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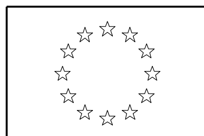

EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate B – Scientific Health Opinions
Unit B3 – Management of scientific committees II

The Modification of Technical Annexes of Council Directive 64/432/EEC to take account of Scientific Developments regarding Tuberculosis, Brucellosis and Enzootic Bovine Leucosis

Report of the
Scientific Committee on Animal Health and Animal Welfare

Adopted 11 October 1999

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SCIENTIFIC COMMITTEE ON ANIMAL HEALTH AND ANIMAL WELFARE

On the modification of the technical annexes of Council Directive 64/432/EEC to take account of scientific developments regarding Tuberculosis, Brucellosis and Enzootic Bovine Leucosis

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1. Request for an Opinion

Council Directive 64/432/EEC on health problems affecting intra-community trade in bovine animals and swine was consolidated and revised by Council Directive 97/12/EC of 17 March 1997.

Article 16 of Directive 97/12/EC requests the Commission on the basis of a report from the Scientific Committee to update and if necessary amend Annexes B, C and D (Chapter II) to adapt them to scientific developments.

2. Introduction

Annex B of the Directive currently sets out the standards for the manufacture and use of bovine and avian tuberculins and also covers such issues as potency testing and the interpretation of the tuberculin tests.

Annex C currently sets out the standards for 8 diagnostic tests for bovine brucellosis. These are the serum agglutination test, complement fixation test, milk ring test, buffered brucella antigen test, plasma ring test, plasma agglutination, micro agglutination test, and the enzyme linked immunosorbent assays.

Annex D (Chapter II) deals with the standards for two tests for Enzootic Bovine leucosis, the agar gel immuno-diffusion test and the enzyme linked immunosorbent assay.

It is important to note that Annex A and Annex D (chapter I) of the Directive set out the rules which determine the status of herds for the three diseases. While not the subject of this report, some comments may be made on these chapters where their provisions affect or conflict with the scientific updating of the other Annexes.

The format adopted in this report is to comment on the broad, important scientific issues. Topics which deserved more detailed discussion, either because of the importance of describing the state of scientific developments (e.g. new tests for tuberculosis) or because of their absence from the present directive (e.g. direct diagnosis of *Brucella abortus* and EBL), have been covered in detail. Possible new chapters drafted in the light of these comments have been annexed for clarity, though

these should be seen as suggestions, in the light of the discussion, not as prescriptions. These new chapters also contain certain minor drafting corrections. Issues which are not the subject of scientific advice from this Committee such as the designation by each member state of National Reference Laboratories have been excluded.

3. General Principles

3.1 Conformity with OIE Guidelines

GATT agreed measures to reduce barriers to international trade in animals. One of these was that the World Trade Organisation would recognise the OIE as the body setting animal health standards and guidelines. Whilst the OIE possesses no legal authority, scientifically based justification is required for deviations from these standards. We have therefore taken the publications (Manual of Standards for Diagnostic Tests and Vaccines, 1996 and The International Animal Health Code) of the OIE into account. In cases where an acceptable test method has been published by the OIE, we propose that reference be made to this method in the Annex, rather than repeating the details of the method. Updates to the Manual should be assessed as to their suitability for incorporation into EU legislation.

3.2 Flexibility

It is important that sufficient flexibility be incorporated into the new legislation to allow for possible modifications to be made rapidly to the legislation in the future to reflect scientific advances, particularly technological developments and modifications. Diagnostic techniques are evolving rapidly and it is important that the legislative structure does not act as a barrier to the use of new high quality tests. The current system is too inflexible in this regard.

A related issue refers to the possibility of new methods of dealing with disease outbreaks becoming available such as the introduction of new deletion or marker vaccines with an accompanying discriminatory diagnostic test. The flexibility to introduce such methods if they are regarded to be of overall benefit should not be excluded.

3.3 Quality Assurance

Whilst test protocols and levels of standardisation can be defined in the Directive or OIE Manual, this does not guarantee that tests are harmonised within the European Union. There is no system of formal Quality Assurance operated at the Community level that can measure the degree of concordance of test results obtained by different laboratories. There is anecdotal evidence of significant variation between different National Laboratories leading to effects on trade if not disease control. The institution of a system of Quality Assurance incorporating the circulation and testing of Quality Control panels, the setting of minimum standards for laboratories and competent laboratory inspections are recommended. Each laboratory should maintain a manual of test procedures.

It should also be noted that several standard sera are now over 20 years old and may need to be replaced in the medium term. There is a need for the qualitative definition of

standards so that the relationship between present and future standards can be established.

3.4 Community Reference Laboratories

The introduction of appropriately funded Community Reference Laboratories for the diseases, the subjects of this report, would assist the evaluation of diagnostic tests and ensure, for example by carrying out proficiency tests (ring trials etc.) that such standards are attained. The needs, in particular, are

- Standardisation and validation of test protocols, especially in relation to the development of new tests.
- Provision of standard reagents for quality control of test procedures in National Laboratories.
- Proficiency testing aimed at harmonisation of National Laboratories.
- Other appropriate functions such as training and storage of isolates.

3.5 Automated Tests and tests using micro techniques

The tests described in this report are generally designed to be carried out manually. However many of these tests, particularly binding assays, can be automated or performed using microtitre techniques. Automated or micro tests should be acceptable for use provided that they produce results with a sensitivity and specificity equivalent to the manual test.

3.6 Pooling of Samples

Tests on blood or milk permit samples from several animals to be pooled thus improving laboratory efficiency. In addition this allows sampling to take place from bulk milk tanks which is cost efficient. As a general principle, samples can be pooled provided that the test is adequate to detect evidence of infection in one of the animals of the group from which the samples were taken. This is explored in more detail when considering specific tests.

3.7 Vaccination and diagnostic tests

Modern disease control methods frequently makes use of vaccination with modified organisms accompanied, where necessary, by companion diagnostic tests to differentiate vaccinated animals from those infected by field strains of organisms e.g. Aujeszky's disease, Infectious Bovine Rhinotracheitis etc.. This trend is set to accelerate with developments in molecular biology and may allow these or similar techniques to be used for the diseases under consideration in this report.

4. Comments on Annex B - Tuberculosis

Standards for the Manufacture and use of Bovine and Avian Tuberculins

4.1 General comments relating to the current text

1. The title of the current annex B is too specific and limiting. It is suggested that it be amended to "Bovine Tuberculosis,".

2. The strains cited are adequate for tuberculin production. However, HCMC tuberculin is no longer used. References to it in the Annex should be deleted.

3. There are likely to be many future developments in TB diagnostic methodologies, which are explored in Section 4.4 below. However at present none are suitable for adoption for use in trade or to establish the official status of a herd. They may, however, be used in the context of eradication programmes, particularly to identify additional infected animals for removal from restricted herds. The use of immunodiagnostic tests should be kept under review, as they may have a much greater role to play in the future when more information becomes available. This may also be the case for defined antigens of improved specificity. There should be sufficient flexibility built into the Annex text to allow these new methods and appropriate antigens to be appropriately used, as they emerge and are evaluated.

4. The method of delivery of the tuberculin need not be specified. The vital point is that the tuberculin must be delivered intradermally. New methods for the delivery of tuberculin have been developed and should be validated.

5. Potency testing is carried out *in vivo,* as there is currently no alternative. Development of alternative methods to *in vivo* testing is recommended.

6. The text specifies that only the Kjeldahl method is used for estimating the protein content of PPD tuberculin. Since any validated method appropriate for protein estimation of PPD can be used, it is not necessary to specify this in the text.

7. The current Annex text uses the term "Community Tuberculin Unit" whereas, the European Pharmacopoeia uses the term "European unit (Eur.Ph.U.)" for bovine tuberculin and I.U. for avian tuberculin. All these units are in fact equivalent and it is therefore preferable that the term I.U. be used consistently.

8. The method for carrying out potency testing in bovines is not described in detail in the Annex text. It is suggested that potency testing should comply with the method specified in the OIE Manual of Standards (1996). Progress needs to be made to eliminate the use of live animals in potency testing.

9. The method for interpreting the tuberculin test for the purpose of establishing and maintaining the tuberculosis status of a herd is set out in the current text at point 32. It was understood that in certain circumstances, e.g. eradication programmes, it is

necessary to modify this interpretation in order to achieve improved test sensitivity (at the expense of specificity). It needs to be clarified that this is allowed under EU legislation.

4.2 Direct identification of the agent

4.2.1 Methods to identify *Mycobacterium bovis* or its DNA..

M.bovis is found within the 'M.tuberculosis complex' of mycobacteria, which is usually considered to encompass *M.tuberculosis, M. africanum, M.bovis, M.bovis* BCG and *M.microti*. There are now other mycobacteria, of uncertain taxonomic status, which are very similar and closely associated with this complex of micro-organisms (Woods *et al.,* 1995, Van Soolingen *et al.,* 1997). There still remains debate about whether or not the 'M.tuberculosis complex' organisms and closely related bacteria should be considered as separate species or species variants

Diagnostic procedures for *M.bovis* differ to some extent in most laboratories but in general, similar principles are followed. Optimum tissues for recovery of *M.bovis* from suspect tuberculous cattle have been identified and specimen processing is adequately described (OIE, 1996, Corner, 1989). Traditionally, primary culture of *M.bovis* is best performed by inoculation of a combination of multiple slopes of supplemented egg based solid agar media (OIE, 1996, Corner, 1989). Inoculation of solid media may be supplemented by radiometric culture in liquid media (Neill *et al.,* 1986, Cousins *et al.,* 1995). Such semi automated liquid systems are perhaps now more widely acceptable since the introduction of alternative monitoring procedures require no radioactively labelled substrates to be employed.

The slow growth of the '*M. tuberculosis* complex' organisms is a useful distinction, excluding the 'fast growers' which are predominantly non-pathogenic mycobacteria, such as those occurring in soil. However, laboratories should be aware of possible concomitant infection involving *M.bovis* with other mycobacteria or non-mycobacterial species. Slow growing mycobacteria isolated from reactor cattle and having cultural characteristics of the '*M. tuberculosis* complex' are often reported as *M.bovis*, although they may not always be definitively identified as such. There are traditional phenotypic tests which can be employed to identify *M.bovis* isolates and distinguish them from the human pathogen *M.tuberculosis* (OIE, 1996, Corner, 1989). In many instances, only reference laboratories will embrace identification of these slow growing mycobacteria. Traditional identification using conventional biochemical tests takes a considerable time and alternative more sophisticated biochemical procedures involving lipid analysis e.g., thin-layer chromatography, gas-liquid chromatography or high-performance liquid chromatography (Torkko *et al.,* 1998, Goodfellow and Magee 1998) are cumbersome and expensive and are undertaken by few laboratories. Bacterial capture using ELISA for detection of *M.bovis* in clinical specimens has been assessed (Duffield, 1990) but this has not had wide acceptance. Advances in molecular biology are now providing alternative rapid and more accurate identification methods for mycobacteria and are becoming more readily available for routine use.

Gene probes offer potential for rapid speciation, however, in practice the sensitivity of these may prevent uptake for direct detection (Butcher *et al.,* 1996). Development and automation of hybridization methodology for rapid detection *of M.tuberculosis* using a Q-Beta replicase assay has been demonstrated (Smith *et al.,* 1997). The Accuprobe system, an alternative molecular identification method, has exploited nucleic acid probes commercially, initially more so for laboratories dealing with human infections than with animal tuberculosis. However, the method, whilst useful and rapid, does not speciate isolates beyond *M.tuberculosis* complex. Direct detection of mycobacteria in human sputum has been demonstrated, using this system (Ichiyama *et al.,* 1997) but the anticipated low numbers of mycobacterial bacilli in many clinical specimens from animals again might limit its use in direct detection.

Nucleic acid amplification such as Polymerase Chain Reaction (PCR) methodology offer potential to enhance the sensitivity of direct detection. Various targets for PCR amplification in the genome of *M.tuberculosis* have been identified in relation to diagnosing human tuberculosis and offer potential for rapid laboratory detection methods, as alternatives to traditional culture and identification (Mangiapan *et al.,* 1996,). However, there are still practical problems in implementing PCR technology routinely (Noordhoek *et al.,* 1996). These include extracting mycobacterial DNA, presence of inhibitors and contamination. Despite these potential problems, PCR has permitted useful developments in *M.bovis* detection and identification.

Despite developments in molecular technology, which now permit direct detection and rapid identification of the *M.tuberculosis* complex bacteria, traditional culture must remain the 'gold standard', as consensus indicates that most alternative methods in reality are less sensitive when used on clinical specimens. Although, these molecular techniques have been applied primarily in relation to *M.tuberculosis,* many can equally well facilitate detection and identification of *M.bovis*.

4.2.2 Molecular Typing

Restriction Enzyme Analysis [REA] of extracted genomic DNA has been used to further differentiate species within the '*M.tuberculosis* complex' of the mycobacteria (Collins and de Lisle 1985). It is evident also that, using REA techniques, isolates of *M.bovis,* like those of *M.tuberculosis*, could be differentiated into strains. This could significantly assist understanding more precisely the epidemiology of bovine tuberculosis (Collins *et al.,* 1988), however the procedure has not attained general routine use because of technical and interpretative difficulties for many laboratories. Other procedures for identifying mycobacterial genomic differences have been developed following recognition of repetitive DNA elements in the '*M.tuberculosis* complex' mycobacteria (Van Embden *et al.,* 1993). Restriction Fragment Length Polymorphism analysis (RFLP) appears to be as discriminatory as REA for *M.bovis* strain typing (Cousins *et al.,* 1993; Skuce *et al.,* 1994; 1996; Van Soolingen *et al.,* 1994) and is simpler to interpret. An alternative typing procedure 'Spoligotyping', developed more recently to discriminate *M.tuberculosis* strains (Groenen *et al.,*, 1993; Kamerbeek *et al.,*, 1996), is simpler again. It is also effective in identifying *M.bovis* isolates and discriminates strains, however it would be less discriminating than RFLP

used with several probes (Roring *et al.,*, 1996, 1998). RFLP and Spoligotyping procedures are more widely employed than REA but in general, are still confined to relatively specialised laboratories. Detection of *M.bovis* strains and simultaneous Spoligotyping directly from clinical specimens is a promising development obviating the need for culture (Kamerbeek *et al.,* 1996). The above mentioned methods, together with Pulsed Field Gel Electrophoresis (PFGE), Amplified Fragment Length Polymorphism (AFLP) and Variable Number Tandem Repeat (VNTR) analyses have been assessed for applicability to *M.bovis* strain typing (E.C. 1999). Comparisons of genomic DNA may assist strain typing in the future.

4.3 Intradermal testing.

Control of bovine tuberculosis initially relied on physical examination and case history of the animal to identify infection, but because of the absence of clinical presentation with many tuberculous animals, intradermal testing was subsequently introduced. The current intradermal tests evolved from various testing procedures tried over the years, simultaneously with attempts to refine the complex tuberculin antigen (O'Reilly, 1992; Monaghan *et al.,* 1994). Intradermal tests using PPD are prescribed for international trade (OIE, 1996). The skin tests commonly used are the single intradermal test and the single comparative intradermal test. National test preferences have been influenced by experience and practicalities of testing and in some countries, a necessity to compensate for exposure to environmental mycobacteria. Intradermal testing is normally performed on the mid-neck region but can be performed in the caudal fold. The single intradermal test performed in the neck however is considered more sensitive (Francis *et al.,* 1978; de Kantor *et al.*, 1994). It is accepted that, when used in tuberculosis-free cattle populations, the single comparative intradermal test has highest specificity (Leslie and Herbert, 1975). There is consensus in recommending measurement of skin fold thickness reactions to tuberculin at 72 hours (Leslie and Herbert, 1975; Lepper and Corner, 1976; Thoen *et al.,* 1976; Lepper *et al.,* 1977). This could be considered somewhat arbitrary when compared to reading tests at 48 hours, however reactions at 72 hours have been reported larger than when read at 96 hours (Francis *et al.,* 1978; Lepper *et al.*, 1977). The size of the reaction appears to plateau about 72 hours, thus reading the test around this period would be acceptable. Repeat testing of animals presenting with inconclusive reactions at 72 hours should be carried out after a further 42 to 60 days (Leslie and Herbert, 1975; Monaghan *et al.,* 1994). Avoidance of desensitisation underpins the rationale for this delay before retest, the precise timing of this relates more to practicalities of testing. Repeat testing at less than 10 days, the 'short-interval re-test' (Wiggins and Essay, cited by Monaghan *et al.,* 1994), however may have high specificity but appears unsuitable for use in areas with existing tuberculosis (Roswurm and Koyna, 1973). Official interpretations of the skin tests (EEC, 1964; OIE, 1996) have been derived primarily from studies by Ritchie (1942) and Leslie and Herbert (1975). The primary tuberculin "antigen" used in these tests is now regulated, and is a purified protein derivative (PPD) from *M.bovis*. A second PPD from *M.avium* is used when the single comparative test is employed. The reaction to *M avium* tuberculin is used to distinguish sensitisation caused by other mycobacteria from that due to *M. bovis*. Strict adherence to testing procedures and attention to tuberculin potency are basic

essentials, as is an awareness of possible animal desensitisation, anergy and attempts to interfere with the test.

4.4 New developments in diagnostic tests for tuberculosis

Primary diagnosis of bovine tuberculosis, has never been laboratory based. On-farm intradermal tests have always had a pivotal role in national eradication programmes employing 'test and slaughter' strategies and have functioned well over the years. Many countries world-wide now indicate freedom from bovine tuberculosis (OIE, 1999). However, it is evident that although most regions with advanced farming practices, have had dramatic success in reducing tuberculosis in cattle, eradication has been inconsistent in some regions.

Identifying asymptomatic animals with disease, in a population of apparently healthy individuals, can be done presumptively by screening and then subsequent testing of suspect animals to confirm infection. Accuracy or validity of the diagnostic test can only be assessed relative to a defined 'gold standard', often only available from necropsy and/or laboratory culture, which in some instances may be difficult and costly to obtain and may have inherent inaccuracies itself. As economic pressures to eradicate bovine tuberculosis intensify, account must be taken of recent scientific developments which might assist towards achieving that goal, e.g., novel diagnostic procedures including improved antigen specificity, for more rapid and accurate detection and identification of *M.bovis*.

4.4.1 Possible candidates for new tests.

Skin tests have inherent practical drawbacks. In some countries with persistent tuberculosis in cattle, tests have come under increasing scrutiny and criticism relating to efficacy, cost and objectivity. Shortcomings in traditional skin testing should be expected especially where disease prevalence is low and infection is primarily recent. Tuberculous cattle exhibit a spectrum of immune responses and therefore potentially detectable parameters do not remain consistent throughout the course of *M.bovis* infections (Ritacco *et al.,* 1991; Neill *et al.,* 1994a). Cell-mediated immunity dominates in early infection with antibody responses developing later, hence the limited potential of alternative serological assays, particularly in countries with widespread and frequent skin test use. The necessity to employ the comparative intradermal test in some countries also demonstrates its lack of absolute specificity. Cross reactivity with environmental mycobacteria can be problematic. Thus, there is a continuing search for alternative antigens to the complex PPD currently used as antigen. It has now also been demonstrated that at specific times during *M.bovis* infection in cattle one or more antigens appear immunodominant and that this antigen profile changes as the infection progresses (Fifis *et al.,* 1994; Pollock and Anderson, 1997a). This might explain cattle with early infection remaining undiagnosed by intradermal testing (Neill *et al.,* 1992; 1994a). It is evident that at this time the precise dynamics of the immune responses in this complex disease in cattle are still not fully understood.

Unfortunately, there is no universally accepted alternative to skin testing with PPD for diagnosing tuberculosis in cattle. However, in recent years, invigorated research on blood parameters for use in potential alternative tests has provided opportunities to enhance disease detection in cattle. Where tuberculosis problems persist, testing strategies should now avail of all laboratory tests with appropriate antigens to improve diagnosis.

4.4.2 Serodiagnostic assays

A variety of serodiagnostic assays have been developed to detect humoral immune responses in cattle. These have been reported to exhibit considerable variation in sensitivity and specificity (Auer, 1987; Hanna *et al.,* 1989; 1992; Plackett *et al*., 1989; Ricatto *et al.,* 1990; Wood and Rothel, 1994; Costello *et al.,* 1997). Much of the sensitivity variation may be accounted for by failure to recognise strong anamnestic responses in cattle following skin testing and by the differing disease profiles within the herds tested. Suboptimium specificity may be due to the complexity of the antigens employed. Because of the early dominance of cell mediated responses in tuberculous cattle serodiagnostic assays are probably of most value in the later stages of infection and can offer diagnostic opportunities in some circumstances. Recognition and better definition of immunodominant mycobacterial antigens (Andersen *et al.,* 1991; Roche *et al.,* 1994; Pollock *et al.,* 1995), together with improved understanding of the precise kinetics of antibody responses in cattle (Lightbody *et al.,* 1998a), should increase the potential for using antibody ELISAs with skin testing to enhance diagnosis in some situations.

4.4.3 *In vitro* Immunodiagnostic assays

The initial tests developed as *in vitro* indicators of bovine cell mediated responses to infection were lymphocyte transformation or proliferation assays (Otteridge and Lepper, 1973; Muscoplat *et al.,* 1974). Such assays proved useful in research and diagnosis (Griffin and Buchan, 1989; Pollock *et al.,* 1994), however they have practical limitations, in handling and performance speed. Such drawbacks would generally preclude large-scale applications, such as in eradication programmes. Additionally they have demonstrated few improvements in sensitivity or specificity over traditional skin testing.

Subsequent recognition of cytokines and their role in tuberculosis immunology has led to the development of an *in vitro* assay for bovine interferon-gamma (IFNγ) as an indicator of tuberculosis (Wood *et al.,* 1990; Rothel *et al.,* 1990). This assay, despite the obvious drawback of time-dependent sampling, could accommodate large scale cattle testing. Kits are commercially manufactured and available and are used in compliance with the manufacturers recommendations Investigating other cytokines may yet yield additional useful diagnostic assays e.g. the IL-2Rα ELISA (O'Nuallain *et al.,* 1997).

It appears that even at this stage, there is a paucity of accurate information on sensitivity and specificity estimations for currently used skin tests (Monaghan *et al.,* 1994). Although these tests may not be perfect assays, they do serve as the standards against which any other test should be compared. Determinations of absolute

sensitivity and specificity for the IFNγ assay are equally difficult to obtain however, comparative evaluations of this assay and traditional skin tests have been undertaken in several countries. One Australian study demonstrated the sensitivity for the IFNγ assay (ranging from 76.8% to 93.6%) to be significantly better than that of the single intradermal caudal fold test, and the specificity ranged from 96.2% to 98.1% (Wood *et al.,* 1991). In another study, sensitivity and specificity estimates of 81.8% and 99.1% respectively, were reported for the IFNγ assay when used with cattle in Australia and New Zealand, compared with corresponding estimates of 68.1% and 96.7% respectively for the Caudal Fold Test (Wood *et al.,* 1992). In Northern Ireland, a preliminary examination of an IFNγ assay (Neill *et al.,* 1994b) demonstrated the sensitivity for the assay to be better than that for the skin test with standard interpretation applied and there was little loss in specificity. A subsequent large-scale study in Northern Ireland, comparing the IFNγ assay and the single comparative tuberculin test, demonstrated similar findings and reported that the sensitivity of the assay can equal that of the skin test with any field interpretation applied (Neill, unpublished data). Maximum reported sensitivity was obtained using the IFNγ assay together with the skin test. The IFNγ assay in both these studies identified tuberculous cattle undiagnosed by the skin test. In the United States of America, an IFNγ assay was reported to match the sensitivity (82%) and specificity (90%) achieved by the Caudal Fold Test (Whipple *et al.,* 1995). In Spain evaluation of the assay indicated that the relative sensitivity range for the IFNγ assay was 81.4%-92.4% compared to 67.7%-81.9% for a single intradermal tuberculin test (Domingo, *et al.,* 1995). In the Republic of Ireland the IFNγ assay was shown to detect the majority of infected cattle identified using the single comparative tuberculin test (Monaghan *et al.,* 1997). From recent studies in New Zealand, the sensitivity and specificity of the IFNγ assay are such that it could be employed usefully as a supplementary assay to the Caudal Fold Test (Ryan, personal communication).

Like the skin test, the IFNγ assay is versatile, usefully permitting trade-off between sensitivity and specificity when required. The relative performance of the IFNγ assay, compared to currently accepted skin tests, commends its use as an official diagnostic test in national eradication or control programmes. However, from most of the evaluations above, the anticipated performance of the IFNγ in the field would have significant impact on considerations of how this test might be best employed. The IFNγ assay could not provide a simple substitution as the sole replacement for the skin tests in existing eradication programmes. However, such a test currently employing PPD, should be available to maximise detection of infected animals in problem herds and could be used without delay following initial skin testing. Acceptance of this diagnostic assay as an official supplementary test procedure for tuberculosis should be encouraged to allow its use under local conditions in individual eradication schemes. Obviously, its deployment will ultimately be governed by the cost benefit to any existing testing programme.

It is of interest to note that the IFNγ assay is now officially accepted as a diagnostic test for use in Australia, New Zealand and the USA. In some European countries the assay is currently employed for diagnostic use in specific situations. The dual advances in understanding early bovine immune responses to *M.bovis* infection and the recognition of novel and specific immunodominant antigens (Pollock and Andersen,

1997a; 1997b) should resolve some of the existing reservations about using *in vitro* assays and also about their specificity.

4.5 Vaccination and Tests

The protracted nature of tuberculosis eradication from cattle in some countries, together with the recognition of significant reservoir hosts for *M.bovis*, has generated interest in cattle and wildlife vaccines (WHO, 1994; MAFF 1998). Available information on cattle vaccination, primarily using BCG (Bacille Calmette Guerin), indicates protection, but this is not consistent in all instances (Buddle *et al.,* 1995a; Buddle *et al.,* 1995b; O'Reilly, 1992; Buddle *et al.,* 1997). As with human vaccination, variation in cattle vaccine efficacy, when using BCG, should be expected, considering cattle exposure to environmental mycobacteria. Such exposure is particularly evident in accounting for specificity variation when skin testing cattle (Monaghan *et al.,* 1994). Vaccine adversaries have concern about the impact of cattle vaccination on current tests prescribed for international cattle trade. From studies in small animal models, subunit vaccines are considered promising (Andersen, 1997) and may possibly provide the solution to some of the problems arising from vaccinating with attenuated *M.bovis* strains. Current research on novel and improved diagnostics could readily take cognisance of the need for the capacity to differentially diagnose naturally infected from vaccinated cattle (Lightbody *et al* 1998b).

5. Suggested new text for Annex B

BOVINE TUBERCULOSIS

5.1 Identification of the agent

5.1.1 Optimum tissues for recovery of *M.bovis* from suspect tuberculous cattle have been identified and specimen processing is adequately described (OIE,1996, p269).

5.1.2 Primary culture of *M.bovis* is best performed by inoculation of a combination of multiple slopes of supplemented egg based and agar based solid media (OIE,1996, p270). Radiometric culture will permit more rapid detection of *M.bovis* in many instances and may be used in conjunction with solid media to facilitate primary culture.

5.1.3. *M.bovis* can be identified by traditional methods (OIE, 1996, p270). However, specific nucleic acid sequence detection and amplification technologies are appropriate for more rapid identification and speciation of mycobacterial isolates. Molecular technology also permits detection and identification of the *M.bovis* directly in clinical specimens but in reality is less sensitive than traditional culture. Further refinements are necessary to improve its sensitivity.

5.2 Tuberculosis testing.

5.2.1 PPD (Purified Protein Derivatives) shall be used for carrying out official tuberculin skin tests

5.2.2. The tuberculin skin test is described in the OIE Manual of Standards (1996, p270)

5.2.3. PPD dosage and volume used, should be those described in the OIE Manual of Standards (1996, p 270)

5.2.4. Bovine and Avian PPD must be manufactured and stored as described in the European Pharmacopoeia (1997 pp 1678-79)

5.2.5. Bovine and Avian PPD must fulfil the technical specifications of the European Pharmacopoeia and have their potency calibrated in international units (IU). (see 5.6 and 5.7 below)

5.3 Intradermal testing technique

5.3.1 The injection sites usually shall be situated at the border of the cranial and middle thirds of the neck. When both avian and bovine tuberculins are used in

one animal, tuberculins are injected in the same side of the neck; the site for injection of avian tuberculins shall be about 10 cm from the crest of the neck and the site for the injection of bovine tuberculin about 12.5 cm lower on a line roughly parallel with the line of the shoulder. If this is not possible because of irritated or inflamed skin or because the neck is too small as for instance in young animals, one injection should be made on each side of the neck at identical sites in the centre of the middle third of the neck.

5.3.2 Injection sites shall be clipped and a fold of skin within each clipped area taken between the forefinger and thumb and measured with callipers and its thickness recorded. PPD shall be injected intradermally using a needle and syringe or by any other method, approved by a Community Reference Laboratory, that ensures that the PPD is delivered intradermally. Usually, a short sterile needle, bevel edge outwards, attached to a graduated syringe charged with tuberculin, is inserted obliquely into the deeper layers of the skin. The dose of tuberculin (see above) shall then be injected intradermally and be confirmed by palpating a small pea-like swelling at each site of injection. The skin-fold thickness of each injection site shall be re-measured and recorded 72 hours (+/- 4h) after injection.

5.4 Interpretation of the tuberculin test

5.4.1 The interpretation of reactions to PPD shall be based on clinical observations and the recorded increases in skin-fold thickness at the sites of PPD injections, as described in the OIE Manual of Standards (OIE, 1996, p 270)

Single Intradermal Test

5.4.2 Animals which are recorded inconclusive in the single intradermal test shall be retested after 42 days.

5.4.3 Animals which are not negative to this second test, shall be deemed to be positive to the test.

5.4.4 Animals positive to the single intradermal test may be subjected to an intradermal comparative test.

Intradermal Comparative Test

5.4.5 The interpretation of reactions to PPD in the intradermal comparative test, for the establishment and maintenance of officially tuberculosis-free herd status, shall be based on clinical observations and the recorded increases in skin-fold thickness at the sites of PPD injections, as described in the OIE Manual of Standards (OIE, 1996, p 270)

5.4.6. Animals inconclusive to the intradermal comparative test shall be subjected to another test after a minimum of 42 days.

5.4.7 Animals, which are not negative to this second test, shall be deemed to be positive to the test.

5.5 Official tuberculosis-free herd status

5.5.1 Officially tuberculosis-free herd status may be suspended and animals from the herd shall not be allowed to enter intra-Community trade until such time as the status of the following animals is resolved:

5.5.1.1 Animals which have been deemed to be inconclusive to the single intradermal tuberculin test

5.5.1.2 Animals which have been deemed to be positive to the single intradermal tuberculin test but are awaiting retest with an intradermal comparative test

5.5.1.3 Animals which have been deemed to be inconclusive to the intradermal comparative test.

(N.B. this section (5.5) should either be located in Annex A, paragraph I,3.c. or be referred to by that paragraph)

5.6 PPD potency testing

5.6.1 The potency of Bovine and Avian PPD must be assessed biologically in comparison with appropriate PPD standards.

5.6.2 Laboratories designated in accordance with Article 17 (*of Directive 64/432/EEC*) will be responsible for the additional examination of routine issue PPD used in Member States to ensure that the potency of each PPD is adequate in relation to the appropriate Community standard. These examinations must be carried out, in tuberculous bovine animals or in suitably sensitised guinea-pigs.

5.6.3 Potency testing of bovine PPD may be carried out using tuberculous cattle, naturally or experimentally infected and must comply with the method specified in the OIE Manual of Standards (1996, p 273).

5.6.4 Potency testing using guinea-pigs should be carried out in accordance with the methodology described in the European Pharmacopoeia (1997, p1678-79)

5.7 PPD standards

5.7.1 The EU standard for bovine PPD is that supplied by the Instituut voor Dierhouderij en Diergezondheid (ID-Lelystad), Lelystad, The Netherlands.

5.7.2 Standard bovine PPD has a potency of 50,000 international units (IU) per mg of PPD

5.7.3 Bovine PPD must be prepared from appropriately identified *Mycobacterium bovis* strains with origin and subsequent history recorded. Usually, *M.bovis* strain AN5 or strain Vallee is used.

5.7.4 The EU standard for avian tuberculin is that supplied by the Veterinary Laboratories Agency, Weybridge, United Kingdom

5.7.5 Standard avian PPD has a potency of 50,000 international units (I.U.) per mg of protein

5.7.6 Avian PPD must be prepared from appropriately identified *Mycobacterium avium* strains with origin and subsequent history recorded. Usually, *M. avium* strain D4ER or strain TB56 are used.

5.8 Supplementary testing.

To enable detection of the maximum number of infected and diseased animals in a herd, Member States may employ appropriate supplementary assays, in addition to skin testing.

6. Comments on Annex C - Brucellosis

6.1 General

The OIE Manual prescribes the following tests for bovine brucellosis; the Enzyme Linked Immunosorbent Assays (ELISAs), the Complement Fixation Test (CFT) and the Buffered Brucella Antigen Tests (BBAT). The two ELISAs mentioned are an indirect ELISA specific for IgG1 using smooth lipopolysaccharide and a competitive (inhibition) ELISA using monoclonal antibodies specific to the O chain polysaccharide position on the smooth lipopolysaccharide. The BBAT tests include the Rose Bengal test and the buffered plate test. The card test is similar to the Rose Bengal test. Other tests mentioned in the manual are the Milk Ring Test and the Brucellin skin test. The Serum Agglutination Test (SAT) is not a prescribed nor an alternative test under the O.I.E. but is specified in legislation and used in the EU.

It is proposed that the order of tests in the Annex be changed to reflect the international downgrading of the SAT, though recognising that the test will continue to be used in some member states.

The Standard Sera to be used should be the appropriate OIE Standards in each case.

Two tests contained in the present Annex C, the plasma ring test and plasma agglutination test are little used today. It is suggested that they not be retained as official tests.

Certain tests are more useful as tests on individual animals (CFT, ELISA) whereas others are more suited for herd screening purposes (e.g. bulk MRT).

6.2 Detection of the Agent

The present Annex C contains no references to the direct identification of the *Brucella abortus* organism. It is suggested that the annex be expanded to include this information. The following is a detailed description of such methods.

B. abortus is the most important aetiological agent of bovine brucellosis, although *B. melitensis* can also be involved in outbreaks of abortion in cattle managed in close contact with sheep and goats. *B. suis* is more rarely incriminated as responsible for the disease in cattle. (Corbel and MacMillan, 1996)

Because brucellosis in animals is generally not characterized by a unique set of symptoms and in ungulates may even be asymptomatic for long periods of time, diagnosis has traditionally relied on the detection of circulating antibodies followed by the bacteriologic isolation of living organisms (Mayfield *et al.*, 1990). In this way, direct diagnosis of Bovine brucellosis is the method to demonstrate the disease with certainty and isolation of *Brucella* spp. is necessary for this purpose (Alton *et al.*,

1988). However, the recently developed polymerase chain reaction and DNA probe methods provide additional means for detection (Corbel and MacMillan, 1996).

6.2.1 Collection of specimens.

Choice and condition of specimens are very important and since *Brucella* may localise in small numbers in tissue or milk, it is important to culture as complete a set of specimens as possible (Mayfield *et al.*, 1990).

For the diagnosis of animal brucellosis by cultural examination, the choice of samples usually depends on the clinical signs observed (Garin-Bastuji and Blasco, 1996). Selection of specimens for *Brucella* isolation should be done considering the expected pathogenesis of brucellosis in the herd under investigation. Placental cotyledon, vaginal discharge, milk and fetal tissues (lung, liver) and abomasal content should be cultured in acute cases of disease, while organs of slaughtered animals should be also selected when a chronic *status* is established in the herd: those of the lympho-reticular system (e.g. spleen, lymph nodes, tonsils), the pregnant or early postparturiant uterus and the udder. According to Alton *et al.* (1988) tissues preferred for culture vary between host species and between different classes of animals within each species, e.g. young and old, male or female.

In mature cows, about 90 % of infections can be detected by culture of the mammary lymph nodes and the inclusion of samples from mandibular, medial iliac lymph nodes and the uterine caruncules, if present, will increase the chance of succesful culture to almost 100 %. In heifers, in the early stage of the incubation period, additional tissues need to be cultured to obtain meaningful results, these are the medial retropharyngeal, parotid, superficial cervical, mesenteric lymph nodes and spleen. In males, samples of testes, prostate, epididymes and seminal vesicles together with their associated lymph nodes must be included (Alton *et al.*, 1988).

Specimens must be cooled immediately after they are taken and frozen if it is going to take more than 12 hours before they are cultured. Containers for specimens should be leak proof and of an appropriate size to avoid containing air (Alton *et al.*, 1988).

6.2.2 Demonstration by staining methods.

Smears of placental cotyledon, vaginal discharge and fetal lung, liver and abomasal contents should be fixed with heat or ethanol and stained by the modified Ziehl-Nielsen (Stamp's), Kösters'or Gram methods (Alton *et al.*, 1988) or by Macchiavello method or with a fluorochrome- or peroxidase-labelled antibody conjugate (Corbel and MacMillan, 1996). However, evidence of microorganisms with characteristics of *Brucella* should be considering only presumptive of brucellosis. DNA probes or polymerase chain reaction (PCR) methods can be used on smear or swabs samples to demonstrate the agent (Corbel and MacMillan, 1996).

6.2.3 Culture media.

With the exception of blood culture, solid media have traditionally been prefered for the isolation of *Brucella* from field specimens (Alton *et al.,* 1988). Culture on solid medium facilities the recognition of *Brucella* colonies, discourages dissociation and limits interference by faster growing extraneous organisms.

The same types of basal medium are used for laboratory manipulations and for culture of specimens but to avoid contaminations such media can be made selective by the addition of antibiotics. Some strains of *Brucella*, e.g. *B. abortus* biovar 2 need serum in the medium for growth.

6.2.4 Basal media.

The development of effective culture methods has progressed steadily over the past 75 years. In this sense, Mayfield *et al.* (1990) briefly reviewed the methods most widely used by diagnostic laboratories around the world. They found that three basal media were commonly used for the isolation of *Brucella.* These were serum-dextrose agar, serum-trytose agar and serum-tripticase soy agar.

A range of commercial dehydrated media is available (Alton *et al.*, 1988). A nutrient agar, serum-dextrose agar, can also be prepared enriched with serum and dextrose (Alton *et al.,* 1988; Corbel and MacMillan, 1996). Either equine or bovine serum which is free from *Brucella* antibodies and has been passed through a sterilising filter can be used.

6.2.5 Selective media.

According to Mayfield *et al.* (1990) a variety of selective media have been developed for the isolation from potentially contaminated tissue sources or milk:

a)Tryptose agar plus three antibiotics (cycloheximide, bacitracin, polymyxin B) and 5% bovine serum (TSA). Brucellae grow very well on this medium, but it is not highly selective;
b) TSA plus ethyl violet (TSAEV). Contamination is not a major problem, but some strains or biovars could be highly inhibited, e.g. *B. abortus* biovar 2;
c) Ewalt's medium (Ewalt, 1983) contains bacitracin, cycloheximide, lincomycin, nystatin, polymyxin B and 5% bovine serum. It has the same advantages and disadvantages that TSAEV;
d) Farrel's medium (Farrel, 1974) contains bacitracin, cycloheximide, nalidixic acid, nystatin, polymyxin B, vancomycin and 5% equine serum. Occcasionaly growth of the colonies is inhibited but is highly selective and is recommended for isolation from heavily contaminated sources (Farrel and Robertson, 1972);
e) Ryan's medium (Ryan, 1967) contains penicillin, ristocetin, polymyxin B, nalidixic acid, cetrimide, nystatin, cycloheximide, and 5 % blood. This medium inhibits *B.*

abortus biovar 2 and it is only effective in controlling moderate contamination (Farrel and Robertson, 1972);
f) Brodie-Sinton's medium (Brodie and Sinton, 1975) contains bacitracin, polymyxin B, nalidixic acid, vancomycin, nystatin, cycloheximide, amphotericin B, cycloserine, and 5 % horse serum. It is a good enrichment broth and is usually used for the isolation of *Brucella* from milk.

Any of the basal media recommended for *Brucella*, containing serum if required, may be used to produce selective media by the addition of various antibiotics (Alton *et al.,* 1988). They recommend two selective media, Kuzdas medium and Morse and Farrel medium.

In most diagnostic situations, contamination is possible, and it is generally recommended that more than one type of selective medium be used (Corbel and Hendry, 1985). *Brucella* may be present in very low numbers, and for that reason at least four selective media should be used per specimen (Mayfield *et al*., 1990), though this may cause difficulties in practice. Moreover, more than one Petri dish should be cultured per specimen (Alton *et al.*, 1988).

6.2.6 Liquid media.

As the numbers of *Brucella* organisms are likely to be lower than in abortion material and as contaminating bacteria may be present in milk, colostrum and some tissue samples, enrichment is advisable in liquid medium consisting of serum-dextrose broth, tryptone soya broth or *Brucella* broth supplemmented with an antibiotic mixture of amphotericin B (1µg/ml), bacitracin (25 µg/ml), polymyxin B (6 µg/ml) and vancomycin (20 µg/ml). The enrichment medium should be incubated at 37°C in air supplemented with 10% (v/v) CO_2 for up to 6 weeks, with weekly subcultures onto solid selective medium. If prefered, a biphasic system of solid and liquid selective medium in the same bottle (Castañeda technique) may be used to minimise subculture (Corbel and MacMillan, 1996).

6.2.7 Quality assurance of culture media.

It is essential to regularly verify the ability of culture media to support growth of the type of *Brucella* organism likely to be found in the material being cultured. For each batch of medium a few plates/containers should be innoculated with a small, known, number or cells (about 100) of the fastidious serum-requiring *B. abortus* biovar 2 or another appropiate culture (Alton *et al.*, 1988; Corbel and MacMillan, 1996).

6.2.8 Identification of *Brucella* organism.

There is no single test by which an organism can be identified as belonging to the genus *Brucella*, but a combination of growth characteristics, serological and bacteriological methods usually enable an organism to be correctly classified (Alton *et al.*, 1988).

Any colonies of *Brucella* type (raised, convex, circular outline, transparent, smooth surface, slow growing) should be checked by examining a Gram stained or Stamp's stained smear. If organisms of *Brucella* morphology (small Gram negative or Stamp's positive coccobacilli or short rods with rounded ends and slightly convex sides) are seen, the colonies should be tested for agglutination with *Brucella* specific antiserum after checking colonial morphology (Corbel and MacMillan, 1996). The latter can be carried out using the direct observation methods by an obliquely reflected light, the acriflavine test and the staining of colonies with cristal violet (Alton et *al.*, 1988).

If the colonies show a smooth morphology, they should be checked against antisera monospecific for A and M surface antigens. In the case of nonsmooth colonies, isolates should be checked with antiserum to *Brucella* R antigen. Agglutination with a *Brucella* antiserum provides presumptive identification and subsequent full identification is best performed by a reference laboratory (Corbel and MacMillan, 1996). Additional identification should be done by using either phage lysis tests or oxidative metabolism tests to identify the species. Identification to biovar level depends on examination for growth in the presence of basic fuchsin and thionin at final concentration of 20 µg/ml; production of H_2S requirements of CO_2 for growth, and agglutination pattern with A-, M- or R-specific antisera. Reference strains should be included in each series of tests to control the quality of reagents and techniques. (Alton *et al.*,1988; Corbel and MacMillan, 1996). Identification of the vaccine strains *B. abortus* strain 19 or *B. melitensis* strain Rev-1 depends on futher tests.

Oxidative metabolic tests expose the operator to the risk of infection, the equipment needed is expensive and special training is required for personnel. Because of this the tests are performed routinely in only a few laboratories in the world. It is suggested, therefore, that if conventional tests and phage typing do not permit a culture to be typed satisfactorily, it should be sent for further examination to one of these laboratories (Alton *et al.*, 1988).

6.2.9 Reference strains and biovars of *Brucella*.

A set of FAO/WHO reference strains is available comprising 6 species of *genus Brucella* and their biovars included in the type collections of both Great Britain (National Collection of Type Cultures-Great Britain) and the USA (American Type Culture Collection) (Alton *et al.*, 1988). These reference organisms should be considered during the typing procedure of *Brucella* species to provide a good quality assurance.

6.2.10 *Brucella melitensis* isolation.

Culture methods suitable for the isolation and identification of *B. melitensis* are very similar to those recommended for other Brucella species (Alton *et al.*, 1988). For Corbel (1985) most of the bacteriological procedures available for the diagnosis of *B. melitensis* infection worked reasonably satisfactorily. However, it should be borne in mind that most of these methods were not developed with this organism specifically in

mind. This applies particularly to the selective media currently in use, most of which were developed for the isolation of *B. abortus* strains and which, for this reason, may give sub-optimal results for the culture of *B. melitensis*.

B. melitensis grows on recommended basal media for *B. abortus* but in a somewhat less luxuriant way (Alton, 1990). Its growth is not dependent on serum in the medium or an atmosphere enhanced by the addition of CO_2 but is somewhat improved by the presence of both of these. The Kuzdas and Morse medium is adequate in most situations (Alton, 1990). Marin *et al.* (1996) compared the sensitivity of Farrel's and modified Thayer-Martin's medium (Brown *et al.*, 1971) for diagnosing *Brucella melitensis* infection in flocks of sheep and goats with chronic brucellosis. The results obtained in this work showed that the sensitivity of Farrel's medium was significantly lower than that obtained by using the less selective Thayer-Martin's medium. These authors concluded that the combined use of both media yielded the largest number of infected specimens and animals.

6.2.11 *Brucella suis* isolation

Media used for other species of brucellae are suitable for *B. suis*. The addition of serum is not essential, but basal medium containing 5 % serum is a satisfactory medium, both for isolation, maintenance of cultures and typing. The addition of CO_2 to the atmosphere is not required (OIE, 1996).

6.2.12 Molecular typing of *Brucella*

The classical methods for the typing of *Brucella* can be extremely effective if carried out correctly. However they require perfectly titrated reagents and high levels of skill and experience if misidentification is to be avoided. Several molecular typing methods, mainly PCR-RFLP and Southern blot analysis of various genes or loci have been employed to find DNA polymorphism which would enable the molecular identification and typing of *Brucella* species and their biovars (Garin-Bastuji *et al.,* 1998). Southern blotting and probing for the IS711 (IS6501) insertion sequence is an effective, though time consuming method capable of the differentiation of the species of *Brucella* (Bricker *et al.*, 1994). Detection of polymorphisms by PCR-RFLP are easier to perform and can more easily be applied to large numbers of samples. The outer membrane protein (omp) genes of *Brucella* have to date proved the best candidates for investigation as they show sufficient polymorphism to allow differentiation between *Brucella* species and some of their biovars. By targetting the genes omp2a and omp2b it is possible to differentiate the six *Brucella* species and some of their biovars (Cloeckaert *et al.*, 1996). The addition of primers for other genes such as omp31and dnaK improve the discrimination still further. Nevertheless, the pronounced homology of the genus hinder the development of genetic typing methods and currently they cannot yet achieve the same degree of discrimination as the classical bacteriological methods. However, it is likely that the situation will improve with the development of new technologies and the identification of other more polymorphic genes.

6.2.13 Other methodologies.

According to Corbel and MacMillan (1996) methods based on latex agglutination, haemagglutination and enzyme-linked immunosorbent assays (ELISA) have been described but are not well-standardised and cannot be recommended for general use.

Mayfield *et al.* (1990), however, stated that other methods than bacterial culture offer advantages in certain situations. Antigen-antibody based detection methods are generally much faster than culture techniques but are one to five orders of magnitude less sensitive. As a general rule, if the number of *Brucella* is less than about $1x10^6$ organisms per gram, histological techniques will require extensive time in screening tissue sections to find a region of infection.

One clear advantage of all of the nonculture techniques is the potential to detect nonliving *Brucella* cells or cell fragments. In that sense, a very useful tool for the near future will be methodologies based on selected sequences of the *Brucella* genome, such as DNA probes (Mayfield *et al.*, 1990) or PCR (Bricker and Halling, 1994 and 1995; Romero et al, 1995a and 1995b; Matar *et al.*, 1996). But to date, they are still under experimental evaluation and methods suitable for general use are not available.

6.3 Standard Reference Sera

The current texts of Annex C sections (A), (B), (D) and (H) are confusing on the identity and use of the standard serum for the standardisation of tests. In practice, the 'Weybridge' serum referred to is the 2nd International anti-*Brucella abortus* Serum (ISABS) and is the primary reference serum to be used for the establishment of national secondary standards. This serum currently acts as the standard for the RBT, SAT, and the CFT, but should now be referred to as OIE International Reference Standard Serum for the RBT, SAT or CFT as appropriate. The OIE has recently approved the establishment of strong positive, weak positive and negative OIE Standards for the indirect and competitive ELISA. These sera are available from the Veterinary Laboratories Agency (VLA), Weybridge, U.K. Such reference sera may also be used for the standardisation of new binding assays in the future.
The definition of the diagnostic threshold for the RBT, SAT and CFT should remain the same as at present, but the definition of the diagnostic threshold for ELISAs and other binding assays should be redefined in terms of the designated OIE sera in line with that stated in the OIE Manual.
It must be made clear that these Standard Sera are International Primary Reference Standards from which National Secondary Reference Standards for each test must be established for use in each member state against which working standards must be calibrated.

6.4 Allergic skin test

The particular advantage of the allergic test for brucellosis is its high specificity, with figures of over 99.8% being reported (Saegermen *et al.,* 1999). However not all infected animals will react and indeed animals negative to the allergic test while at the

same time excreting brucella organisms have been reported (Alton *et al.,* 1988). However, the test can be useful, particularly as a supplementary tool in interpreting serological results in herds where the brucellosis status is not clear. The test has been shown to be capable to clearly discriminating brucellosis from false positive serological reactions caused by infection with *Yersinia enterocolitica O9* (Saegerman *et al.,* 1999). The allergic test cannot be used in animals that have been vaccinated with vaccines that cause prolonged sensitivity to brucella allergens.

6.5 New tests under development

The Brucellosis IFN-γ test was developed because of the emergence of False Positive Serological Reaction (FPSR) in brucellosis tests (Weynants *et al*., 1995). This phenomenon has become the major problem as far as brucellosis epidemiosurveillance is concerned in Northern European Countries. Research is continuing and no conclusions can yet be drawn.

Other binding assays e.g. the Fluorescent Polarisation Assay test, are currently being developed and may prove useful in the future. However such tests will need to be validated before official use.

The use of vaccines may also have an important role to play in certain circumstances in the eradication of the disease. The further development of vaccines and, in particular, appropriate differential diagnostic tests is necessary before vaccination can be recommended in the current European epidemiological situation.

6.6 Pooling of sera and milk

The current directive requires that bulk milk samples are classified as negative to the ELISA if they give a reaction less than 50% of that given by a 1 in 10,000 dilution of the second international brucellosis standard serum made up in negative milk.

No requirements are currently given for bulk milk ring tests (MRT) except to state that if ring tests are carried out on samples from bulk tanks the number of tests required shall be doubled and the interval between them shall be halved. (EC 1997, Annex A.I.13)

The MRT is a useful test, particularly because it is suitable for field use and does not require complicated techniques to carry it out. It is however less accurate than the ELISA which would be a preferable test for the testing of bulk samples

20 International Units have been classically accepted as the cut off for positive samples for the MRT. This value seems reasonable. A standard giving 20 I.U. can be prepared by diluting the OIE International Reference Standard Serum for the SAT or CFT in negative milk. Bulking of milk for use in the MRT may present a problem in that individual low positive cows may not be detected when a large number of otherwise negative samples are bulked together. In the past bulk MRTs were carried out in eradication programmes where it was more likely that infection would be present in

several animals in a herd and the test performed quite well in this context. Now, however, with much of the EU free from the disease, it is more likely that cases of brucellosis would result from new introductions into previously free herds and thus would appear as single positive animals.

Therefore, the upper limit for the number of samples that can be bulked together should be set so that a single animal with a titre of 20 I.U. in the milk should be detected. This upper limit should be set by the national reference laboratory. This upper limit should be such that the test should be capable of detecting a single animal with a titre of 20 I.U. in a pool of milk from negative cows and should be set according to the sensitivity of a given antigen.

A similar situation applies to the bulk ELISA which may be used to test pooled serum or milk samples, though little work has been carried out to test the efficacy of testing pooled serum samples. The upper limit to the number of samples that can be pooled should be such that the OIE Weak Positive Standard would still be detected by the test if diluted in negative serum or milk at the dilution of the 'pool'.

The current Directive specifies that the ELISA may be used on bulk milk samples provided that 30% of the cows in the herd are in milk at that time. This proportion appears to based on the practical possibility of covering all animals in the herd when the test is repeated at intervals throughout the year. When used in this manner, therefore, the ELISA is suitable for monitoring herd status but not for indicating the status of an individual animal.

There is a need for further research to define the capabilities and limitations of diagnostic tests when used on pooled samples.

7. Suggested new text for Annex C

7.1 Identification of the agent

The demonstration by modified acid-fast or immunospecific staining of organisms of Brucella morphology in abortion material, vaginal discharges or milk provides presumptive evidence of brucellosis, especially if supported by serological tests. After isolation, the species and biovar should be identified by phage lysis and/or oxidative metabolism tests, cultural, biochemical and serological criteria.

7.2 Immunological Tests

7.2.1 Standards

The standard reference serum for the RBT, SAT or CFT is the OIE International Reference Standard Serum, available from from the Veterinary Laboratories Agency (VLA), Weybridge, United Kingdom.

The Standard reference sera for the ELISA and other binding immunoassays are the Strong Positive, Weak Positive and Negative OIE Standards, available from from the Veterinary Laboratories Agency (VLA), Weybridge, United Kingdom.

The reference sera for the MRT should be prepared by diluting the OIE International Reference Standard Serum for the SAT or CFT in negative milk to give 20 international units.

7.2.2. Enzyme-linked immunosorbent assays (ELISAs) or other binding assays for detecting bovine brucellosis

The technique used, its standardisation and the interpretation of results must conform to that specified in the OIE Manual of Standards (1996, pages 248 and 249). ELISAs may be used for the testing of milk, plasma or serum and must detect the OIE Positive and Weak Positive reference sera. When samples are pooled, the number of samples included in each bulking shall be such that the OIE Weak Positive Standard Serum shall be detected as positive when diluted in negative milk or sera by the number of samples making up the pool. This upper limit shall be determined by the national reference laboratory.

7.2.3. Complement fixation test

The technique used, its standardisation and the interpretation of results must conform to that specified in the OIE Manual of Standards (1996, page 247). Other methodologies of the CFT may be used provided that they also detect the appropriate dilution of the reference serum.

The OIE International Standard contains 1,000 international CFT units (ICFTU). A serum containing 20 or more ICFTU per ml, is considered to be positive.

7.2.4. Milk Ring test

The technique used, its standardisation and interpretation must conform to that specified in the OIE Manual of Standards (1996, page 250).

The number of samples included in each bulking shall be such that a single animal with a titre of 20 I.U. (see 7.2.1) in the milk should be detected. The upper limit shall be determined by the national reference laboratory according to the sensitivity of the test.

7.2.5. The buffered brucella antigen test

The technique used, its standardisation and interpretation must conform to that specified in the OIE Manual of Standards (1996, page 246).

7.2.6. Serum agglutination tests

The technique used, its standardisation and interpretation must conform to that specified in the OIE Manual of Standards (1996, page 250).

EDTA (10mM final dilution in preparation of the antigen suspension) may be added to reduce the level of false positives to the serum agglutination test.

7.3. National Reference Laboratories

7.3.1 Specific Tasks

National Reference Laboratories will be responsible for checking the quality of the test method used, and in particular for determining, for each production batch, the maximum number of samples to be pooled .

The National Reference Laboratories will also be responsible for calibrating the standard working antigen of the laboratory against the standard sera mentioned above.

All antigens must be checked for quality in the National Reference Laboratories.

7.3.2 List of National Reference Laboratories

To be completed by the relevant authorities

8. Comments on Annex D - Enzootic Bovine Leucosis

8.1 General

The current annex describes two serological tests, ELISA and Agar Gel Immunodiffusion test (AGID). Most ELISA and AGID tests in routine use detect antibodies to the glycoprotein gp-51 of Bovine Leukaemia Virus (BLV), as these antibodies appear early in the infection stage and more consistently. In addition, some assays also detect antibodies to the core protein p24 of BLV. Comparative studies have revealed that the immunological response of cattle to BLV infections is characterised by an anti-glycoprotein titre at least 10-fold higher than the titre of antibodies against protein p24. The level of antibodies against protein p24 is higher in the tumor phase of the disease (Miller and Van der Maaten, 1977; Bex *et al.,* 1979)

Generally, antibodies can be detected 3-16 weeks after infection. However, the presence of detectable antibodies depends on the course of the infection. Maternally derived antibodies may take up to 6 or 7 months to disappear and there is at present no way of distinguishing passively transferred antibodies from those resulting from active infection. In infected cows and heifers the serum antibody level may drop during the periparturient period, due to a shift from blood circulating antibodies to colostral antibodies. Negative test results 2 – 3 weeks pre- and post-partum are therefore not conclusive and should be repeated, especially when using the AGID test.

AGID tests use antigen produced in tissue culture. Many of these cultures are contaminated with Bovine Viral Diarrhoea (BVD) virus and this can give rise to specificity problems due to crossreactions with antibodies to BVD. The AGID test is only suitable for use for individual animals and not for pooled samples. It should not be used in milk samples.

ELISAs are superior to AGID in terms of automation and accordingly high-throughputs of samples. Furthermore, both serum and milk can be used as samples in ELISA (Hoff-Jørgensen, 1989), but usually require separate kits. Either indirect or competitive ELISAs may be used. ELISA kits based on either principles are commercially available. Other binding assays may become available in the future

8.2 Direct detection of Bovine Leukaemia virus

This may be necessary in order to confirm or rule out that suspect tumours identified at post mortem inspection result from BLV infection and for the identification of infected calves and their distinction from uninfected calves with maternal antibodies.

Culturing of BLV in cell-cultures is a both time and labour consuming process and therefore not suitable for routine laboratory diagnosis of enzootic bovine leucosis.

A bioassay in sheep for detecting the virus has been used, but this approach also has time limitations, as it can take up to 2 to 4 weeks before the development of a specific immune response (Djilali *et al.,* 1987). This type of assay also has expense and animal welfare problems so only very restricted use is recommended.

In the recent years several assays based on detection and amplification of proviral DNA or RNA specific for BLV, PCR has been described (Naif *et al.,* 1990;1992; Brandon *et al.,* 1991; Berg-Rasmussen *et al.,* 1991 Murtaugh *et al.,* 1991; Ballagi-Pordany *et al*., 1992, Sherman *et al.,* 1992; Agresti *et al.,* 1993; Kelly *et al.,* 1993; Rasmussen *et al.,* 1994; Dube *et al*., 1997). Such assays are able to detect viral or proviral RNA/DNA in peripheral blood mononuclear cells from infected animals and from tumors. Proviral DNA is present in all stages of the disease and positive results can be obtained on an earlier stage of infection by use of PCR than by the hitherto described serological assays, which makes PCR an attractive supplemental tool in detecting infected animals. Detection of perinatal BLV infection by using PCR is also described (Agresti *et al.,* 1993) and seems to be independent of the serological status of the calves. However, variations in the genomic sequence of BLV isolated from cattle from different geographic areas (Dube at al., 1997) have been described, which can make the PCR method a valuable epidemiological tool, but also must encourage laboratories to be aware of the variations when choosing the PCR primers. These problems can perhaps be avoided by choosing the highly conserved envelope region as the target sequence for the PCR (Beier *et al.,* 1998; Klintevall 1994). An interesting observation was that several cows were infected with multiple BLV strains whose divergence ranged from 1.5 - 5% (Dube *et al*., 1997).

Furthermore, some of the PCR methods have been automated and can be carried out within 24 hours, which make them possible to use in large scale screening or surveillance programmes.

In general, the PCR methods described, seem to have very high analytical sensitivities, e.g some amplification protocols allow detection of 1 viral genome in 100,000 cells.
However, evaluations of diagnostic sensitivity (Dsen) and specificity (Dspe) described in the references, have some limitations. The evaluations of test results in the publications are all based on a limited number of animals, which give estimates of sensitivity and specificity with very wide confidence intervals.
In some of the evaluations, the animals are divided into diseased and non-diseased based on their serological status, which allow errors in the estimates of Diagnostic sensitivity and Diagnostic specificity of the serological assays to be carried over into those estimates of the PCR methods.
However, the above mentioned problems are common for evaluations of new laboratory assays used for infections where the selection of a 'gold standard' is difficult.
Other publications (Naif *et al.,* 1992; Kelly *et al.,* 1993) describe PCR and serological results from experimentally infected animals, but evaluation of Diagnostic sensitivity of the PCRs is not possible due to limitations in the number of animals used and because the animals might not be representative of the naturally exposed populations.

Reliable estimates of the test characteristics as they apply to naturally infected animals are needed, both on the individual animal level and on the herd level, for comparison to

be made with serological assays and for recommendation of implementation of this type of assay.

However, the PCR methods described seem promising in detecting infected animals in the early stage of disease where the serological assays are not yet able to detect antibodies to BLV or when it is not possible to distinguish from maternally derived antibodies. One approach could be recommendation of the serological assays for surveillance of infection status of a population (e.g. herd/country) and the use of PCR for determining the individual infection status (e.g. in eradication programmes) or verifying a non-infected serological diagnosis in animals originating from infected populations (e.g. for trade).

Basic research on the cell mediated immunity after infection with BLV has also been published (Keefe *et al.,* 1997a, 1997b). By using reverse-transcriptase PCR (RT-PCR), IL-2, IL-10 and IFNγ messager was detected in the T-cell fraction of BLV infected animals, but not in non-infected controls. Furthermore consistently elevated levels of IL-2 and IL-12 were detected in BLV-positive aleukaemic and BLV-positive persistently lymphocytotic cattle. IFN-gamma was solely elevated in the BLV-positive aleukaemic cattle.

Standardised PCR protocols have to be agreed at the international level before this test is accepted for official purposes. Methods for proficiency testing for these tests also need to be developed.
Positive PCR results might support a diagnosis of BLV infection, but at present they are not sufficiently specific and therefore not conclusive for the infection.

8.3 Standard Sera

E4 serum is considered the reference serum standard to be used in serological assays for diagnosis of enzootic bovine leucosis within EU Reference Laboratories. The E4 reference serum standard is obtainable from the Danish Veterinary Laboratory, Bülowsvej 27, DK-1790 Copenhagen, Denmark. This serum is also available for reference laboratories in the OIE Member countries. National standards should be produced and calibrated against E4 so that a standard serum is available for routine use within Member States.

The E4 reference serum standard is a pool of serum from two cows infected as calves, prior to ingesting colostrum, with BLV infected lymphocytes derived from a naturally infected donor cow, diluted 1:10 in negative serum which originated from uninfected cows. Serum from the infected cows was collected before the animals developed tumours.

The E4 serum standard contains antibodies against BLV glycoproteins, including the envelope glycoprotein gp51 and core protein p24.

In assays where samples (serum and milk) are tested individually, E4 reference serum standard diluted 1:10 in negative serum or 1:250 in negative milk must be scored positive when tested in the same assay dilution as used for individual test samples.

8.4 Pooling of Samples

Some binding assays are sufficiently sensitive to be used with pooled samples of sera or of milk.
Where individual serum samples are tested as a pool, E4 reference serum standard must test positive when diluted ten times more than the number of individual serum samples included in the pool (e.g. for a pool composed of 50 individual samples, E4 diluted 1 in 10 x 50, i.e. 1:500, must give a positive result). Similarly, for tests on pooled milk or whey samples, E4 must be found positive when diluted in milk 250 times more than the number of individual samples comprising the pool.

The current Directive specifies that the ELISA may be used on bulk milk samples provided that 30% of the cows in the herd are in milk at that time. As in the case of brucellosis, this proportion appears to based on the practical possibility of covering all animals in the herd when the test is repeated at intervals throughout the year. When used in this manner, therefore, the test is suitable for monitoring herd status but not for indicating the status of an individual animal.

9. Suggested new text for Annex D, Chapter II

TESTS FOR ENZOOTIC BOVINE LEUCOSIS

Diagnostic tests for enzootic bovine leucosis by the detection of Bovine Leukaemia Virus (BLV) antibodies shall be carried out by the enzyme-linked immunosorbent assay (ELISA) under the conditions described in 9.2 or by the agar gel immuno diffusion test (AGID) under the conditions described in 9.3. The immuno-diffusion method may not be used on bulked samples.

9.1 Serum/standards

The standard reference serum for the ELISA and the AGID test is the standard serum (E4 serum) presently supplied by the Danish Veterinary Laboratory, Bülowsvej 27, DK-1790 Copenhagen, Denmark.

9.1.1. Standardisation and sensitivity of the ELISA

In assays where samples (serum and milk) are tested individually, E4 reference serum standard diluted 1:10 in negative serum or 1:250 in negative milk must be scored positive when tested in the same assay dilution as used for individual test samples.
Where individual serum samples are tested as a pool, E4 reference serum standard must test positive when diluted ten times more than the number of individual serum samples included in the pool (e.g. for a pool composed of 50 individual samples, E4 diluted 1 in 10 x 50, i.e. 1:500, must give a positive result). Similarly, for tests on pooled milk samples, E4 must be found positive when diluted 250 times more than the number of individual samples comprising the pool.

9.1.2. Standardisation and sensitivity of the AGID

The sensitivity of the AGID must be of such a level that E4 reference serum or derived standard is scored positive when diluted 1:10 in negative serum. The antigen used in the test must contain bovine leukaemia virus glycoprotein (gp51).

9.2. Enzyme-linked immunosorbent assays (ELISAs)

The technique used, its standardisation and interpretation must conform to that specified in the OIE Manual of Standards (1996, pages 278 and 279).

9.3. Agar gel immuno diffusion test for enzootic bovine leucosis (AGID)

The technique used, its standardisation and interpretation must conform to that specified in the OIE Manual of Standards (1996, page 277-278). This test may only be used on individual serum samples. Negative results to the test must not be read before 72 hours.

9.4. Method for antigen standardisation

The BLV antigen must be standardised in accordance with the technique specified in the OIE Manual of Standards (1996, page 278).

9.5. National Reference Laboratories

9.5.1 Specific Tasks

The National Reference Laboratories listed in 9.5.2 below will be responsible for checking the quality of the ELISA method, and in particular for determining, for each production batch, the maximum number of samples to be pooled on the basis of the count obtained for the E4 serum.

The National Reference Laboratories will also be responsible for calibrating the standard working antigen of the laboratory against the official EEC standard serum (E4 serum) provided by the Danish Veterinary Laboratory, Copenhagen and for carrying out quality control, as necessary, on antigens.

9.5.2 List of National Reference laboratories

To be completed by the relevant authorities

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11. Acknowledgements

This report of the Scientific Committee on Animal Health and Animal Welfare is based on the work of a working group established by the Committee. The working group was chaired by Dr. Michael Gunn. The members of the group are listed below.

Dr. D. Baier, Dr M. Duran-Ferrer Dr J. Godfroid, Dr M. Gunn, , Dr T. Heneghan, Dr A. MacMillan, Dr. S. Neill, Dr. V. Sorensen, Dr Hoff-Jorgensen, Dr. C. Staak, Dr. F. VanZijderveld,