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REVIEW PAPER

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Standardization of BSE rapid test performances and experiences gathered during the implementation of large-scale testing

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Abstract The accumulation of pathological prion protein is used as a diagnostic marker for transmissible spongiform encephalopathies. According to European Union (EU) regulations cattle older than 30 months of age (Germany, France, Italy, and Spain by national law >24 months) and slaughtered for human consumption must be tested by using rapid tests for bovine spongiform encephalopathy (BSE). Likewise fallen stock and clinically affected animals must be tested. This article gives a short overview of the incidence of BSE in Europe. The diagnostic hierarchy, i.e., the officially approved methodology for the confirmation of suspect rapid test cases, and the organization of the numerous laboratories involved in this large-scale testing for BSE are described. Special emphasis is given to necessary quality control measures currently in place for BSE rapid testing laboratories and to measures intended to assure a consistent performance of the commercially available rapid test kits.

Keywords Bovine spongiform encephalopathy · Rapid tesing · Standardization · Reference material · Proficiency testing · Quality assurance

Introduction

Bovine spongiform encephalopathy (BSE) belongs to the group of transmissible spongiform encephalopathies (TSEs) (Table 1), which are believed to be caused by infectious proteins, the so-called prions. BSE was first diagnosed in 1986, in the United Kingdom and, since then, has led to a huge epidemic with possibly more than 2.5 million animals having been infected. While the majority of these infected animals were slaughtered and used for human consumption before the onset of clinical symptoms, about 180,000 cattle grew old enough to develop clinical signs and could thus be diagnosed. Until 2000, indigenous BSE cases were only found in a few European countries. However, after the introduction of obligatory BSE rapid testing in January 2001, cases were found in almost every European state (Table 2) and the incidence rate in countries with previously confirmed cases rose substantially (Fig. 1). Countries like Germany, which were considered BSE free until then, confirmed 125 cases in 2001 and 106 cases in 2002.

The host encoded cellular prion protein (PrP^{C}) plays a crucial role in the pathogenesis and infection cycle of TSEs. Following infection, it is converted into the pathological, assumably infectious isoform designated (PrP^{Sc}). The conversion is characterized by an increase in the β-sheet content and hydrophobicity of the protein which results in an agreggration of PrP^{Sc} into amorphous or plaque-like depositions in vivo and into fibrillar structures (so-called scrapie-associated fibrils or SAFs) in vitro. In contrast to PrP^{C}, PrP^{Sc} partially resists proteinase K (PK) cleavage, i.e., only about 70 amino-terminal residues are cleaved off, leaving behind a resistant fragment whose molecular mass is reduced by 6–7 kDa. PrP^{C} has been found in more than 60 mammalian species which have been analyzed to date, and also in fish and avian species.

Table 1 Transmissible spongiform encephalopathies in animals and humans

Disease	Origin	Host species	First report
Scrapie	Transmissible	Small ruminants	1,732
Creutzfeldt-Jakob Disease (CJD)	Sporadic hereditary transmissible	Man	1,920
Gerstmann-Sträussler-Scheinker Syndrome (GSS)	Hereditary	Man	1,936
Transmissible mink encephalopathy (TME)	Transmissible	Mink	1,947
Chronic Wasting Disease (CWD)	Transmissible	Cervids	1,967
Kuru	Transmissible	Man	1,957
Fatal familial insomnia (FFI)	Hereditary	Man	1,986
Bovine spongiform Encephalopathy (BSE)	Transmissible	Ruminants	1,986
Feline spongiform E.	Transmissible	Felidae	1,991
Variant CJD (vCJD)	Transmissible	Man	1,996

Table 2 BSE cases and incidence in cattle in Europe in 2002

Countries	Number of cases	Relative incidence (number of cases per mill. cattle >30 month of age)
Austria	0	
Belgium	38	25
Czech Republic	2	
Denmark	3	3
Finland	1	
France	239	21
Japan	7	
Israel	1	
Germany	106	16
Ireland	333	97
Liechtenstein	0	
Luxembourg	1	
Netherlands	24	13
Poland	4	
Portugal	86	129
Slovakia	6	
Slovenia	1	
Switzerland	24	26
United Kingdom	1,018	>200

All European countries that are listed have experienced at least one BSE case in the past. The relative incidence is calculated by taking into account the number of heads of cattle that are kept in the respective country.

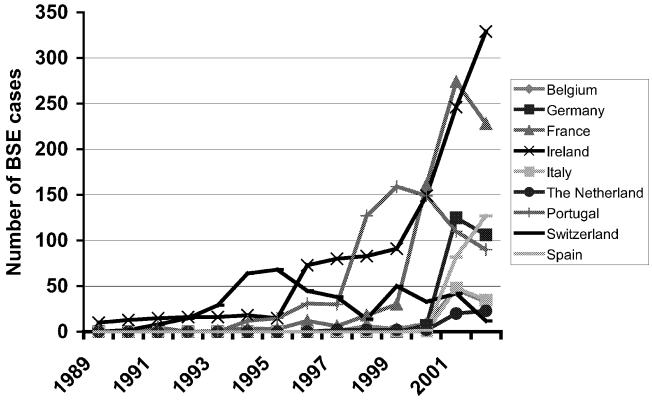

Fig. 1 Number of confirmed BSE cases per year in EU member states. Note the sharp increase almost in all countries after the introduction of BSE testing in January 2001

PrP^C is largely composed of two structural areas, the flexible non-structured amino-terminus (up to amino acid—aa—120) and the structured carboxy-terminal region (aa 121–230). The carboxy-terminal region comprises three α-helices (spanning aa 144–154, 174–192, and 199–218) and two short anti-parallel β-sheets (aa 127–130, 160–163). Helices II and III are connected by a disulfide bond (C178 and C213), and helix II bears a N-glycosylation at N180. The second N-glycosylation is sited at N196, with a heterogeneous distribution of glycans at both sites.

The diagnostic hierarchy for BSE is defined by the Office Internationales des Epizooties (OIE) which is the corresponding organization for animal diseases as the world health organization (WHO) is for human health. This diagnostic hierarchy is based on the scientific knowledge about the available diagnostic methods and the experience of using these techniques, and particularly takes into account their specificity and sensitivity. According to the OIE regulations and to the EU legislation (EU decision 999/2001), all suspect cases found by BSE rapid testing must be retested using confirmatory methods by a national reference laboratory (NRL) in the particular country or directly by the community reference laboratory (CRL), which is located at the veterinary laboratories agency (VLA) in Weybridge, UK. In Germany, the NRL is situated at Institute for Novel and Emerging Infectious Diseases at the Federal Research Centre for Virus Diseases of Animals located on the isle of Riems. Confirmatory methods for BSE are

- The histopathological examination of the brain stem (obex region) and detection of spongiform lesions particularly in the solitary tract nucleus and/or the spinal nucleus of the trigeminal nerve (HE)
- The immunohistochemical detection of pathological PrP^{Sc} depositions in tissue sections of the above mentioned brain stem areas (IHC)

– The immunochemical detection of pathological PrP^{Sc} in fibril preparations from brain stem tissue (SAF-immunoblot)
– The electron-microscopical detection of BSE-associated fibrils in brain stem extracts

To date, five different BSE rapid tests have been successfully evaluated by the EU commission and approved for use in monitoring studies and for testing slaughtered animals. These tests include a colorimetric sandwich ELISA, a luminescense sandwich ELISA, an indirect ELISA using chemilumenscense, a rapid western blot assay and a conformational dependant immunoassay for the detection of the pathological prion protein.

There are three crucial parameters which characterize the performance of a rapid test:

– Diagnostic sensitivity, percentage of correctly classified positive samples
– Diagnostic specificity, percentage of correctly classified negative samples
– Analytical sensitivity, determination of the minimal concentration of an analyte to give a positive result

It is difficult, however, to determine the first parameter for rapid tests. In contrast to most other tests applied for the diagnosis of infectious diseases in animals and humans, BSE rapid tests do not use body tissue fluids like serum, plasma, or urine in which the analyte is dissolved homogeneously. Instead, brain stem samples are collected at abbatoirs and in rendering plants from which the region of highest PrP^{Sc} concentration (region around the obex) is sampled. As a consequence of the inhomogeneity of PrP^{Sc} deposition in the brain, test results for one single brain stem sample can vary considerably due to collection and homogenization artefacts. This is one reason why standard operating procedures (SOPs) for rapid tests need to be strictly followed and only sections close to the obex can be used. Any deviation could lead to false or unreliable results. However, the inhomogeneity also prohibits collecting a set of BSE positive samples for which the assay results can be predicted. In addition each testing means a substantial consumption of brain stem tissue despite of its finite availability. Therefore, the analytical sensitivity of an assay can only be determined by using borderline reactive BSE tissue dilutions, meaning BSE brain stem macerates blended in consecutively higher proportions of uninfected cattle brain stem macerate (as a matrix). Many difficulties were initially encountered when reference material was produced that could be used in proficiency testing rounds with rapid testing laboratories as well as for the evaluation of commercial rapid test kits. A major bottleneck was the lack of suitable quantities of bovine brain stem material, particularly in countries like Germany which had only a very few BSE cases at the beginning of the rapid testing program. Moreover, most available samples were of poor quality, i.e., derived from fallen stock animals in rendering plants, and, therefore, were frequently heavily autolyzed. In the first experiments to prepare dilution series of a positive brain macerate, distilled water or sucrose solution was used as a dilution matrix. However, the positive signal dropped disproportionately fast in the diluted samples. This is thought to be caused by the fact that the PK concentration that is applied in the rapid tests is adjusted to a certain amount of brain material. If less tissue material is present in the solution, the enzyme is heavily overdosed and will start to digest the partly PK resistant PrP^{Sc}, resulting in too low or even false-negative test results. This problem was overcome as soon as a negative brain macerate was used as a dilution matrix.

However, the preparation of these macerates turned out to be rather difficult. At the beginning of the rapid testing program, when brain stem homogenates or lysate dilutions were produced, they suffered from a steep decline in their PrP^{Sc} antigenicity. This problem was eventually overcome by a colleague at the Danish NRL (hence the technique was named “Lind” protocol), who used crude macerates instead of fine ground dounce homogenates or lysates (also containing detergens), which allow for better tissue and antigen preservation. In the amended protocol used at the German NRL, macerates (defined as coarse brain homogenates) are produced by adding an equal amount of a 5% sucrose solution (in deionized water) to precut brain stem pieces (for example 5 g brain material plus 5 ml sucrose solution). This mixture is ground and quenched mechanically through a commonly used metal household sieve using a plunger. In this way 50% tissue/volume macerates of BSE as well as non-infected brain stems are produced and blended subsequently in appropriate proportions. In our experience such macerate blends are stable if stored at either −20°C or −70°C and retain PrP^{Sc} antigenicity for months, if not several years. One drawback of this method is that the homogeneity of such coarse tissue grindings may be insufficient so that heavily PrP^{Sc} containing areas are not dispersed. However, lacking better solutions to this problem, such heterogeneity was eventually considered acceptable and, indeed, such effects were hardly observed during practical use.

As all inactivation methods applied to reduce BSE infectivity also destroy the antigenicity of the pathological prion proteins, any inclusion of an authentic positive control sample in the rapid test kits would mean including infectious material. As this is impossible for logistical and labour safety reasons, rapid tests contain auxiliary controls such as recombinant PrP^{C} or synthetic peptide analogues. However, although such controls are useful to confirm the functionality of the enzymatic detection of PrP, they cannot compensate properties of PrP^{Sc} which are important for the purification and diagnostic discrimination in the rapid tests: PK resistance and hydrophobicity/fibrillogenicity.

By the middle of 2003 more than 23 million rapid tests had been conducted in Europe and more than 1,500 BSE cases had been discovered by this testing program alone. Direct rapid testing costs add up to 575 million Euros (calculation based on 25 € average testing cost per cattle) in the EU. In total, about 8 million tests were carried out in Germany. Following the introduction of obligatory rapid testing in December 2000, more than 100 regional BSE rapid testing laboratories carried out the testing scheme after the initial shortage of testing capacity had been overcome. However, when sale revenues dropped rapidly and when the high quality control standards that are strictly enforced became obvious, half of the laboratories dropped out of the market. About 50 rapid testing laboratories remained in service in Germany, 35 of which belong to the public veterinary services, and 15 are run by private contractors.

Any initially reactive result in the regional rapid testing laboratories needs to be verified by the NRL and in cases of unclear results they are further examined by and discussed with the CRL. The introduction of such a large-scale testing program was only possible in combination with a variety of quality assurance (QA) measures. These included:

(a) The implementation of suitable "quality management systems for TSE laboratories" belonging to all stages in the diagnostic hierarchy (regional testing laboratory—RTL, NRL, CRL)
(b) The "evaluation and approval of the rapid tests" to be used
(c) The strict and continuous supervision and control of the "performance of the production batches of the rapid tests" to be placed on the market by the producer as well as by state veterinary authorities

The measures in all three areas are outlined below. However, as the most practical issues of the implementation of EU legislation are not fully harmonized in all member states, the following description of the QA systems for the German TSE rapid testing program is only exemplary and must not apply for control measures currently in use in other EU member states.

Quality management system for TSE laboratories

Each laboratory performing diagnostic investigations on TSEs (RTL, NRL, CRL) needs to define all administrative and laboratory activities including detailed and unmistakable method collections, and control measures in place in a quality management (QM) manual. This manual contains all SOPs for the sampling and testing, the decision tree and responsibilities of personnel in the laboratory, the kind and maintenance of the equipment to be used, the interpretation and reporting of all test results, and the education and training of personnel. It also defines which continuous process controls should be carried out and which correction measures must be applied in case of deviations from predetermined values. Moreover, it regulates, how all QM system (QMS) data and test results must be documented. The QMS is monitored by an internal (continuously) as well by external QA agent (regular visits). Internal and external QA audits with all involved personnel are carried out on a regular basis, too. External audits are held by officially appointed accreditation bodies, which eventually certify that the QMS functions according to ISO 17025 ("*General Requirements for the Competence of Calibration and Testing Laboratories*"), meaning that individual laboratories are accredited. Moreover, commercial RTL are controlled by the competent veterinary authorities on a biannual basis (minimum) and in the context of the implementation of TSE decision EU 999/2001. All commercial and state RTL laboratories have to participate in (initial and biannual follow-up) proficiency testing rounds (ring trials), which are conducted under the supervision of the NRL. In these ring trials the analytical sensitivities of the individual BSE rapid testing laboratories are determined. Any laboratory which does not meet the general standard has to either discontinue the service or improve its testing performance by getting advice and training by the NRL and the rapid testing companies. Improvements are verified by repeated proficiency testing rounds with the laboratory in question. The NRL itself also participates in proficiency testing rounds carried out by the CRL. Only laboratories which have successfully passed such proficiency testing trials are allowed to carry out rapid testing.

Evaluation and approval of the rapid tests

The EU commission has organized the evaluation of BSE rapid tests in the past, by calling together a scientific working group which outlined the general setup of a study to determine the candidate tests' performance. The Institute for Reference Materials and Measurements (IRRM), Geel, Belgium was in charge of carrying out this study together with the rapid test companies. Five rapid tests have passed this stage to date and have been approved by the EU commission for monitoring diseased or fallen ruminants, as well as for testing healthy slaughter animals. However, it must be kept in mind that, so far, all rapid tests have only been evaluated on bovine samples but have been approved by the EU authorities for the testing of ruminant samples (cattle, sheep, and goats). It must be stressed that the currently available BSE rapid tests are capable of detecting BSE infections in cattle only as early as 6 months before the onset of clinical disease, but presumably not earlier. This is due to the particular pathogenesis of BSE in cattle, i.e., the

infection seems to be very much restricted to the central nervous system. Even in the target area (obex), infectivity has not yet been detected earlier than that.

The EU approval of the first three rapid tests concerned only the general capability and performance of the laboratory methods but did not regulate the criteria and standards to be met by the commercial products developed thereof. In Germany, BSE rapid tests fall under the legal veterinary diagnostics regulation, which require an official national approval for all products on the domestic market. This national approval process is unique in Europe. In this process it is checked that the commercial product is identical with the EU approved method and still has the same performance. Moreover, the clarity and comprehensiveness of the (German) test instruction and the correctness of the German product designation and labeling are ensured.

Performance of individual production batches of BSE rapid tests kits

The wide use of rapid tests makes it necessary that the companies manufacture their products continuously. Hence, for a given test, the actual kits for sale can belong to different production batches. Altogether, the rapid test kits that have been used in Germany have belonged to more than 150 different batches to date. The German situation is unique in the sense that it is legally required that each single production batch of a test kit must be quality tested and approved by the NRL before being allowed to be placed on the market. Moreover, rapid test producers also make remarkable efforts to assure that all subsequently produced batches are of the same performance as their initially approved product and that this is well documented. There are two elements to assure this:

Verification of identical production quality

The first key step for this is that all relevant chemical ingredients, buffers, and recipes, etc., are defined and deposited at the German NRL. Moreover, a QMS for the production of batches is fixed and notified. Any change to either of the aforementioned depositions must be notified and agreed on by the German NRL. This also includes all SOPs for all QA measurement steps in the production process, which must be written down and deposited. All target values and their acceptable deviation for these measurements are determined and notified.

Measurement of the test performance of individual production batches

The currently approved BSE rapid tests combine a sample preparation and PrP^{Sc} extraction step with the immunochemical detection of PrP^{Sc}. While the first part solely depends on parameters which can be measured chemically and physically, it is more difficult to QC and document the immunochemical assay. Therefore, any new production batch is challenged in the following ways:

Analytical sensitivity (Table 3). Test producers have defined one of their production batches for which sufficient performance data are available as their reference kit batch. Depending on the products' shelf life, this reference can be replaced on the producers own judgement by a newer reference kit batch, if necessary, and if comparative testing data verify a similar performance. All production batches to be released are checked against a reference BSE macerate dilution series, which has been produced by the company following the suggestions of the German NRL. This series contains well reactive as well as borderline reactive samples. The same samples are also tested using the reference kit, and the results are compared. All results are reported to the German NRL. Moreover, the companies provide sufficient quantities of reference kits as well as reference macerate dilutions to the German NRL, so that the results can be reproduced there. Finally, the most critical compounds in the assay such as the primary and secondary antibody dilutions are crosschecked by replacing each of them in the test batch and in the reference batch. Such a replacement should not alter the analytical sensitivity of the kits.

Intra-plate and inter-plate variability (Table 3). Immunochemical results can vary considerably even in a given assay, for instance if not exactly the same amounts of the capture antibodies are coated onto the reaction wells, or coating difficulties prevented the even charging of all plastic surfaces with immunglobulins. To determine the intra-plate and inter-plate variation, 384 reaction wells must be assayed using a defined antigen—preferably the authentic positive control. Coated wells should come from different time points in the production line, at best by collecting 48 different 8-well strips, or ,if this is not possible, by using 4 fully coated plates. The variation of the results (CV) of the 8-well strips should not exceed 30% and the variation within and between plates should not exceed 20%.

Specificity control (Table 3, Fig. 2). BSE rapid tests possess a high analytical sensitivity to discover even trace amounts of the analyte, which is indicative for an upcoming BSE disease in bovines. This is highly desirable to minimize the risk of exposure of consumers to BSE infectivity. However, misleading false-positive assay re-

Table 3 Summary sheet on the test kit performance data which are requested for each new production batch prior to approval for market release in Germany. In the first step, the performance of the newly produced batch is compared to a standard reference kit. This standard reference kit comprises an earlier batch with well-documented performance, which has been named by the test kit producer themselves. In the second step, individual test kit components from the new batch are used in the standard reference kit, e.g., the primary antibody or the secondary antibody (antibody-enzyme conjugate) and vice versa. In the third step, the intra- and inter-plate repeatability is tested by using the kits own positive control in severalfold repetitions to mimic reactive sample.

Component/test	Criteria	Result
New production batch	Negative control	Below set value
	Blank	Below set value
	Positive control	Above set value
	BSE-Reference 20%	Reactive result
	BSE-Reference 10%	Reactive result
	BSE-Reference 5%	Reactive result
	BSE-Reference 2.5%	Reactive result
	BSE-Reference 1.25%	Reactive result
	Total no. field samples tested	~800
	Mean	Within set range (cut-off ratio)
	Standard deviation	Within set range (cut-off ratio)
	% CV of negative population	Within set range
Standard reference kit	Negative control	Below set value
	Blank	Below set value
	Positive control	Above set value
	BSE-Reference 20%	Reactive result
	BSE-Reference 10%	Reactive result
	BSE-Reference 5%	Reactive result
	BSE-Reference 2.5%	Reactive result
	BSE-Reference 1.25%	Reactive result
	Total no. field samples tested	~800
	Mean	Within set range (cut-off ratio)
	Standard deviation	Within set range (cut-off ratio)
	% CV of negative population	Within set range
Primary antibody (anti-PrP)	Negative control	Below set value
	Blank	Below set value
	Positive control	Above set value
	BSE-Reference 20%	Reactive result
	BSE-Reference 10%	Reactive result
	BSE-Reference 5%	Reactive result
	BSE-Reference 2.5%	Reactive result
	BSE-Reference 1.25%	Reactive result
Secondary antibody (anti-IgG)	Negative control	Below set value
	Blank	Below set value
	Positive control	Above set value
	BSE-Reference 20%	Reactive result
	BSE-Reference 10%	Reactive result
	BSE-Reference 5%	Reactive result
	BSE-Reference 2.5%	Reactive result
	BSE-Reference 1.25%	Reactive result
Positive control (repeatability test)	Plate A mean	Above set value
	Standard deviation	Within set range
	CV	<20%
	Strips mean	Above set value
	Strips CV	<35%
	Plate B mean	Above set value
	Standard deviation	Within set range
	CV	<20%
	Strips mean	Above set value
	Strips CV	<35%
	Plate C mean	Above set value
	Standard deviation	Within set range
	CV	<20%
	Strips mean	Above set value
	Strips CV	<35%
	Plate D mean	Above set value
	Standard deviation	Within set range
	CV	<20%
	Strips mean	Above set value
	Strips CV	<35%

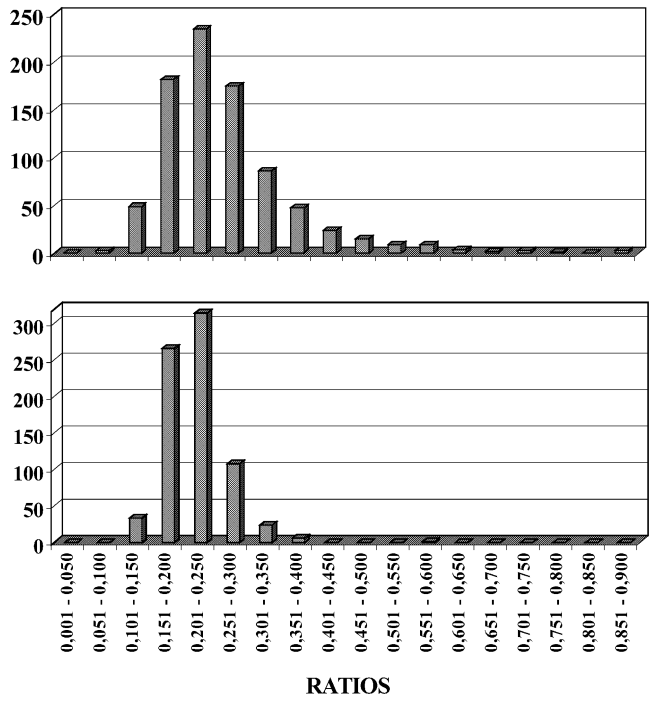

Fig. 2 Specificity: distribution of test negative samples (~800 samples each, ratios to cut-off → initial reactive rate)

sults must also be avoided, as this means serious restrictions for the slaughterhouse/rendering plant as well as for the farm where the animal originated that cause high financial losses for these institutions. Therefore, BSE rapid tests must also be of the highest specificity. Field testing has shown that in the initial testing rounds an average of 3–4 false-positive results turn up, almost independently of the rapid test used. Most of these false positive reactions are corrected by instant re-testing. However, if rapid tests are even unintentionally shifted towards the farthest edge of analytical sensitivity, larger numbers of false-positive results are experienced. In order to have a good balance between the assay sensitivity and specificity, rapid test producers are asked to evaluate new production batches on a larger number of previously negative tested samples and to determine whether these results match those of previous studies (preferentially obtained with the reference kit) in terms of their distribution and variation.

Conclusion

A large-scale BSE rapid testing scheme started in the EU in January 2001 which meant that several hundreds of state and privately run laboratories set up the necessary testing capacity. Although not realized immediately, it eventually became obvious that a high QC system for all areas of this testing scheme is of key importance to regain consumer safety and confidence by this measure. Therefore, at least some of the regulations for the QC of the applied rapid tests and for the correct performance of the testing laboratories were fixed at a later stage. However, as sufficient experience with the critical points was gained, the actually implemented regulations could be very detailed, strict, and precise but also driven by a practical knowledge. It is important to note that standardization of these QC measures in all EU member states would be useful, but has not been realized to date. By implementing control measures such as those described in this article, an excellent quality standard for rapid testing programs could be achieved.

Acknowledgements The standardization of the BSE rapid testing would not have come thus far, if the three rapid test kit producers, namely Abbot (distributor of the Enfer Test), Biorad (Platelia test), and Prionics (Check Western and LIA tests) (in alphabetical order of producers names) had not cooperated so well to assure the correct use of their products. Moreover, they shared their experience and—driven by the pride in their products—attempted to fulfill all set criteria for the German test approval and batch releasing process with enthusiasm and correctness.