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Exploring PMCA as a potential in-vitro alternative method to mouse bioassays for the highly sensitive detection of BSE prions

Evaluierung der PMCA als In-vitro-Ersatzmethode zum Maus-Bioassay zur hochsensitiven Detektion von BSE-Prionen

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Summary

Classical bovine spongiform encephalopathy (C-BSE) belongs to the transmissible spongiform encephalopathies (TSE), which are also designated prion diseases since they are caused by the conversion of the host-encoded cellular prion protein PrP^{C} to its pathological isoform PrP^{TSE}. BSE carries a zoonotic potential as BSE prions cause variant Creutzfeldt-Jakob disease in humans. To date, C-BSE infectivity can only be detected by bioassay, e.g. highly sensitive bovine PrP transgenic mice (e.g. Tgbov XV mice). Recently, highly sensitive in-vitro prion seeding activity assays, such as the Protein Misfolding Cyclic Amplification (PMCA), have been developed, which work particularly well for the template-assisted prion conversion of scrapie prions, while a similarly efficient bovine C-BSE-prion amplification remained unavailable. In the here described study, we have therefore compared the analytical sensitivities of the transgenic Tgbov XV mouse bioassay and our C-BSE PMCA protocol by analysing serial dilutions of a BSE-positive bovine brainstem homogenate pool. As both methods were shown to possess comparable sensitivities, we propose the C-BSE PMCA as a potential in-vitro replacement method, allowing the reduction and refinement of mouse bioassays for the detection of cattle derived classical BSE prions by reducing them to only specific analytical applications.

Keywords: Bovine spongiform encephalopathy, infectivity, PrP^{BSE}, Protein Misfolding Cyclic Amplification, replacement of animal experiments

Zusammenfassung

Die klassische Bovine Spongiforme Enzephalopathie (C-BSE) gehört zu den Transmissiblen Spongiformen Enzephalopathien (TSE), welche auch als Prion-Erkrankungen bezeichnet werden, da ihnen die Konversion des wirtseigenen zellulären Prion-Proteins PrP^{C} in seine pathologische Isoform PrP^{TSE} zugrunde liegt. BSE birgt ein zoonotisches Risiko, da die neue Variante der Creutzfeldt-Jakob-Krankheit beim Menschen durch BSE-Prionen hervorgerufen wird. Bislang kann C-BSE-Infektiosität nur mittels Bioassay, z. B. in Rinder-PrP-transgenen Mäusen (z. B. Tgbov XV Mäusen) nachgewiesen werden. Unlängst wurden auf der Keimbildungsaktivität der Prionen basierende hochsensitive In-vitro-Methoden, wie die Protein Misfolding Cyclic Amplification (PMCA), entwickelt. Diese sind besonders effektiv in der In-vitro-Konversion von Scrapie-Prionen, während eine ähnlich effiziente Amplifikation von bovinen C-BSE-Prionen bisher nicht zur Verfügung stand. In der hier beschriebenen Studie wurde daher die analytische Sensitivität des transgenen Tgbov XV-Maus-Bioassays und unseres C-BSE-PMCA-Protokolls mittels Untersuchung einer seriellen Verdünnungsreihe eines BSE-positivem Hirnstamm-Homogenat-Pools verglichen. Aufgrund der so nachgewiesenen vergleichbaren Sensitivitäten beider Methoden schlagen wir die C-BSE-PMCA als potentielle In-vitro-Ersatzmethode vor, die die Reduktion und Verbesserung (Refinement) des transgenen Maus-Bioassays zum Nachweis von Rinder-C-BSE-Prionen erlaubt, da dieser auf spezielle analytische Anwendungen reduziert werden kann.

Schlüsselwörter: Bovine Spongiforme Enzephalopathie, Infektiosität, PrP^{BSE}, Protein Misfolding Cyclic Amplification, Ersatzmethoden zum Tierversuch

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Introduction

Prion diseases, or transmissible spongiform encephalopathies (TSE), are fatal neurodegenerative diseases of the central nervous system, including Creutzfeldt-Jakob disease (CJD) in humans as well as classical bovine spongiform encephalopathy (C-BSE) in cattle, scrapie in small ruminants and chronic wasting disease (CWD) in cervids. Moreover, BSE is a zoonotic disease, as the ingestion of BSE-contaminated food may cause the variant form of CJD (vCJD) (Bruce et al. 1997, Hill et al. 1997). Prusiner (1982, 1997) postulated that the TSE agent is a "proteinaceous infectious particle that lacks nucleic acid", referred to as prion. Prion diseases are caused by the conversion of the host-encoded cellular membrane-bound glycoprotein (PrP^{C}) to its abnormal isoform, the pathological prion protein (PrP^{TSE}). This involves a conformational change where the protein β-sheet content increases, due to partial refolding of the α-helical structures (Prusiner 1982, Pan et al. 1993, Prusiner 1998). According to the model, this newly formed PrP^{TSE} provides a template for the refolding of PrP^{C} into nascent PrP^{TSE} further during this conversion process (Prusiner 1982). Strong evidence supporting the protein-only hypothesis as well as the infectious and autocatalytic features of PrP^{TSE} was achieved by the in-vitro generation of prions, using the Protein Misfolding Cyclic Amplification (PMCA) method, where the in vitro generated PrP^{TSE} induced clinical TSE disease in Syrian hamsters (Castilla et al. 2005a).

Three decades ago the livestock industry was shocked by the emergence of BSE in cattle and other animal species, ten years later followed by the detection of the first vCJD cases in humans in a variety of countries. Due to the extremely long TSE incubation times in animals and humans, drastic public health protection methods were implemented especially since the year 2000, in order to reduce the human exposure risk to potentially infectious bovine tissues in food and pharmaceutical products. WHO has issued 'Guidelines on Transmissible Spongiform Encephalopathies in relation to Biological and Pharmaceutical Products' in which body tissues and fluids were classified into risk groups based on the detection of infectivity by mouse bioassay (as gold standard) and PrP^{TSE} by biochemical and immunohistological methods. These guidelines were put into force by the EU commission by the 'Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products' (EMA/410/01 rev.3) which are still valid to date. The European Pharmacopoeia contains a monograph addressing'Products with risk of transmitting agents of animal spongiform encephalopathies'. This monograph defines quality standards for the manufacture of human and veterinary medicinal products in the light of a possible TSE contamination. As a consequence, only products and extracts thereof can be used in pharmaceutical products for which the absence of BSE prion infectivity can be shown either by biological evidence or excluded by their geographical origin. A number of raw materials from bovines have been excluded as possible sources / starting material for the manufacturing of pharmaceuticals because there is still no scientific evidence available that would prove that they are reliably free of BSE infectivity. These restrictions concern all pharmaceutical companies worldwide. Thus, despite of the recent substantial decline of new BSE cases, producers of bovine material derived pharmaceuticals must still comply with regulations that demand sound scientific justifications if potential "high-infectivity tissues" from cattle (e. g. brain, spinal cord or eye tissue) are used.

Due to its high sensitivity, the bovine PrP transgenic mouse bioassay is the only accepted proof for the absence of BSE infectivity to date, and is therefore considered as gold standard to reveal infectivity in bovine tissues. However, assays using the prion seeding activity, like PMCA (Saborio et al. 2001) as well as quaking-induced conversion (QuIC) (Atarashi et al. 2008), have also enabled the detection of low quantities of prions, with sensitivity levels that are comparable or even higher than scrapie bioassays in Syrian hamsters and bank voles (Saá et al. 2006, Wilham et al. 2010, Boerner et al. 2013, Morales et al. 2013, Chianini et al. 2015). Recently published protocols for BSE prion amplification indicated similar PMCA improvements, targeting sensitivities that are at least comparable to those of bioassays in transgenic mice (Murayama et al. 2010, Balkema-Buschmann et al. 2011). Therefore, the here described study intends to show that the PMCA method is an alternative to the bioassay for this purpose.

Initially, the PMCA has been described by Saborio et al. (2001), providing a method that involves the cyclic amplification of a misfolded protein, which for the first time showed a highly efficient in-vitro replication of the pathological prion protein. Briefly, a PrP^{TSE} aggregate (seed) provides a template that enlarges by the conversion of excess PrP^{C} (substrate) in the reaction, followed by cycles of sonication and incubation (Saborio et al. 2001). Sonication breaks up the newly formed PrP^{TSE} aggregates, which then makes new seeds available for the conversion of PrP^{C} to PrP^{TSE} during the following incubation cycle (Saborio et al. 2001). Successive improvements were made by the introduction of programmable sonicators to automate the PMCA, as well as by the development of serial PMCA (Bieschke et al. 2004, Castilla et al. 2005a,b, 2006, Saá et al. 2006). This is achieved by the dilution of an aliquot of the reaction mix after one round of amplification and sonication cycles into fresh substrate for a new round of amplification and sonication cycles, which can be repeated numerous times, increasing the sensitivity of the assay. By this approach, the PMCA method enables prion detection in samples with even minor amounts of the agent by the amplification of PrP^{TSE} to levels which can be detected by standard protein biochemical methods, such as immunoblot (Saborio et al. 2001, Saá and Cervenakova 2015). Moreover, based on the relation between the number of PMCA rounds or cycles needed for the PrP^{TSE} detection and the PrP^{TSE} amount in a predefined sample, quantitative PMCA allows an estimated quantification of the PrP^{TSE} concentration in samples (Chen et al. 2010).

Besides the above mentioned merits for its use in basic prion research, the detection of PrP^{TSE} during the clinically silent phase in CJD-affected humans is a further perspective of the PMCA, which however has not yet been adjusted as a routine procedure for disease diagnostic (Saá and Cervenakova 2015). Using PMCA, PrP^{CJD} was detectable in urine (Moda et al. 2014) as well as in white blood cells and buffy coat (Lacroux et al. 2014) of patients affected with vCJD. Furthermore, PMCA also

TABLE 1: *Results of titration of the BSE brainstem pool in Tgbov XV mice and by PMCA*

Dilutions of BSE brainstem pool	End-point titration in Tgbov XV mice[1]	PMCA
$10^{-3.4}$	8/8, 340; ± 17.68	+++
$10^{-4.1}$	6/8, 444; ± 66.14	+++
$10^{-4.8}$	4/8, 442; ± 31.50	++
$10^{-5.5}$	5/8, 493; ± 35.33	++
$10^{-6.2}$	2/8, 574; ± 156.27	++
$10^{-6.9}$	2/8, 501; ± 145.66	+
$10^{-7.6}$	0/8, >621; n.a.	(+)
$10^{-8.3}$	4/8, 523; ± 69.50	+
$10^{-9.0}$	not done	–
negative control	0/20, >720; n.a.	–

[1] positive/inoculated mice, mean incubation time in days; ± standard error of the mean (SEM), n.a.: not applicable; PMCA: Protein Misfolding Cyclic Amplification, +++: positive from the first round, ++: positive from the second round, +: positive from the third round, (+): positive only in the fourth round, –: negative

contributed to the improvement of the non-invasive diagnostic methods of animal prion diseases (Saá and Cervenakova 2015), as prions were detected in the urine (Rubenstein et al. 2011), in white blood cells (Thorne and Terry 2008) and plasma (Rubenstein et al. 2010) of scrapie-infected sheep and in the urine of preclinical and clinically CWD-infected deer (Rubenstein et al. 2011). However, in contrast to other prions and animal species, the PMCA amplification of bovine BSE prions was not as successful for a long time, and only recently Murayama et al. (2010) and Franz et al. (2012) reported successful studies, albeit the essential sensitivity and the reproducibility of this method still remained to be optimised.

For these reasons, we aimed to determine whether in our hands, the PMCA provides a comparable sensitivity as the Tgbov XV mouse bioassay. Hence we used both methods to analyze dilutions of a BSE-positive brainstem homogenate pool of clinically diseased cattle. Our data support PMCA as a potential method for the replacement of the transgenic mouse bioassays in studies aiming for a sensitive detection of BSE prions.

Material and methods

Brainstem pool of clinically BSE-diseased cattle

In the frame of an early pathogenesis study, aiming to track the first eight months after an oral BSE infection, 20 unweaned Simmental calves were orally challenged with classical BSE using a 100 g dose of a BSE-positive brainstem homogenate, as described in more detail before (Ackermann et al. 2017). This inoculum was prepared from a brainstem pool of >50 clinically diseased cattle (kindly provided by APHA, Weybridge, United Kingdom).

End-point titration by Tgbov XV mouse bioassay

The infectivity load (LD_{50}) of the brainstem pool was determined as described before (Ackermann et al. 2017) using an end-point titration experiment in transgenic Tgbov XV mice over-expressing bovine PrP (Buschmann and Groschup 2005). Briefly, eight mice per group were intracerebrally inoculated with 30 µl of the following dilutions of the brainstem pool: $10^{-3.4}$, $10^{-4.1}$, $10^{-4.8}$, $10^{-5.5}$, $10^{-6.2}$, $10^{-6.9}$, $10^{-7.6}$ and $10^{-8.3}$.

All mice were monitored for the onset of clinical signs at least twice per week. Animals showing at least two clinical symptoms indicative of a BSE infection, such as hind limb paresis, abnormal tail tonus, behavioural changes and weight loss over several consecutive days (Buschmann and Groschup 2005) were sacrificed and brain samples were taken. The brains were analysed for the presence of PrP^{BSE} by digestion with 50 µg/ml Proteinase K at 55°C for 1 h followed by Western blot using mab L42 (r-biopharm, Darmstadt, Germany) at a concentration of 0.4 µg/ml as detection antibody. Any inconclusive results were verified by PTA immunoblotting (Gretzschel et al. 2005). Results of mice incubating at least 100 dpi were taken into evaluation.

Sensitivity assay using Protein Misfolding Cyclic Amplification (PMCA)

The earlier described PMCA protocol (Balkema-Buschmann et al. 2011, Franz et al. 2012, Ackermann et al. 2017) was applied with some modifications. Briefly, brain tissue from Tgbov XV transgenic mice (Buschmann et al. 2000) was used as the PrP^C source for the PMCA reaction. Brain samples were homogenised to a concentration of 10% (w/v) in PMCA conversion buffer to prepare the substrate solution. The template for the positive control PMCA reaction was a 10% (w/v) homogenate of bovine brain tissue in PBS. Dilutions of a confirmed BSE positive brain (10^{-3}, 10^{-6} and 10^{-9}) were prepared as positive controls in substrate solution. A brain sample of a confirmed BSE negative cattle served as a negative control. All controls and all samples were analysed in duplicate.

A sensitivity assay was performed to compare the analytical sensitivities of the used PMCA protocol and the Tgbov XV mouse bioassay. The same dilutions used in the above described end-point titration experiment in Tgbov XV mice of the brainstem pool were prepared and then tested by PMCA: $10^{-3.4}$, $10^{-4.1}$, $10^{-4.8}$, $10^{-5.5}$, $10^{-6.2}$, $10^{-6.9}$, $10^{-7.6}$, $10^{-8.3}$ and $10^{-9.0}$.

In this sensitivity assay, these dilutions were subjected to four rounds of PMCA with each 48 cycles of sonication for 20 s at a potency of 210–250 W (level 8), followed by a 30 min incubation.

The experiment was considered valid if at least the 10^{-3} and 10^{-6} dilutions of the positive control sample were clearly identified as positive after three rounds of amplification, and the negative control gave a negative result.

The obtained results were interpreted as follows: a clear PrP^{BSE} signal in all PMCA rounds was interpreted as +++ positive, a signal from the second PMCA round was interpreted as ++ positive, and a signal from the third PMCA round was interpreted as + positive. The results were reported as (+) if only the fourth round gave a positive result.

Results

To compare the analytical sensitivities of the applied PMCA protocol and the highly sensitive transgenic Tgbov XV mouse bioassay, we assayed a serial dilution of the brainstem pool used for a BSE challenge experiment of calves (Table 1).

End-point titration by Tgbov XV mouse bioassay

The infectivity load of the brainstem pool was determined by end-point titration in the transgenic mice, which revealed an LD_{50}-titer of $10^{-5.730}$ (95% confidence interval, $10^{-6.569}$–$10^{-4.891}$) (Ackermann et al. 2017).

Detailed mouse bioassay results of each dilution are shown in Table 1. In the group of mice inoculated with a $10^{-3.4}$ dilution of the brainstem pool, all mice developed BSE (100% attack rate) after a mean incubation time of 340 days post infection (dpi), indicating a high infectivity titre, which is between the values observed for $10^{4.4}$ ID_{50} g^{-1} (≈280 d) and 10^{3} ID_{50} g^{-1} (≈400 d) dilutions in earlier Tgbov XV bioassay studies (Buschmann and Groschup 2005, Balkema-Buschmann et al. 2011). For mice inoculated with the dilutions $10^{-4.1}$, $10^{-4.8}$ and $10^{-5.5}$, we observed 50% to 75% attack rates with incubation times between 444 and 493 dpi, in analogy to earlier Tgbov XV studies. The lower attack rates in TgbovVX mice inoculated with dilutions $10^{-6.2}$ and $10^{-6.9}$ as well as the longer incubation periods of >500 dpi (mice from groups challenged with dilutions $10^{-6.2}$, $10^{-6.9}$, $10^{-8.3}$) indicate low infectivity titers of less than $10^{2.5}$ ID_{50} g^{-1} (Buschmann and Groschup 2005, Balkema-Buschmann et al. 2011). It should be noted that in the dilutions close to the end-point, we observed variable attack rates (Table 1), which is a frequent observation in end-point infectivity studies.

Sensitivity assay using PMCA

PrP^{BSE} seeding activity was detectable by PMCA for all dilutions up to $10^{-8.3}$ (Table 1; Fig. 1). We observed highly positive reactions for low dilutions of the brainstem homogenate, identified as +++ positive reaction in dilution $10^{-3.4}$ and $10^{-4.1}$ (Fig. 1A, B). Intermediate seeding activity levels were revealed by ++ positive results between dilutions of $10^{-4.8}$, $10^{-5.5}$ and $10^{-6.2}$ (Fig. 1C–E). In the $10^{-6.9}$ and $10^{-8.3}$ dilutions, a + positive reaction revealed prion seeding activity (Fig. 1F, H). For the dilution of $10^{-7.6}$ a (+) positive results was obtained, as PrP^{BSE} was only detectable in the fourth round (Fig. 1G). In this study, the performance of a fourth round of PMCA clearly enhanced the sensitivity of the protocol, as compared to the protocol using three rounds of PMCA (Balkema-Buschmann et al. 2011, Franz et al. 2012). The negative control was clearly negative in all assays.

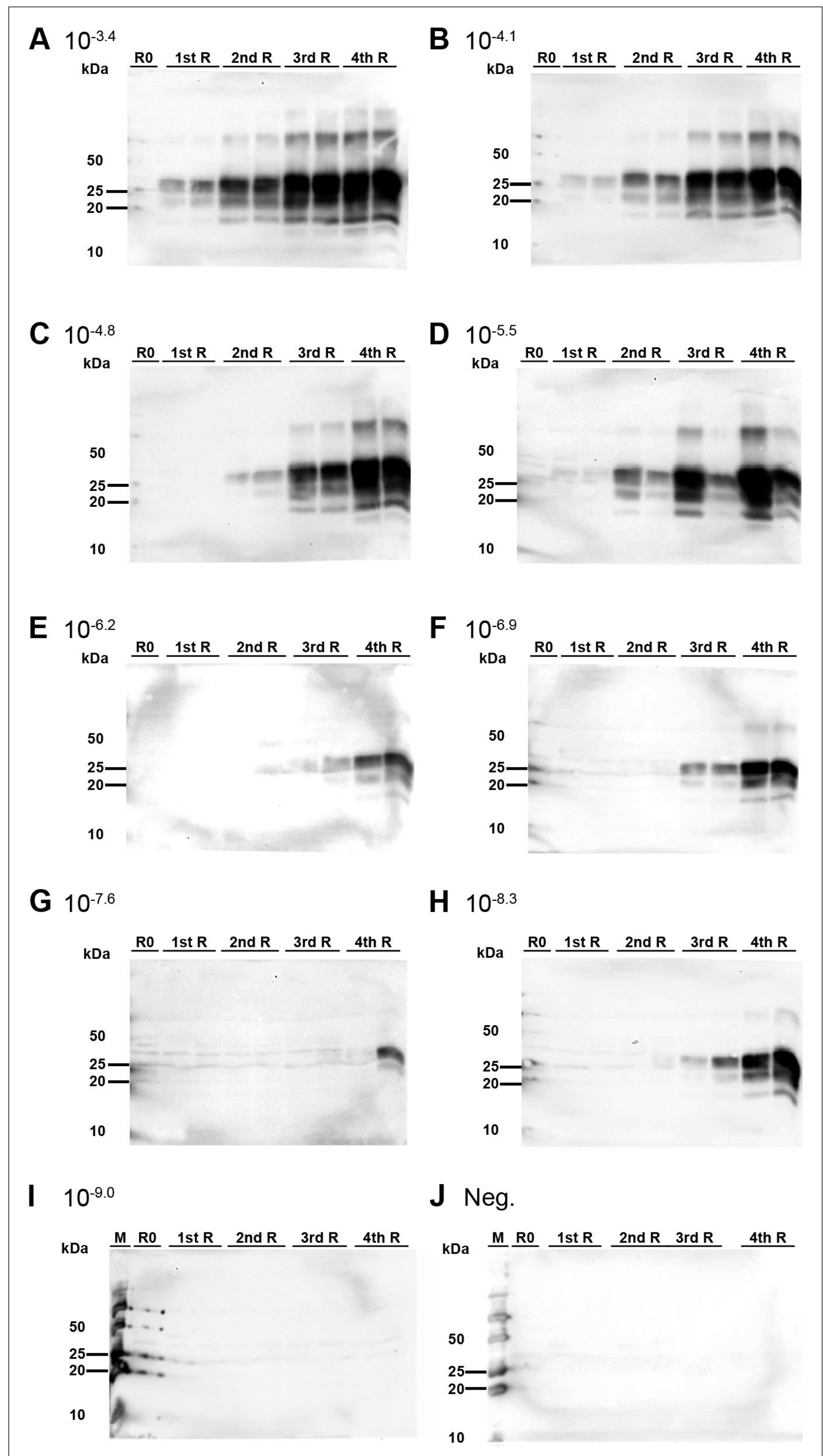

FIGURE 1: *PrP^{BSE} amplification by PMCA in $10^{-3.4}$ to $10^{-8.3}$ dilutions of a BSE positive brainstem homogenate pool. Seeding activity was present in the tested dilutions of $10^{-3.4}$ (A), $10^{-4.1}$ (B), $10^{-4.8}$ (C), $10^{-5.5}$ (D), $10^{-6.2}$ (E), $10^{-6.9}$ (F), $10^{-7.6}$ (G) and $10^{-8.3}$ (H). A, B: +++ positive PMCA reactions determined for the low dilutions of $10^{-3.4}$ (A) and $10^{-4.1}$ (B); C, D, E: intermediate levels of seeding activity (++ positive reactions) were revealed for the dilutions of $10^{-4.8}$ (C), $10^{-5.5}$ (D) and $10^{-6.2}$ (E); F, G, H: + positive reactions showed PrP^{BSE} amplification in the higher dilutions of $10^{-6.9}$ (F) and $10^{-8.3}$ (H), while in the $10^{-7.6}$ dilution (G) PrP^{BSE} was only detectable in one duplicate of the fourth PMCA round, representing a (+) positive result; I: no amplification was revealed in the $10^{-9.0}$ dilution; J: absence of amplification in the negative control (Neg.); all dilutions were analysed in duplicate and subjected to four rounds of PMCA; M: marker, R0: analyte homogenate diluted 1:10 in Tgbov XV brain substrate without sonication, R: round.*

Discussion

Given that the PMCA method was initially developed for the detection of the hamster-adapted scrapie strain 263K (Saborio et al. 2001), for which it still displays the highest diagnostic sensitivity, publications reporting a higher sensitivity of the PMCA as compared to hamster bioassays (Saá et al. 2006, Boerner et al. 2013, Morales et al. 2013) indicated that this in vitro test may also be suitable as an alternative to animal bioassays for other species. A protocol for PMCA detection of CWD prions provided a 10^5-fold higher sensitivity than a cervid PrP transgenic mouse bioassay (Johnson et al. 2012). In the case of C-BSE, prion detection by PMCA has been reported by our own earlier studies as well as by others to be a method with a sensitivity that is at least comparable to a bovine PrP transgenic mouse bioassay (Murayama et al. 2010, Balkema-Buschmann et al. 2011, Franz et al. 2012, Yoshioka et al. 2013). We aimed to confirm this for our currently applied PMCA protocol, in order to validate if PMCA may function as an alternative method to the mouse bioassay in future studies. Therefore we analysed serial dilutions of the brainstem homogenate pool used for a C-BSE challenge of calves by PMCA as well as by an end-point titration study in Tgbov XV mice.

The sensitivity assay revealed an overall good agreement between PMCA and Tgbov XV mouse bioassay, as indicated by BSE positive mice also in the group inoculated with the $10^{-8.3}$ dilution of the brainstem pool and clearly positive PMCA results up to a dilution of $10^{-8.3}$. Moreover, in our earlier study on ileal Peyer's patch samples of the calves from the same experiment, the PMCA results were in good accordance with those of the Tgbov XV mouse bioassay (Ackermann et al. 2017). The reliable agent detection shown by positive results obtained with these two methods up to a $10^{-8.3}$ dilution of a positive brainstem homogenate support the PMCA as an optimal method for analysing tissue samples with even low infectivity titers. Nevertheless, we did on some occasions observe transmission to transgenic mice after challenge with tissue homogenates of BSE-infected calves when the PMCA yielded negative results for the same sample (Ackermann et al. 2017, Ackermann et al. unpublished results). This observation prompts to reassess PMCA-negative or inconclusive samples by transgenic mouse bioassay, whereas in case of a positive result the PMCA proofs the presence of the BSE agent.

Comparable sensitivities of Tgbov XV mouse bioassay and a similar PMCA protocol were reported before by Balkema-Buschmann et al. (2011). Recently, O'Connor et al. (2017) also reported a protocol enabling C-BSE prion detection up to a $10^{-6}/10^{-7}$ dilution of a brain homogenate. In a different experiment, the addition of potassium dextran sulfate as enhancing reagents enabled the detection of a $10^{-9}/10^{-10}$ dilution of a cattle C-BSE brain sample, which made this PMCA 10^5-fold more sensitive than a bioassay in a different transgenic mouse line, namely Tg(BoPrP)4092 mice (Murayama et al. 2010). However, in our current titration experiment using the well established Tgbov XV mouse bioassay, we obtained solid detection up to a dilution of $10^{-6.9}$, indicating a higher sensitivity of Tgbov XV mice as compared to Tg(BoPrP)4092 mice. As discussed by Franz et al. (2012), the exclusion of additives, to avoid potential non-specific amplification effects, may restrict the analytic sensitivity of the PMCA to levels comparable to the bioassay (Balkema-Buschmann et al. 2011, Franz et al. 2012). Therefore, as concluded from our current results as well as from other studies, the PMCA can be considered as a highly sensitive detection method with sensitivities that are even comparable to those of animal bioassays. This implies that the PMCA may allow to considerably reduce the number of mice used in bioassays, which solely aim to prove the presence or absence of the BSE agent in a tissue sample.

However, concluded from the observations described above for BSE prion detection, the PMCA does not yet seem adequate to completely replace BSE mouse bioassays for the analysis of samples possibly containing minute amounts of BSE infectivity. Establishing protocols with high sensitivities for the detection of PrP^{BSE} seems more difficult as compared to PMCA detection of scrapie prions, for which protocols with higher sensitivities have been described. For sheep scrapie, detection of a 10^{-8} dilution (Thorne and Terry 2008, Rubenstein et al. 2010, Lacroux et al. 2012, Murayama et al. 2012) up to a 10^{-10} dilution (Murayama et al. 2012) of a 10% brain homogenate have been reported. Moreover, for 263K hamster scrapie, the detection of a 10^{-12} dilution after seven rounds, making the PMCA 4000-fold more sensitive than the bioassay, has been shown (Saá et al. 2006). In another experiment the same dilution was detectable already after two rounds of PMCA (Morales et al. 2013). Boerner et al. (2013) described an in-vitro end-point titration experiment using a quantitative PMCA, which is 300-fold more sensitive than the hamster bioassay, when after two rounds 1 x 10^{-11} g of a 263K stock were detectable. In that study, only one normal hamster brain was needed as PMCA substrate compared to a bioassay requiring five hamsters for the titration of one sample (Boerner et al. 2013). Thus, based on those studies it seems reasonable to suggest a replacement of the hamster bioassay for the detection of the laboratory strain 263K.

Regarding PMCA implementation, it has to be considered that increasing numbers of amplification rounds may favour spontaneous de novo generation of PrP^{TSE}, which argues for the restriction of the numbers of rounds performed (Saá et al. 2006, Franz et al. 2012, Morales et al. 2012). The importance of relevant controls when performing PMCA experiments is underlined by an experiment, in which the spontaneous formation of PrP^{TSE} from bacterially expressed recombinant prion protein without any addition of a PrP^{TSE} seed was reported after multiple cycles of amplification, which upon inoculation caused prion disease in wild-type mice (Wang et al. 2010). In addition to that, adequate negative controls are also of particular importance in order to monitor the risk of cross-contamination during the performance of such an ultra-sensitive method which enabled detection of a single infectious PrP^{TSE} unit (Saá et al. 2006, Saá and Cervenakova 2015). Controls monitoring the absence of cross-contamination and spontaneous prion formation are highly important for PMCA studies, which provide data relevant for risk assessments of bovine tissues. Thus, negative controls are absolutely essential in each PMCA run performed, thereby ensuring the reliability of PrP^{BSE} amplification in tested samples. We propose preparing analyte tissue sample homogenates in 0.9% saline solution, as implemented in our PMCA protocol. This enables the use of the same homogenate for PMCA

application as well as for inoculation of mouse bioassays to exclude sampling artefacts when comparing both methods. First, the sample should be tested by three rounds of PMCA, which in case of a positive reaction can reveal the final result. When the third round of PMCA still provides a negative result, a fourth round should be performed to provide ultra-sensitive analysis of low titre homogenates. In case of a PMCA-negative or inconclusive result, subsequently the same homogenate should be inoculated into transgenic bioassay mice over-expressing bovine PrP, as in this study used Tgbov XV mice (Buschmann et al. 2000, Buschmann and Groschup 2005). Using our protocol (performing three rounds of PMCA), one Tgbov XV mouse brain provides sufficient substrate for analysing four samples by PMCA compared to the use of 40 Tgbov XV mice necessary for bioassaying one bovine sample with an expected negative result. Using this approach would be an improvement regarding the reduction of the number of mice used for BSE bioassays, whilst ensuring reliable final results, given that comparable sensitivities of both methods protocols have been demonstrated by parallel titration of a BSE-positive reference homogenate. Another clear advantage of the PMCA is the time frame necessary to obtain a result, which is one week for the PMCA, in contrast to two years in case of the transgenic mouse bioassay.

In conclusion, the PMCA is a promising alternative method to the transgenic mouse bioassays, even if a full replacement of BSE bioassays by PMCA currently remains a goal to aim for in the long term. Our current PMCA protocol enables the reliable prion detection in samples with even low titers. Thereby, indeed the PMCA can significantly reduce the number of mice used in bioassays aiming only at the detection of the presence of the BSE agent. For samples with extremely low titers, which cannot be completely excluded in case of a negative IHC or PMCA result, we suggest the further verification by mouse bioassay to ensure the reliability of negative results. This is particularly important for studies providing data relevant for risk assessments regarding public health protection. Thereby, only few mice are likely to develop clinical BSE-symptoms due to the extremely low titers in such samples, resulting in a refinement of the still necessary bioassays. Finally, efforts to further improve the sensitivity of BSE-PMCA protocols are desirable, in order to extend the C-BSE-PMCA to a full in-vitro replacement method of mouse bioassays.

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Conflict of interests

The authors have no potentially competing interests.

Ethical approval

The challenge experiments in cattle and mice described in this manuscript were approved by the competent authority of the Federal State of Mecklenburg-Western Pomerania, Germany on the basis of national and European legislation, namely the EU council directive 2010/63/EU for the protection of animals used for experiments (file number: 7221.3-1.1-037/13).

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Authors contribution

This study was designed by ABB, IA and MHG and the experimental work was carried out by IA, ABB, MK, JCS and OIF. Data were analysed by IA, ABB and MHG. The manuscript was written by IA, ABB and MHG and proofread by all coauthors.

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