Molecular diagnostics of transmissible spongiform encephalopathies

Loredana Ingrosso, Vito Vetrugno, Franco Cardone and Maurizio Pocchiari

Clinical criteria for the diagnosis of sporadic, iatrogenic and variant Creutzfeldt–Jakob diseases are now available and show an excellent sensitivity and specificity (~98%). Post-mortem diagnosis, based upon the identification in the brain of the pathological conformer of the prion protein (PrPSc), is also very accurate, and several diagnostic kits are now available that facilitate the immunoochemical measurement of PrPSc. Several new molecular diagnostic techniques aimed at increasing the sensitivity and specificity (~98%) of PrPSc detection, and at identifying markers of disease that are other than PrPSc, are the subject of ongoing studies. The aim of these studies is to develop preclinical screening tests for the identification of infected, but still healthy, individuals. These tests are also badly needed to check the safety of blood or blood-derived products, and to ensure meat safety in European countries.

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Table 1. European criteria for sporadic CJ D classification

<table>
<thead>
<tr>
<th>Definite</th>
<th>Probable</th>
<th>Possible</th>
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<tr>
<td>Neuropathologically confirmed and/or PrPSc detection by immunocytochemistry or by western blot</td>
<td>Progressive dementia with at least two of the four clinical features: Myoclonus, visual or cerebellar signs, pyramidal/extrapiramidal signs, and akinetic mutism</td>
<td>Clinical features as above</td>
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<tr>
<td>or</td>
<td></td>
<td>No PSWC in EEG</td>
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<tr>
<td>14-3-3 proteins in cerebrospinal fluid (CSF) and disease duration of &lt;2 years</td>
<td>or</td>
<td>No 14-3-3 detection in CSF</td>
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<tr>
<td>or</td>
<td>Duration of &lt;2 years</td>
<td></td>
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PrPSc: marker of disease and infectivity

The formation of PrPSc occurs only in TSEs and therefore its presence is a specific marker for these
disorders. The prevailing belief among TSE researchers is that the infectious agent is made only of proteins and that PrPSc is the major or only component [9]. After experimental inoculation of rodents with TSE agents, PrPSc is usually detectable in the CNS weeks before the appearance of disease, and its level increases until the animal dies [23]. As the rise of PrPSc parallels that of infectivity [23], PrPSc is often used as a surrogate marker for measuring the amount of infectivity in biological samples (Box 2).

The ratio of infectivity and PrPSc is relatively constant in the hamster brain experimentally infected with the 263K strain of scrapie at a mean value of $10^5$–$10^6$ protein molecules for one lethal dose 50 (LD50) (~1–10 attomoles of PrPSc in 50-µl volume used for the intracerebral inoculation, which is equivalent to 20–200 femtomolar) [23,24]. This ratio, however, might not apply to other experimental or ‘natural’ TSEs or tissues and body fluids other than brain. However, there are exceptions; under specific experimental conditions, the brain of TSE-affected rodent is infectious without containing any measurable PrPSc [25]. Moreover, in some cases [e.g. in patients with fatal familial insomnia (FFI)], the amount of PrPSc is often 5–10 fold lower than in sporadic CJD [26].

**The need for sensitive methods**

From the perspective of pre-clinical diagnosis, the sensitivity of diagnostic methods and the procedures to concentrate PrPSc become crucial because the amount of PrPSc outside the CNS might be extremely small. Concentration of PrPSc can be realized by chemophysical precipitation protocols, affinity chromatography or affinity precipitation techniques. The improved extraction method for PrPSc with

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**Box 2. Infectivity bioassay**

Experimental transmission to laboratory animals is the only established method for the identification and measurement of infectivity. The level of infectivity in a sample can be estimated either by end-point titration or by incubation period [a]. Calculation of infectivity titre by the end-point method requires inoculation of at least six animals per group with ten-fold dilutions of the sample. The dilution able to kill half of the inoculated animals contains 1 lethal dose 50 (LD50). Calculation of infectivity by incubation period is done by referring the mean incubation period of the inoculated animals to a dose incubation curve (Box 2).

Transmission of disease to an animal of the same species yields the maximum efficiency of infection, whereas interspecies transmission is hampered by a ‘species barrier’ effect [b]. Estimation of infectivity in natural TSEs is further complicated by the existence of multiple ‘strains’ of scrapie and CJD agents with a variety of clinical and pathological properties in different host animals. Each strain is characterized by a stable incubation period in a given host, a typical pattern of histological lesions, and a given PrPSc glyctype. Characteristics of a strain can change, however, after passage in different host species suggesting either a strain mutation of an ‘informational molecule’ present in the agent itself or a strong pressure that changes the conformation of novel PrPSc in the host. More than 20 scrapie strains have been isolated in mice [c], while only one in cattle (BSE), which is identical to that identified in vCJD [d]. The number of strains in sporadic CJD is unknown.

**References**


d. Bruce, M.E. et al. (1997) Transmissions to mice indicate that ‘new variant’ CJD is caused by the BSE agent. *Nature* 389, 498–500

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**Table 2. Criteria for variant CJD classification**

<table>
<thead>
<tr>
<th>Category</th>
<th>Criteria</th>
</tr>
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<tbody>
<tr>
<td><strong>I History</strong></td>
<td>Progressive neuropsychiatric disorder</td>
</tr>
<tr>
<td>A</td>
<td>Duration of illness &gt;6 months</td>
</tr>
<tr>
<td>B</td>
<td>No alternative diagnosis available</td>
</tr>
<tr>
<td>C</td>
<td>No history of potential iatrogenic exposure</td>
</tr>
<tr>
<td>D</td>
<td>No evidence of a familial form of transmissible spongiform encephalopathy (TSE)</td>
</tr>
<tr>
<td>E</td>
<td>Early psychiatric symptoms</td>
</tr>
<tr>
<td>B</td>
<td>Persistent painful sensory symptoms</td>
</tr>
<tr>
<td>C</td>
<td>Ataxia</td>
</tr>
<tr>
<td>D</td>
<td>Myoclonus or chorea or distonia</td>
</tr>
<tr>
<td>E</td>
<td>Dementia</td>
</tr>
<tr>
<td><strong>III Neurological investigations</strong></td>
<td>Electroencephalogram (EEG) does not show the typical appearance of sporadic CJD (or no EEG performed)</td>
</tr>
<tr>
<td>B</td>
<td>Magnetic resonance imaging (MRI) brain scan shows bilateral symmetrical pulvinar high signal</td>
</tr>
<tr>
<td><strong>IV Laboratory investigations</strong></td>
<td>Characteristic neuropathological and immunopathological findings</td>
</tr>
<tr>
<td>A</td>
<td>Presence of PrPSc in tonsillar tissue taken at biopsy</td>
</tr>
<tr>
<td><strong>Definite</strong></td>
<td>IA and IVA</td>
</tr>
<tr>
<td><strong>Probable</strong></td>
<td>I and 4/5 of II and III or I and IVB</td>
</tr>
<tr>
<td><strong>Possible</strong></td>
<td>I and 4/5 of II and IIIA</td>
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sodium phosphotungstate [5] and newly discovered molecules (plasminogen [27] and protocadherin-2 [19]) binding with high affinity to PrPSc, might boost new hopes for preclinical diagnosis of TSEs. An original approach to increase the minimum detectable level of PrPSc comes from Saborio et al. [28], who, starting from the hypothesis that production of PrPSc in vivo occurs via a nucleation-dependent mechanism, developed an efficient protocol for the amplification of PrPSc (see Fig. 1). This technique appears promising, the sensitivity is increased by 10–100 fold, and opens a conceptually new research area to improve the sensitivity of PrPSc-based assays.

Sensitive methods are not required for post-mortem diagnosis, as great amounts of PrPSc are usually present in the brain of affected individuals. In recent years, great attention has been paid to the possible use of PrPSc detection in peripheral and accessible tissues (such as tonsil) or body fluids [such as the cerebrospinal fluid (CSF) or blood] for preclinical in vivo diagnosis of TSEs. Two recent reports have also shown the presence of PrPSc in urine of CJD patients and animals with natural or experimental TSEs [29], and in the skeletal muscle of mice experimentally infected with laboratory strains of TSE [30]. These studies are potentially of great diagnostic value, but obviously need to be confirmed by other laboratories.

The detection of PrPSc in the brain by immunochemical techniques is the most accurate procedure for the diagnosis of these disorders, and is more accurate than classical histological examination of the brain. Many polyclonal and monoclonal anti-PrP antibodies have been developed in the past 20 years with the aim of increasing the sensitivity and specificity of immunological tests. However, none of them discriminates between PrPc and PrPSc, despite the fact that these two isoforms have different three-dimensional structures and should, in theory, express different epitopes. As a consequence, all immunological methods require a pre-treatment of the sample (usually a proteolytic step) to remove or degrade the normal PrPc. Unfortunately, these pre-treatments might also hydrolyse PrPc or even small amounts of PrPSc, thus reducing the sensitivity of the immunological tests.

Among the immunological methods of PrPSc detection, western blotting is the best characterized and widely validated method (see Table 3). It offers the advantage of recognizing different forms of PrPSc through the analysis of the molecular mass and the relative abundance of di-, mono- and non-glycosylated bands. These parameters characterize the so-called PrP glyctype, a kind of ‘PrP signature’, which varies among different forms of TSEs. PrPSc glyotyping has been proposed for distinguishing various forms of TSEs (e.g. scrapie from BSE [31], sporadic from variant CJD [32]) and for improving the classification of human TSEs [32–34]. In sporadic CJD, for example, the combination of the two most frequent PrPSc glycotytes (I and II) with the three possible genotypes of PrP at the polymorphic codon 129 (methionine homozygous, valine homozygous, or heterozygous) enables the subclassification of this form into six distinct groups, each of which presents distinct clinical and pathological features [35]. This grouping might need revision after the results obtained with two-dimensional gel electrophoresis, which allows a more detailed analysis of the PrPSc glycoform population [8,36]. Disadvantages of western blot as a screening test are that few samples can be processed in a single gel, the technique is time-consuming and requires experienced personnel. Many of these limitations are overcome by latest-generation sandwich enzyme-linked immunosorbent assay (ELISA) [37,38] (Table 3), which can discern PrPSc in samples containing less than 1 LD50 of BSE infectivity (as measured by the mouse bioassay) [37], or detect PrPSc in the brain of BSE-infected cows before clinical onset [39]. Both ELISA and western blot are now commercially

![Fig. 1.](http://tmm.trends.com)
Table 3. Summary of currently available PrP\textsuperscript{Sc} detection methods

<table>
<thead>
<tr>
<th>Technique</th>
<th>Principle of operation</th>
<th>Detection method</th>
<th>Detection limit\textsuperscript{b}</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western blot\textsuperscript{c}</td>
<td>SDS-PAGE, electroblot on a membrane, incubation with anti-PrP specific primary and anti-PrP\textsuperscript{c} enzyme-linked secondary antibodies.</td>
<td>Chemiluminescence</td>
<td>10–20 pM</td>
<td>[5,8,66]</td>
</tr>
<tr>
<td>ELISA\textsuperscript{a}</td>
<td>PrP\textsuperscript{Sc} absorption (with or without a capture antibody) onto a plastic well; incubation with anti-PrP specific primary and anti IgG enzyme-linked secondary antibodies.</td>
<td>Chemiluminescence</td>
<td>2 pM</td>
<td>[37–39]</td>
</tr>
<tr>
<td>DELFIA/CDI\textsuperscript{a}</td>
<td>PrP\textsuperscript{Sc} absorption (with or without a capture antibody) onto a plastic well; incubation with anti-PrP specific primary and anti IgG enzyme-linked secondary antibodies.</td>
<td>Fluorescence</td>
<td>0.2–2 pM</td>
<td>[40–42]</td>
</tr>
<tr>
<td>FCS</td>
<td>PrP is tagged by two fluorescent antibodies, highly fluorescent aggregates made of PrP\textsuperscript{Sc} are detected by confocal microscopy.</td>
<td>Fluorescence</td>
<td>2 pM</td>
<td>[45,46]</td>
</tr>
<tr>
<td>MUFS</td>
<td>Conformation-specific fluorescence signature of aromatic residues of PrP.</td>
<td>Fluorescence</td>
<td>In the picomolar range</td>
<td>[44]</td>
</tr>
</tbody>
</table>

\textsuperscript{a}These tests can not discriminate between PrP\textsuperscript{c} and PrP\textsuperscript{Sc}; they require the removal of PrP\textsuperscript{c}.

\textsuperscript{b}In the brain of 263K strain of scrapie-infected hamsters, one LD\textsubscript{50} is equivalent to ~0.02–0.2 pM of PrP\textsuperscript{Sc}

Abbreviations: DELFIA/CDI, dissociation-enhanced lanthanide fluorescence immunoassay/conformation-dependent immunoassay; FCS, confocal dual-color fluorescence correlation spectroscopy; MUFS, multi-spectral ultraviolet fluorescence spectroscopy.

available as ready-to-use kits, and have been validated by the European Community as screening test for BSE in slaughtered cattle (so-called 'rapid tests').

Dissociation-enhanced lanthanide fluorescence immunoassay/conformation-dependent immunoassay (DELFIA/CDI) is the latest-generation immunoassay with an ELISA format, where the detection system is sensitive time-resolved lanthanide fluorescence instead of chemiluminescence [40–42]. It can measure picograms (10\textsuperscript{-12} grams) of PrP\textsuperscript{Sc} per ml and thus represents one of the most sensitive technique for the detection of PrP\textsuperscript{Sc} (Table 3). In association with a mild denaturation pre-treatment of samples, the DELFIA/CDI can distinguish PrP\textsuperscript{Sc} from different forms of TSEs with a precision claimed to be greater than that of one-dimensional western blots [40]. However, similar to other immunological tests, it cannot differentiate between normal and pathological PrP and this might reduce the sensitivity and the specificity of this test.

The claim that PrP\textsuperscript{Sc} can be detected in blood of scrapie-affected sheep and elks with chronic wasting disease by capillary immunoelectrophoresis (CIE) [43] has not been reproduced in other laboratories, and any effort to measure PrP\textsuperscript{Sc} in blood from CJD patients has so far failed [19].

Only two methods exist so far that can overcome the lack of specific anti-PrP\textsuperscript{Sc} antibodies, as they operate in near-native conditions and can therefore appreciate secondary, tertiary (and quaternary) structure of PrP conformers. One is multi-spectral ultraviolet fluorescence spectroscopy (MUFS) [44] and the other is confocal dual-color fluorescence correlation spectroscopy (FCS) [45,46]. MUFS identifies proteins by their specific fluorescence pattern of emission when they are excited by ultraviolet radiation and bypasses the need for pre-treatment steps to eliminate PrP\textsuperscript{c} or for antibody binding (Table 3). It has the potential to discriminate cellular from pathological prion protein, and various forms of PrP\textsuperscript{Sc} from different strains. Its description, however, dates back to 1998 and no further development has been so far reported [19]; therefore, practical application does not look imminent.

FCS recognizes single fluorescent molecules in solution as they pass between the exciting laser beam and the objective of a confocal microscope, equipped with a single-photon counter; it is performed quickly and requires only small amounts of samples. The assay solution is mixed with anti-PrP antibodies tagged with fluorophores that bind strongly to PrP\textsuperscript{Sc} aggregates, which become highly fluorescent and easily visible against the background of monomeric PrP [45]. This technique is ~20-fold more sensitive than western blot (see Table 3) and was able to detect, for the first time, PrP\textsuperscript{Sc} in the CSF of ~20% of CJD patients [46]. Although these results have not yet been validated in other laboratories because the instrument was commercially unavailable until very recently (all the work was done with a prototype by Carl Zeiss, Jena, Germany), they are similar to those obtained by measuring CSF infectivity in non-human primates (~15%) [47]. Taken together, these experiments suggest that CSF might not be suitable for the diagnosis of CJD based upon the direct (bioassay) or indirect (PrP\textsuperscript{Sc} detection) measurement of infectivity.

**Markers of disease other than PrP\textsuperscript{Sc}**

The search for markers of disease other than PrP\textsuperscript{Sc} has been ongoing for the past 20 years. In particular, signals of cerebral suffering in the relatively accessible CSF have been extensively investigated as potential surrogate markers of disease.

Among them, the search for 14-3-3 proteins represents a valuable resource and, in fact, they are formally included in the European diagnostic criteria for sporadic CJD [48]. 14-3-3 proteins belong to a family of highly conserved regulatory proteins ubiquitously expressed in all eukaryotic cells, including neurons and glia. These proteins are highly specific and predictive, and constitute a significant
improvement in the diagnosis of sporadic CJD when used in a correct clinical setting; that is, in a cohort of patients with rapidly progressive dementia (duration < 2 years) where encephalitis, stroke and paraneoplastic disorders have been excluded. In this context, the diagnostic value of the 14-3-3 proteins has significantly increased the sensitivity of case definition, especially when the EEG does not show a typical periodic pattern [48]. Recently reported criticisms of false positive results are simply related to the use of unselected cohorts of demented patients as controls [49]. An increase of 14-3-3 proteins in the CSF testifies of a massive neuronal destruction and therefore is also associated with encephalitis (particularly, herpes encephalitis), hypoxic brain damage (stroke), subarachnoid haemorrhage, or paraneoplastic syndromes ([48] and M. Pocchiari, unpublished).

14-3-3 proteins were also detected in the CSF of patients with genetic CJD carrying the codon 200 or the codon 210 mutation of the PRNP gene [48], in ~50% of CSF samples taken from patients with vCJD ([50], but not in the CSF of patients with FFI and seldom in those with Gerstmann–Sträussler–Scheinker syndrome (GSS) ([48] and M. Pocchiari, unpublished).

Proteins released in the CSF after cerebral damage, such as tau, neuron-specific enolase (NSE), and S-100, are also increased in CJD patients, although their specificity, sensitivity and predictive values are always inferior, or at most equal, to those of 14-3-3 proteins [51].

Special mention should be made of Fourier transform infrared (FT-IR) spectroscopy, which has been shown to discriminate between scrapie-infected and uninfected brain material simply by comparing spectra analyses [52]. The origins of these differences are unknown but, if applicable in tissues other than brain (CSF, blood), might represent an entirely new and exciting approach to the pre-clinical diagnosis of TSEs.

The progressive decreased expression of the erythroid differentiating related factor (EDRF) during the course of disease in TSE-affected mice, sheep and bovine, coupled with its easy measurement by northern blot in blood, give hope for early diagnosis of CJD and related diseases [53].

Predictive genetic markers of disease

For the majority of TSEs there are no markers that can foresee the appearance of disease. The only exceptions are the familial forms that are always associated with a point or insertion mutation of the prion gene [1]. Within these families, it is of course possible to identify mutated members, but, depending upon the mutation they carry, it is not always feasible to predict whether they will develop the disease and, if they do, at what age. Each mutation is generally correlated with a characteristic clinical and pathological feature, but there are exceptions, and it is possible that affected members of the same family bearing the same mutation show a remarkable phenotypic variability of the disease. For example, point mutations at codon 200 or 210 of the PRNP gene do not always produce the disease ([54,55]; it is relatively common within the same family to observe some carriers who develop CJD in their 50s, some 10–20 years later, and some others who are still healthy over the age of 80 ([56] and M. Pocchiari, unpublished). The probability that individuals carrying the PRNP codon 200 mutation develop the disease during their life span varies from 100% to 60% from one geographical area to another, suggesting that other genetic or environmental factors might act as additional risk determinants [54]. Other PRNP mutations (e.g. codon 102 or certain insertion mutations) are more aggressive in terms of penetrance and early age at onset of disease [1].

These uncertainties make it difficult to carry out an objective evaluation of the significance, in terms of prognostic value, for a healthy person to have a PRNP mutation and, as a consequence, genetic counseling can often be arduous.

Genetic susceptibility to the disease is also present in sporadic and iatrogenic CJD where ~70% of affected individuals are homozygous for methionine at the polymorphic 129 site of PrP [57}; in vCJD, 100% of patients are methionine homozygous [58]. This susceptibility, however, can not be used to forecast who will develop the disease since ~40% of the Caucasian population are methionine homozygous [57].

The discovery of a PrP-like gene (PRND gene) located downstream from the PrP gene in mammalian species has provided hope that PRND and its encoded protein (Doppel, Dpl) contributes to the pathogenesis of TSEs, and be of help in the recognition of at-risk individuals [59]. However, there is no evidence that the PRND gene plays a role in the susceptibility for human ([60,61] and M. Pocchiari, unpublished) or animal TSEs [62].

In a recent paper, human leukocyte antigen (HLA) typing of vCJD patients revealed a reduced frequency of the HLA class-II type DQ7 compared with sporadic CJD cases and unaffected Caucasian British controls [63]. However, the role of HLA typing in the pathogenesis of vCJD, as well as other diseases, is far from understood and its potential predictive value is uncertain because of the small number of tested patients.

Conclusions and perspectives

In the past three years, there has been great progress in the diagnosis of TSEs. Obviously, the development of a sensitive, rapid, economical and non-invasive screening test that is able to discriminate between TSE-infected, but still clinically unaffected individuals, and uninfected individuals is important for both the scientific and economic world. These tests would greatly benefit measures of public health turned to guarantee the safety of blood, tissues and organs for donation, the protection of people from
bovine and ovine TSE-contaminated meat, and the safety of pharmaceutical products derived from animal tissues. CJD patients, on the other hand, would only marginally benefit from such preclinical tests because, despite the substantial improvement of research in experimental therapy [64], it is still too optimistic to think that an early or a preclinical diagnosis might save the life of a person incubated with the disease. Finally, epidemiological studies would also be enormously beneficial for such screening tests and might finally produce a convincing forecast for the dimension of vCJD epidemics in Europe.

While waiting for such tests, a short-term achievement for improving the diagnosis of TSEs is the support of the World Health Organization in its efforts to establish a panel of international reference materials that would be an essential tool in the validation of diagnostic procedures [65].

References

Molecular diagnostics in infectious diseases and public health microbiology: cottage industry to postgenomics

Gwendolyn L. Gilbert

Molecular methods have been used increasingly over the past ten years to improve the sensitivity and speed of diagnosis in infectious diseases. Although their routine use is still limited to the detection of pathogens that are difficult to culture in vitro, ‘real-time’ methods, commercial kits, quantification and automation will increase potential applications. Molecular methods are now widely used for epidemiological fingerprinting of isolates of public health importance. Sequence-based identification and strain typing, together with the development of tools that can probe for thousands of markers, will allow detailed strain fingerprinting to assist in disease management and control.

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Laboratory diagnosis of an infectious cause of disease is a complex process that ideally starts with identification of the causative agent in an appropriate clinical specimen. The slow multiplication or fastidious growth requirements of many important pathogens limit culture-based diagnosis. Culture of specimens containing normal commensal flora might require a separate selective medium for each likely pathogen. Automated systems, which use non-selective enriched media to culture blood, allow fairly rapid isolation of the common, known causes of bacteremia. However, only a minority of infections is associated with bacteremia and contaminants might be difficult to exclude.

Identification and antibiotic susceptibility testing of significant isolates take extra time. If culture is difficult or specimens are not collected at the appropriate time, the diagnosis of infection is often made retrospectively, if at all, by demonstrating a serum antibody response. These delays and limitations mean diagnostic microbiology has been regarded as something of a ‘cottage industry’. Treatment of a suspected infectious disease is usually started on the basis of a clinical diagnosis and an educated guess as to the cause. This can lead to inappropriate antibiotic use in patients who have viral infections or use of unnecessarily broad-spectrum agents, which contributes to increasing rates of antibiotic resistance. If empirical treatment is ineffective, the patient might deteriorate or transmit infection to others.

Current use of molecular methods in infectious diseases

In vitro amplification of a pathogen-specific nucleic acid sequence allows rapid diagnosis with a high degree of sensitivity and specificity. Over the past ten years, there has been increasing use of nucleic acid amplification tests, such as PCR, in routine diagnosis of infectious disease [1]. In the short term, these tests are unlikely to replace culture for normally sterile specimens, but they are...