METHOD ARTICLE

A sensitive 301V BSE serial PMCA assay [version 1; referees: awaiting peer review]

Kevin C. Gough[1], Keith Bishop[2], Robert A. Somerville[3], Nora Hunter[3], Ben C. Maddison[2]

[1]School of Veterinary Medicine and Science, The University of Nottingham, Loughborough, UK
[2]ADAS-UK Biotechnology group, School of Veterinary Medicine and Science, The University of Nottingham, Loughborough, UK
[3]The Roslin Institute, The University of Edinburgh, Easter Bush, UK

v1 **First published:** 18 Oct 2016, **5**:2529 (doi: 10.12688/f1000research.9735.1)
Latest published: 18 Oct 2016, **5**:2529 (doi: 10.12688/f1000research.9735.1)

Abstract
The prion strain 301V, is a mouse passaged form of bovine spongiform encephalopathy (BSE). It has been used as a model of BSE for more than 20 years, in particular in the investigation of tissue distribution of infectivity, the molecular phenotype and transmission properties of BSE, strain typing assays and prion inactivation studies. Most 301V experiments have required murine bioassay as a method for the quantitation of infectivity. To date this model strain has not been studied with the protein misfolding cyclic amplification assay (PMCA) which detects prion-associated PrP^{Sc} protein. The detection of BSE PrP^{Sc} by PMCA can be more sensitive than mouse bioassay and is carried out in a much shorter time frame of days as opposed to months/years. Here, we describe the development of a new highly sensitive and specific PMCA assay for murine 301V and assess the sensitivity of the assay in direct comparison with murine bioassay of the same material. This *in vitro* assay detected, in a few days, 301V at a brain dilution of at least $1x10^{-9}$, compared to bioassay of the same material in VM mice that could detect down to a $1x10^{-8}$ dilution and took >180 days. The 301V PMCA may therefore offer a faster and more sensitive alternative to live animal bioassay when studying the BSE agent in VM mice.

Open Peer Review

Referee Status: *AWAITING PEER REVIEW*

Discuss this article

Comments (0)

Corresponding author: Ben C. Maddison (ben.maddison@adas.co.uk)

How to cite this article: Gough KC, Bishop K, Somerville RA *et al.* **A sensitive 301V BSE serial PMCA assay [version 1; referees: awaiting peer review]** *F1000Research* 2016, **5**:2529 (doi: 10.12688/f1000research.9735.1)

Copyright: © 2016 Gough KC *et al*. This is an open access article distributed under the terms of the Creative Commons Attribution Licence, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Data associated with the article are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

Grant information: This work was funded by DEFRA under project SE1433 (Robert Somerville) and by BBSRC Institute Strategic Grant BB/J004332/1 (The Roslin Institute).
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: No competing interests were disclosed.

First published: 18 Oct 2016, **5**:2529 (doi: 10.12688/f1000research.9735.1)

Introduction

The transmissible spongiform encephalopathies (TSE or prion diseases) form a group of infectious and fatal neurodegenerative diseases affecting several species of mammals for which there is no available treatment or cure. The cause is thought to be a novel infectious agent (the prion), itself a misfolded isomer (PrP^{Sc}) of a benign cell associated protein known PrP or PrP^{C}. This group of diseases include scrapie in sheep and goats, Creutzfeldt Jakob disease (CJD) in humans and bovine spongiform encephalopathy (BSE) in cattle. The UK BSE epizootic of the mid-1980s to early 1990s was the result of recycling BSE through the cattle food chain via a high protein feed additive known as meat and bone meal. It is thought that over 460,000 BSE infected UK cattle entered the human food chain before a ban on feeding specified risk materials to cattle came into force[1]. As a consequence of this, a new human disease referred to as vCJD, the human form of BSE, began to present in a number of young adults from the mid-1990s. This demonstration of the zoonotic potential of prion diseases generated an accelerated program of research into these diseases and much of this has required animal models. The murine passaged BSE strain known as 301V was first described by Bruce and colleagues[2] during transmission studies of cattle BSE to wild type mice. BSE 301V is the product of serial passage within the VM mouse line and this combination of 301V/VM has been well characterised and used in numerous studies, including those aimed at understanding the fundamental brain pathology during neuropathogenesis[3]. In addition, the 301V/VM model has also been important in experiments analysing the effectiveness of various decontamination measures for BSE infectivity. For example, Taylor *et al.*[4] demonstrated the effectiveness of formic acid in inactivating both 301V and scrapie in the context of occupational exposure to histological samples. This BSE model has also been used to show the lack of sufficient inactivation of BSE prions during historical rendering processes which resulted in the BSE outbreak in UK cattle[5]. For development of safe procedures in the context of human health, 301V has been used to model vCJD in the fractionation of plasma and the safe manufacture of blood products[6], and additionally has been used to estimate BSE infectivity that is likely to remain after processes in the derivation of bone gelatine from bovine products[7]. A further study by McLeod *et al.*[8] screened a number of different proteases for their ability to reduce the infectious titre of 301V as a novel method for the decontamination of sensitive surgical instruments. More recently, the availability of transgenic mice expressing the bovine *PRNP* transgene have become available, and with this their high susceptibility to bovine BSE prions has complemented the use of 301V in these types of experiments. A study published by Giles *et al*.[9] directly compared the effectiveness of decontamination of both bovine BSE and 301V in transgenic and VM mice, respectively. 301V was more sensitive to both heat and chemical denaturation than cattle BSE, suggesting that the physical properties of the 301V BSE strain have diverged slightly from those of cattle BSE. Despite the more recent availability of these transgenic rodent strains the 301V/VM infection system remains a useful, well characterised model for BSE in TSE research and allows direct comparison with numerous previous studies.

In the last 15 years or so prion research has been revolutionised by the demonstration of *in vitro* assays that are thought to replicate the molecular events occurring *in vivo* during prion infection and the conversion of PrP^{C} to the disease isomer PrP^{Sc}. First reported by Saborio and colleagues in 2001[10] the protein misfolding cyclic amplification (PMCA) assay is able to replicate prions *in vitro* within a source of PrP^{C} (generally produced from a healthy brain homogenate) during cycles of PrP^{C} to PrP^{Sc} seeded conversion followed by sonication with high frequency sound waves that break up aggregates of PrP^{Sc} to form new seeds or sites of nucleation. The products of this sensitive *in vitro* assay retain the biochemical characteristics of the prion seed and are infectious[11]. The sensitivity of the PMCA assay was improved by including the dilution of the reaction into fresh PrP^{C} substrate after an optimal period of amplification. This modification, known as serial PMCA (sPMCA)[12] has been widely adopted by the research community and has been applied to several rodent prion strains[13], scrapie in sheep[14,15], BSE in cattle[16], and CWD of cervids[17]. sPMCA can achieve levels of sensitivity significantly beyond that of animal bioassay[18] and these experiments take days or weeks to perform compared to the months to years of animal bioassay, and at a fraction of the cost. As such, amplification of prions by sPMCA can be used as a surrogate for measuring infectivity *in vivo*. To date, the 301V strain of BSE has not been used in sPMCA based studies. Here, we describe a high sensitivity 301V sPMCA that can, over a period of 5 days detect higher dilutions of infectivity than are attained by a 170–200 day bioassay within the VM mouse line.

Results and discussion

Dataset 1. Raw uncropped images of the Western blots shown in Figure 1

http://dx.doi.org/10.5256/f1000research.9735.d138638

A pool of 301V mouse brain homogenate was used to assess the efficacy of a new sPMCA assay alongside conventional 301V/VM bioassay. For the *in vitro* assay we used a 5 day amplification method and a murine VM substrate. This 301V sPMCA assay demonstrated assay sensitivity to 1×10^{-9} dilution of brain homogenate (Figure 1A). The batch-to-batch variability test of a further 3 substrate preparations detected 301V to at least the same level(Figure 1B). The observed variations in sensitivity within these limiting dilution experiments (between 10^{-9} and 10^{-11}) is likely a reflection of differences in individual substrate preparations that will be seen when making small volume preparations from limited numbers of brains.

The same 301V sample was also analysed in a VM mouse bioassay by limiting dilution and this bioassay detected infectivity in 1 out of 12 mice at the 10^{-8} dilution of 301V brain material (Table 1), equating to a 301V titre of the original brain pool of $10^{8.5}$ LD_{50}/g (as determined by Karber methodology[19]).

The 301V sPMCA assay can therefore detect PrP^{Sc} at a level at least tenfold more sensitive than the VM mouse bioassay, in a

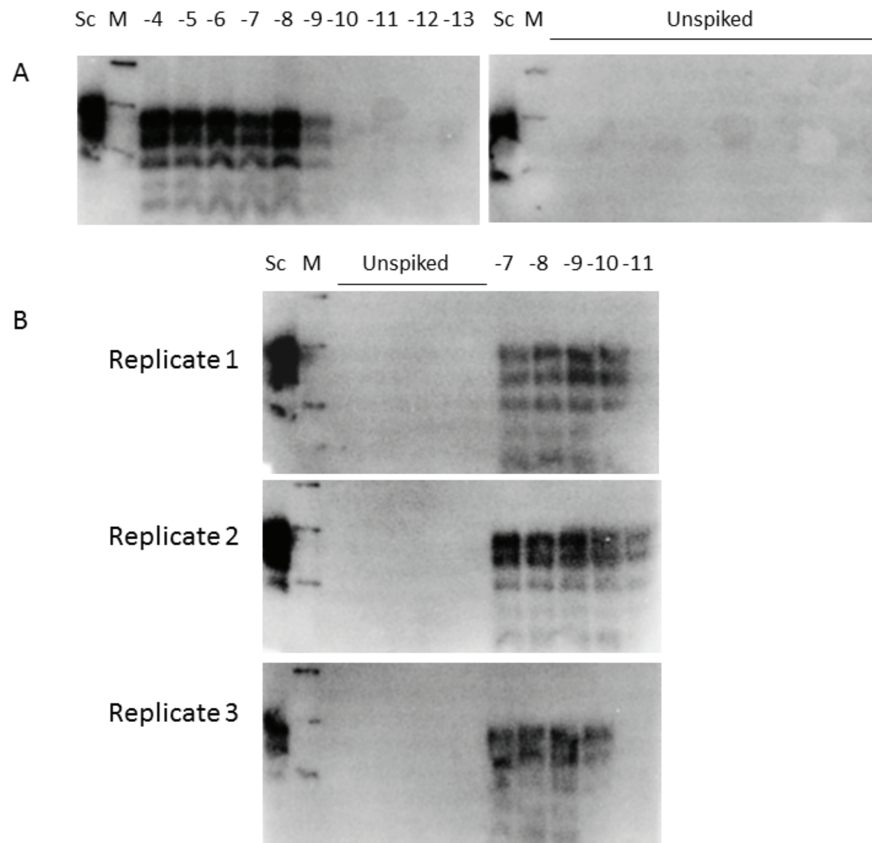

Figure 1. sPMCA amplification of 301V BSE. A. Reactions were seeded with 10 µls of 10^{-4} to 10^{-13} dilution of 301V brain (as indicated). Unspiked samples had 10 µl VM brain substrate only. Sc, scrapie positive brain sample was used as a blotting control. **B**. The assessment of reproducibility of the 301V sPMCA using three separate batches of VM substrate, each were seeded with 301V brain dilutions 10^{-7} to 10^{-11} (as indicated). Unspiked PMCA samples were always negative (a total of 15 replicates are shown). Western blots were probed with the anti-PrP antibody SHa31, M, molecular mass markers at 41, 30 and 22 kDa.

Table 1. Titration of 301V infected mouse brain. *Number of clinical and pathology positive mice/total injected. Mouse numbers exclude intercurrent deaths i.e. animals dying earlier than the 1st clinical case (there was one intercurrent death of a mouse receiving 10^{-7} brain dilution, and two receiving the 10^{-4} dilution). Total number of challenged mice were 6 per group for 10^{-4} and 10^{-5}, 12 per group for 10^{-6} to 10^{-9}.

301V Brain Dilution	No. mice positive*	Incubation period (days)	SD
10^{-4}	4/4	142	10
10^{-5}	5/6	149	14
10^{-6}	10/12	170	12
10^{-7}	4/11	191	18
10^{-8}	1/12	184	-
10^{-9}	0/12	-	-

total assay time taking little over a week. Whilst we report sensitivity of the assay at 5 days of amplification, it is very likely that much higher levels of sensitivity could be attained with additional rounds of amplification. The highest dilution of 301V infectivity that could be detected within the VM bioassay was a $1{\times}10^{-8}$ dilution of brain at 184 days post inoculation, or 26 times longer than the sPMCA assay. Maintaining animals within bioassay, including their category 3 containment make these kind of titration experiments very costly and time consuming to carry out. That, coupled with the ethical implications of use of animals means sPMCA could be the method of choice unless there is a good scientific reason for requiring to demonstrate infectivity (the ability to cause disease), or a requirement to monitor strain phenotype, as opposed to the surrogate marker of disease, PrP^{Sc} protein. A useful way of incorporating these two assays into future studies, could be to assess 301V seeding activity within a wide range of samples to identify those that contain PrP^{Sc}. Bioassay could then be used on a limited number of sPMCA-positive samples to confirm the presence of BSE infectivity. Another example of the application of sPMCA that has been routinely used for the detection of prions in a rodent

prion model is with cervid CWD[17]. In this instance, CWD amplification within cervid CNS tissue substrate is notoriously inefficient, and transgenic mice have been used as an animal bioassay model for infectivity studies and also to provide substrate to facilitate efficient *in vitro* amplification by sPMCA.

In summary, we have developed a reliable *in vitro* method (sPMCA) for the detection of PrP^{Sc} resulting from infection with 301V (mouse passaged BSE). The assay is at least as sensitive as mouse bioassay and can derive data on the presence of PrP^{Sc} in a fraction of the time. This will be useful in studies such as those looking at BSE decontamination where the screening of large numbers of samples is required.

Materials and methods

All use of animals, the collection of animal tissues and the use of such tissue was carried out in accordance with the Animal (Scientific Procedures) Act (ASPA) 1986, under licences from the UK Government Home Office (Project licence 60/2544). All animal experiments were subject to review and approval (01-124) by The Roslin Institute Ethical Review Committee and euthanasia methods were approved by the UK Home Office.

Bioassay

A serial dilution of pooled murine VM brains that were taken from 301V challenged animals was made as previously described[20]. A dilution series of this brain homogenate from 10^{-1} to 10^{-10} was made up in saline and used to inoculate groups of VM mice, bred in house at The Roslin Institute and of mixed sex, 6 weeks old (groups of 6 mice at 10^{-4} and 10^{-5}, 12 mice from 10^{-6} to 10^{-10}) with 20 µl of each dilution intracerebrally, as previously described[20]. Animals were observed daily for signs of ill health and euthanised by cervical dislocation when clinical signs of neurological disease or any intercurrent illness were observed. After euthanasia, brain tissue was confirmed as 301V positive or negative by detection of brain tissue vacuolation by light microscopy after Haematoxylin and Eosin staining[20]. This analysis was carried out blinded to the identity of the tissue in each case.

sPMCA

VM brains from healthy animals were supplied frozen, before preparation of the 10 % (w/v) homogenate substrate. Preparation of 10 % brain homogenates as substrates for sPMCA has been previously described[21]. Here, we included the sPMCA additive digitonin[22] (Sigma-Aldrich) which was added to reactions at 50 µg/ml. sPMCA reactions were assembled in 200 µl thin wall PCR tubes, and comprised 90 µl brain homogenate substrate with digitonin, three 2.4 mm Teflon beads (Precision plastic ball co. Ltd) and 10 µl of 301V sample to be amplified (10^{-4} to 10^{-13} dilution of 301V brain). Unspiked negative control samples were set up substituting the 301V seed with 10 µl VM brain substrate only. Reaction tubes were placed in a Misonix S3000 sonicating water bath set on a program of 10 seconds sonication every 30 mins, for 24 hours at a power setting of 190–200 W at 37°C. Every 24 hours, samples were diluted 1 in 10 into fresh VM brain substrate and sonicated for a further 24 hour round of repeated sonication and incubation retaining the same three Teflon beads throughout the 5 rounds of sPMCA. Amplifications were carried out for a total of 5 days. Dilutions of 301V brain homogenate are recorded as the dilution of brain spike before addition to the amplification reaction, ie 10^{-1} is 10 µl of a 10 % (w/v) preparation of brain, 10^{-2} is 10 µl of a 1 in 10 dilution of the 10^{-1} preparation of brain etc. All sPMCA was carried out at 37°C in a Misonix S3000 microplate horn.

Western blotting

sPMCA reaction products (10 µl) were digested using a final concentration of 50 µg/ml proteinase K (Sigma-Aldrich), for 90 minutes at 40°C. Samples were then boiled for 5 minutes in 1X LDS buffer (Invitrogen) and electrophoresed through a NuPAGE SDS-PAGE gel system (Invitrogen) using 12% (w/v) acrylamide gels. Molecular mass markers (prestained Seeblue plus2, Invitrogen LC5925) were run alongside samples. As a blotting control an aliquot of proteinase K digested (50 µg/ml proteinase K (Sigma-Aldrich), 60 minutes at 40°C) scrapie positive ovine brain (equivalent to 2 µl of a 10% w/v brain homogenate) was also loaded onto each SDS-PAGE gel. Proteins were transferred to PVDF (Roche) membrane by electroblotting, and the membranes were then blocked for 1 hour with 3 % (w/v) skimmed milk. Western blots were probed with the anti-PrP mouse monoclonal antibody SHa31 (SpiBio A03213) diluted to 1/80,000 and a polyclonal goat anti-mouse immunoglobulins Horse Radish Peroxidase conjugate (Dako P04477), diluted to 1:20,000, as previously described[21]. Blots were imaged after the addition of EZ-ECL HRP substrate (Geneflow) using an ICCD225 photon counting camera system and IFS32 image software (Photek Ltd).

Data availability

F1000Research: Dataset 1. Raw uncropped images of the Western blots shown in Figure 1. 10.5256/f1000research.9735.d138638[23]

Author contributions

RAS and NH conceived the study and provided both the 301V and VM biological material, BCM and KCG devised and directed the experiments, KB carried out the experiments. BCM wrote the manuscript, all authors proof read the manuscript before submission.

Competing interests

No competing interests were disclosed.

Grant information

This work was funded by DEFRA under project SE1433 (Robert Somerville) and by BBSRC Institute Strategic Grant BB/J004332/1 (The Roslin Institute).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Valleron AJ, Boelle PY, Will R, *et al.*: **Estimation of epidemic size and incubation time based on age characteristics of vCJD in the United Kingdom.** *Science.* 2001; **294**(5547): 1726–1728.
PubMed Abstract | Publisher Full Text
2. Bruce M, Chree A, McConnell I, *et al.*: **Transmission of bovine spongiform encephalopathy and scrapie to mice: strain variation and the species barrier.** *Philos Trans R Soc Lond B Biol Sci.* 1994; **343**(1306): 405–411.
PubMed Abstract | Publisher Full Text
3. Williams A, Lucassen PJ, Ritchie D, *et al.*: **PrP deposition, microglial activation, and neuronal apoptosis in murine scrapie.** *Exp Neurol.* 1997; **144**(2): 433–438.
PubMed Abstract | Publisher Full Text
4. Taylor DM, Brown JM, Fernie K, *et al.*: **The effect of formic acid on BSE and scrapie infectivity in fixed and unfixed brain-tissue.** *Vet Microbiol.* 1997; **58**(2–4): 167–174.
PubMed Abstract | Publisher Full Text
5. Taylor DM, Fernie K, McConnell I, *et al.*: **Solvent extraction as an adjunct to rendering: the effect on BSE and scrapie agents of hot solvents followed by dry heat and steam.** *Vet Rec.* 1998; **143**(1): 6–9.
PubMed Abstract | Publisher Full Text
6. Reichl HE, Foster PR, Welch AG, *et al.*: **Studies on the removal of a bovine spongiform encephalopathy-derived agent by processes used in the manufacture of human immunoglobulin.** *Vox Sang.* 2002; **83**(2): 137–145.
PubMed Abstract | Publisher Full Text
7. Grobben AH, Steele PJ, Somerville RA, *et al.*: **Inactivation of the bovine-spongiform-encephalopathy (BSE) agent by the acid and alkaline processes used in the manufacture of bone gelatine.** *Biotechnol Appl Biochem.* 2004; **39**(Pt 3): 329–338.
PubMed Abstract | Publisher Full Text
8. Mcleod AH, Murdoch H, Dickinson J, *et al.*: **Proteolytic inactivation of the bovine spongiform encephalopathy agent.** *Biochem Biophys Res Commun.* 2004; **317**(4): 1165–1170.
PubMed Abstract | Publisher Full Text
9. Giles K, Glidden DV, Beckworth R, *et al.*: **Resistance of bovine spongiform encephalopathy (BSE) prions to inactivation.** *PLoS Pathogens.* 2008; **4**(11): e1000206.
PubMed Abstract | Publisher Full Text | Free Full Text
10. Saborio GP, Permanne B, Soto C: **Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding.** *Nature.* 2001; **411**(6839): 810–3.
PubMed Abstract | Publisher Full Text
11. Weber P, Giese A, Piening N, *et al.*: **Generation of genuine prion infectivity by serial PMCA.** *Vet Microbiol.* 2007; **123**(4): 346–57.
PubMed Abstract | Publisher Full Text
12. Castilla J, Saá P, Soto C: **Detection of prions in blood.** *Nat Med.* 2005; **11**(9): 982–5.
PubMed Abstract | Publisher Full Text
13. Yoshioka M, Imamura M, Okada H, *et al.*: **Sc237 hamster PrP^{Sc} and Sc237-derived mouse PrP^{Sc} generated by interspecies *in vitro* amplification exhibit distinct pathological and biochemical properties in tga20 transgenic mice.** *Microbiol Immunol.* 2011; **55**(5): 331–40.
PubMed Abstract | Publisher Full Text
14. Thorne L, Terry LA: ***In vitro* amplification of PrP^{Sc} derived from the brain and blood of sheep infected with scrapie.** *J Gen Virol.* 2008; **89**(Pt 12): 3177–84.
PubMed Abstract | Publisher Full Text
15. Thorne L, Holder T, Ramsay A, *et al.*: ***In vitro* amplification of ovine prions from scrapie-infected sheep from Great Britain reveals distinct patterns of propagation.** *BMC Vet Res.* 2012; **8**: 223.
PubMed Abstract | Publisher Full Text | Free Full Text
16. Franz M, Eiden M, Balkema-Buschmann A, *et al.*: **Detection of PrP^{Sc} in peripheral tissues of clinically affected cattle after oral challenge with bovine spongiform encephalopathy.** *J Gen Virol.* 2012; **93**(Pt 12): 2740–8.
PubMed Abstract | Publisher Full Text
17. Kurt TD, Perrott MR, Wilusz CJ, *et al.*: **Efficient *in vitro* amplification of chronic wasting disease PrP^{RES}.** *J Virol.* 2007; **81**(17): 9605–8.
PubMed Abstract | Publisher Full Text | Free Full Text
18. Saá P, Castilla J, Soto C: **Ultra-efficient replication of infectious prions by automated protein misfolding cyclic amplification.** *J Biol Chem.* 2006; **281**(46): 35245–52.
PubMed Abstract | Publisher Full Text
19. Kärber G: **Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche.** *Archives of Experimental Pathology and Pharmacology.* 1931; **162**(4): 480–483.
Publisher Full Text
20. Grobben AH, Steele PJ, Somerville RA, *et al.*: **Inactivation of transmissible spongiform encephalopathy agents during the manufacture of dicalcium phosphate from bone.** *Vet Rec.* 2006; **158**(11): 361–366.
PubMed Abstract | Publisher Full Text
21. Maddison BC, Baker CA, Terry LA, *et al.*: **Environmental sources of scrapie prions.** *J Virol.* 2010; **84**(21): 11560–2.
PubMed Abstract | Publisher Full Text | Free Full Text
22. Gonzalez-Montalban N, Makarava N, Ostapchenko VG, *et al.*: **Highly efficient protein misfolding cyclic amplification.** *PLoS Pathogens.* 2011; **7**(2): e1001277.
PubMed Abstract | Publisher Full Text | Free Full Text
23. Gough K, Bishop K, Somerville R, *et al.*: **Dataset 1 in: A sensitive 301V BSE serial PMCA assay.** *F1000Research.* 2016.
Data Source