this study demonstrate that this is not the case, at least with the BSE and
452 scrapie combinations used; all tests and models, except the sPMCA, could
453 correctly classify both of the strains contributing to each of the mixtures,
454 including the successful isolation, in mice, of all the component isolates of
455 each mixture, with retention of the biological phenotypes of the unmixed
456 controls.
457 The bioassay data, particularly from the bovinised mice, also suggest that if
458 sheep are exposed to both BSE and scrapie, the two agents will most likely
459 propagate as independent entities according the dynamics of titre, time of
460 exposure to each agent and ovine PrP genotype. Therefore exposure to both
461 agents is unlikely to result in a novel agent with previously undetected
462 biochemical or biological properties, although this possibility should always be
463 considered when a new or unusual isolate is identified. Under these
464 circumstances exposure to both agents would give rise to a mixture in which
465 BSE can be detected with the current biochemical and biological tests
466 provided that the titre of scrapie is not overwhelming. The data also show that
467 the choice of diagnostic test, and which scrapie strain is present, both dictate
468 the level of scrapie that “overwhelms” the detection of BSE. For BSE mixed

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with atypical scrapie the presence of 10% scrapie masked BSE detection by ELISA whereas, at the other extreme, BSE mixed with atypical scrapie and detected by WB required the presence of above 90% scrapie to mask BSE.

Additional unknowns, if trying to use an *in vivo* challenge model to recreate possible co-infection, are the age of the animal at challenge, the order in which the challenges occur, and possibly the length of time between the challenges [38,39,40].

The main purpose of this study was to attempt the identification of the BSE component of the mixture by using the approved discriminatory tests (EU regulation 36/2005), followed by discriminatory bioassay in tg338 mice to selectively propagate the scrapie strains, and tg110 mice to selectively propagate the BSE agent, in mixtures in which the identifiable biochemical signature of BSE has been lost or obscured. We conclude that the biochemical rapid tests can discriminate BSE in the presence of scrapie to varying degrees depending on the scrapie source, but this variation does not appear to be attributable to the amount of PrP^{res}. The WB was more reliable than ELISA in discriminating BSE in the presence of scrapie, regardless of the scrapie isolate.

The bioassay was capable of resolving cases of co-infection even where BSE represented just 1% of the total TSE infectivity. In order to achieve this, bioassay systems should include a bovinised mouse line that favours propagation of BSE over scrapie, and an ovinised line with complementary properties, i.e. a line that preferentially facilitates the propagation of scrapie prions over the BSE agent. In addition to the ability to identify BSE in mixed infections, bioassay continues to be the only validated method available that

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enables the comprehensive phenotypic characterisation of an unknown isolate.

It is probably not possible to source 'ideal' mouse lines with the above properties particularly as classical scrapie consists of various strains with widely variable properties. However, the selected mouse lines (tg338 and tg110) are as close as possible to that ideal situation and could be used in combination to resolve co-infection cases in a surveillance context if they arise. In addition, IHC may be useful to resolve a small proportion of bioassays in which the relative mouse line susceptibility is not, by itself, conclusive.

Within this study, the new discriminatory sPMCA approach was the only *in vitro* method which consistently detected BSE when it was present in these mixtures, even at very low concentration (down to 0.1%). This sensitivity could potentially be exploited to screen pooled ovine TSE brain samples for the presence of BSE, greatly increasing the throughput, and decreasing the costs of such screening programmes in the future. When the purpose of an investigation is solely to determine the presence or absence of BSE (as opposed to characterising whatever is in the isolate), this assay would appear to offer the potential for a fast and cost-effective alternative to bioassay, and will be proposed to the EURL Strain Typing Expert Group as a useful addition to the panel of tests currently used for the screening of unknown isolates.

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Table 1. Estimation of PrPres concentration in the brains of the sources that contributed to the mixtures

TSE source	PrPres (µg/ml)	Normalised for BSE	Log10
BSE[1]	2.2	1	0.00
VRQ/VRQ classical scrapie[2]	1.0	0.45	-0.34
ARQ/ARQ classical scrapie[3]	12.0	5.45	0.74
1-4-7 ARQ/ARQ classical scrapie[4]	9.3	4.23	0.63
Atypical scrapie (AHQ/ARQ)[5]	4.1	1.86	0.27

[1] Experimental ovine BSE produced and characterised at APHA
[2] Classical scrapie case supplied by Dr Olivier Andreoletti and characterised in tg338 mice (PG 127 Classical scrapie [41])
[3] Classical scrapie field case characterised at APHA (Scrapie 67 [35])
[4] Classical scrapie field case characterised at APHA (Scrapie 19 [35]) The designation 1-4-7 indicates a lesion profile in RIII mouse bioassay with peaks in areas 1,4 and 7, the same as seen with BSE isolates.
[5] Atypical scrapie field case (UK active surveillance) characterised and titrated ($10^{6.92}$ LD50/gr) at APHA (unpublished data)

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Table 2 Representative descriptions for 14 blinded samples, illustrated in Figure 1

WB lane number	mAb SHa31 detection affinity	mAb SHa31 Banding pattern	mAb SHa31 Molecular mass[a]	mAb P4 detection affinity	mAb P4 Banding pattern	'blind' interpretation	Sample details
1	++	Atypical	NA	+	Atypical	Atypical scrapie	100% atypical scrapie
2	+++	Classical[b]	High	+++	Classical	Classical scrapie	100% ARQ 147 scrapie
3	+++	Classical	Low	+	NA[c]	BSE	100% ovBSE
4	+++	Classical	High	+++	Classical	Classical scrapie	100% VRQ scrapie
5	+++	Classical	High	+++	Classical	Classical scrapie	100% ARQ scrapie
6	+++	Classical	Low	-	NA	BSE	99% ovBSE, 1% atypical scrapie
7	+++	Classical	Low	+++	Classical	Scrapie with BSE	50% ovBSE, 50% ARQ147 scrapie
8	++	Classical	Low	++	Atypical	BSE with atypical	10% ovBSE, 90% atypical scrapie
9	+++	Classical	High	+++	Classical	Scrapie	10%ovBSE, 90% VRQ scrapie
10	+++	Classical	Low	+	Classical	BSE with low dilution of scrapie or CH1641	99% ovBSE, 1%ARQ147 scrapie
11	+++	Classical	Low	+	Classical	BSE with low dilution of scrapie or CH1641	75%ovBSE, 25% atypical scrapie
12	+++	Classical	High	+++	Classical	Scrapie	25%ovBSE,75%VRQ scrapie
13	+++	Classical	Low	++	Classical	BSE with scrapie	90% ovBSE, 10% ARQ scrapie
14	++	Atypical	NA	++	Atypical	Atypical	1% ovBSE, 99% atypical scrapie

a. Molecular mass of the unglycosylated band of a classical 3-band pattern
b. Three band pattern associated with classical scrapie and BSE.
c. Sample too weak to determine the banding pattern

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Table 3 Ability of biochemical tests to identify BSE when mixed with different scrapie sources (Panel 1)

Scrapie source	Ovine BSE source (%) in the mixture	BSE detection		
		WB	ELISA	sPMCA
	100 (C)	+	+	+
Classical (VRQ/VRQ)	99	+	+	+
	90	+	+	+
	75	+	+	+
	50	+	-	+
	25*	-	-	+
	10	-	±	+
	1*	-	-	+
	0*	-	-	-
Classical (ARQ/ARQ)	99	+	+	+
	90	+	+	+
	75	+	+	+
	50	+	-	+
	25	+	-	+
	10*	-	-	+
	1*	-	-	+
	0*	-	-	-
Classical (1-4-7 ARQ/ARQ)	99	+	+	+
	90	+	+	+
	75	+	-	+
	50	+	-	+
	25*	-	-	+
	10	-	-	+
	1*	-	-	+
	0*	-	-	-
Atypical (AHQ/ARQ)	99	+	+	+
	90	+	-	+
	75	+	-	+
	50	+	-	+
	25	+	-	+
	10	+	-	+
	1*	-	-	+
	0*	-	-	-

(C) indicates the BSE source (100% BSE) that was used to create the pools.. The percentage in the other cells in the same column indicates the percentage of BSE in the mixture. The difference reflects the percentage of scrapie material in the mixture.
Dotted lines indicate the limit below which both WB and ELISA failed to identify the BSE component in the mixture.
+ BSE detected; - BSE not detected; ± inconclusive result
* Indicates mixtures selected for bioassay in transgenic mice.

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Figures

FIG 1. Representative Western Blot for blinded samples, using mAbs SHa31 (upper panel) and P4 (lower panel). This is an original diagnostic blot, selected to illustrate the nature of the blots on which the initial blind interpretation was made. The samples represented in lane 1-14 contained either 100% of a pure form of each TSE strain used in the study (lanes 1-5) or varying mixtures of the TSE strains (lanes 6-14). Mixed samples exhibit multiple band patterns dependent on the actual TSE strains contained in each mix. Interpretation of each sample was based on the observation of the main characteristics described in the WB method section. Detailed description of the samples, and their interpretation, in lanes 1-14 can be found in Table 2.. B=classical bovine BSE control, O=classical ovine scrapie control, M=molecular mass marker.

FIG 2 Discriminatory ELISA. The mixed samples were tested in duplicates in blinded conditions. Ovine BSE was mixed with A) atypical scrapie B) classical scrapie (VRQ/VRQ) C) classical scrapie (1-4-7 ARQ/ARQ) or D) classical scrapie (ARQ/ARQ). The normalised ratio for classical scrapie samples, which are highly PK resistant, is less than 0.3, intermediate scrapie samples present a normalised ratio comprised between 0.3 and 0.7 and experimental ovine BSE samples have a normalised ratio between 0.7 and 1.3. Atypical scrapie samples have ratio greater than 1.3. According to these values, the blinded samples were categorised as "scrapie" (grey), "intermediate scrapie" (hatched), "BSE" (black) or "atypical scrapie" (white).

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FIG 3 Bioassay data in tg338 and tg110 mice of 25% and 1% BSE mixtures with VRQ/VRQ classical scrapie. The original BSE and VRQ/VRQ classical scrapie sources that were used to produce the mixtures were also bioassayed. (A) Incubation periods; (B) lesion profiles in tg338 mice; (C) lesion profiles in tg110 mice. At least 5 clinically and histopathologically positive mice contributed to each lesion profile (solid lines) unless indicated (dashed line).

FIG 4 Bioassay data in tg338 and tg110 mice of 10% and 1% BSE mixtures with ARQ/ARQ classical scrapie. The original BSE and ARQ/ARQ classical scrapie sources that were used to produce the mixtures were also bioassayed. (A) Incubation periods; (B) lesion profiles in tg338 mice; (C) lesion profiles in tg110 mice. At least 5 clinically and histopathologically positive mice contributed to each lesion profile (solid lines) unless indicated (dashed line).

FIG 5 Bioassay data in tg338 and tg110 mice of 25% and 1% BSE mixtures with 1-4-7 ARQ/ARQ classical scrapie. The original BSE and 1-4-7 ARQ/ARQ classical scrapie sources that were used to produce the mixtures were also bioassayed. (A) Incubation periods; (B) lesion profiles in tg338 mice; (C) lesion profiles in tg110 mice. At least 5 clinically and histopathologically positive mice contributed to each lesion profile (solid lines) unless indicated (dashed line).

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FIG 6 Immunohistochemistry of tg110 mice challenged with (A) 1-4-7 ARQ/ARQ classical scrapie, (B) ovine BSE, C) mixture with relative BSE: scrapie ratio 1:3 (25% BSE) and (D) mixture with relative BSE:scrapie ratio 1:99 (1% BSE). All photos show rostral medulla at the same magnification. Black rectangles in A, B and D indicate areas that have been further magnified and presented as insets in the corresponding images. The mice inoculated with the BSE:scrapie mixtures show BSE associated coalescing PrPSc patterns even at the lowest ratio of BSE:scrapie (D). Red arrowheads show examples of neurons with intraneuronal PrPSc deposits; blue arrowheads point to patterns of coalescing PrPSc deposits; black arrowheads illustrate examples of neurons devoid of intraneuronal PrPSc deposits.

FIG 7 Bioassay data in tg338 and tg110 mice of a 1% BSE mixture with atypical scrapie. The original BSE and atypical scrapie sources that were used to produce the mixtures were also bioassayed. (A) Incubation periods; (B) lesion profiles in tg338 mice; (C) lesion profiles in tg110 mice. At least 5 clinically and histopathologically positive mice contributed to each lesion profile.

FIG 8 Western blot of PK digested sPMCA products. Samples of BSE brain homogenate were diluted into 4 isolates of ovine scrapie positive brain homogenate of VRQ, ARQ, Atypical and 1-4-7 ARQ types, as indicated, at 1/10, 1/100, 1/1000 or 1/10000 dilutions (labelled as 1-4 respectively). Samples were amplified in duplicate including an equal number of scrapie only samples. Positive sPMCA samples were then further analysed: single sPMCA sample replicates were re-digested with PK and immunoblotted using the antibodies SHa31 and P4 (as shown). Blotting controls

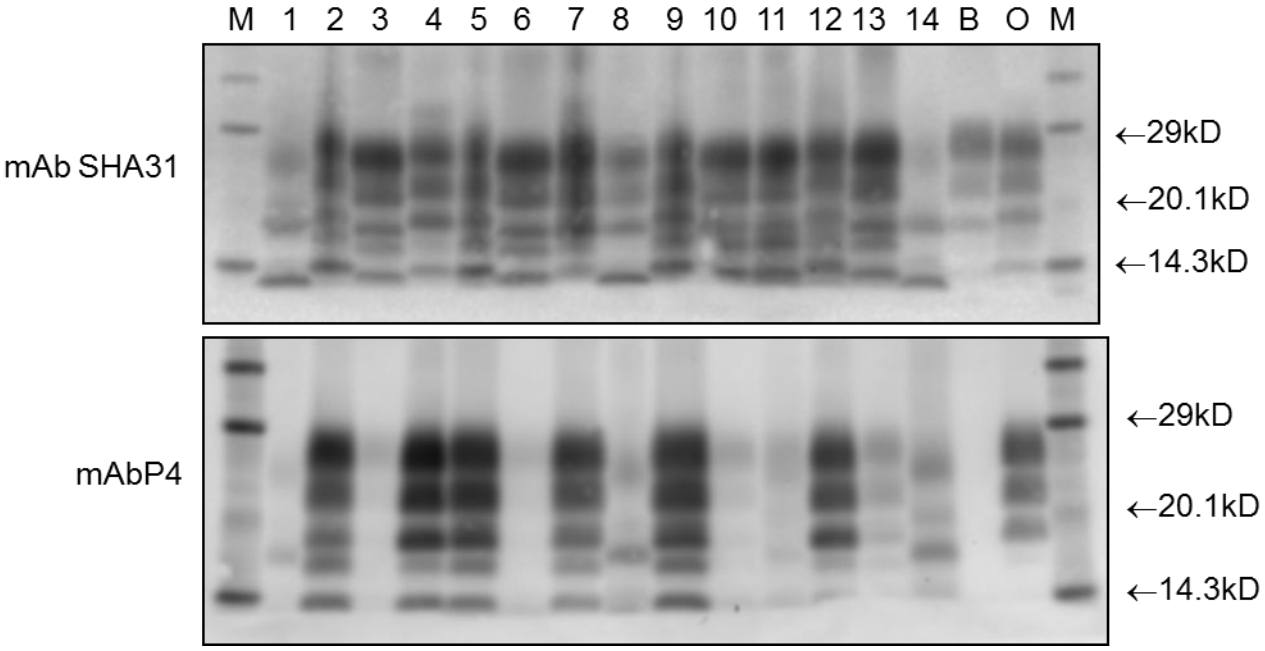
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were positive sheep scrapie (+S) or BSE (+B) brain homogenates and an ovine BSE sPMCA positive sample ($+B^P$). Molecular mass markers (M) at 20 and 30kDa are shown. Blots were probed with either SHa31 or P4 monoclonal antibodies. Samples were scored positive for BSE (as indicated) if over a signal threshold on the WB and using a SHa31/P4 WB ratio as previously described [32].

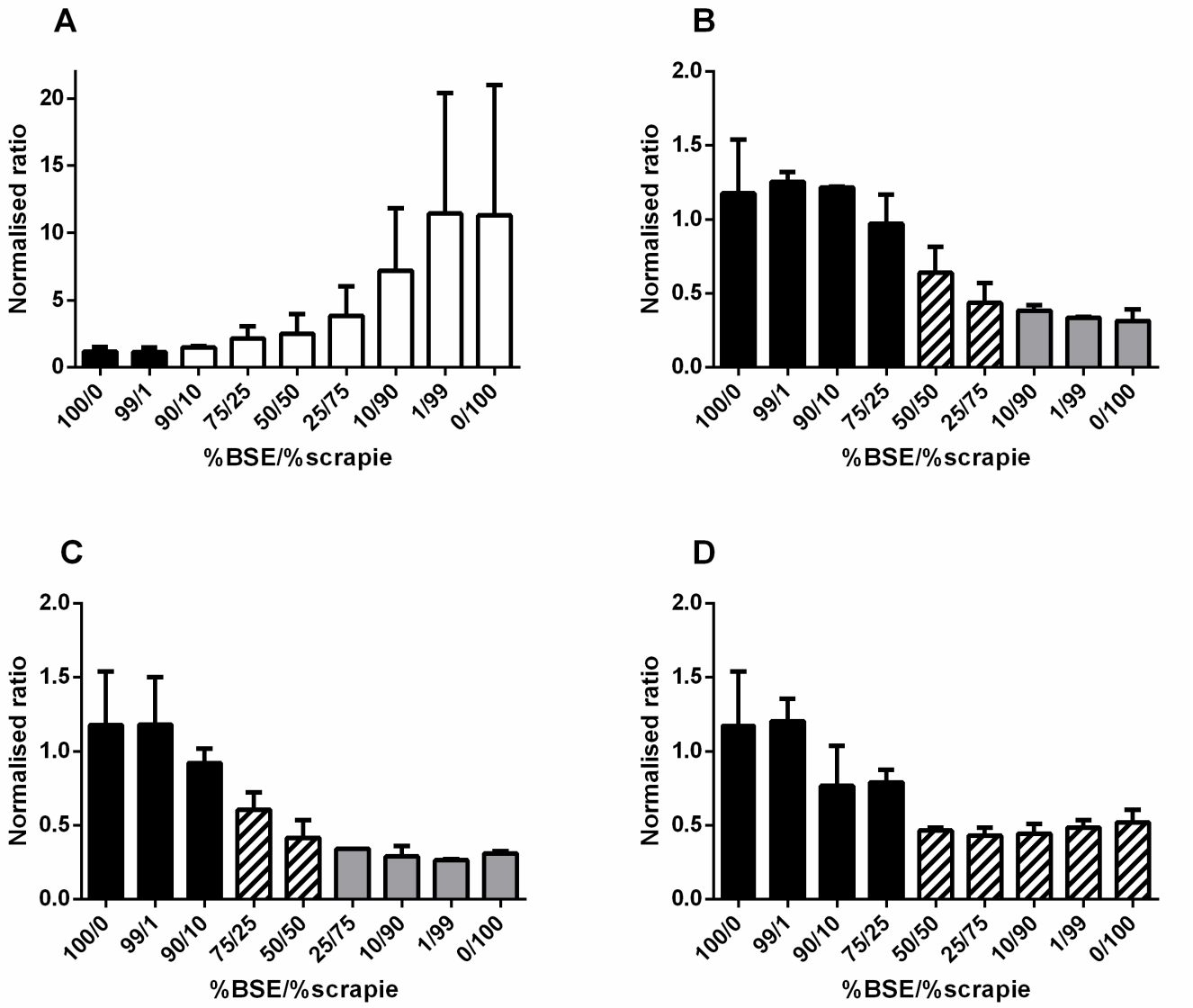
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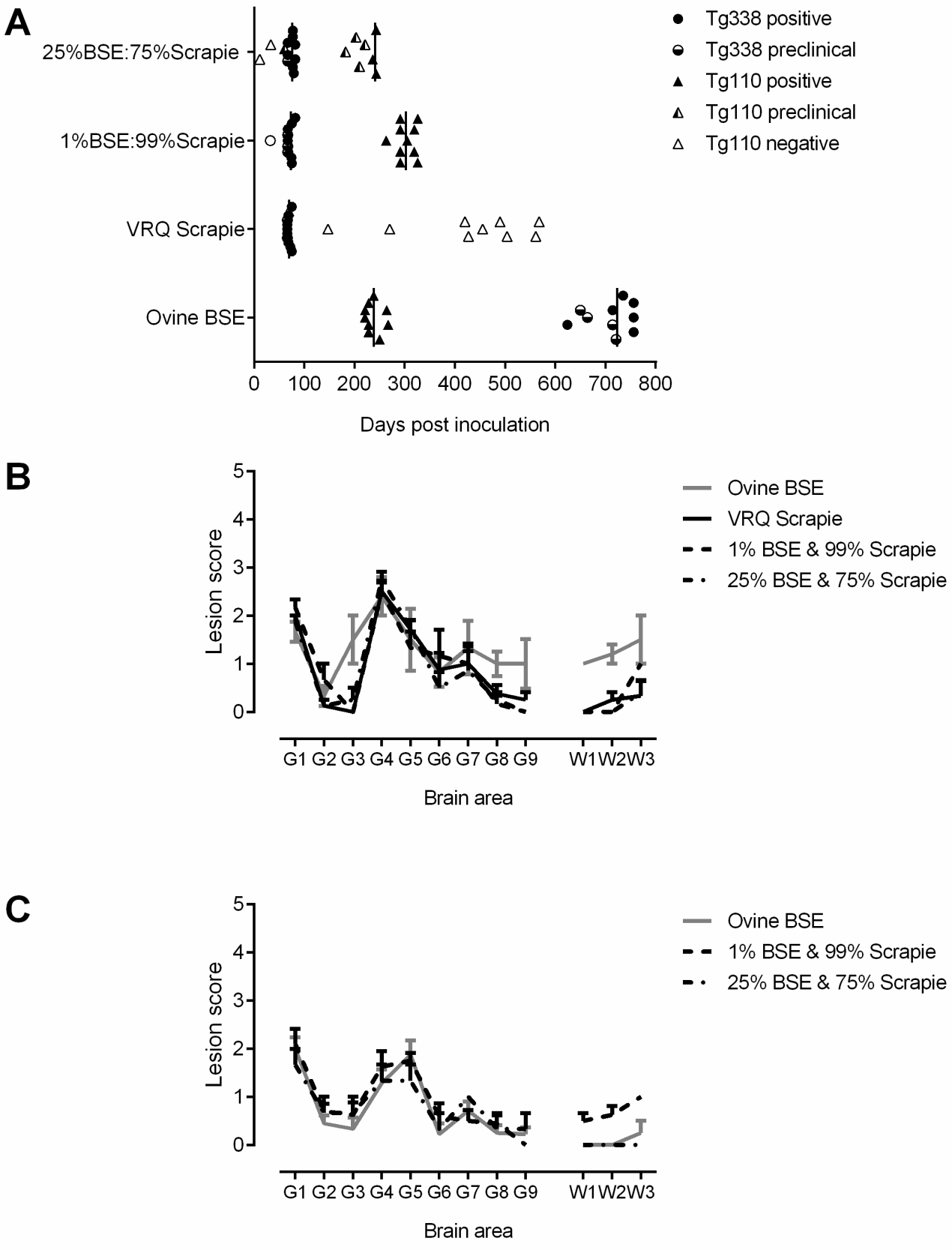
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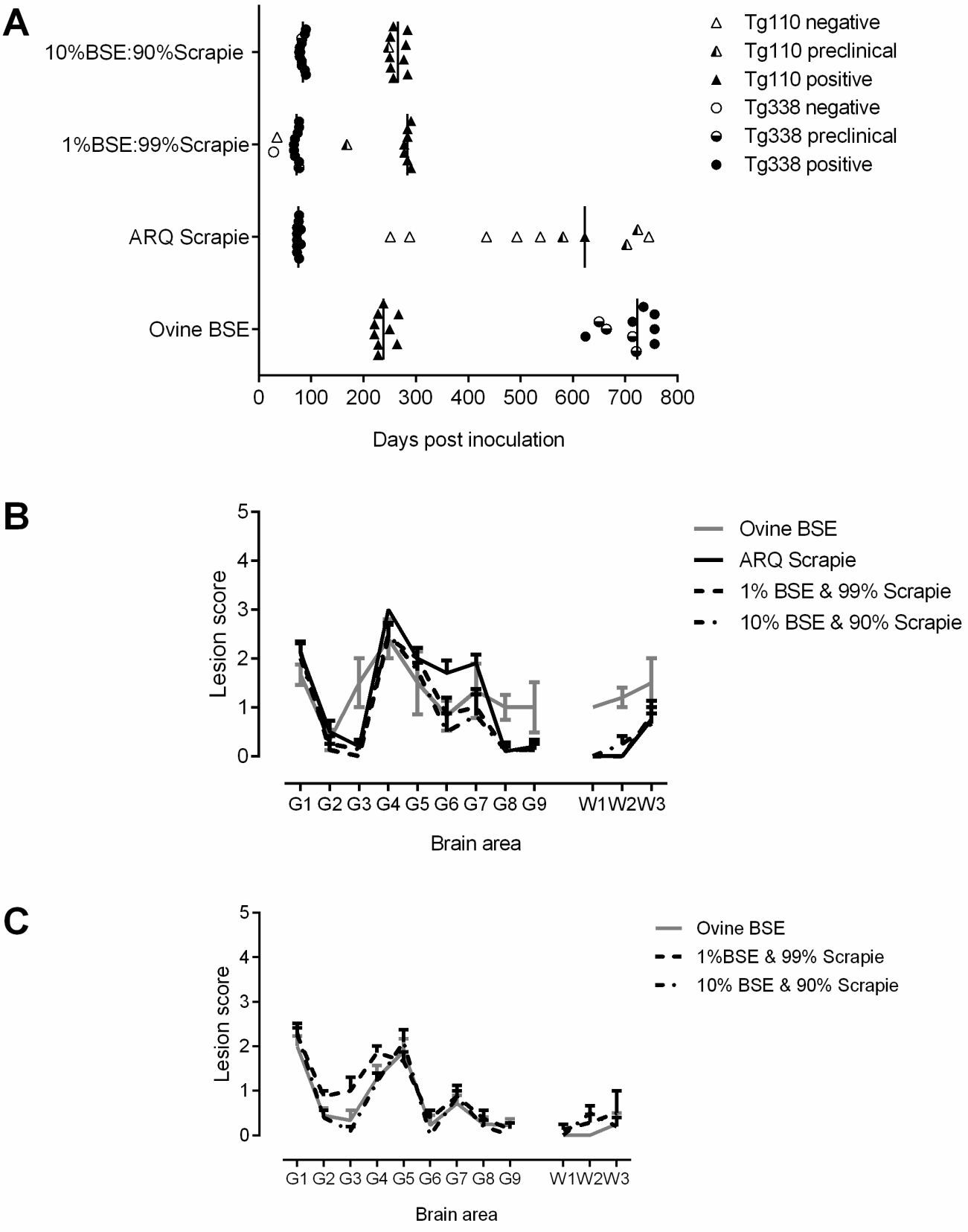
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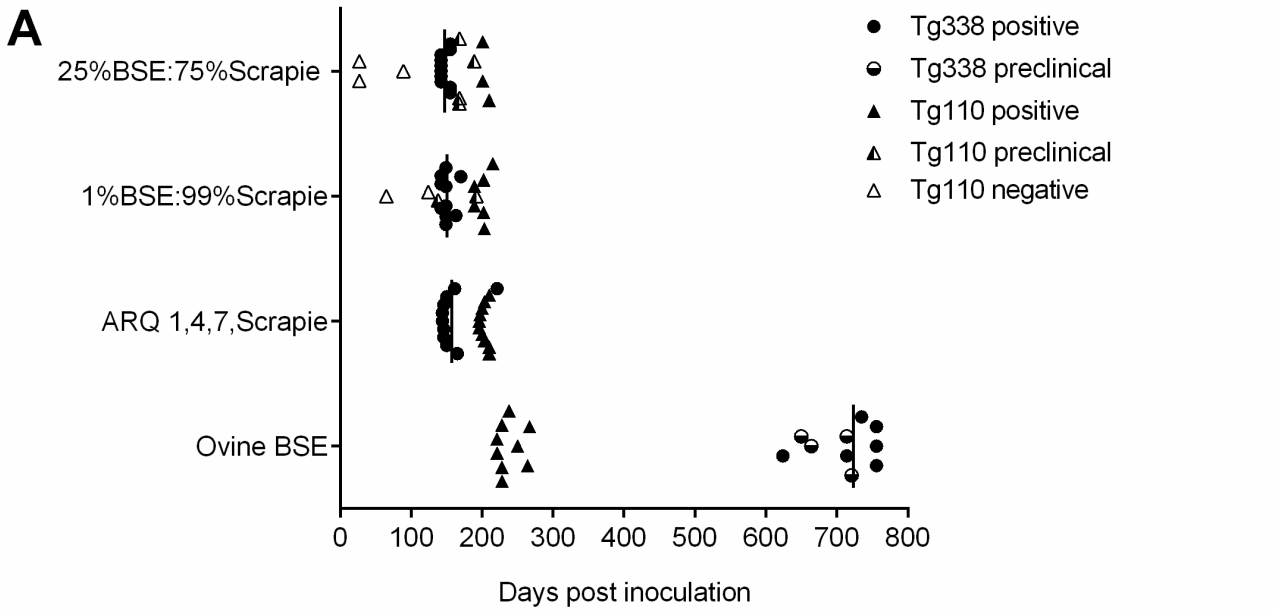
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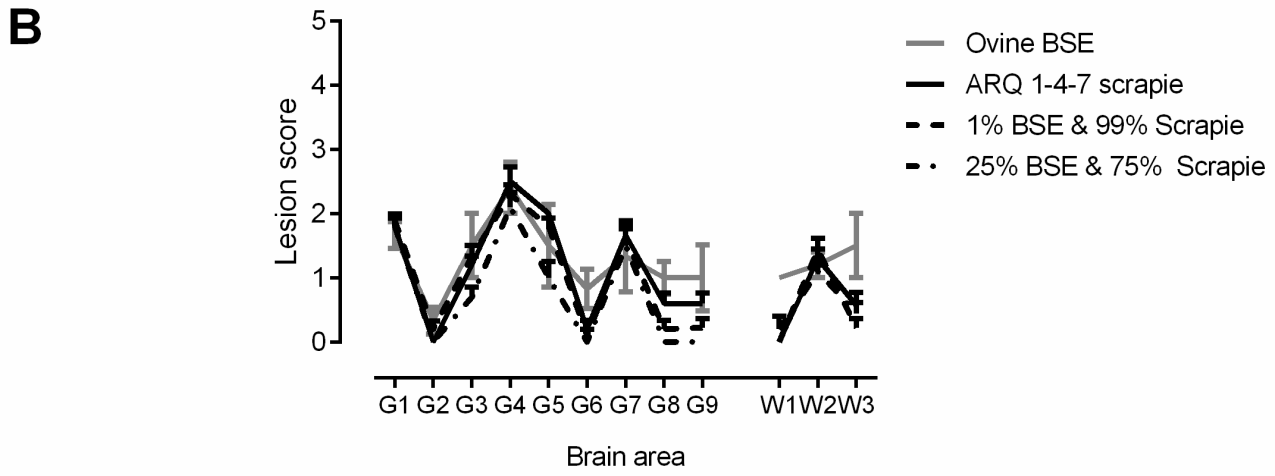
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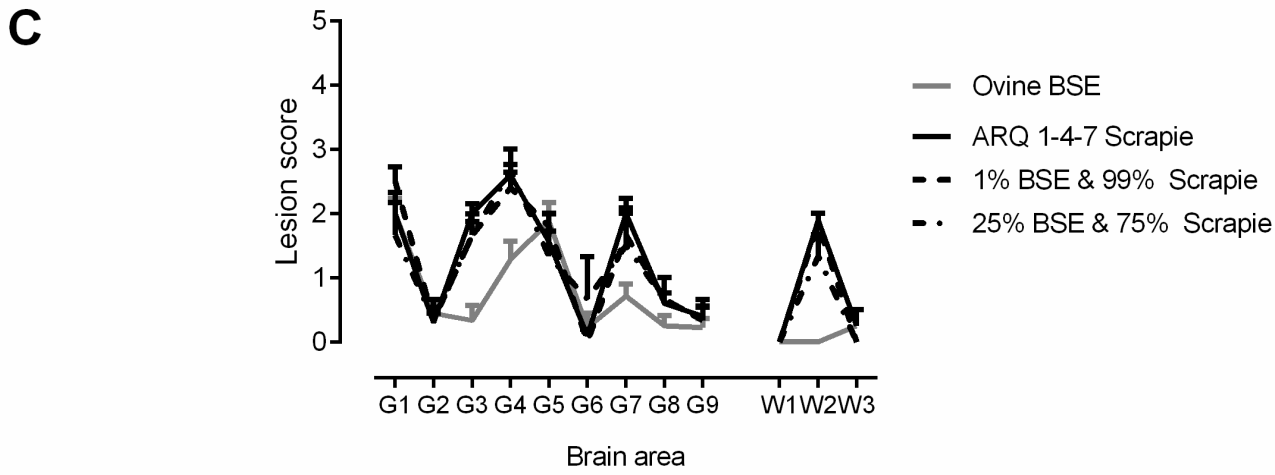


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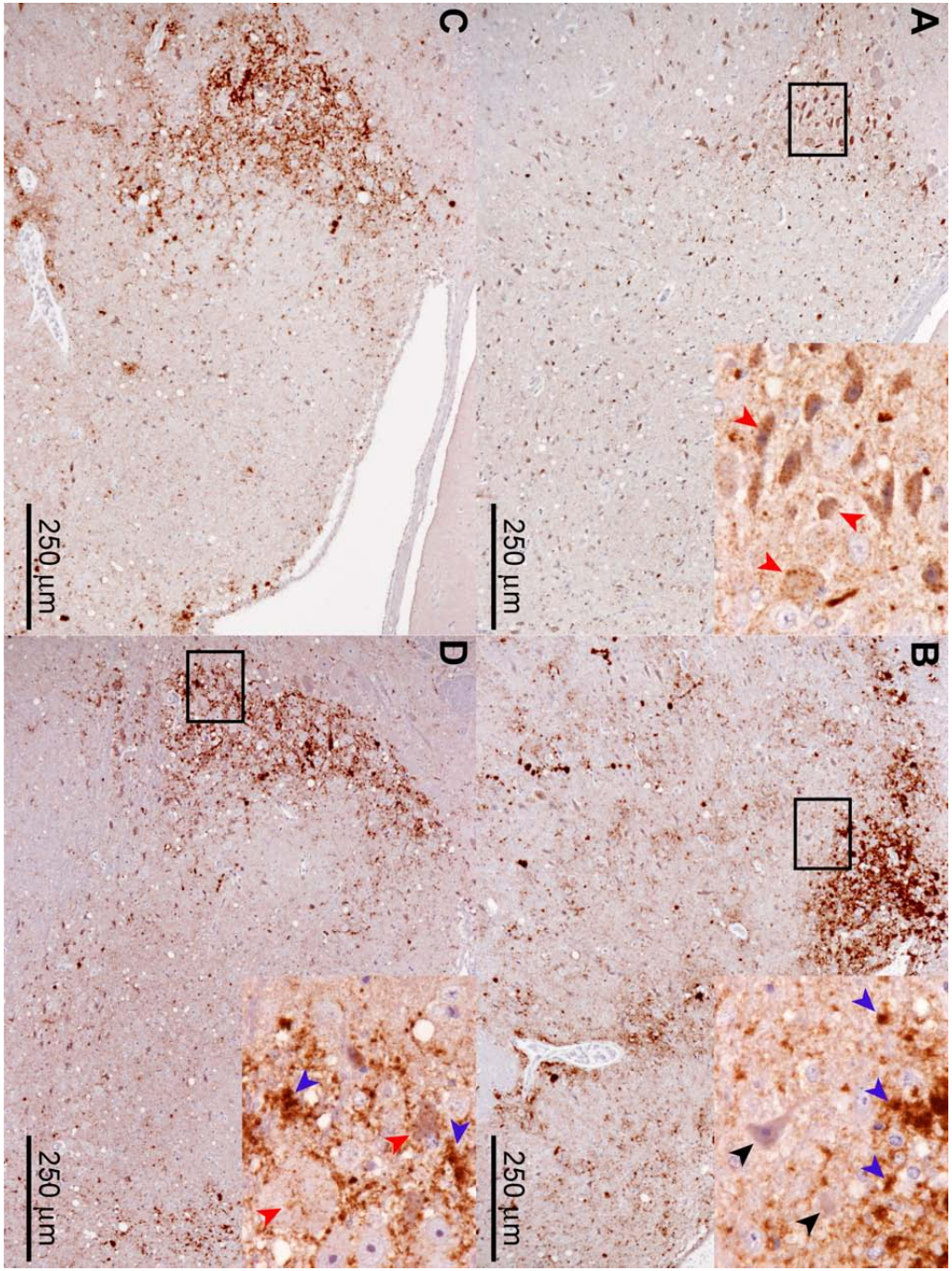
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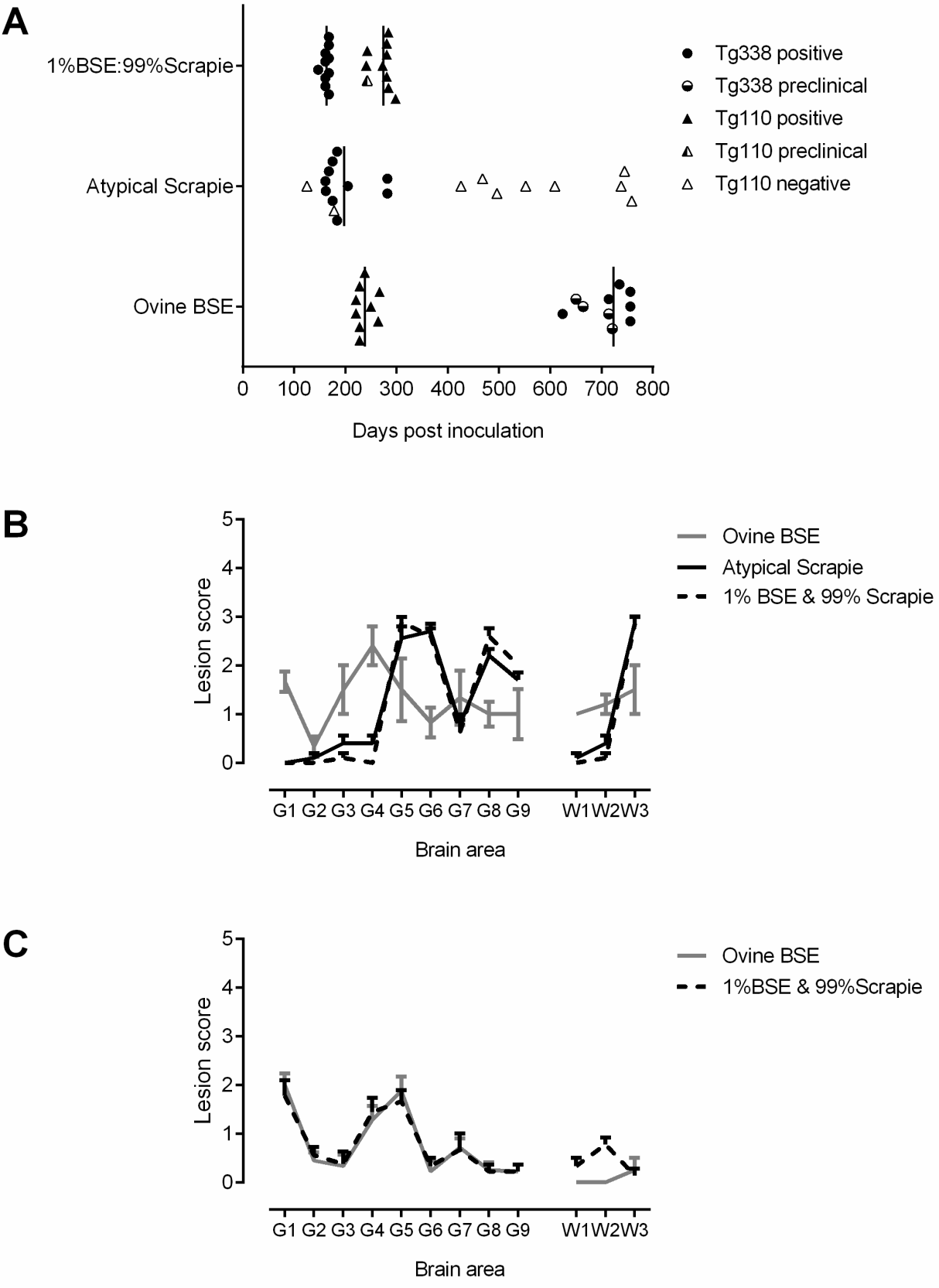




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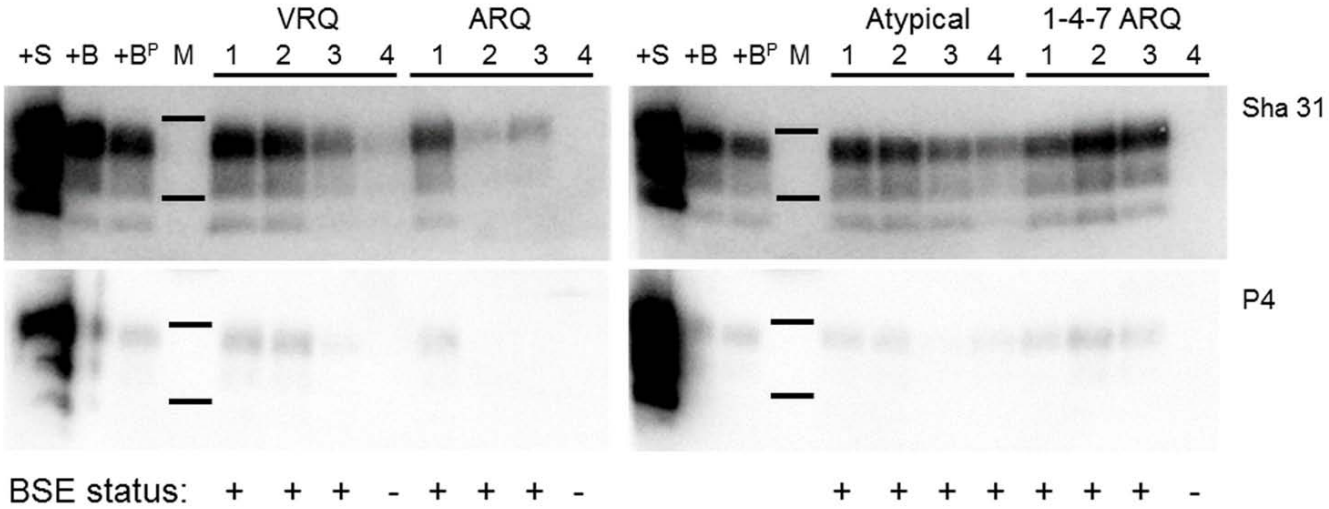

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