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Fast broad-range disinfection of bacteria, fungi, viruses and prions

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5) The affiliation of Karin Lemmer has changed since completion of the study and now is: ZBS 2 – Highly Pathogenic Microbial Pathogens – Bacteria and Fungi, Robert Koch-Institut, Nordufer 20, 13353 Berlin, Germany.

Abstract

Effective disinfectants are of key importance for the safe handling and reprocessing of surgical instruments. We tested whether new formulations containing SDS, NaOH and 1-propanol (n-propanol) are simultaneously active against a broad range of pathogens including bacteria, fungi, non-enveloped viruses and prions. Inactivation and disinfection were examined in suspension and on carriers, respectively, using coagulated blood or brain homogenate as organic soil. Coomassie blue staining was used to assess whether formulations did undesirably fix proteins to rough surfaces. A mixture of 0.2% SDS and 0.3% NaOH in 20% n-propanol achieved potent decontamination of steel carriers contaminated with PrP^{TSE}, the biochemical marker for prion infectivity, from 263K scrapie hamsters, or patients with sporadic or variant Creutzfeldt-Jakob disease. 263K scrapie infectivity on carriers was decreased by $\geq$5.5 $\log_{10}$ units [logs]. Furthermore, the formulation effectively inactivated poliovirus, hepatitis A virus and caliciviruses (including murine norovirus) in suspension tests. It also yielded significant titre reductions of bacteria (*E. faecium*, *M. avium*; >6 logs), fungi (spores of *Aspergillus niger*; >5 logs) or poliovirus ($\geq$4 logs) embedded in coagulated blood on carriers. The formulation was not found to fix proteins more than was observed with water as cleaning reagent. SDS, NaOH and n-propanol can synergistically achieve fast broad-range disinfection.

Introduction

Effective disinfection is of utmost importance in the maintenance of re-usable surgical instruments. In this context, an ideal disinfectant applicable also to heat sensitive devices should have a fast and broad-range activity on bacteria, viruses, fungi as well as prions while being easy to use and free of effects that fix (i. e. bind) organic soil containing proteins to the surface of instruments. Such requirements cannot be met readily since a variety of pathogens such as mycobacteria, fungal spores, non-enveloped viruses (like poliovirus) and last but not least prions are known for their unusually high tolerance to inactivation (Beekes *et al.*, 2004; Fernie *et al.*, 2007; Rutala *et al.*, 2008; Taylor, 2004).

Prions, “proteinaceous infectious particles” (Prusiner, 1982), are considered as the causative agents of transmissible spongiform encephalopathies (TSEs) such as scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, or Creutzfeldt-Jakob disease (CJD) and its variant form (vCJD) in humans. According to the prion hypothesis these agents are composed essentially – if not entirely – of misfolded prion protein (PrP) which is derived from a host-encoded cellular precursor (PrP^{C}) (Prusiner, 1998). The deposition in the central nervous system of disease-associated prion protein, referred to as PrP^{Sc} (Prusiner, 1998) or PrP^{TSE} (Brown & Cervenakova, 2005), with an abnormal folding and/or aggregation structure is a pathological hallmark of TSEs, and PrP^{TSE} has been used in many studies as a molecular marker for prion infectivity (Beekes & McBride, 2007).

The study described in this report emanated from previous work in which we tested various formulations for their ability to detach, destabilize or degrade PrP^{TSE} attached to steel wires and to inactivate prion infectivity on such carriers. Similar steel wire models for prion disinfection of surgical instruments (Flechsig *et al.*, 2001; Zobeley *et al.*, 1999) have also been employed by other research groups (Fichet *et al.*, 2004; Fichet *et al.*, 2007; Yan *et al.*, 2004). In our previous studies we identified a formulation of 0.2% SDS and 0.3% NaOH (in the following referred to as SDS/NaOH) as a highly potent disinfectant against 263K scrapie

agent from hamsters (Lemmer *et al.*, 2004; Lemmer *et al.*, 2008). We have now explored whether, by adding different concentrations of ethanol or n-propanol to this formulation, its protein-destabilizing and prion inactivating activity may be extended to disinfection of bacteria, viruses and fungi without causing effects that would undesirably fix proteins.

Methods

Efficacy testing of formulations against bacteria, fungi and viruses

Test organisms and nutrient media (in brackets)

Enterococcus faecium DSM 2146 (Brain Heart-Infusion-Agar), *Mycobacterium avium* DSM 44156 (Middlebrook 7H10 Agar with OADC [Oleic acid, albumin fraction V / bovine, dextrose, catalase / beef] enrichment), *Aspergillus niger* ATCC 16404 (Malt Extract Agar); *Feline calicivirus* (FCV) strain F-9 in CrFK cells, *Poliovirus* type I LSc-2ab (PV) in L20B cells, *Hepatitis A virus* (HAV) strain HM 175cyt in RHK cells (MEM [minimum essential medium] with Hanks salt solution for FVC, PV and HAV), *Murine Norovirus* (MNV) strain S99 in RAW 264.7 cells (modified Dulbecco´s MEM (Park *et al.*, 2007)).

Formulations used for disinfection

a) 0.2% (w/v) SDS, 0.3% (w/v) NaOH (in double distilled water [ddH_2O]),

b) 0.2% (w/v) SDS, 0.3% (w/v) NaOH, 50% (v/v) ethanol (in ddH_2O),

c) 0.2% (w/v) SDS, 0.3% (w/v) NaOH, 30% (v/v) n-propanol (in ddH_2O),

d) 0.2% (w/v) SDS, 0.3% (w/v) NaOH, 20% (v/v) n-propanol (in ddH_2O),

e) 50% (v/v) ethanol (in ddH_2O),

f) 20% (v/v) n-propanol (in ddH_2O),

g) 2% (v/v) glutardialdehyde (in ddH_2O),

h) 0.35% (v/v) peracetic acid (in ddH_2O),

i) sodium hypochlorite (NaOCl) solution with 20.000 ppm available chlorine (in ddH_2O).

Microbial test suspensions

E. faecium

Subcultured preparations of *E. faecium* were pelleted, washed and diluted in ddH_2O to achieve suspensions containing at least 10^9 colony forming units (CFU)/ml.

M. avium

Subcultured preparations of *M. avium* were pelleted and washed in 0.1% (v/v) Tween 80 in ddH_2O. After resuspension using a glass/Teflon homogenizer the bacterial solution was adjusted to a concentration of at least 10^9 CFU/ml.

A. niger

After sporulation spores were harvested with 0.05% (v/v) polysorbate 80 in distilled water (dH_2O). The fungal suspensions were adjusted to 10^8 - 10^9 CFU/ml.

Viruses

Confluent monolayers of CrFK-, L20B-, RHK- or RAW 264.7 cells in culture flasks were infected with FCV, PV, HAV or MNV, respectively. Supernatants were harvested after 5 days (PV, MNV and FCV) or 14 days (HAV) and concentrated by ultracentrifugation. Infectious titers of virus suspensions were in the range of 10^8 – 10^9 $TCID_{50}$/ml.

Quantitative suspension tests

Formulations a–f were applied in an Eppendorf Thermomixer at 20°C to preparations of all test organisms specified above. Suspensions of the respective test organisms and disinfectants were mixed at a ratio of 1:10. Suspension tests with poliovirus and hepatitis A virus were also performed with an additional organic burden of 10% (v/v) fetal calf serum, and all solutions were incubated for 20 minutes under constant shaking (300 rpm). As controls, suspensions of the test organisms were exposed to ddH_2O only.

Recovery of bacteria and fungi

After incubation for 20 minutes samples were immediately centrifuged (12000 x g, 1 minute); pellets were harvested and resuspended in equivalent quantities of 0.1 M phosphate buffer pH 7 (containing 3 % [v/v] Tween 80 in case of mycobcteria and *A. niger*). Subsequently,

samples were serially diluted 1:10 in the respective phosphate buffer. From the undiluted test mixtures and their serial dilutions, 0.1 ml aliquots were spread on nutrient media, or in the case of enterococci mixed with nutrient media (1 ml per petri dish). Incubation was performed at 36°C for bacteria, and at 30°C for *A. niger*.

The efficacy of disinfection was indicated by the reduction factor (RF): From the average number of CFUs observed after exposure of test organisms to the respective disinfectant (log N) and the number of CFUs observed for the control samples exposed to ddH_2O (log N_0) the reduction factor was calculated as follows: $RF = \log N_0 - \log N$.

Recovery of viruses

After incubation for 20 minutes 1:10 serial dilutions of samples were performed using nutrient cell culture media as diluents. Inactivation of viruses was determined using $TCID_{50}$-assays on 96 well plates. 100 µl of the various dilutions each were added to confluent monolayers and cytopathic effects were determined after 5 days (PV, FCV, MNV) or 14 days (HAV). Virus titers were calculated according to the method of Spearman and Kärber (Kärber, 1931;Spearman, 1908).

Quantitative carrier tests

Quantitative carrier tests were performed according to a method established at the Robert Koch-Institut, Germany (1995). Briefly: Sterilized frosted glass strips were used as carriers. Suspensions of test organisms were mixed with heparinised sheep blood. Immediately before application onto the carriers, Protamin 1000 had been added to the blood suspensions for coagulation. When coagulation of the blood was accomplished carriers were incubated in 10 ml of the different disinfectant formulations. After exposure to disinfectant formulations, residual contaminations of test organisms in blood were carefully harvested from the carriers.

Recovery of bacteria, fungi and viruses and the calculation of reduction factors were performed as described for quantitative suspension tests.

Efficacy testing of formulations against prions

In vitro carrier assays with PrP^{TSE}

Decontamination of steel carriers from PrP^{TSE} of 263K scrapie agent was tested in an *in vitro* carrier assay as described previously for SDS/NaOH (Lemmer *et al.*, 2004). Similar *in vitro* studies on the decontamination of steel carriers from PrP^{TSE} of patients with sporadic CJD (sCJD) or vCJD were performed with the following modifications: Stainless steel wire grids (DIN 1.4301; Spörl, Sigmaringendorf, Germany) measuring about 100mm x 5mm were contaminated with 25% (w/v) brain tissue homogenate from a patient with sporadic CJD (type MM1; kindly provided by W. Schulz-Schaeffer, Universitätsklinikum Göttingen, Germany) or from a patient with vCJD (kindly provided by the CJD Surveillance Unit, Edinburgh, UK). After drying, grids were incubated in 45 ml of the disinfectant formulations, or water, for 5 min (vCJD) or 20 min (sCJD). Subsequently, grids were washed and dried (Lemmer *et al.*, 2004), coiled up and treated with 300 µl Proteinase K solution (PK, 20 µg/ml) in TBS-Sarkosyl (50 mM Tris/HCl, 150 mM NaCl pH 7.5, 1% Sarcosyl) for 10 min (vCJD) or 20 min (sCJD) at 37°C. After PK digestion, samples were mixed with 100 µl of 4 x electrophoresis sample loading buffer, boiled for 10 min, and analysed by SDS-PAGE and Western-blotting for the presence of PrP^{TSE} using the monoclonal antibody 3F4 as previously described (Lemmer *et al.*, 2004). If not otherwise specified, 25 µl aliquots were used for Western blotting. The studies on tissue samples from human donors were performed in compliance with informed consent and German legal and ethical regulations.

Bioassays with 263K scrapie agent

The disinfection of steel wires contaminated with 263K prion infectivity was examined *in vivo*. For this purpose contamination of wires, processing for decontamination in SDS/NaOH containing 20% or 30% n-propanaol, bioassays in hamsters using a dose-response relationship established by end-point titration, and PET blotting were performed as recently described (Lemmer *et al.*, 2008). The bioassays on the SDS/NaOH/n-propanol formulations were carried out in duplicate (bioassay group 1 and bioassay group 2). The studies in animals complied with German legal regulations and were approved by the responsible ethic committees and regulatory authorities.

Analysis of whether formulations used for disinfection fix protein to rough surfaces

We examined whether formulations a, d, g, h and i - as compared to water - did additionally fix protein to rough test surfaces, using frosted glass strips as carriers. The carriers were contaminated with 10% (w/v) 263K scrapie brain homogenate or sheep blood capable of coagulation (see quantitative carrier test). After contamination, carriers were separately immersed in 10 ml each of the different disinfectant formulations at 20°C for 30 minutes. After rinsing with dH_2O, the glass strips were immersed at room temperature for 45 minutes in 15 ml Coomassie blue solution. Finally, the glass strips were rinsed 4-5 times in destaining solution (50% [v/v] methanol, 10% [v/v] acetic acid in dH_2O). In each series of stainings a non-contaminated glass strip was processed similarly as a negative control for comparison.

Results

Efficacy of the tested formulations against bacteria, fungi and viruses

The efficacy of candidate formulations was tested using a broad range of conventional pathogens known to be highly tolerant to conventional disinfectants, like enterococci, *Mycobacterium avium*, norovirus, poliovirus, HAV, and spores of *Aspergillus niger*. Since the activity against infective agents embedded in proteinacous matrices was of utmost interest, tests using carriers contaminated with pathogens in coagulated blood were performed. Additional suspension tests (some samples of which contained an additional organic burden of 10% fetal calf serum [FCS]) were carried out to corroborate findings obtained with carrier assays.

Efficacy against enterococci and mycobacteria

The efficacy against mycobacteria and enterococci was examined in a quantitative carrier test using coagulated blood as test soil (Table 1). While SDS/NaOH without alcohol showed no relevant inactivating effect, the addition of 50% ethanol or 20% n-propanol resulted in reduction factors of > 6 logs. All formulations found to be effective in the carrier test were also at least as active when assessed in quantitative suspension tests (data not shown).

Efficacy against non-enveloped viruses

Virucidal activities of the various formulations were initially assessed for a broad range of viruses including vacciniavirus (VV), simian virus 40 (SV40), adenovirus (AV), caliciviruses (feline calicivirus and murine norovirus) or poliovirus in quantitative suspension tests. Caliciviruses, poliovirus and HAV were identified as the most tolerant viruses in these assays (data not shown for other viruses), and poliovirus was subsequently also used in quantitative carrier tests with coagulated blood as test soil. The findings with poliovirus, HAV and caliciviruses are summarized in Table 2.

In contrast to the results with bacteria, ethanol or propanol alone did not exert a relevant inactivating effect on these viruses. However, when 20% n-propanol was used in a mixture with SDS/NaOH reduction factors of 4 logs or more were observed in quantitative suspension tests for caliciviruses, hepatitis A virus (with 10% FCS as additional organic burden) or poliovirus (with or without 10% FCS as additional organic burden), and in the quantitative carrier test with coagulated blood for poliovirus.

Efficacy against spores of Aspergillus niger

Neither SDS/NaOH, nor 50% ethanol or 20% n-propanol alone were able to achieve a significant disinfection of carriers contaminated with *Aspergillus niger* spores (Table 3). However, strong synergistic effects in terms of disinfection were observed, yielding reduction factors of > 5 logs, when formulations of SDS/NaOH in 50% ethanol or 20% n-propanol were applied.

Efficacy of the tested formulations against prions

Alcoholic mixtures of SDS/NaOH were tested *in vitro* for their potential to decontaminate steel carriers from PrP^{TSE} that had been coated with brain homogenates of scrapie hamsters or patients with sporadic or variant Creutzfeldt-Jakob disease. The disinfection by these formulations of steel wires contaminated with 263 K scrapie agent was monitored in hamster bioassays.

Decontamination of steel wires from PrP^{TSE} of 263K scrapie hamsters

As described previously (Lemmer *et al.*, 2004) the efficacy of the decontamination of steel wires from PrP^{TSE} was assessed *in vitro* by comparing the initial load of contamination on the wires with the amount of total PrP and PrP27-30 (the proteinase K resistant core of PrP^{TSE}) residually attached to the carriers after processing in the test solutions. According to the

established sensitivity of our assay a complete disappearance of PrP^{TSE} staining in eluates from the wires with or without PK-treatment indicated a 500-1000 fold reduction of PK-resistant PrP or total PrP, respectively.

When contaminated steel wires were processed in a mixture of SDS/NaOH containing 50% ethanol a strong signal for residual PrP was observed after incubation for 5 min (Fig. 1A, lane 1), and after 20 min of incubation weak staining for PrP was still visible without PK digestion (Fig. 1A, lane 3). After PK digestion, a complete disappearance of residual PrP^{TSE} could be observed (Fig. 1A, lanes 2 and 4). When 50% n-propanol was used instead of 50% ethanol in the mixture, residual staining of PrP was less intense after 5 min (Fig. 1B, lane 1) and nearly absent after 20 min of incubation (Fig. 1B, lane 3). After digestion with PK, immunostaining of residual PrP^{TSE} completely disappeared (Fig. 1B, lanes 2 and 4). These findings indicated that 50% n-propanol and particularly 50% ethanol in the solution caused effects that fixed PrP^{TSE} as compared to SDS/NaOH alone [14].

We therefore focussed subsequently on n-propanol and lowered its concentration in SDS/NaOH to 30% or 20%. This led to a virtually complete disappearance of specific PrP immunostaining both without and after PK treatment after incubation for 5 or 10 min (Fig. 1C and D). Obviously, lowering the concentration of n-propanol in SDS/NaOH to 30% or 20% counteracted effects of the alcoholic component that fixed proteins and restored the efficacy of wire decontamination to the level previously observed for SDS/NaOH without alcohol [14]. Thus, in the next step, we validated the prion disinfecting activity of SDS/NaOH in 20 % or 30 % n-propanol by steel wire bioassays.

Bioassay of the disinfection of steel wires contaminated with 263K scrapie agent

For bioassay validation we used a recently published *in vivo* carrier assay (Lemmer *et al.*, 2008). Contaminated wires were implanted intracerebrally into hamsters after reprocessing in the test mixtures and monitored for their potential to trigger clinical or subclinical (i. e.

asymptomatic) infection within an observation period of 500 days. The animals of the positive control group challenged with implanted contaminated wires that had been rinsed in dH_2O only developed terminal scrapie after a survival time of 86±3 (mean±SD) days. According to our previously established dose-response relationship (Lemmer *et al.*, 2008) this confirmed an initial infectivity load of $\geq 3x10^5$ $LD_{50i.c.imp}$ per wire. In contrast, all animals which received wires treated in SDS/NaOH containing 20% or 30% n-propanol stayed free of clinical scrapie symptoms until termination of the experiment at 503 days after wire implantation (Table 4; a total of n=5 animals was excluded from the titration assay due to intercurring death unrelated to scrapie). No subclinical infection could be detected by PET blotting for cerebral PrP^{TSE} in any of the animals that survived without scrapie symptoms until 503 days post implantation (not shown). These findings indicated a reduction in infectivity of ≥ 5.5 log_{10} units (logs). Thus, the addition of 30% or 20% n-propanol did not compromise the prion disinfecting activity previously observed for SDS/NaOH alone (Lemmer *et al.*, 2008).

Decontamination of steel wires from PrP^{TSE} of patients with sporadic and variant CJD

In order to examine the effect SDS/NaOH containing 30% or 20% n-propanol on human PrP^{TSE} *in vitro*, we used steel wire grids coated with brain homogenates from patients with sCJD or vCJD as test carriers. For contamination of grids with sCJD material, brain homogenate from a patient with the most frequent subtype of sporadic CJD (MM1, i. e. PrP^{TSE} type 1, homozygous genotype for methionine at codon 129 of the prion protein gene (Heinemann *et al.*, 2007)) was used. By using steel wire grids (that provided a larger surface for PrP^{TSE} binding than steel wires) as test carriers we were able to monitor depletion of PrP^{TSE} over a range of 3.3 logs (Fig 2, lanes 1-5 in A, and lanes 1-4 in B). With this modified *in vitro* assay no residual immunostaining for PrP^{TSE} could be detected after processing in the SDS/NaOH solution containing 20% n-propanol (Fig 2A, lane 6 and Fig 2B, lane 5) for 20 min (sCJD) or even only 5 min (vCJD). This indicated an at least 2000-fold reduction of the

PrP^{TSE} load on the carriers by this formulation. However, when the SDS/NaOH mixture used for decontamination contained 30% instead of 20% n-propanol, a residual signal of sCJD-associated PrP^{TSE} was observed (Fig 2A, lane 7), indicating a binding or stabilization of this specific form of PrP^{TSE} by the higher concentrated alcohol which did not occur with vCJD material (Fig 2B, lane 6). This binding/stabilizing effect could be omitted by incubation of the sCJD brain homogenate on the grids for 20 min in SDS/NaOH alone (Fig 2A, lane 8), with or without subsequent treatment, again for 20 min, in SDS/NaOH containing 30% n-propanol (Fig 2A, lane 9).

Assessment of whether formulations used for disinfection fix protein to rough carrier surfaces

The formulations were further assessed in a qualitative assay for effects that fix proteins to rough test surfaces. For this purpose, residual protein from hamster brain homogenate or coagulated sheep blood on frosted glass strips was stained with Coomassie blue after processing in disinfectant formulations (Fig 3).

Findings for blood: The visual examination of our assay results revealed that incubation in glutardialdehyde (Fig 3, lower panel, lanes 1&2) or peracetic acid (Fig 3, lower panel, lanes 3&4) resulted in the detection of more coloured organic material on the carrier surfaces as found for water (Fig 3, lower panel, lanes 11&12). The blood sample treated with glutardialdehyde did not show a blue, but a red/black staining. NaOCl produced only a very weak blue staining (Fig 3, lower panel, lanes 5&6), and blood contaminated carriers which were incubated in SDS/NaOH (Fig 3, lower panel, lanes 7&8) or SDS/NaOH containing 20% n-propanol (Fig 3, lower panel, lanes 9&10) did not show a stronger coomassie blue staining than that observed after cleaning with water (Fig 3, lower panel, lanes 11&12).

Findings for hamster brain homogenate: When brain homogenate was used as organic soil instead of blood, only a faint blue staining was found after treatment in glutardialdehyde (Fig

3, upper panel, lanes 1&2) while incubation in peracetic acid (Fig 3, upper panel, lanes 3&4) again produced a strong blue staining signal. With NaOCl or SDS/NaOH no residual staining was observed (Fig 3, upper panel, lanes 5&6 and 7&8, respectively). A weak blue staining of glass carriers after incubation in the formulation of 0.2%SDS, 0.3% NaOH and 20% n-propanol (Fig 3, upper panel, lanes 9&10) indicated residual binding of brain homogenate proteins on the carrier surface, but as for blood this formulation did not produce a stronger coomassie blue staining than that observed with water (Fig 3, upper panel, lanes 11&12). Similarly processed non-contaminated glass strips consistently produced negative results (Fig 3, upper and lower panel, lane 13).

Discussion

Efficacy of tested formulations for broad-range disinfection

We found that a formulation of 0.2% SDS and 0.3% NaOH in 20% n-propanol (pH 13.0 ± 0.05) exerts a strong disinfecting activity against bacteria including mycobacteria, fungal spores, non-enveloped viruses and - prions. This formulation was also effective in the presence of challenging substrates for disinfection such as dried brain homogenate or coagulated blood.

While SDS/NaOH (pH 12.8) alone (Lemmer *et al.*, 2004) was virtually inactive on *E. faecium* and *M. avium*, these pathogens were efficiently inactivated by the formulation containing 20% n-propanol. On the other hand, 20% n-propanol alone did not exert a relevant inactivating effect on poliovirus, HAV or caliciviruses (including murine norovirus), whereas in combination with SDS/NaOH effective inactivation of these viruses was observed. While vegetative forms of fungi are usually quite sensitive to conventional disinfectants spores of *Aspergillus niger* are more tolerant (Rutala *et al.*, 2008). However, we observed a pronounced synergistic effect of SDS/NaOH and 20% n-propanol against spores of *Aspergillus niger*.

The presence of 20% n-propanol did not compromise the activity against 263K scrapie prions previously established for SDS/NaOH alone (Lemmer *et al.*, 2004; Lemmer *et al.*, 2008). The latter finding is of particular importance since alcohols may potentially bind proteins to metal surfaces (Prior *et al.*, 2004), stabilize PrP^{TSE} and enhance the tolerance of prions to inactivation (Taylor, 1999). In cases where higher alcohol concentrations would be required in disinfectant formulations for specific purposes, our findings suggest that adverse effects that fix or stabilise PrP^{TSE} could be avoided by a two-step procedure: Initial treatment in SDS/NaOH alone, and subsequent incubation in formulations containing SDS/NaOH as well as higher concentrated alcohol.

By assessing the decontamination of steel wire grids contaminated with human brain homogenates from a patient with the most frequent subtype of sporadic CJD (PrP^{TSE} type 1,

homozygous for methionine at codon 129 of the prion protein gene) that accounts for about 63% of the sCJD cases in Germany (Heinemann *et al.*, 2007), or from a patient with variant CJD, an at least 2000-fold reduction of the PrP^{TSE} load was demonstrated for SDS/NaOH in 20% n-propanol. This is consistent with the data observed in our study for the disinfection of steel wires from PrP^{TSE} of 263K scrapie agent. Based on recent findings it has been recommended, that any prion inactivation procedures should be validated by bioassay against the prion strain for which they are intended to be used (Giles *et al.*, 2008). This would require comprehensive experiments in animals. Furthermore, even the best bioassay models commonly available for vCJD, familial CJD or the six different forms of sCJD (Heinemann *et al.*, 2007) appear sub-optimal for this purpose in terms of their sensitivity and the rather limited ranges of titre reduction they would allow to demonstrate only. In order to promote the further development of effective disinfectants it might be therefore helpful to comparatively validate candidate formulations against human prions with a focus on the most relevant human TSEs such as sCJD/subtype MM1 or vCJD. Additionally, alternative techniques such as protein misfolding cyclic amplification (PMCA) (Castilla *et al.*, 2006;Jones *et al.*, 2007) or novel cell culture assays may be considered as potential surrogate methods for bioassays in animals.

Protein binding to surfaces by formulations used for disinfection

We performed a qualitative assessment of whether our new disinfectant formulation fixes organic soil containing proteins to carrier surfaces, and whether the formulation does this more, or no more, than water or conventional disinfectants such as peracetic acid. In order to mimick challenging conditions in terms of protein retention, frosted glass strips with a rough surface were used as test carriers in this assay.

By visual examination our coomassie blue assay indicated that glutardialdehyde and peracetic acid fixed blood proteins to carrier surfaces as compared to cleaning by water alone, which is

in accordance with previously reported findings using metal carriers (Kampf *et al.*, 2004) In contrast, blood contaminated carriers which were incubated in the formulation of 0.2% SDS, 0.3% NaOH and 20% n-propanol did not show a stronger coomassie blue staining than that observed after cleaning with water.

When brain homogenate was used as organic soil instead of blood, incubation in peracetic acid again produced a strong staining signal with coomassie blue, while no staining was found after treatment in glutardialdehyde. Since glutardialdehyde is well known to fix protein (Kampf *et al.*, 2004) this finding may indicate that glutardialdehyde interferes with the staining reaction by masking binding sites for coomassie blue. Such potentially interfering effects, which may also explain that the blood samples treated with glutardialdehyde did not show a blue coomassie- but rather their original reddish staining, have to be taken into account when interpreting the findings from our assay. However, they do not seem to account for the absence of coomassie staining after processing of brain homogenate on glass strips with NaOCl or SDS/NaOH, since for these formulations efficient cleaning of steel wire carriers contaminated with 263 K scrapie brain homogenate was already previously demonstrated by electron microscopy (Lemmer *et al.*, 2008). Thus, with respect to the observed weak coomassie blue staining of glass carriers after incubation in the formulation of 0.2%SDS, 0.3% NaOH and 20% n-propanol the most obvious conclusion would be that – as compared to SDS/NaOH alone - some protein was fixed to the carrier surface due to the presence of alcohol. It has been already previously reported that alcohols may potentially bind protein to metal surfaces (Prior *et al.*, 2004). However, despite this potential adverse effect of alcohols, also with brain homogenate the formulation of 0.2% SDS and 0.3% NaOH in 20% n-propanol was not found to fix proteins more to carrier surfaces than was observed with water as cleaning reagent.

Practical considerations and outlook

Our findings suggest a mixture of 0.2% SDS and 0.3% NaOH in 20% n-propanol as a potent candidate formulation for the general elimination of infectious agents in the routine maintenance of surgical steel instruments (including drills used in dentistry). The components of our formulation are inexpensive and the mixture is simple to prepare. The solution was found to be effective within 20 min (the uniform exposure time tested on the examined bacteria, viruses and fungi) or even shorter exposure times tested on prions. As compared to standard formulations commonly recommended for prion disinfection (e. g. 1-2 M NaOH or 2.5-5 % NaOCl) our mixture appears as a rather mild reagent which much higher compatibility to steel surfaces, although the alkaline nature of the formulation would still be corrosive in some situations. N-propanol is easily flammable in higher concentrations and not odorless, but in the light of the widespread use of alcohol-based hand- and instrument disinfectants in hospitals these factors do not appear as critical. SDS, NaOH and n-propanol are potentially toxic, but our formulation can be handled easily and safely under appropriate conditions of use.

Addressing the disinfection of TSE agents not only in its own right but in combination with conventional pathogens as recently also reported by others (Lehmann *et al.*, 2009) may open new avenues for the effective broad-range disinfection of surgical instruments and heat sensitive medical devices.

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Legends to Tables

Table 1

Efficay of the tested disinfectant formulations on bacteria in a quantitative carrier test with coagulated blood.

Reduction factors are indicated in log_{10} units (logs) and were calculated from the difference between the bacterial load on carriers after processing in the tested formulations and the bacterial load on carriers after similar processing in dH_2O only. Red., reduction in logs.

Table 2

Efficay of the tested disinfectant formulations on viruses as determined by different test formats.

Reduction factors are indicated and were calculated as specified for table 1. The disinfecting activities of examined formulations was determined for polio-, hepatitis A- and caliciviruses by quantitative suspension tests (†). The virucidal activity against poliovirus and hepatitis A virus in the suspension test was also monitored with an additional organic burden of 10% fetal calf serum (‡). Inactivation of poliovirus was additionally measured in a quantitative carrier test with coagulated blood (*). The data for caliciviruses refer to both feline calicivirus and murine norovirus; however, where specifically indicated values refer to murine norovirus because of higher observed tolerance (¶). ND, not determined; Red., reduction in logs.

Table 3

Efficay of the tested disinfectant formulations on spores of *Aspergillus niger* in a quantitative carrier test with coagulated blood.

Reduction factors are indicated and were calculated as specified for table 1. Red., reduction in logs.

Table 4

Bioassay for prion infectivity on wires coated with 263K scrapie brain homogenate: Findings after reprocessing in formulations of 0.2% SDS / 0.3% NaOH containing 20% or 30% n-propanol.

For contamination, wires were incubated in 150 µl 10% 263K scrapie brain homogenate, providing an initial infectivity load of $\geq 3x10^5$ $LD_{50i.c.imp}$ per wire. Survival times until the development of terminal scrapie are provided in days post implantation (p.im.; mean±SD), and survival times in bold (>500 days p.im.) refer to hamsters that were sacrificed at the indicated time points without having developed clinical signs of scrapie. Residual wire infectivity was deduced from attack rates and survival times using a dose-response relationship previously established in an end-point titration experiment (Lemmer *et al.*, 2008). Titre reductions were calculated by comparing the residual infectivity with the contamination load prior to decontamination. Bioassays were carried out in duplicate (bioassay group 1 and bioassay group 2). Two (†) or one (‡) out of six challenged animals died for reasons unrelated to scrapie (accordingly, the number animals in the respective groups was set to n=4 or n=5, respectively). Reduction due to rinsing in dH_2O could not be quantified in our experimental setup (*). ND, not determined; Red., reduction in titre [logs]; Res. inf., residual infectivity; RT, room temperature; UD, undetectable.

Legends to Figures

Figure 1

Efficacy of formulations containing 0.2% SDS and 0.3% NaOH in ethanol or n-propanol for the decontamination of steel surfaces from PrP^{TSE} of 263K scrapie agent.

Western blot detection of full-length PrP and PrP27-30, the proteinase K–resistant core of PrP^{TSE}, in eluates from contaminated steel wires after incubation in SDS/NaOH formulations containing 50% ethanol (A) or 50%, 30% or 20% n-propanol (B, C and D, respectively) without (- PK) or after proteinase K (+ PK) digestion.

(A-D) Lanes 10^{-6} or 10^{-7}, internal standards: PK-digested brain homogenate from scrapie hamsters corresponding to $1x10^{-6}$ g or $1x10^{-7}$ g brain tissue. Lane M, molecular mass marker. (A-B) Numbered lanes 1-4 represent protein eluates from 30 contaminated wires incubated for 5 min (lanes 1 and 2) or 20 min (lanes 3 and 4) at 23°C in the formulations. Samples not subjected to PK digestion (lanes 1 and 3) correspond to 46.2 mm^2, PK treated samples (lanes 2 and 4) correspond to 23.1 mm^2 of wire surface. (B) Lanes 5 and 6: Eluates (1:10 diluted in LPP/Urea) from 30 contaminated wires incubated in dH_2O for 20 min at 23°C. These samples correspond to 4.62 mm^2 (lane 5) and 2.31 mm^2 (lane 6) of wire surface.

(C-D) Numbered lanes 1-4 represent protein eluates from 30 contaminated wires incubated for 5 min (lanes 1 and 2) or 10 min (lanes 3 and 4) at 23°C in the formulations. Samples not subjected to PK digestion (lanes 1 and 3) correspond to 46.2 mm^2, PK treated samples (lanes 2 and 4) correspond to 23.1 mm^2 of wire surface. (D) Lanes 5 and 6: Eluates (1:10 diluted in LPP/Urea) from 30 contaminated wires incubated in dH_2O for 20 min at 23°C. These samples correspond to 4.62 mm^2 (lane 5) and 2.31 mm^2 (lane 6) of wire surface.

Figure 2

Efficacy of formulations containing 0.2% SDS, 0.3% NaOH and n-propanol for the decontamination of steel surfaces from PrP^{TSE} of CJD- or vCJD agent.

Western blot detection of PrP27-30, the Proteinase K-resistant core of PrP^{TSE}, in eluates from contaminated steel wire grids after incubation in the formulations and proteinase K digestion. (A) Findings for PrP^{TSE} from sCJD (type MM1) brain homogenate. Lanes 1-5, dilution series of protein eluate from a contaminated steel wire grid (25 µl out of a total sample volume of 400 µl per grid were diluted 1:100, 1:300, 1:500, 1:1000 and 1:2000). Lanes 6-8, protein eluates from contaminated grids incubated for 20 min at 23°C in the following formulations: SDS/NaOH in 20% n-propanol (lane 6), SDS/NaOH in 30% n-propanol (lane 7), and SDS/NaOH without alcohol (lane 8). Lane 9, protein eluate from a grid incubated initially for 20 min at 23°C in SDS/NaOH without alcohol, and subsequently, again for 20 min at 23°C, in SDS/NaOH in 30% n-propanol. Samples were digested with proteinase K (20 µg/ml) for 20 min. 25 µl of the different dilutions and test samples were applied onto lanes 1-9.
(B) Findings for PrP^{TSE} from vCJD brain homogenate. Lanes 1-4, dilution series of protein eluate from a contaminated steel wire grid (25 µl out of a total sample volume of 400 µl per grid were diluted 1:40, 1:100, 1:1000, 1:2000). Lanes 5-6, protein eluates from contaminated grids incubated for 5 min at 23°C in the following formulations: SDS/NaOH in 20% (lane 5) or 30% (lane 6) n-propanol. Samples were digested with proteinase K (20 µg/ml) for 10 min. 4 x 25 µl of the different dilutions and test samples were successively applied onto lanes 1-6 (with intermittent electrophoresis for concentrating applied material in the stacking gel prior to subsequent loading).

Figure 3

Protein binding to rough carrier surfaces by various formulations used for disinfection applied on 10% (w/v) dried hamster brain homogenate (upper panel) or coagulated sheep blood (lower panel).

Contaminated frosted glas strips were tested in duplicate for residual protein by Coomassie blue staining after treatment for 30 minutes at 20 °C in 2% glutardialdehyde (GDA, lanes 1 and 2), 0.35% peracetic acid (PAA, lanes 3 and 4), sodium hypochlorite (NaOCl) with 20.000 ppm available chlorine (lanes 5 and 6), formulations of SDS/NaOH (lanes 7 and 8) and SDS/NaOH in 20% n-propanol (lanes 9 and 10), and ddH_2O (lanes 11 and 12). Lane 13: Non-contaminated glas strip incubated for 30 minutes in ddH_2O. Neg. Ctrl., negative control.

Table 1

Formulation	Concen-tration	Time [min]	Tempera-ture [°C]	*E. faecium* Red. (log_{10})	*M. avium* Red. (log_{10})
SDS / NaOH	0.2% / 0.3%	20	20	0	0
Ethanol	50%	20	20	> 6	> 6
SDS / NaOH in ethanol	0.2% / 0.3 % in 50%	20	20	> 6	> 6
n-propanol	20%	20	20	> 6	> 6
SDS / NaOH in n-propanol	0.2% / 0.3 % in 20%	20	20	> 6	> 6

Table 2

Formulation	Concen-tration	Time [min]	Tempera-ture [°C]	Poliovirus		Hepatitis A Virus		Caliciviruses
				Red.[†] [and] [‡] (log_{10})	Red.[*] (log_{10})	Red.[†] (log_{10})	Red.[‡] (log_{10})	Red.[†] (log_{10})
SDS / NaOH	0.2% / 0.3%	20	20	> 4	≤ 4	≤ 3	ND	> 4
Ethanol	50%	20	20	< 1	<1.5	< 1	ND	< 2[¶]
SDS / NaOH in ethanol	0.2% / 0.3 % in 50%	20	20	> 4	> 4	≥ 4	> 4	> 5
n-Propanol	30%	20	20	< 0.5	ND	< 1	ND	> 4
	20%	20	20	< 0.5	<1.5	< 1	ND	< 1[¶]
SDS / NaOH in n-propanol	0.2% / 0.3 % in 30%	20	20	> 4	ND	≥ 4	ND	> 5
	0.2% / 0.3 % in 20%	20	20	> 4	> 4	> 3 to < 4	> 4	> 5

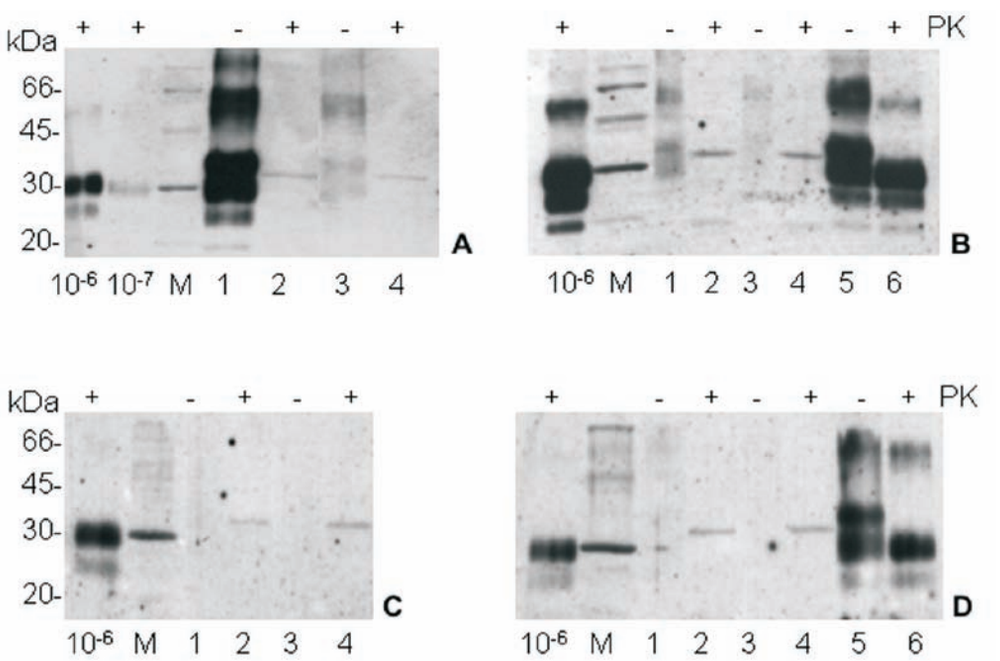
Table 3

Formulation	Concen-tration	Time [min]	Tempera-ture [°C]	*A. niger* Red. (log_{10})
SDS / NaOH	0.2% / 0.3%	20	20	< 0,5
Ethanol	50%	20	20	< 1
SDS / NaOH in ethanol	0.2% / 0.3 % in 50%	20	20	> 5
n-propanol	20%	20	20	< 1
SDS / NaOH in n-propanol	0.2% / 0.3 % in 20%	20	20	> 5

Table 4

Formulation	Concen-tration	Time [min]	Tempera-ture [°C]	Bioassay group 1				Bioassay group 2			
				Attack rate	Survival time (days p.im.)	Res. inf./wire ($LD_{50i.c.imp}$)	Red. (log_{10})	Attack rate	Survival time (days p.im.)	Res. inf./wire ($LD_{50i.c.imp}$)	Red. (log_{10})
None, rinsed in distilled water only			RT	3/3	86±3	≥ $3x10^5$	-*	ND	ND	ND	ND
SDS / NaOH in n-propanol	0.2% / 0.3 % in 20%	10	23	0/4†	*503*	UD	≥ 5.5	0/6	*503*	UD	≥ 5.5
SDS / NaOH in n-propanol	0.2% / 0.3 % in 30%	10	23	0/5‡	*503*	UD	≥ 5.5	0/4†	*503*	UD	≥ 5.5

Figure 1


kDa
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45-
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20-
+ + - + - +
10-6 10-7 M 1 2 3 4
A
+ - + - + - + PK
10-6 M 1 2 3 4 5 6
B
kDa
66-
45-
30-
20-
+ - + - +
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C
+ - + - + - + PK
10-6 M 1 2 3 4 5 6
D

Figure 2

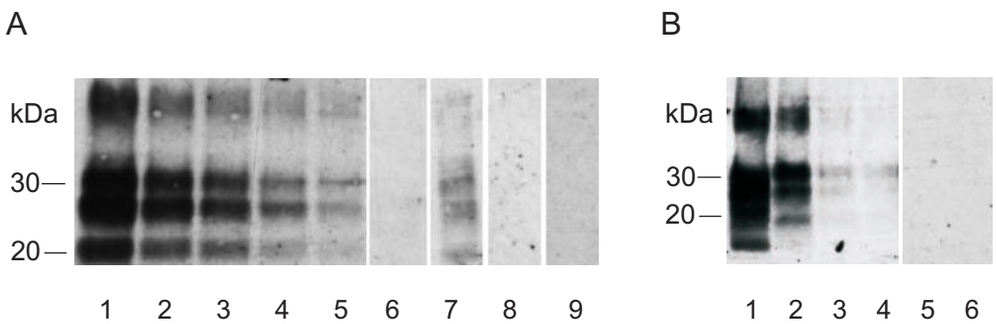
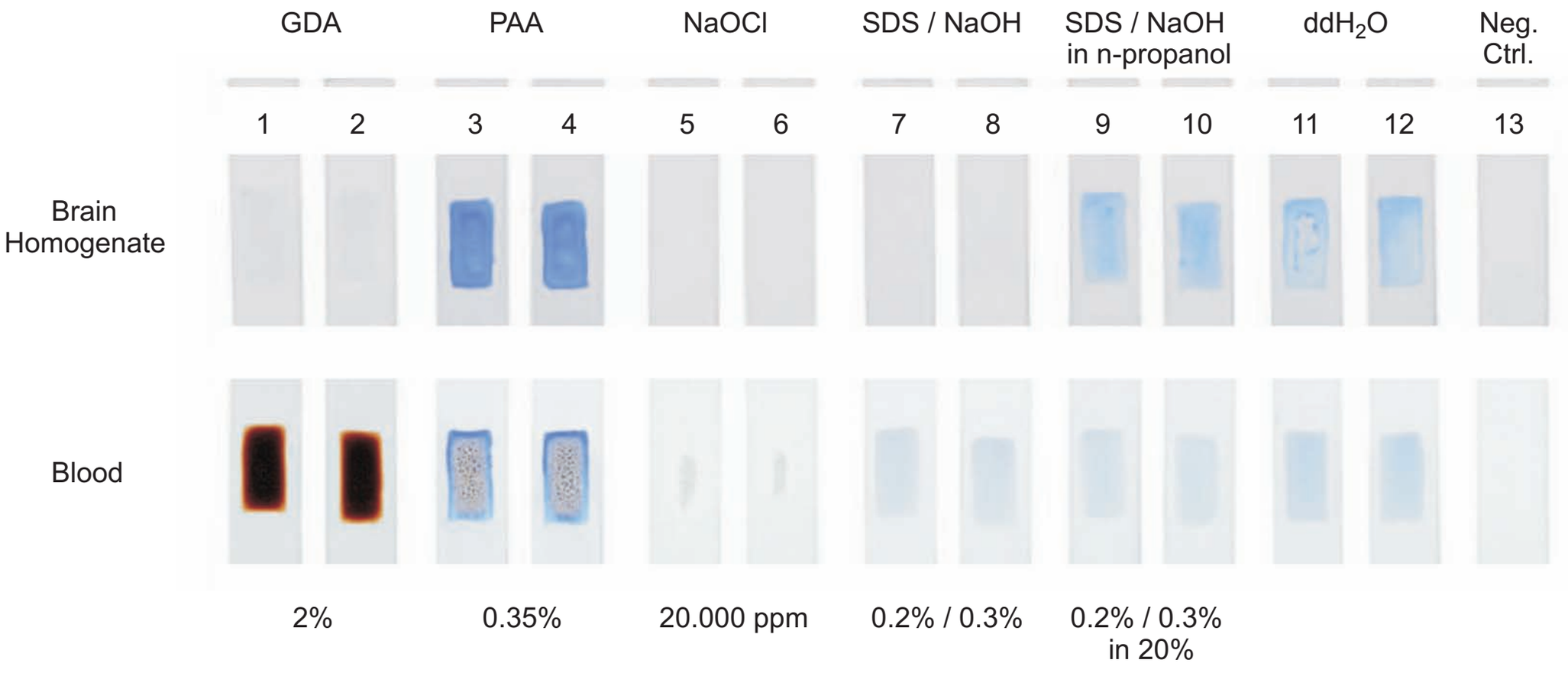


Figure 3


GDA
PAA
NaOCl
SDS / NaOH
SDS / NaOH in n-propanol
ddH2O
Neg. Ctrl.
1
2
3
4
5
6
7
8
9
10
11
12
13
Brain Homogenate
Blood
2%
0.35%
20.000 ppm
0.2% / 0.3%
0.2% / 0.3% in 20%