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Risk assessment of laboratories involving the manipulation of unconventional agents causing TSE

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Cover image : NMR structure of hamster PrP-C fragment

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RISK ASSESSMENT OF LABORATORIES INVOLVING THE MANIPULATION OF UNCONVENTIONAL AGENTS CAUSING TSE

A. Leunda Casi, K. Pauwels, P. Herman, C. Verheust, W. Zorzi, O. Thellin, S. Roels, B. Van Vaerenbergh

The present document aims at summarizing the biosafety recommendations and the containment level required for laboratories where animal and human tissues potentially contaminated by a TSE (Transmissible Spongiform Encephalitis) causing agent are manipulated. A particular attention will be paid to decontamination procedures, as the prion protein[1] is remarkably resistant to conventional inactivation methods and may stay infectious for long periods of time. We will discuss large surface decontamination procedures of facilities handling TSE causing agents. This is of specific concern for laboratories that have been manipulating TSE causing agents, sometimes for years, but wish today dedicate the facilities to another activity.

[1] *For convenience, in this document, the term "prion" will be used for the abnormal form of the prion protein*

1| Introduction

Prions are proteinaceous infectious particles that cause invariably fatal neurodegenerative diseases also called transmissible spongiform encephalopathies (TSE) including Creutzfeldt-Jakob disease (CJD) and kuru in humans, bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep and goats. The prion is a product of a specific gene, namely the *PrP* gene. Transcription of this gene and translation of the mRNA generate the protein PrP (or PrP^{c} for cellular PrP) which is the precursor of the prion protein. The protein PrP (or PrP^{c}) undergoes several post-translational events to become the prion, also designated as PrP^{Sc} in recognition of scrapie, which is the earliest described prion-associated disease. Other names are used to designate the prion: PrP^{Res}, which indicates the protein that shows high resistance and PrP^{Dis}, which indicates the protein that is responsible for the disease.

No differences could be found in chemical composition between PrP^{c} and PrP^{Sc}. This means that the amino acid sequence, the glycosylation, and lipid anchor of both isoforms are identical. However, they differ significantly in secondary structure. PrP^{c} contains α-helices predominantly and has only a little β-sheet content, whereas PrP^{Sc} is characterized by similar amounts of α-helices and β-sheets. This conformational change has significant consequences as it imparts to the prion protein the ability to induce the same conformation in the normal PrP protein leading to the aggregation of β-sheet-forming peptides and form amyloid fibrils. The amyloid fibrils are finally responsible of the destruction of the host cells, neurons among others, which destruction is irreversible. The enigma of how PrP^{Sc} multiplies and causes disease is still largely unknown.

2| Biological hazards of TSE causing agents

CJD, the most commonly encountered prion-associated disease in humans, may result from spontaneous or inherited mutations in the gene encoding PrP (sporadic or familial CJD) or after medical and surgical procedures such as administration of prion-contaminated growth hormones, transplantation of prion-contaminated dura mater and corneal grafts (iatrogenic CJD). Human-to-human transmission of the disease has also occurred through ingestion such as in the kuru disease.

Prion transmission is generally believed to be species specific. Infection of one organism with prions generated in another species often results in delayed, if any, onset of disease. However, after clinical disease has been established in the foreign host, subsequent passaging of prions isolated from this host through further individuals often results in adaptation to this new species with a corresponding decrease in incubation times (Cobbs and Surewicz, 2009). On the other hand, a growing body of evidences suggests that ingestion of the agent of bovine spongiform encephalopathy (BSE) results in neurodegenerative disease in a variety of mammals, including humans. The feeding of BSE-contaminated meat to livestock may be responsible for the outbreak of BSE in Great Britain in the nineties. The subsequent consumption of diseased cattles by humans is believed to be responsible for the emergence of variant CJD (vCJD) in humans. A hypothesis advanced to explain the prion capacity to cross the species barrier is that a single prion protein, even though unaltered by genetic modification, may give rise to different strains of prions. These strains could be accounted for by different misfolded conformations of the same protein. The generation of different strains could sometimes enable prions to jump the “species-barrier” to infect a species other than the one originally infected (Casalone et al., 2004, Collinge and Clarke, 2007).

Major concerns still remain with regard to TSEs: the not yet well-understood behaviour of prion proteins, the underestimation of prevalence of the disease in the population (animal and human) and the difficulties in detecting it in organic material and diagnose the disease in living species. In Belgium, several laboratories perform prion protein research and routine diagnostics on animal and human tissues. In 2000, the WHO published guidelines for the prevention of iatrogenic and nosocomial exposure to TSE agents (WHO, 2000). These guidelines were essentially intended for healthcare workers and patient care facilities. The BMBL (5^{th} ed, 2007) proposes then specific biosafety measures for laboratories and facilities manipulating prion proteins.

3| Risk assessment and risk management

Unconventional agents associated with TSEs are classified under class of risk 3 in Belgium (Belgian Biosafey Server, 2009). The evolution of prion-related diseases is slow but always fatal. There are neither medicines nor vaccines against prions. The most probable ways of contamination during laboratory activities are the accidental parenteral inoculation and the ingestion of infected material. To date, there is no indication that the prion protein is transmitted by aerosols. However, the possibility of contamination occurrence by inhalation or indirect ingestion of infectious aerosols could not be totally discarded. According to the Canadian Food Inspection Agency, no occupational infections have been recorded from working with prion proteins (PHA Canada, 2005). The highest concentration of prions is found in the central nervous system. However, prions can also be found in other tissues such as cerebrospinal fluid, peripheric nervous system, muscles, lung, liver, kidney, lymphoid tissue (spleen, lymph nodes, tonsils) and placenta. The BMBL (5^{th} ed, 2007) recommends for research and diagnostic laboratories to manipulate prions at a containment level 2 or 3 depending on the type of tissue handled, the nature of the manipulation and the amount of material handled. However, most activities manipulating human prions are conducted in containment level 3 facilities.

In Belgium, most activities handling prions are related to the epidemiology surveillance program of TSE in animals using their brain material. Laboratories of veterinary TSEs diagnosis are accredited under the ISO norm 17025 and their activities are under the supervision of the Federal Agency for the Safety of the Food Chain (FASFC, Belgium). Except the National Reference Laboratory VAR (Veterinary and Agrochemical Research Centre), most of these laboratories only use one rapid technique. This technique is based on an immuno-enzymatic test (ELISA), mainly starting with grinding and homogenization of the samples, followed by treatment with proteinase K which degrades totally the normal PrP protein and partially the prion protein (PrP^{Res}). By applying a PrP specific antibody to the protease treated homogenate, the remaining PrP^{Res} can be detected and visualized (BIO-RAD). One (out of the 17) laboratory uses an ELISA in which no treatment of proteinase K is needed but instead they use a specific marker, called the “ligand” (IDEXX).

This type of activity must be performed in a containment level 3 facility adapted to BSE work (also noted L3-BSE). In a L3-BSE, some L3 containment criteria may not be applied, such as:

- a negative pressure differential between the room and the adjacent area;
- the necessity to filter exhaust air through a HEPA filter;
- the necessity of an airtight room allowing gas fumigation.

The L3-BSE requirements take into account the non-airborne nature of the prion protein and the resistance of the prion to commonly used disinfectants such as formaldehyde. To perform some of the method steps of TSE detection which are of particular biosafety concern because they may generate infectious aerosols (crushing, homogenization, centrifugation or heating) the facility must be equipped with closed systems allowing the complete containment of the infectious material during these manipulations. One of the most essential aspects when performing laboratory work in a L3-BSE consists in a good training of the personnel, good work practices and an adapted waste management. Annex 1 summarizes the technical characteristics and equipment, the work practices and waste disposal management required in Belgian laboratories that perform rapid BSE detection testing.

4| Decontamination procedures

The conformational change in PrP protein which lead to a high β-sheet content renders the prion protein particularly resistant to commonly used chemical and physical decontamination methods. The proteinaceous infectious particle is an insoluble and partially protease-resistant particle. Moreover, the degree of resistance to protease and to thermal inactivation varies between different strains of prions (Jackson et al., 2005). It is recommended to validate all prion inactivation procedure by bioassays against the prion strain for which it is intended to be used (Giles et al, 2008). This is of major concern for the inactivation studies of different prion proteins or strains specific to humans and other large mammals. The necessity appeared for prion infectivity studies to develop host models with short incubation period and also prion strains initially isolated from another species but adapted to the new host model. Therefore, the recommendations for prion inactivation are based on rodent-passaged prion isolates. The development of transgenic mice expressing a chimerical human/mouse PrP, the human PrP, the bovine PrP or the mouse PrP that are highly susceptible to specific prion strains provide sensitive model systems (Korth et al., 2003; Béringue et al., 2008).

The commonly used autoclave cycle of 121°C for 18 minutes or six successive cycles of 3 minutes each, are ineffective to decontaminate material. Procedures which result in “fixing” the prion agent, such as drying, treatment with organic solvents or cross-linking with aldehyde based disinfectants result in an increase in the proportion of prion protein demonstrating resistance to inactivation. The fixing properties of the agent probably modulate resistance to inactivation by either preventing access to the inactivation solution (i.e. drying) or by preventing denaturation of the aggregated prion protein (i.e. aldehyde fixation). So, formaldehyde or formalin-fixed, glutaraldehyde-fixed and paraffin-embedded tissues, particularly of the brain, remain infectious for long periods, if not indefinitely and gases such as ethylene oxide and formaldehyde are ineffective, as are most chemical disinfectants in standard conditions. Table 1 summarizes chemicals and processes used generally for decontamination and their effectiveness against prions.

Annex 2 describes recommended BMBL methods for effective prion decontamination of surfaces and wastes. Aside from these recommendations, it is now recognized that decontamination processes that combine physical and chemical procedures are the most effective to decontaminate material or waste. Examples of such combination are autoclaving in sodium hydroxide 1M at 121°C during 30 minutes or immersing in sodium hydroxide 1M or sodium hypochlorite 2% during one hour followed by autoclaving at 121°C during one hour and in more secure way, at 134°C during one hour (Oberthür et al., 2007). The processes effective to completely inactivate prions are particularly harmful for human health and are extremely corrosive for some commonly used surface finishes. So care should be taken when handling these chemicals at work concentrations. Personal protective equipment such as use of gloves, eye and face protections and lab coats should be worn.

Incineration at a temperature higher than 800°C is the preferred method that completely destroys the prion protein in infectious biological waste and disposable material. However, other alternatives must be applied for reusable material and for large surface decontamination. Concerning the reusable material and instruments, it is highly recommended to avoid the use of them as much as possible and to prefer disposable material. When unavoidable their sterilization is indeed a major issue for the prevention of iatrogenic CJD in hospitals where surgical instruments and devices that could still be contaminated by a TSE causing agent are re-applied on new patients. Medical reusable material should be extremely well sterilized because it is known that transmission of disease is achieved with tiny amounts of infected tissue. Specific practice recommendations of the CDC exist that should be followed in order to increase the effectiveness of the disinfection procedure and to decrease the risk contamination with prions. The reusable instruments:

- should be kept wet until cleaned and decontaminated;
- should be cleaned as soon as possible to prevent drying of material;
- do not mix instruments used on materials potentially contaminated with prions with instruments used for other purposes:
- Instruments that will be cleaned in a dishwasher must first be decontaminated and the dishwasher must be run through an empty cycle before being used for other instruments.

It has to be mentioned that biomedical and biotechnology companies as well as public health services make today important efforts to develop novel and safer disinfectants and decontamination methods able to effectively decontaminate surfaces, which are not resistant to the highly corrosive treatments with NaOH or sodium hypochlorite. In the case of surgical instruments, it is observed that prion proteins have a high affinity and bind tightly to stainless steel material. This prion propriety renders the decontamination procedure particularly challenging to achieve. In addition, the material is sensitive to the corrosive NaOH or NaOCl solutions usually used as well as to high temperatures. Surfaces of devices usually used in laboratories must also be considered in this sensitive material category. Thus, the need to find alternatives rapidly appears. Ways of research focus on sterilization methods using oxidizing agents such as ozone (TSO3, Johnson et al., 2009) or a mix of hydrogen peroxide and peracetic acid, the hydrogen peroxide with copper (Lehmann et al., 2009), the hydrogen peroxide gas plasma system (Rogez-Kreuz et al., 2009), the protease enzymes, detergents and a combination of both, or the phenolic disinfectants (Fichet et al., 2004) in particular conditions of humidity, temperature, pH and contact time (Jackson et al., 2005). By this way, they also have demonstrated that treatments, which were considered ineffective against prion, may be indeed effective but under different conditions of use or when used in specific combinations.

5| Decontamination of large areas

Fumigation of facilities by gaseous disinfectants is generally performed in situations of definite and widespread contamination of the laboratory surfaces or the biosafety cabinet with an airborne and virulent infectious agent. Despite its less disinfection effectiveness than the liquid form, the gaseous form has indeed the advantage to reach locations that are normally inaccessible to decontamination with liquids disinfectants. In Belgium, the general design features of containment level 3 facilities must allow fumigation with gaseous disinfectant to decontaminate the facility in case of large or uncontrolled area contamination (spills). This is achieved most of the time with formaldehyde. However, prions are resistant to formaldehyde and the L3-BSE technical characteristics, as it is, could not allow fumigation. The decontamination procedure against prions for large surfaces is clearly challenged. Moreover, due to evidence-based considerations, the requirement and benefits of decontamination by fumigation could be questioned. So, in a situation of accidental spill or splash with an infectious liquid or tissue in the room or in the biosafety cabinet, the contaminated surface must be firstly treated by a strong liquid disinfectant such as NaOH 1M before considering fumigation.

Studies suggest that hydrogen peroxide in its vapour-form may provide the expected efficiency for prion protein decontamination. Hydrogen peroxide is a disinfectant and sterilizer, as well as a potent but relatively safe antimicrobial agent. It acts as an oxidant by producing hydroxyl radicals, which attack essential cellular components including proteins that are broken into smaller peptides (McDonnell & Russell, 1999). Two companies, Bioquell and Steris, specialized in bio-decontamination in R&D, clinical and pharmaceutical facilities, have developed a surface decontamination system based on the use of hydrogen peroxide. Researches conducted by these companies in association with EPA (US) and HPA (UK) indicate that the hydrogen peroxide gas would be efficient against prion proteins. These low temperature processes leave no residuals or toxic by-products, only water vapour and oxygen. Both systems have been validated and are effective against a wide range of micro-organisms, including bacterial spores, bacteria, viruses and fungi. Bioquell proposes the HPV (Hydrogen Peroxide Vapour) system, a wet process in which the produced gas is subsequently condensed. The decontamination of a room is achieved by depositing a layer of 'micro-condensation' of hydrogen peroxide over all surfaces. Steris commercializes the VHP system (Vaporized hydrogen peroxide), a dry process in which the produced gas is maintained in its gaseous phase and does not condense.

Bioquell and Steris claim that their systems are efficient for prion inactivation, but no studies are currently reported for large area decontamination process. Most of the studies on the hydrogen peroxide gas efficiency against prion contamination focus on the decontamination of re-usable devices and surgical instruments in hospitals. In this case, cleaning surfaces with a detergent before fumigation is the first step in the decontamination procedure. It will always guarantee the remove of organic and inorganic material increasing the gas efficiency. This is illustrated in a study on the decontamination of wires infected with prions (Fichet et al., 2004). It shows among others that the

combination of an enzymatic cleaner followed by VHP performs successfully a 5 to 6 log reduction of infectivity in the used model, the same reduction was reached by the recommended chemicals (NaOH 1M or NaOCl 20000 ppm for 1 hour).

The hydrogen peroxide gas may also be combined with other elements or used in processes that increase the production of free radicals and its oxidising powerful, the principle of the peroxide disinfection property. The combination of peroxide hydrogen gas (as well as liquid) with copper, iron or manganese results in the production of free radicals, including the hydroxyl radical $^{\cdot}OH$. Same results are obtained combining the use of the hydrogen peroxide with ozone, UV, peracetic acid or plasma (McDonnell, 2007). It has also to be mentioned that repeated fumigations also increase the decontamination efficacy.

Fumigation with the oxidant agent chlorine dioxide, a broad-spectrum biocide, may also provide an alternative for large area decontamination. The inactivation of prions by liquid chlorine dioxide has been studied. Chlorine dioxide (50 ppm) exhibited moderate to substantial inactivation and it was suggested that doubling or tripling its concentration would be more effective in inactivating the agent of CJD (Block, 2001). However, at the present time, there is no study available reporting on the potential capacity of chlorine dioxide to inactivate prion-contaminated surfaces.

6| Conclusion

TSE causing agents present a particular resistance to conventional decontamination agents. Current protocols include treatment of materials and wastes with high concentrations of corrosive sodium hydroxide solution or the sodium hypochlorite solution followed by autoclaving. For large-scale decontamination of facilities handling prion or biosafety cabinets, formaldehyde fumigation should not be used as this agent may increase the prion resistance. Studies indicate that hydrogen peroxide or other oxidizing molecules in their gas form or included in aerosols may represent an alternative. The hydrogen peroxide oxidant agent when used as a fumigant provides an efficient prion inactivation of contaminated surgical material.

To date there is no evidence for a validated fumigation procedure for the decontamination of facilities that are potentially contaminated with prions. More in dept studies are still required. On the other hand, one could wonder whether the criterion for effectiveness and outcome of a fumigation procedure of these facilities should meet "evidence-based" risk assessment considerations or "zero-risk tolerance" considerations. To examine "evidence-based risk assessment" considerations, following questions should be raised:

A| Biological risks associated to the nature of prion proteins

Though prion proteins are particularly resistant to commonly used inactivation methods, it should be emphasized that prion proteins are no air-borne agents and that there is no indication that prions are transmitted by aerosols. Even though this way of transmission could not be discarded, the most probable ways of contamination during occupational exposure are still the parenteral inoculation and ingestion.

B| Biological risks associated to the nature of the contained use activities

The applied work practices, the lab design and the technical characteristics of the L3 were adapted to contain the prion during manipulations. It is of relevance however to consider the number of positive specimens as well as concentrations of infectious material that have been handled in the laboratory during the time of prion activity. The higher the number and the concentrations of positive TSE samples, the greater is the probability to contaminate the room. The number and the type of spills that occurred during the years of prion activity is also a factor that may increase this probability. In case of a diagnostic laboratory, the quality system in place (generally ISO 17025) will allow tracing of the number of positive samples and incidents that occurred and cleaning procedures in the laboratory.

Sampling of selected surfaces (wall, floor, door, and equipment) could be a way to determine the contamination of the facility. In this case, the surface to sample must allow to take down the contaminating prion and, one should keep in mind that standard detection methods based on immunoassays will only allow detecting high concentrations of contaminating agent and may thus give false negative results. On the contrary, methods based on bioassays, as far as the adapted biological model is available, will allow to detect low level of prion contamination. However, these methods need a long time (sometimes a year) before getting results.

C| Biological risks associated to the nature of the future activity

It may be important to consider what will be the new activity of the laboratory and which containment level it will be adopted. In case the future activity will continue to need a containment level 3, the work environment and particularly the work practices will remain stringent and will therefore reduce the possibility of exposure to remaining contaminating prions. However, one should keep in mind that standard decontamination methods usually used in L3 containments for conventional micro-organisms do not insure effective decontamination of prions and may even reinforce the resistance of prions to disinfectants. In case the TSE laboratory is aimed to be declassified to a laboratory of containment level 1 or even an office (without assignment of a containment level), only surface strong treatment

with concentrated liquid NaOH or sodium hypochlorite during an appropriated time exposure, followed by an oxidizing agent fumigation, will insure an effective decontamination. All devices and equipment of the laboratory should then be dismantled and destroyed by incineration.

In conclusion, it is important to evaluate the remaining biological risk associated to the potential contamination of the facility and the type of the future activity that will take place in this facility in order to evaluate and to decide if the large surface decontamination is necessary. In case the evaluation shows a measurable prion contamination, it is recommended in the current state of knowledge, to apply a strong liquid disinfectant before fumigation with an oxidizing agent such as the hydrogen peroxide. However, more studies should demonstrate the efficacy of the hydrogen peroxide or other process against the non-conventional agent of the TSE.

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Annex 1: Design features, technical characteristics, safety equipment, work practices and waste disposal management required in Belgian laboratories that perform rapid BSE detection testing

Lab design and technical characteristics

1. The laboratory is physically separated from other facility areas in the same building or is located in a separate building. The laboratory is exclusively dedicated to BSE manipulation.
2. The entry into the laboratory occurs through an airlock or a L2 laboratory.
3. The entrance door is self-closing and lockable.
4. Windows are sealed.
5. Furniture is designed to facilitate room cleaning and decontamination and also a pest control management program.
6. An observation window or alternative must allow observation inside the laboratory.
7. The contained area has a hands-free or automatically operated sink for hand washing and decontamination. The sink is located in the airlock or near the room exit door.
8. A locker room or coat hooks must be provided for clothing. Lab coats and city clothes must be separated.
9. Floor and bench tops are easy to clean, impervious to water and are resistant to acids, alkalis, organic solvents, and those disinfectants and chemicals used for decontamination.
10. The laboratory is equipped with a fire detection and alarm system.
11. The laboratory is equipped with an interphone, a phone or any other system for external communication.

Safety equipment

12. The laboratory has at least one class II biological safety cabinet (BSC). All open manipulations of infectious materials are performed under the BSC. The BSC is installed in order to avoid disturbing airflow equilibrium inside the work area. It is located away from doors, windows, room supply and exhaust air louvers, and from heavily travelled laboratory areas. It is controlled and certified when placed, after each move and at least once a year.
13. In case the air from the BSC is recirculated into the laboratory, it is recommended but not mandatory to filtrate the exhaust air through 2 HEPA filters considering the small size and the particular nature of prions and the inability to inactivate prions with a standard gaseous agent such as formaldehyde.
14. An autoclave is located in contained area.
15. The laboratory must be equipped with centrifuges. Biological material must be contained in leak proof tubes and centrifuges should be equipped with safety cups in order to contain any aerosol, which could be produced in case of tube damage.

Work practices and waste management

16. Access to laboratory is restricted to authorized personnel. Personnel must be informed about the risk. An access control system must be placed.
17. On the lab access door, the following information must be displayed:
    - Biohazard sign
    - Containment level
    - Coordinates (name and phone number) of the person responsible for the contained area,
    - The nature of the biological risk
    - The list of authorized personnel
    - Required procedures for entering the contained area.
18. Dedicated equipment is assigned to the laboratory.
19. A protective coat, preferably disposable, is permanently worn in the laboratory and should not be worn outside the laboratory.
20. Disposable gloves are available for the personnel and must be worn during manipulation.
21. A Mask, eye protection or a face shield are worn during manipulations likely to create splashes or aerosols.
22. Any skin damage must be well protected by an occlusive waterproof dressing.

23. When not manipulated, all infectious material is contained in closed systems (tubes, boxes,…).
24. Infectious splashes created during operations such as mixing, homogenization, centrifugation must be avoided, preferable by the use of closed systems (rotors or screw cap buckets and BSC or similar).
25. All manipulations likely to produce infectious aerosols or involving potential risks are conducted within a class II BSC.
26. The use of needles and other sharp instruments should be avoided. If this is impossible, the instruments should be adequately managed to prevent or reduce the risk of percutaneous injuries.
27. Mechanical pipetting devices are used. Mouth pipetting is prohibited.
28. Hygienic rules are strictly respected. Eating, drinking, smoking, handling contact lenses, applying cosmetics, and storing food for human consumption are not permitted in the laboratory.
29. An updated register must be kept with all pathogenic agents manipulated and stored.
30. Control measures and control and protection equipment must be regularly checked.
31. Handwashing is mandatory when leaving the laboratory and each time it appears to be necessary.
32. Instructions for correct use of an appropriate disinfectant are available to the personnel. Depending on the purpose, instructions precise the kind of disinfectant to use, its concentration and contact time.
33. Instruction of personnel on biosafety aspects is conducted as well as a follow up and regular updates. The personnel are specifically trained to work in an area with containment level 3.
34. A biosafety manual is prepared and adopted. Personnel are advised of special risks they are exposed to and are required to read instructions on work practices. Behaviour in case of an accident is clearly posted in the laboratory. All exposure accidents to BSE or BSE waste must be reported.
35. Specific decontamination and inactivation procedures must be applied. It is recommended to use disposable material. If large equipment is used, all constitutive elements must be exclusively dedicated to the BSE activity.
36. The biohazard sign must be posted on incubators, fridges, freezers, cryogen tanks, where biological material is stored
37. A pest control program is implemented.
38. Contaminated waste and /or residual biological material are inactivated by an appropriate and validated method before disposal.
39. Contaminated material (glass, microscopic slides,…) must be inactivated by an appropriate and validated method before being washed, re-used and/or destroyed.

Concerning decontamination procedures and waste management, specific inactivation procedures are required because the BSE is resistant to commonly used physical and chemical inactivation procedures. The following procedures are recommended:

- Chemical inactivation with sodium hypochlorite at 6° or with sodium hydroxide 1M during one hour. However, this method seems not to be totally effective. (or this method does not seem totally effective)
- Physical inactivation by autoclaving at 134°C during at least 18 minutes. This method seems also not to be totally effective.

In addition to the inactivation methods, the following precautions are taken:

40. Material and instruments must be well washed before inactivation.
41. BSE contaminated material must be autoclaved separate from other material.
42. Autoclaves must be regularly controlled and validated.
43. Work surfaces should be covered with an absorbent material that can be incinerated. This type of material is also used to recover spills.
44. To eliminate waste, leak proof containers must be used: e.g. two bags/recipients placed one in another avoiding contamination of the outside. The container is closed and labeled with the biohazard sign before leaving the contained zone.
45. All contaminated waste and disposable material must be removed by a specialized and accredited company for incineration.

Table 1: Chemicals and processes ineffective, partially ineffective and effective against TSE causing agents (based on WHO infection control guidelines for TSE, 2000)

Chemical disinfectants	Gaseous disinfectants	Physical processes
Ineffective Alcohols Ammonia β-propiolactone Hydrogen peroxide Phenolics SDS 5% **Variably or partially effective** Glutaraldehyde Chlorine dioxide Iodophores Guanidium thiocyanate 4M Urea 6M Sodium dichloro-isocyanurate Sodium metaperiodate Hydrochloric acid Peracetic acid **Effective** Sodium hypochlorite 2% during 1hour Sodium hydroxide 1M during 1 hour	**Ineffective** Ethylene oxide Formaldehyde	**Ineffective** Dry heat (<180°C) Boiling Ionizing, UV or microwave radiation **Variably or partially effective** Autoclaving at 121°C for 15 minutes Boiling in SDS 3% **Effective** Autoclaving at 134°C for 18 minutes

Annex 2: Effective inactivation of prions, based on the guidelines from BMBL 2007 and the CDC

1. Contaminated surfaces: may be treated with a bleach solution in a concentration of 20000 ppm available chlorine for 1 hour **or** with NaOH 1N for 1 hour.
   Repeated wetting with the disinfectant over 1 hour will be required. After the treatment period, surfaces should be thoroughly rinsed with clear water.
2. Contaminated reusable instruments: immerse in 1N NaOH or sodium hypochlorite (20000 ppm available chlorine) for 1 hour, transfer to water, autoclave (gravity displacement) at 121°C for 1 hour **or** immerse in 1N NaOH or sodium hypochlorite (20000 ppm available chlorine) for 1 hour, rinse with water, autoclave (gravity displacement) at 121°C for 1 hour or at 134°C for 1 hour (porous load).
3. Liquid waste: mix with NaOH for a **final** concentration of 1N NaOH and keep at room temperature for 1 hour **or** mix with bleach for a **final** concentration of 20000 ppm available chlorine and keep at room temperature for 1 hour. After treatment, liquid waste may be neutralized and discharged to the sewer or disposed of as chemical waste.
4. Contaminated dry waste: should be removed for incineration.