Journal of Hospital Infection 132 (2023) 125–132

Available online at www.sciencedirect.com

Journal of Hospital Infection

journal homepage: www.elsevier.com/locate/jhin

Kinetics of the reduction of Creutzfeldt–Jakob disease prion seeding activity by steam sterilization support the use of validated 134°C programmes

K.A. Schwenke [a,b,*,†], K. Wagenführ [a,†,‡], M. Thanheiser [b], M. Beekes [a]

[a] *Prion and Prionoid Research Unit, Division Proteomics and Spectroscopy, Centre for Biological Threats and Special Pathogens, Robert Koch Institute, Berlin, Germany*

[b] *Division Hospital Hygiene, Infection Prevention and Control, Department of Infectious Diseases, Robert Koch Institute, Berlin, Germany*

ARTICLE INFO

Article history:
Received 13 June 2022
Accepted 13 August 2022
Available online 7 October 2022

Keywords:
Creutzfeldt–Jakob disease (CJD)
Prions
Steam sterilization
Seeding activity
Protein misfolding cyclic amplification (PMCA)

SUMMARY

Background: Prions are renowned for their distinct resistance to chemical or physical inactivation, including steam sterilization. Impaired efficacy of inactivation poses a risk to patients for iatrogenic transmission of Creutzfeldt–Jakob disease (CJD) via contaminated surgical instruments.

Aims: Most established prion inactivation methods were validated against scrapie agents, although those were found to be generally less thermostable than human prions. Thus, knowledge gaps regarding steam-sterilization kinetics of CJD prions should be filled and current guidelines reviewed accordingly.

Methods: Prion inactivation through widely recommended steam sterilization at 134°C was assessed for several holding times by analysing the residual prion seeding activity using protein misfolding cyclic amplification (PMCA).

Findings: Scrapie 263K was found to be the least thermoresistant prion strain showing no seeding activity after 1.5 min at 134°C, while variant CJD was the most stable one demonstrating some seeding activity even after 18 min of steam sterilization. Sporadic CJD subtype VV2 exhibited residual seeding activity after 3 min, but no detectable activity after 5 min at 134°C.

Conclusion: Validated steam sterilization for 5 min at 134°C as previously recommended for the routine reprocessing of surgical instruments in contact with high-risk tissues is able to substantially reduce the seeding activity of CJD agents, provided that no fixating chemical disinfection has been performed prior to sterilization and that thorough cleaning has reduced the protein load on the surface to less than 100 μg per instrument.

DOI of original article: https://doi.org/10.1016/j.jhin.2022.09.020.

* Corresponding author. Address: Robert Koch Institute, Nordufer 20, 13353 Berlin, Germany. Tel.: +49 30 187542637.
E-mail address: SchwenkeK@rki.de (K.A. Schwenke).
† These authors contributed equally to this work.
‡ Present address: Central Authority of the Laender for Health Protection with regard to Medicinal Products and Medical Devices, Bonn, Germany.

https://doi.org/10.1016/j.jhin.2022.08.014

Introduction

Prions — the causative agents of transmissible spongiform encephalopathies (TSEs) — are infectious, self-replicating protein seeds that are thought to essentially consist of pathologically misfolded and aggregated conformational isomers of the cellular prion protein (PrP^{C}), referred to as PrP^{Sc} or PrP^{TSE} [1,2]. Due to their molecular structure, prions exhibit a remarkable resistance to common chemical and physical decontamination procedures that effectively inactivate conventional pathogens. While for chemical disinfection, protein denaturizing agents such as sodium hydroxide, sodium hypochlorite or guanidine thiocyanate are effective against prions, many common disinfectants based on, e.g., alcohols, glutaraldehyde or peracetic acid fail to inactivate or even fixate prions [3]. Furthermore, prions are renowned for their particular strong heat resistance exceeding even that of bacterial endospores. The standard autoclaving programmes for sterilization of medical instruments, 15 min at 121°C or 3 min at 134°C under saturated steam pressure, sufficiently inactivate all micro-organisms including endospores [4,5], but subpopulations of prions can withstand especially the first condition [6—9]. Over time, while the unusual prion agent and the nature of its infectivity have been deciphered, at least 500 cases of iatrogenic Creutzfeldt—Jakob disease (iCJD) occurred worldwide since the first report in 1974 [10—12]. The majority of cases resulted from treatments with prion-contaminated human-derived growth hormones and dura mater grafts; however, stereotactic electrodes and surgical instruments have also been reported in low numbers as transmission sources of iCJD. The prolonged pre-symptomatic phase of prion diseases, difficulties in early diagnosis of CJD and the distribution of prions in extraneural tissues [13—15] harbour a risk for prion contaminations on non-disposable instruments used in patients with sub- or preclinical CJD. Because prions have a high tenacity to stainless-steel surfaces [16,17], and drying or heat-fixation of tissue can further stabilize the thermoresistant subpopulations [18], persistent concerns were raised for iatrogenic prion transmission via medical devices, despite the few cases actually observed so far. Steam sterilization has been established as a highly relevant and effective method of countering such risk, which can be further improved by chemical cleaning and disinfection beforehand to achieve optimal decontamination. The World Health Organization (WHO) recommends in its guideline *WHO Infection Control Guidelines for Transmissible Spongiform Encephalopathies* [19] a thorough chemical disinfection through immersion in sodium hydroxide or sodium hypochlorite for tolerant instruments prior to steam sterilization. If medical devices cannot be chemically treated, a prolonged steam sterilization for 18 min at 134°C is recommended with the caveat that prion infectivity will not be completely but only largely reduced. The German guideline *Hygiene Requirements for the Reprocessing of Medical Devices* [20] (for English translation see http://go.nature.com/2vjyysf) suggests a combination of (pre-)cleaning, chemical disinfection and subsequent steam sterilization at 134°C for at least 5 min; in case of insufficient chemical cleaning the holding time should be increased to 18 min. French authorities generally recommend steam sterilization of 18 min at 134°C for all medical instruments [21]. However, most of the guidelines for prion decontamination were not validated against CJD but scrapie prions due to the wider availability of sensitive animal bioassays [18,22—24]. Scrapie prions, though, were found to be substantially less resistant to steam sterilization than other TSE agents, especially bovine spongiform encephalopathy (BSE) [7,25,26] and BSE-derived variant CJD (vCJD) [9]. Thereby, vCJD prions are of increased concern because of their widespread tissue distribution in affected individuals, which is not limited to neural and lymphatic tissues only but also extends to peripheral ones such as blood or muscles [27]. Accordingly, many studies addressing safe reprocessing of medical devices focused on vCJD but not the much more frequent sporadic forms of CJD (sCJD), which account for approximately 85% of all CJD cases [28]. Based on current knowledge, it seems rather unlikely that new vCJD cases will occur frequently in the future, while the efficacy of steam sterilization against sCJD prions remains to be examined in more detail. In this study we evaluated the sensitivity of one type of sCJD in relation to 263K scrapie and vCJD towards steam sterilization for different holding times at 134°C. For this, we determined the reduction of their proteinaceous seeding activity, the basic principle underlying prion replication, using highly-sensitive protein misfolding cyclic amplification (PMCA). PMCA mimics the nucleation-dependant polymerization of prions in a cyclic and accelerated process, and allows a direct titration of the seeding activity of several prion strains *in vitro* without the need for animal bioassays [29—33].

Methods

Brain tissue samples

All applicable international, national, and institutional guidelines for the care and use of animals for scientific purposes were followed. Euthanasia of Syrian hamsters and transgenic mice was performed under isofluorane anaesthesia and was acknowledged by the institutional and local governmental authorities (Landesamt für Gesundheit und Soziales Berlin, Germany; registration IDs: T 0191/17, TN 0001/20). 263K brain tissue was taken from stock obtained from terminally ill Syrian hamsters at Robert Koch Institute (approval ID: G0085/00 [33]).

Human tissue samples were used in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Sampling and use of tissues from CJD patients for scientific purposes were undertaken with the understanding and written consent of each donor or authorized caregiver. Brain tissue of a patient diagnosed with vCJD was kindly provided by National Creutzfeldt—Jakob Disease Surveillance Unit, Edinburgh, UK, and brain tissue of one donor diagnosed with sCJD VV2 by Prof. Walther Schulz-Schaeffer, Saarland University, Homburg, Germany. Experimental use of these tissues was approved by the East of Scotland Research Ethics Service (No. 16/ES/0084) and the Ethics Committee of the University Medical Centre Göttingen (No. 11/11/93), respectively.

Steam sterilization

Of each tissue sample 10% (w/v) brain homogenates in phosphate-buffered saline (PBS) (pH 7.4) were prepared by sonication. Ten microlitres each of 10% TSE-brain homogenate or 10-fold dilutions (10^{-1} to 10^{-9}) in normal brain homogenate (NBH) as reference values were surface-dried in microtubes using a SpeedVac concentrator Servant DNA 120 (Thermo Fisher, USA) running for 20 min at medium dry rate.

Samples comprising undiluted brain homogenate were subjected to pre-vacuum steam sterilization at 134°C and 3000 mbar for the indicated holding times in a pre-heated custom-made autoclave (resistometer HP, #1/0459, MMM Münchener Medizin Mechanik, Germany). One thermo-pressure and seven thermo data loggers (calibrated with ±0.1°C/1 mbar, ebro, Xylem Analytics, Germany) enabled precise parameter control.

Protein misfolding cyclic amplification

Steam-sterilized samples and their respective dilutions as well as negative controls (NBH only) and positive controls (untreated and undiluted brain homogenate) were directly analysed by PMCA as described elsewhere [31,33,34] with modifications [32,35]. Due to a change of PMCA devices from Misonix 3000 (Misonix, US; original protocol) to Q700 (QSonicator, USA; updated protocol) ultrasonication processor and the use of a heating bath circulation thermostat (CC–304B, Peter Huber Kältemaschinenbau AG, Germany) for more precise temperature control, protocols required adaptations between the experiments. Reproducibility of data from the new setup was strictly evaluated and confirmed. Complete data sets were repeated twice for selected holding times, whereas incomplete data sets were filled up and the results compared with existing data.

As conversion substrate for PMCA 10% (w/v) NBH of perfused brains from Syrian hamster or transgenic mice expressing human PrP with codon 129 polymorphisms MM or VV (tg_hu(129M) or tg_hu(129V), respectively [36]) in conversion buffer (PBS, 1% Triton X-100, protease-inhibitor cocktail cOmplete (Roche, Switzerland), pH 7.4; partly supplemented with Chondroitin sulphate (CHS; Sigma-Aldrich, USA) or heparin (LKT Labs, USA)) were used. For dissolving the dried samples, the respective PMCA substrate was added to each microtube of either control, reference or steam-sterilized sample and sonicated at 300 W for 30 s prior to transfer to the PMCA microtubes equipped with beads (either 20-mg glass beads (0.75–1 mm; Roth, Germany) or two Teflon-coated beads (1/16″, McMaster-Carr, USA)). The conditions for all PMCA reactions are listed in detail in Table I.

Western blot

PMCA products collected from each round were digested with 75 μg/mL (VV2 and vCJD) or 150 μg/mL (263K) proteinase K (Roche, Switzerland) for 45 min or 1 h, respectively, at 55°C in the presence of 1% sarcosyl and 0.06% SDS, subsequently centrifuged at 18,700 g for 1 min, and the supernatants denatured in sample loading buffer at 110°C for 10 min. Ten microlitres per sample were run on Mini-PROTEAN 4–12% TGX protein gels (Bio-Rad, USA) for SDS-PAGE and electrotransferred on PVDF membranes. Western blot was performed with anti-PrP monoclonal antibody 3F4 (1:2000; in-house production) and anti-mouse IgG alkaline phosphatase-linked secondary antibody (1:5000; Dako, USA). Stained proteins were visualized with CDP-Star chemiluminescent substrate (Thermo Fisher, USA) for alkaline phosphatase chemiluminescence reaction on Amersham Hyperfilm™ ECL films (GE Healthcare, USA). Images were created in Illustrator 2021 (Adobe, USA) and graphs of analysed data in GraphPad Prism 9 (USA).

Results

Establishment and optimization of the experimental setup

A direct correlation between the molecular seeding activity of animal or human prions *in vitro* and their biological infectivity *in vivo* has been demonstrated in different studies [31,32,37,38]. On this basis, infectivity can be determined by fast and easy titration of their seeding activity *in vitro* by, e.g., PMCA. Because the sensitivity of PMCA assays for prion seeding activity is generally higher than that of animal bioassay for prion infectivity [32,37,38], such an *in vitro* approach is particularly useful for assessing residual infectivity after inactivation procedures.

In this study 10 μL of 10% (w/v) brain homogenates containing 100 μg of total protein [39] were used as standardized samples. This amount of total protein reflects the maximum of residual proteinaceous load found on surgical instruments after chemical cleaning [40] and also corresponds to the

Table I
Overview on protein misfolding cyclic amplification (PMCA) reaction conditions of the tested prion isolates and comparison of the previous and the current PMCA protocols

Prion isolate	PMCA version	NBH substrate	Conversion buffer supplements	Beads	Reaction volume	Temperature	Sonication	Passaging	Number of rounds
263K	Original (unmodified)	Hamster	4 mM EDTA	Glass	150 μL	38°C	200 W 40 s/59.3 min	1:5 after 24 cycles	4
vCJD	Original	tg_hu (129M)	4 mM EDTA 400 μg/mL CHS	Glass	150 μL	37°C	200 W 40 s/59.3 min	1:2 after 24 cycles	8
vCJD	Updated	tg_hu (129M)	6 mM EDTA 100 mM NaCl 400 μg/mL CHS	Teflon	100 μL	37°C	170 W 30 s/29.5 min	1:6.67 after 48 cycles	8
VV2	Original	tg_hu (129V)	4 mM EDTA 400 μg/mL CHS	Glass	150 μL	37°C	200 W 40 s/59.3 min	1:3 after 48 cycles	8
VV2	Updated	tg_hu (129V)	6 mM EDTA 100 mM NaCl 100 μg/mL heparin	Teflon	100 μL	37°C	170 W 30 s/29.5 min	1:4 after 48 cycles	5

CHS, chondroitin sulphate; NBH, normal brain homogenate; vCJD, variant Creutzfeldt–Jakob disease.

maximum acceptable amount on reprocessed medical devices prior to steam sterilization according to German guidelines [20]. Although brain macerates might be more challenging for decontamination procedures [9], brain homogenates have long been considered to represent a worst-case paradigm in prion decontamination, especially when fixed on surfaces by air-drying as in this study [3,41–43]. Furthermore, other than macerates, brain homogenates have the advantage of providing homogeneous and standardizable samples in experimental series, which is another reason why they have been frequently used in prion inactivation studies [23,44–47].

In order to examine variations of different prion strains with regard to their tolerance to steam sterilization, three representatives were chosen: the hamster-adapted scrapie strain 263K as a frequently used model agent reaching high titres but being considered as non-pathogenic to humans [48], sCJD type VV2 as a human prion isolate of ongoing practical relevance, and vCJD as it is known to exhibit a particular strong heat resistance among prions next to BSE [9].

For inactivation kinetics the reduction after holding times of 0, 0.5, 1.5, 3, 5 and 18 min at 134°C were determined. Thereby, 0 min refers to the heating phase of the autoclave including the evacuation time of the chamber, 3 min represents the standard holding time for sterilization of surgical instruments [4], while 5 min follows the aforementioned German guideline [20] for routine reprocessing of medical devices in contact with high-risk tissues.

In our study PMCA conditions for the three isolates 263K, VV2 and vCJD were adjusted at first, such that a precise gradation for 10-fold dilutions was obtained after each round. Therefore, a sufficient sensitivity comparable between the strains was as essential as a moderate amplification rate to avoid indistinguishable PMCA results for adjacent dilutions. Furthermore, PMCAs had to be highly reproducible between the independently performed experiments, which could also be ensured after updating our PMCA setup by the described adjustments.

Prion seeding activity of the PMCA products from steam-sterilized samples and reference brain homogenates was determined by the amplified proteinase K-resistant PrP^{res} in Western blot. Analysis of the results was carried out based on the occurrence and intensity of PrP^{res} bands and was independently performed by two operators. Western blot signals of the steam-sterilized samples were then correlated to the matching 10-fold dilutions of their respective reference sample. In case of signals lying between two serial dilutions of the reference sample, the lower dilution was counted. The respective negative and positive controls allowed monitoring of the correct performance of the PMCA runs. By correlating the residual seeding activity of steam-sterilized samples to the defined 10-fold dilutions of reference brain homogenates, the $\log_{10}$-reduction factors could be calculated. An exemplary Western blot of one experiment with sCJD VV2 is shown in Figure 1 to illustrate the analytical comparison of reference and steam-sterilized samples after PMCA.

sCJD VV2 prions exhibit a lower resistance to steam sterilization than vCJD prions

The special autoclave (resistometer) used in this study exhibits a highly precise and fast heating capacity, enhancing the reproducibility between experiments, in particular for short holding times, which cannot be achieved by standard autoclaves. The resistometer was pre-heated according to the manufacturer's instructions before performing the experiments to ensure equal heating curves to the final temperature of 134°C for each run. This heating time was defined as 1 min as depicted in Figure 2, although the exact time varied by seconds between different runs. An exemplary temperature profile of a steam-sterilization run is depicted in Supplementary Figure S1. Inactivation of each prion isolate was examined in at least three independent experiments run in duplicate (except for 263K, 3 and 5 min: $N = 2$). In Table II the means of the $\log_{10}$-reduction factors for each holding time as well as the number of samples and experiments are presented. Figure 2 shows the reduction of seeding activity over holding time for the three prion agents.

It is clearly visible that during the heating to 134°C a substantial amount of seeding active material already became inactivated. In general, the first minutes account for the strongest inactivation until the reduction slows down. Thereby, for 263K the heating time already led to a reduction of seeding activity of 7 $\log_{10}$ (holding time 0 min), while in two of three experiments this agent was fully inactivated after 0.5 min holding time only and in the third after 1.5 min – corresponding to a mean reduction factor of 8.75 and 9.5 $\log_{10}$, respectively. VV2 shows a strong reduction until a holding time of 1.5 min of about almost 8 $\log_{10}$. After 5 min a mean reduction of 8.9 $\log_{10}$ was achieved, which constitutes a complete inactivation of detectable seeding activity in all tested samples, depending on the achieved sensitivity in the respective run. Until 1.5 min holding time, the seeding activity of vCJD prions showed a similarly shaped reduction curve as that for VV2, though with a somewhat lower reduction of about 7 $\log_{10}$. However, other than VV2 the reduction profile became increasingly static with a final reduction of 8 $\log_{10}$ only. In contrast with 263K and VV2, a resistant fraction of vCJD withstood steam sterilization at 134°C even for 18 min, with a residual seeding activity equal to the 10^{-9} dilution of untreated vCJD brain homogenate in all samples.

A substantial variation in the detected residual seeding activities was observed for all three prion agents between the experiments, in particular for samples subjected to short holding times less than 1.5 min. However, these deviations increasingly diminished with longer holding times. Because variations occurred between experiments and not between the replicates within one run, the surface drying process and hence stabilization of prions seemed to differ despite intense efforts for standardization. In addition, the detection limits of seeding activities were found to vary up to one order of magnitude between independent runs, resulting in further variation of the reduction factors despite equally complete inactivation of detectable seeding activity.

Discussion

In this study, resistance of prions to pre-vacuum steam sterilization at 134°C was examined over different holding times between 0 and 18 min. In order to address the well-known strain differences in this regard, we compared the three prion isolates scrapie 263K, sCJD subtype VV2 and vCJD. Our results confirm previous studies: 263K was found to be the least thermoresistant strain showing no detectable seeding activity after 1.5 min at the latest, while vCJD was the most resistant one with residual fractions of seeding activity

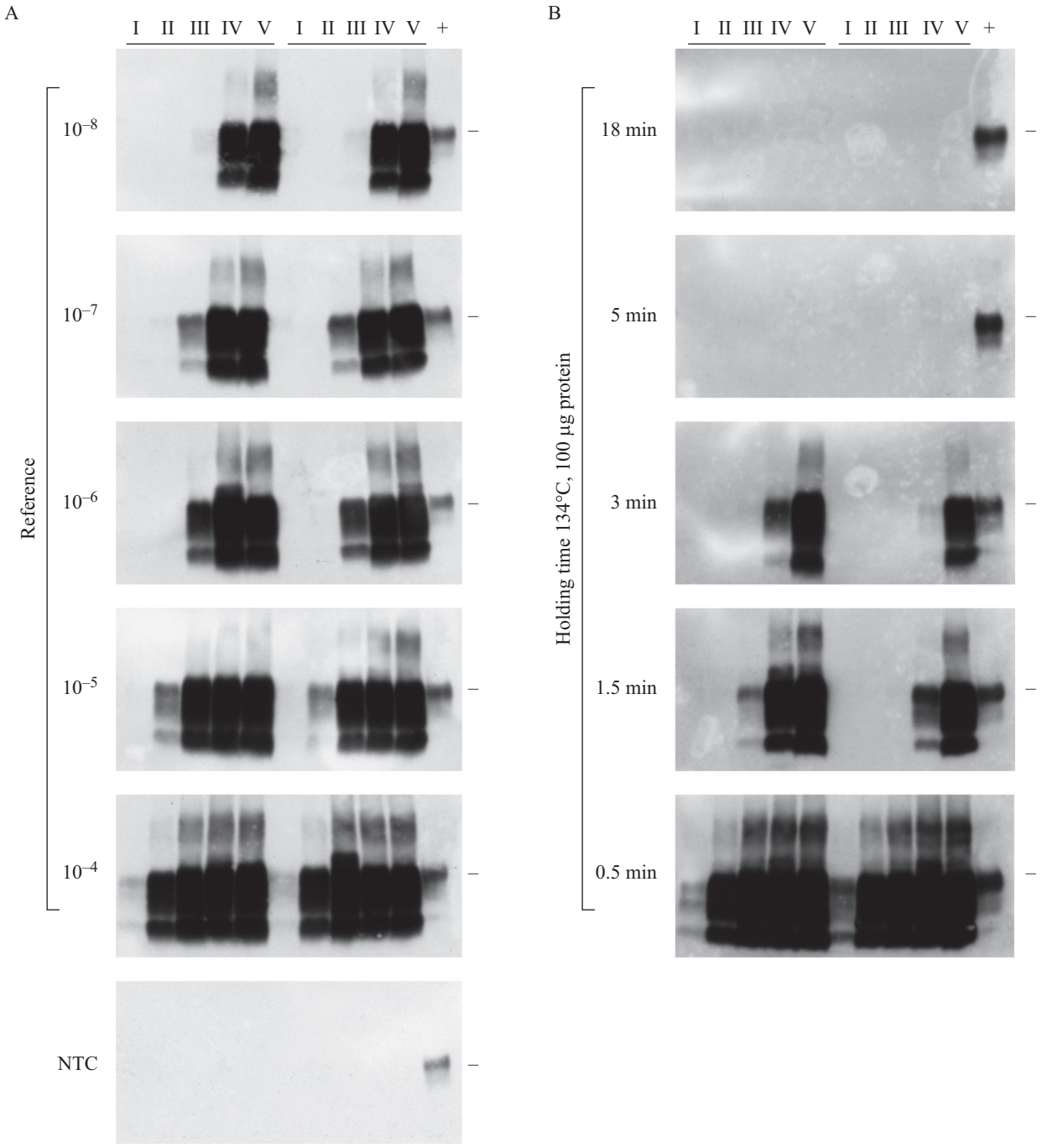

Figure 1. Exemplary illustration of one steam sterilization experiment with sporadic Creutzfeldt–Jakob disease (sCJD) VV2. Western blots of duplicate protein misfolding cyclic amplification (PMCA) samples are shown from PMCA rounds I–V. Reference samples in serial 10-fold dilutions of untreated sCJD VV2 brain homogenate are depicted on the left (a), while samples subjected to steam sterilization for different holding times at 134°C are shown on the right (b). Steam-sterilized samples were then correlated with the matching dilution of the reference samples (here: residual seeding activity after 0.5 min: $\geq 10^{-4}$, 1.5 min: $\leq 10^{-7}$, 3 min: $< 10^{-8}$, 5 and 18 min: $\ll 10^{-8}$). NTC, negative control. +, loading control (10% 263K brain homogenate, diluted 1:1000); black lines indicate 30 kDa.

persisting even after 18 min of steam sterilization in all experiments. In particular, the lack of data for sCJD previously raised concerns of a thermostability equal to vCJD and thus a risk for transmission via surgical instruments. Here we could demonstrate that the analysed VV2 isolate as a relevant example for sCJD prions ranged clearly below vCJD but above 263K in terms of heat resistance: at 134°C complete inactivation of detectable seeding activity was achieved after 5 min but not after 3 min. It can be assumed that the single VV2 and vCJD isolates examined in our study are representative of the thermostability of their strains, because according to the prion hypothesis prion strain characteristics are molecularly enciphered in the conformation of PrP^{TSE} of the respective strain. Thus, the differences observed between both prion

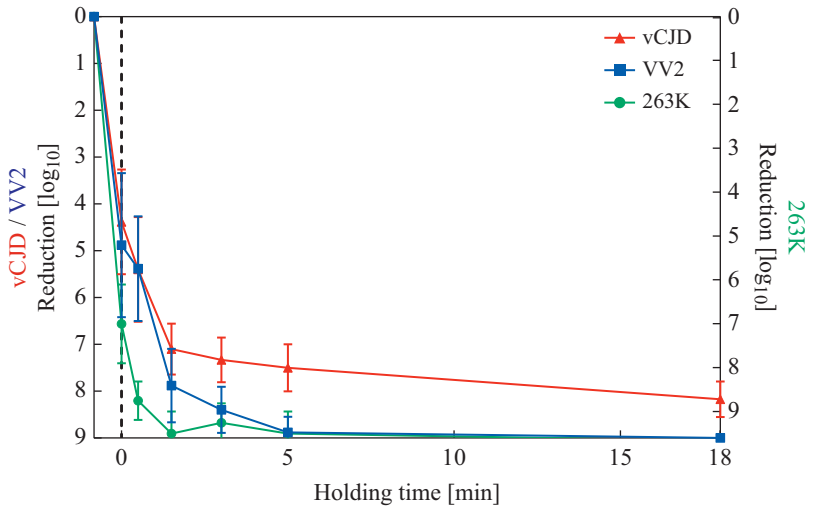

Figure 2. Reduction kinetics of the three prion isolates variant Creutzfeldt–Jakob disease (vCJD) (red/triangle), sporadic form of CJD (sCJD) VV2 (blue/squares) and scrapie 263K (green/dots). The dashed line indicates the start of the holding time from 0 to 18 min; the time prior to that represents the heating time which was defined as 1 min for illustration. The log_{10}-reduction factor for vCJD and VV2 is shown on the left y-axis, while the right y-axis presents it for 263K. Both y-axes cover the complete range to the detection limit of each strain. For 263K and sCJD VV2 a complete reduction of detectable prion seeding activity could be achieved, whereas for vCJD residual seeding activity was detected even after a holding time of 18 min.

agents most plausibly resulted from their intrinsic molecular characteristics rather than varying individual factors of the donors.

Our findings mitigate concerns regarding re-using neurosurgical instruments even in cases of unknown contaminations with sCJD prions, if they were properly cleaned and sterilized. In addition, the results confirm the rationale of the current German guideline for the routine reprocessing of medical devices and accentuate the need for a prolongation of steam-sterilization holding times from the standard 3 min to at least 5 min for possibly prion-contaminated instruments [20]. None the less, a thorough cleaning and non-fixating chemical disinfection is required to minimize the risk of transmission and to achieve a sufficient sterilization. Chemical cleaning, particularly alkaline-based, efficiently removes tissue lumps and fatty components that contribute to prion stabilization. Thereby, residual protein loads on instrument surfaces are reduced below 100 μg and thus potential infectious prion contaminations too. Additionally, it is evident that prion-containing material dried on surfaces fixate prions and increase their thermostability [18]; hence, it is essential to keep surgical instruments moist after use and to pre-soak and pre-clean them prior to sterilization [26,49]. In this study, the prion-containing material was fixated on polypropylene instead of surgical steel. Different surface materials can have an influence on the decontamination of prions as described recently [50]. However, an impaired access of the disinfectant to the prion contamination due to porous or hydrophobic surfaces rather than prion stabilizing effects of the materials are thought to be causative for varying disinfection efficacy. There seems to be no evidence that carrier materials alter the tenacity of prions during the surface drying process, other than the fixation by drying itself [16–18].

Safety of sterilized surgical instruments is an ongoing concern in prevention of nosocomial infections. Systematic and regular evaluation of sterilization efficacy is important, for

Table II
Reduction factors in log_{10} for each tested prion isolate per holding time at 134°C, presented as mean of all samples and experiments performed, including standard deviation and total number of samples and experiments

	vCJD				VV2 (sCJD)				263K			
Holding time at 134°C (min)	Reduction factor (log_{10})	Standard deviation	No. of experiments	No. of samples	Reduction factor (log_{10})	Standard deviation	No. of experiments	No. of samples	Reduction factor (log_{10})	Standard deviation	No. of experiments	No. of samples
–	–	–	6	12	–	–	4	8	–	–	4	8
0	4.38	1.11	4	8	4.88	1.54	4	8	7.00	0.89	3	10
0.5	5.40	1.11	5	10	5.38	1.11	4	8	8.75	0.43	3	8
1.5	7.10	0.54	5	10	7.88	0.78	4	8	9.50	0.50	3	6
3	7.33	0.47	3	6	8.40	0.49	5	10	9.25	0.43	2	4
5	7.50	0.50	5	10	8.88	0.33	4	8	9.50	0.50	2	4
18	8.17	0.37	3	6	9.00	0.00	4	8	9.67	0.47	3	6

sCJD, sporadic Creutzfeldt–Jakob disease; vCJD, variant Creutzfeldt–Jakob disease.

which pathogens with the possibly highest resistance to inactivation provide the most appropriate test species. Prions exhibit such a strong heat resistance that they even exceed *Geobacillus stearothermophilus* spores, the commonly used model pathogen for autoclave testing. Hence, prions appear to represent ideal test agents for reliable validation of steam-sterilization procedures. In this context, PMCA provides an easy, fast and ultrasensitive tool for analysing prion inactivation.

Our data provide evidence that VV2 as the second most frequent sCJD subtype [51] exhibits a less strong resistance to steam sterilization at 134°C than vCJD. The tenacity of vCJD was confirmed to be remarkably strong, and a prolongation of the holding time from 5 to 18 min did not further reduce remaining heat-resistant vCJD species as observed in several previous studies [8,18]. Therefore, concerns regarding vCJD contaminations on medical devices appear plausible, as highlighted by Fernie *et al.* [9]. However, the heat-resistant fraction of vCJD prions that withstood steam sterilization in our study represented a seeding activity corresponding to the 10^{-9} dilution of untreated vCJD brain homogenate only. Due to the apparently minor risk of new vCJD patients in future, the recommended steam sterilization at 134°C for 5 min from 2012 [20] can be regarded to be sufficiently safe for the routine reprocessing of heat-resistant devices unknowingly used in sCJD carriers, at least those with the disease subtype VV2. Further, our study highlights that 263K and presumably also other scrapie strains are an imperfect surrogate for human prions concerning steam sterilization.

Acknowledgements

The authors are grateful to Prof. Walther Schulz-Schaeffer, Saarland University, Homburg, Germany, and the National Creutzfeldt—Jakob Disease Surveillance Unit, Edinburgh, UK, for providing us with CJD brain tissue. We thank the developing scientists of tg_hu(129M) and tg_hu(129V) mice (The Roslin Institute, University of Edinburgh, UK) for providing access to breeding stock of the animals. We are also grateful to Marion Joncic and the animal caretakers at Robert Koch Institute for excellent support, to Prof. Martin Mielke for valuable advice on this project, and to Joo-Hee Wälzlein for comments on the manuscript. Valuable scientific support by the research platforms BB3R (www.bb3r.de) and Berlin Einstein Center 3R (www.ec3r.org) with respect to the refinement, reduction and replacement of animal experiments is gratefully acknowledged.

Author contributions
Study conceptualization, analysis and interpretation of data: K.A.S., K.W., M.T. and M.B. Acquisition of data: K.A.S. and K.W. Acquisition of funding for K.A.S. and K.W.: M.B. Drafting the manuscript: K.A.S. All authors revised and approved the final manuscript.

Conflict of interest statement
The authors declare no competing interests.

Funding sources
K.A.S. was financially supported by Alberta Prion Research Institute (Alberta, Canada, project number PEX12015) and Robert Koch Institute; K.W. was financially supported by The German Federal Ministry of Health (Project IIA5-2512NIK004//321-4471-02).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2022.08.014.

References

[1] Brown P, Cervenakova L. A prion lexicon (out of control). Lancet 2005;365(9454):122.
[2] Prusiner SB. Prions. Proc Natl Acad Sci U S A. 1998;95(23):13363—83.
[3] Lemmer K, Mielke M, Pauli G, Beekes M. Decontamination of surgical instruments from prion proteins: in vitro studies on the detachment, destabilization and degradation of PrPSc bound to steel surfaces. J Gen Virol 2004;85:3805—16.
[4] Deutsches Institut für Normung. DIN EN 285:2021-12: Sterilization — Steam sterilizers — Large sterilizers; EN 285:2015+A1:2021. 2021. Beuth Verlag.
[5] Rutala WA, Weber DJ. Guideline for disinfection and sterilization in healthcare facilities, 2008. Centers for Disease Control and Prevention 2008. Update 2019.
[6] Kimberlin RH, Walker CA, Millson GC, Taylor DM, Robertson PA, Tomlinson AH, et al. Disinfection studies with two strains of mouse-passaged scrapie agent. Guidelines for Creutzfeldt—Jakob and related agents. J Neurol Sci 1983;59(3):355—69.
[7] Taylor DM, Fraser H, McConnell I, Brown DA, Brown KL, Lamza KA, et al. Decontamination studies with the agents of bovine spongiform encephalopathy and scrapie. Arch Virol 1994;139(3—4):313—26.
[8] Taylor DM. Inactivation of prions by physical and chemical means. J Hosp Infect 1999;43(Suppl):S69—76.
[9] Fernie K, Hamilton S, Somerville RA. Limited efficacy of steam sterilization to inactivate vCJD infectivity. J Hosp Infect 2012;80(1):46—51.
[10] Brown P, Brandel JP, Sato T, Nakamura Y, MacKenzie J, Will RG, et al. Iatrogenic Creutzfeldt—Jakob disease, final assessment. Emerg Infect Dis 2012;18(6):901—7.
[11] Bonda DJ, Manjila S, Mehndiratta P, Khan F, Miller BR, Onwuzulike K, et al. Human prion diseases: surgical lessons learned from iatrogenic prion transmission. Neurosurg Focus 2016;41(1):E10.
[12] The National CJD Research, Unit Surveillance. 29th annual report 2020 creutzfeldt—jakob disease surveillance in the UK. 2020.
[13] Sutton JM, Dickinson J, Walker JT, Raven ND. Methods to minimize the risks of Creutzfeldt-Jakob disease transmission by surgical procedures: where to set the standard? Clin Infect Dis 2006;43(6):757—64.
[14] Hamaguchi T, Noguchi-Shinohara M, Nozaki I, Nakamura Y, Sato T, Kitamoto T, et al. The risk of iatrogenic Creutzfeldt-Jakob disease through medical and surgical procedures. Neuropathology 2009;29(5):625—31.
[15] Douet J-Y, Huor A, Cassard H, Lugan S, Aron N, Arnold M, et al. Wide distribution of prion infectivity in the peripheral tissues of vCJD and sCJD patients. Acta Neuropathol 2021;141(3):383—97.
[16] Zobeley E, Flechsig E, Cozzio A, Enari M, Weissmann C. Infectivity of scrapie prions bound to a stainless steel surface. Molec Med 1999;5(4):240—3.
[17] Flechsig E, Hegyi I, Enari M, Schwarz P, Collinge J, Weissmann C. Transmission of scrapie by steel-surface-bound prions. Molec Med 2001;7(10):679—84.
[18] Taylor DM, Fernie K, McConnell I, Steele PJ. Observations on thermostable subpopulations of the unconventional agents that cause transmissible degenerative encephalopathies. Vet Microbiol 1998;64(1):33—8.
[19] World Health Organization. WHO infection control guidelines for transmissible spongiform encephalopathies. 2000.
[20] Kommission für Krankenhaushygiene und Infektionsprävention. Anforderungen an die Hygiene bei der Aufbereitung von

Medizinprodukten. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz 2012;55(10):1244—310.
[21] Le ministre de la santé. INSTRUCTION N° DGS/RI3/2011/449 du 1er décembre 2011 relative à l'actualisation des recommandations visant à réduire les risques de transmission d'agents transmissibles non conventionnels lors des actes invasifs. SAS - Santé et sports; 2011.
[22] Yan ZX, Stitz L, Heeg P, Pfaff E, Roth K. Infectivity of prion protein bound to stainless steel wires: a model for testing decontamination procedures for transmissible spongiform encephalopathies. Infect Control Hosp Epidemiol 2004;25(4):280—3.
[23] Lemmer K, Mielke M, Kratzel C, Joncic M, Oezel M, Pauli G, et al. Decontamination of surgical instruments from prions. II. In vivo findings with a model system for testing the removal of scrapie infectivity from steel surfaces. J Gen Virol 2008;89(Pt 1):348—58.
[24] Beekes M, Lemmer K, Thomzig A, Joncic M, Tintelnot K, Mielke M. Fast, broad-range disinfection of bacteria, fungi, viruses and prions. J Gen Virol 2010;91(Pt 2):580—9.
[25] Peretz D, Supattapone S, Giles K, Vergara J, Freyman Y, Lessard P, et al. Inactivation of prions by acidic sodium dodecyl sulfate. J Virol 2006;80(1):322—31.
[26] Fernie K, Steele PJ, Taylor DM, Somerville RA. Comparative studies on the thermostability of five strains of transmissible-spongiform-encephalopathy agent. Biotechnol Appl Biochem 2007;47(Pt 4):175—83.
[27] Douet JY, Lacroux C, Aron N, Head MW, Lugan S, Tillier C, et al. Distribution and quantitative estimates of variant Creutzfeldt—Jakob disease prions in tissues of clinical and asymptomatic patients. Emerg Infect Dis 2017;23(6):946—56.
[28] Watson N, Brandel J-P, Green A, Hermann P, Ladogana A, Lindsay T, et al. The importance of ongoing international surveillance for Creutzfeldt—Jakob disease. Nat Rev Neurol 2021;17(6):362—79.
[29] Chen B, Morales R, Barria MA, Soto C. Estimating prion concentration in fluids and tissues by quantitative PMCA. Nature Meth 2010;7(7):519—20.
[30] Wilham JM, Orrú CD, Bessen RA, Atarashi R, Sano K, Race B, et al. Rapid end-point quantitation of prion seeding activity with sensitivity comparable to bioassays. PLoS Pathog 2010;6(12):e1001217.
[31] Pritzkow S, Wagenfuhr K, Daus ML, Boerner S, Lemmer K, Thomzig A, et al. Quantitative detection and biological propagation of scrapie seeding activity in vitro facilitate use of prions as model pathogens for disinfection. PLoS One 2011;6(5):e20384.
[32] Makarava N, Savtchenko R, Alexeeva I, Rohwer RG, Baskakov IV. Fast and ultrasensitive method for quantitating prion infectivity titre. Nat Commun 2012;3(1):741.
[33] Boerner S, Wagenfuhr K, Daus ML, Thomzig A, Beekes M. Towards further reduction and replacement of animal bioassays in prion research by cell and protein misfolding cyclic amplification assays. Lab Animals 2013;47(2):106—15.
[34] Paul L, Kirsch P, Thomzig A, Thöne-Reineke C, Beekes M. Practical approaches for refinement and reduction of animal experiments with bank voles in prion research. Berl Munch Tierarztl Wochenschr 2018:131.
[35] Lyon A, Mays CE, Borriello F, Telling GC, Soto C, Pritzkow S. Application of PMCA to screen for prion infection in a human cell line used to produce biological therapeutics. Sci Rep 2019;9(1):4847.
[36] Bishop MT, Hart P, Aitchison L, Baybutt HN, Plinston C, Thomson V, et al. Predicting susceptibility and incubation time of human-to-human transmission of vCJD. Lancet Neurol 2006;5(5):393—8.
[37] Matsuura Y, Ishikawa Y, Bo X, Murayama Y, Yokoyama T, Somerville RA, et al. Quantitative analysis of wet-heat inactivation in bovine spongiform encephalopathy. Biochem Biophys Res Commun 2013;432(1):86—91.
[38] Bélondrade M, Jas-Duval C, Nicot S, Bruyère-Ostells L, Mayran C, Herzog L, et al. Correlation between bioassay and protein misfolding cyclic amplification for variant Creutzfeldt—Jakob disease decontamination studies. mSphere 2020;5(1):1—11.
[39] Banay-Schwartz M, Kenessey A, DeGuzman T, Lajtha A, Palkovits M. Protein content of various regions of rat brain and adult and aging human brain. Age 1992;15(2):51—4.
[40] Murdoch H, Taylor D, Dickinson J, Walker JT, Perrett D, Raven ND, et al. Surface decontamination of surgical instruments: An ongoing dilemma. J Hosp Infect 2006;63(4):432—8.
[41] Köhnlein J, Schmidt V, Staffeldt J, Werner HP. Analysis of different test soilings for verification of cleaning performance. Hygiene & Medizin 2004;29(1—2):13—9.
[42] Taylor DM. Resistance of transmissible spongiform encephalopathy agents to decontamination. Contrib Microbiol 2004;11:136—45.
[43] McDonnell G, Dehen C, Perrin A, Thomas V, Igel-Egalon A, Burke PA, et al. Cleaning, disinfection and sterilization of surface prion contamination. J Hosp Infect 2013;85(4):268—73.
[44] Fichet G, Comoy E, Duval C, Antloga K, Dehen C, Charbonnier A, et al. Novel methods for disinfection of prion-contaminated medical devices. Lancet 2004;364(9433):521—6.
[45] Belondrade M, Nicot S, Béringue V, Coste J, Lehmann S, Bougard D. Rapid and highly sensitive detection of variant creutzfeldt-jakob disease abnormal prion protein on steel surfaces by protein misfolding cyclic amplification: application to prion decontamination studies. PLoS One 2016;11(1):e0146833—.
[46] Marín-Moreno A, Aguilar-Calvo P, Moudjou M, Espinosa JC, Béringue V, Torres JM. Thermostability as a highly dependent prion strain feature. Sci Rep 2019;9(1):11396.
[47] Pinder P, Thomzig A, Schulz-Schaeffer WJ, Beekes M. Alpha-synuclein seeds of Parkinson's disease show high prion-exceeding resistance to steam sterilization. J Hosp Infect 2021;108:25—32.
[48] EFSA PoBH. Joint Scientific Opinion on any possible epidemiological or molecular association between TSEs in animals and humans. EFSA J 2011;9(1):1945.
[49] Lipscomb IP, Pinchin H, Collin R, Keevil CW. Effect of drying time, ambient temperature and pre-soaks on prion-infected tissue contamination levels on surgical stainless steel: concerns over prolonged transportation of instruments from theatre to central sterile service departments. J Hosp Infect 2007;65(1):72—7.
[50] Eraña H, Pérez-Castro MÁ, García-Martínez S, Charco JM, López-Moreno R, Díaz-Dominguez CM, et al. A novel, reliable and highly versatile method to evaluate different prion decontamination procedures. Front Bioeng Biotechnol 2020;8:589182.
[51] Gambetti P, Kong Q, Zou W, Parchi P, Chen SG. Sporadic and familial CJD: classification and characterisation. Br Med Bull 2003;66:213—39.