

# Thermostability of mouse-passaged BSE and scrapie is independent of host PrP genotype: implications for the nature of the causal agents

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Five experimentally maintained strains of scrapie and BSE agents have been passaged in two PrP genotypes of mice. Brain macerates were autoclaved at 126 °C and the levels of surviving infectivity were measured by titration. There was a large difference in the survival properties of transmissible spongiform encephalopathy (TSE) infectivity between TSE strains. PrP genotype had little effect. Phenotypic properties of the TSE strains were not affected with the exception that with one strain (ME7), incubation periods of the heated sample were longer than the controls given equivalent doses. It is concluded that PrP is probably not responsible for differences in thermostability between strains. More likely, a host-independent molecule which differs in covalent structure between strains accounts for these properties.

## Introduction

The transmissible spongiform encephalopathies (TSEs) are a group of progressive neurological diseases of mammals. They include scrapie in sheep, bovine spongiform encephalopathy (BSE) and Creutzfeldt–Jakob disease (CJD) in humans, which is manifested sporadically, familiarly and iatrogenically. The recent discovery of variant CJD (vCJD) has been associated with BSE infection of humans, probably through dietary exposure (Bruce *et al.*, 1997; Will *et al.*, 1996).

TSEs are caused by an infectious agent whose replication is under the genetic control of the host (Dickinson *et al.*, 1965). Many different TSE strains have been typed according to a series of phenotypic properties which include relative incubation periods in several mouse strains and the amount and distribution of neuropathological lesions. The structural elements of the causal agents responsible for specifying differences between TSE strains have not been identified.

The biochemical structure of the agent is poorly understood and remains a matter of considerable controversy. The debate is centred on what genetic information the infectious agent carries, how it is carried and the structure of the components of the agent which carry that information. The prion hypothesis in its protein-only formulation proposes that conformational change of the protein PrP encodes the information (Prusiner,

1998); the virino hypothesis proposes that an informational molecule (probably a nucleic acid) encodes the information (Farquhar *et al.*, 1998); and the virus hypothesis suggests that a structure akin to other conventional viruses constitutes the infectious agent (Chesebro, 1998).

In mice the gene *Sinc* controls replication of infectivity (Dickinson *et al.*, 1968). The *Sinc* gene, also subsequently called *Prn* or the PrP gene, encodes a host glycoprotein, PrP (Carlson *et al.*, 1986; Moore *et al.*, 1998). PrP is found in uninfected brain and many other tissues. Its normal function is unknown. In infected brain, an abnormal form, PrP<sup>Sc</sup>, is found as well. PrP<sup>Sc</sup> tends to co-purify with infectivity (Diringer *et al.*, 1983) although some of the PrP in this fraction can be separated from the bulk of infectivity under certain conditions (Manuelidis *et al.*, 1987; Shaked *et al.*, 1999; Somerville & Dunn, 1996; Wille *et al.*, 1996). Nevertheless it is probable that a proportion of the PrP in the PrP<sup>Sc</sup> fraction is a component of the infectious agent.

TSE agent strains show variability in their sensitivity to heat (Kimberlin *et al.*, 1983; Somerville *et al.*, 2002) although they are all difficult to inactivate by heating in an aqueous environment such as an autoclave. Even a fairly thermolabile strain (22C) is stable up to 70 °C while others, e.g. 22A, have been shown to be stable to near 100 °C (Somerville *et al.*, 2002). TSE strains derived from BSE show even greater thermostability (Taylor *et al.*, 1994), although their properties have not yet been studied as extensively. Although it is possible to achieve full inactivation (Rohwer, 1984), in some circumstances significant amounts of infectivity survive (Taylor

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**Table 1.** Titrations of TSE agent strains before and after autoclaving

The number of mice diagnosed with TSE infection per number injected is shown.

Dilution group (log <sub>10</sub> )	-1	-2	-3	-4	-5	-6	-7	-8	-9	Titre*	Titre loss
SV 22C Unheated			18/18	18/18	17/18	4/18				7.4	
SV 22C Heated	0/18	0/18	0/18	0/18						≤ 2.2	≥ 5.2
VM 22C Unheated			16/16	17/17	14/17	6/17				7.4	
VM 22C Heated	0/18	0/18	0/18	0/18						≤ 2.2	≥ 5.2
SV ME7 Unheated			18/18	18/18	18/18	18/18				≥ 8.2	
SV ME7 Heated	3/10	0/17	0/17	0/18						2.5	≥ 5.7
VM ME7 Unheated			17/17	18/18	16/16	18/18				≥ 8.2	
VM ME7 Heated	6/17	1/17	0/18	0/18						2.7	≥ 5.5
SV 139A Unheated			18/18	18/18	18/18	16/17				≥ 8.1	
SV 139A Heated	6/14	2/18	1/18	0/18						2.7	≥ 5.4
VM 139A Unheated			18/18	18/18	18/18	14/17				≥ 8.0	
VM 139A Heated	4/16	2/16	0/19	0/19						2.6	≥ 5.4
SV 22A Unheated			18/18	18/18	14/18	4/11				7.3	
SV 22A Heated	10/14	3/13	0/17	0/16						3.1	4.2
VM 22A Unheated			18/18	18/18	16/16	14/18				≥ 8.0	
VM 22A Heated	16/18	11/16	3/17	0/17						4.0	≥ 4.0
SV 301V Unheated					17/18	6/18	0/18	0/18	0/18	7.5	
SV 301V Heated	18/18	17/17	17/17	8/18	0/17	0/17				5.6	1.9
VM 301V Unheated					18/18	16/17	7/17	2/18	0/18	8.7	
VM 301V Heated	18/18	18/18	18/18	13/18	0/17	0/17				5.9	2.7

\* Titres are calculated per injected dose of inoculum (ID<sub>50</sub>/g), according to the Karber (1931) method. In titrations where an end-point was not obtained it is assumed that no cases would have occurred in higher dilutions that were not assayed.

*et al.*, 1998). Differences in sample preparation may affect survival (Taylor *et al.*, 1998). In particular dehydration (Taylor *et al.*, 1996) and protein fixation (Taylor & McConnell, 1988) convert the agent into more thermostable forms and it is predicted that partial aqueous heat inactivation may also create a protected, more thermostable form (Somerville *et al.*, 2002).

The 22A and 301V strains are the most thermostable mouse-passaged strains characterized so far (Dickinson & Taylor, 1978; Kimberlin *et al.*, 1983; Taylor, 2000). These strains were originally isolated and have subsequently routinely been passaged through VM/Dk mice (VM mice), a mouse strain carrying the *Sinc*<sup>P7D7</sup> allele which encodes PrP with phenylalanine at codon 108 and valine at codon 189. Most TSE strains have been isolated by passage in *Sinc*<sup>S7S7</sup> mice (Carlson *et al.*, 1986) which encode PrP with leucine at codon 108 and threonine at codon 189. Both 22A and 301V have longer incubation periods in *Sinc*<sup>S7S7</sup> mice. Their particular resistance to heat inactivation posed the question as to whether the high thermostability of 22A and 301V might have been acquired as a result of their passage through VM mice. This paper describes the experiments that were carried out to answer this question and the more general question of whether PrP genotype affects TSE agent thermostability. Five TSE strains were passaged in congenic mice which differ only in their PrP genotype (Bruce *et al.*, 1991). Infected brain macerates

from them were autoclaved at 126 °C for 30 min and the residual infectivity was titrated in mice. The data imply that differences in thermostability are due to structural properties of the TSE agent which are not associated with differences in the mouse sequence of PrP.

## Methods

■ **Preparation of the starting materials.** The TSE strains used were derived as follows: ME7 was derived from natural sheep scrapie by serial passage through C57BL (*Sinc*<sup>S7S7</sup>) mice; 22C and 22A were derived by serial passage of the experimental sheep scrapie source, SSBP/1, through C57BL and VM/Dk (*Sinc*<sup>P7D7</sup>) mice respectively; 139A was derived by passage of the drowsy goat source of experimental scrapie through *Sinc*<sup>S7S7</sup> mice (Dickinson, 1976); and 301V by passage of BSE through VM (*Sinc*<sup>P7D7</sup>) mice (Bruce *et al.*, 1994). Groups of weanling SV/Dk (*Sinc*<sup>S7S7</sup>) mice (SV mice) and VM mice were injected intracerebrally (i.c.), 20 µl per mouse, with 10% (w/v) sterile saline homogenates of mouse brain-tissue infected with the five strains of TSE. These mice were culled when they developed clinical signs of terminal neurological disease; brains were removed aseptically and allocated to the appropriate pools. The brains in these pools were blended without dilution using sterile scalpels until homogeneous macerates were obtained.

■ **Autoclaving.** From each of the pooled brain macerates representing the different mouse-strain/agent-strain combinations, two 50 mg samples were each placed on the smooth glass surface of the upper body

of a sterile, newly obtained, Griffiths tube. These were then placed in a Boxer BA852 Sovereign portable autoclave (Denley Instruments Ltd, Billingshurst, UK) that had already been run empty at 126 °C for an hour. The tubes were laid flat with their mouths facing outwards to avoid any cross-contamination through 'sputtering' of the samples, even though the possibility of this type of cross-contamination had already been investigated, and was found not to occur (D. M. Taylor, unpublished data). The samples were then subjected to gravity-displacement autoclaving at 126 °C for 30 min.

■ **Titration of infectivity in the unheated and autoclaved brain-pools.** Samples taken from the various unheated macerated brain-pools, and the samples that had been autoclaved, were initially homogenized (10%, w/v) in sterile injection saline in Griffiths tubes. The autoclaved samples were homogenized in the tubes in which they had been autoclaved and a series of tenfold dilutions (v/v) was prepared in saline. Various dilutions were injected i.c. (20 µl per mouse) into groups of anaesthetized SV or VM mice, as shown in Table 1. Formal scoring of the mice for clinical signs of neurological disease was initiated at an appropriate time depending upon the agent strain/mouse strain combinations. Scoring was based upon a system developed by A. G. Dickinson (Dickinson *et al.*, 1968), and continued through to the stage of humane culling because of terminal neurological disease or until unaffected mice were culled routinely at the end of the experiments. Unaffected SV mice injected with 22C were culled 851 days post-injection (p.i.), 700 days p.i. with 139A and 892 days p.i. with ME7. Unaffected VM mice injected with 22A were culled 800 days p.i. and 675 days p.i. with 301V. By comparing the ratios of affected and unaffected mice in the different dilution groups (excluding those that died from intercurrent disease), the infectivity titre expressed as a  $\log_{10}$  value per gram can be calculated using the statistical method of Karber (1931). The brains of all mice were examined histopathologically for the presence of spongiform encephalopathy and brain-lesion profiles were constructed for each mouse. This is a well-established semi-quantitative method of measuring the targeting of vacuolation to different brain regions, and reliably discriminates between TSE strains in mice (Fraser & Dickinson, 1973).

## Results

Five TSE strains (22C, ME7, 139A, 22A and 301V) were passaged by i.c. challenge through SV and VM congenic mice to create pools of infected brain material. Table 2 shows their incubation periods. It should be noted that the incubation periods vary according to both TSE strain and mouse strain.

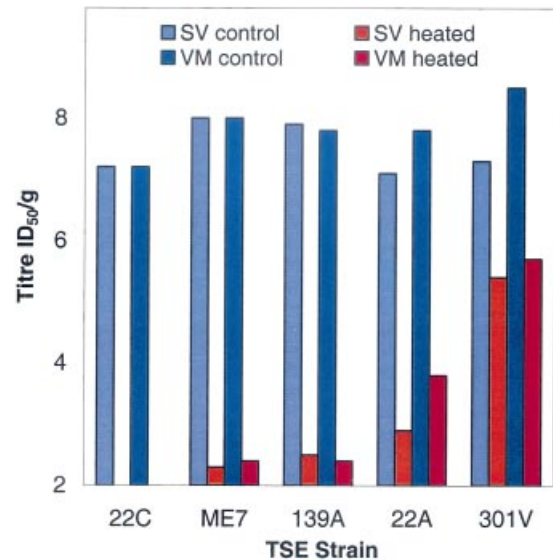


Fig. 1. Inactivation of TSE strains with heat. Comparison of titre ( $ID_{50}/g$ ) between autoclaved (126 °C for 30 min) and unheated agents in mouse-passaged BSE and scrapie brain macerate.

Three of the strains, 22C, 139A and ME7 have shorter incubation periods in SV mice while the other two, 22A and 301V, are shorter in VM mice (Bruce *et al.*, 1991).

Brains were collected after humane culling at clinical endpoint, frozen, thawed, macerated and one aliquot heated at 126 °C. These conditions were previously used (Kimberlin *et al.*, 1983) and were known to result in survival of some infectivity with some TSE strains but not others. Another aliquot was retained frozen. These samples were then homogenized in saline and titrated in SV (ME7, 22C and 139A) or VM (22A and 301V) mice, the mouse strains in which they are normally passaged and incidentally give the shorter incubation periods. Results of the titrations show that titres are substantially reduced by autoclaving. (Table 1 and Fig. 1). The major differences in recovered infectivity after autoclaving correlate with the strain of agent: no infectivity was recovered from 22C after passage in either SV or VM mice, about

Table 2. Incubation periods in donor mice

Incubation periods (days) and standard error of the mean (SEM) of each group of mice whose brains were used to create the macerates for autoclaving.

	SV		VM	
	Inc. period	SEM	Inc. period	SEM
ME7	160.7	0.9	333.8	1.1
22C	178.0	0.9	535.2	16.5
139A	131.6	0.6	182.8	2.8
22A	470.4	12.9	206.8	2.9
301V	244.0	2.3	119.0	0

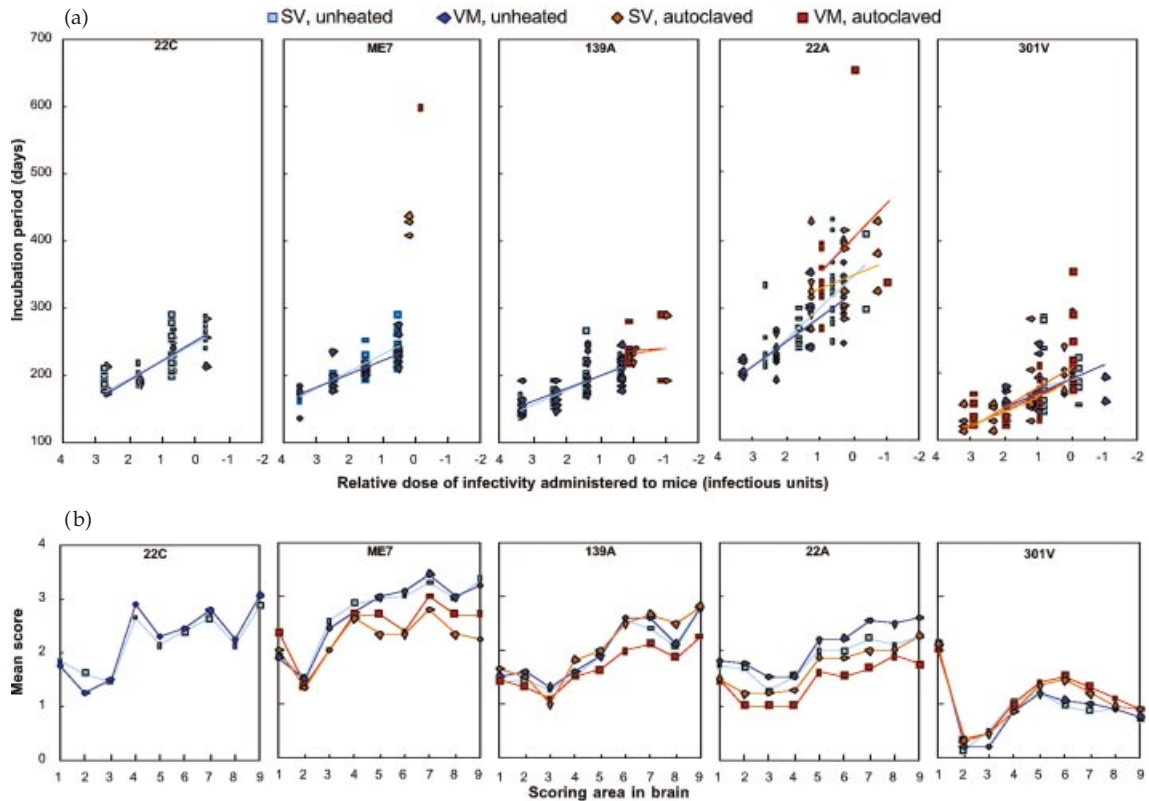


Fig. 2. Comparison of dose-response curves (a) and lesion profiles (b) of autoclaved and unheated agents in mouse-passaged BSE and scrapie brain macerates.

$10^5$ – $10^6$   $ID_{50}/g$  were recovered with both 301V samples, and recovery of infectivity from the other strains gave intermediate values. By contrast, for each strain the differences in recovered titre after autoclaving were little affected by PrP genotype.

To test whether heating altered the interaction between TSE agent and the host, the dose-response curves and lesion profiles of samples before and after autoclaving were compared. The dose-response curves for 301V, 22A and 139A after heating were similar to control dose-response curves (Fig. 2a), indicating no detectable change in the intrinsic incubation period properties of these strains. However, the four mice to succumb to autoclaved ME7 with defined incubation periods all had long incubation periods, consistent with previous experiments with ME7 in which incubation periods were extended after heating (Dickinson & Fraser, 1969). Lesion profiles of heated and control samples were very similar (Fig. 2b), indicating that no significant change in the neurological targeting of the five strains had occurred, i.e. there were no changes in the properties of the agent which specify targeting.

## Discussion

The results show that there are large differences in the thermostability between TSE agent strains in hydrated systems. By contrast, there are only small differences between

samples of the same TSE strain from brains of mice differing in *Sinc* (PrP) genotype.

The patterns of severity of spongiform change in different areas of the mouse brain vary according to the strains of agent with which the mice have been challenged. The resulting brain-lesion profiles have been shown to be reliable phenotypic markers for different strains (Fraser & Dickinson, 1973). Accordingly, the distribution of neuropathological lesions, as assessed by the lesion profile system, was examined to test whether any changes in strain properties had occurred as a result of the exposure to heat. Any differences between strains could have indicated changes in the pathogenesis of the infection hence resulting in differences in titre after autoclaving. In no case was any major difference detected between control and heated samples. It is also worth noting that there was little difference between TSE strains passed in the two PrP genotypes, whether autoclaved or not. Similarly, the dose-response curves were assessed. No significant differences were observed for 139A, 22A or 301V. The four mice that succumbed after injection with autoclaved ME7 had longer incubation periods than is normally seen at limiting dilution after i.c. injection with this strain. This phenomenon for ME7 has been known for many years (Dickinson & Fraser, 1969) and has been observed with other strains including 263K (Taylor & Fernie, 1996) and 301V (R. A. Somerville, unpublished), usually

when only residual amounts of infectivity can be detected. The reasons for this phenomenon are unknown. The effect may be due to changes in the agent's structure such that infectivity is sequestered and/or it does not initiate infection in its normal target cells but is re-routed to other cells, possibly in other parts of the body. In another experiment where heating extended incubation periods, the phenotypic change was not retained on further subpassage of ME7 in mice (results not shown). It is concluded that in these experiments there was little or no change in the observed phenotypic properties of the agent strains caused by heating, with the exception of ME7. Accordingly, differences in thermostability can be ascribed to differences in the structure of the causal agent.

These results provide complementary evidence to those from the study of the passage of TSE agents through different PrP genotypes, usually in different species (Bruce *et al.*, 1994). Most notably, BSE from cattle has retained similar phenotypic properties on direct passage to mice to those experimentally passed into sheep and then mice and to isolates from other species, including nyala, cat and man, despite substantial differences in amino acid sequences of PrP between these species.

Any differences detected in lesion profiles might have been due to differences in survival of TSE strains with different thermostability properties. With the exception of 139A, the strains of agent used had all been purified by cloning; this involves the serial passage through mice of brain material obtained from mice succumbing to disease after i.c. challenge with limiting dilutions of infectivity. The use of these cloned strains in a wide variety of experiments has confirmed their purity. Brain-lesion profiles show no major differences for all unheated and heated agent-strain/mouse-strain combinations, including the 139A profiles. Thus there is no evidence of a change or a selection of strains within these experiments.

PrP may be a component of the agent. However, differences in its sequence contributed little to differences in thermostability between these models. Nevertheless, it is possible that in some combinations of agent and PrP genotype the PrP sequence may contribute to the differences (Somerville *et al.*, 2002). Since in some cases at least (including these examples) PrP sequence differences have little effect, it is concluded that other properties of the structure of the infectious agent must be responsible for differences in thermostability between strains of TSE agent. It is difficult to envisage different conformations of PrP which are each thermodynamically stable at near denaturing temperatures, as would be required by the prion hypothesis. A much more likely proposal is that a host-independent molecule which differs in covalent structure between strains is responsible for differences in thermostability between them (Farquhar *et al.*, 1998; Somerville *et al.*, 2002).

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