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Diagnostic Tests for Contagious Bovine Pleuropneumonia (CBPP)

Report of the

Scientific Committee on Animal Health and Animal Welfare

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1. REQUEST FOR OPINION

The Scientific Committee on Animal Health and Animal Welfare is requested to review diagnostic tests for contagious bovine pleuropneumonia (CBPP).

In particular, the Committee is requested to advise on uses of diagnostic tests for CBPP, with particular emphasis on the role of immunoblotting. It is also requested to comment on the most suitable confirmatory test.

2. BACKGROUND

The Scientific Veterinary Committee (SVC) previously addressed the issue of CBPP in 1993 when it reported on the epidemiology, pathogenesis and methods of diagnosing the disease (SVC, 1993). Since then, the disease appears to have been eradicated in the EU. However it is important to update the available diagnostic methods to ensure that the most up to date tools are available and are used in the correct context. In particular, the immunoblotting test has been introduced since the previous report of the Committee in 1993, and other test techniques have been developed and refined.

The Committee also reviewed the earlier report of the Committee and has commented on items (definition of outbreak, standard sera) where an updating is now appropriate.

3. INTRODUCTION

3.1. The agents

Mycoplasma mycoides subsp. *mycoides* small colony type (*Mmm*SC), the causative agent of CBPP, belongs to the genus *Mycoplasma*, class *Mollicutes*, order *Mycoplasmatales* and family *Mycoplasmataceae*. *Mmm*SC is a self- replicating and wall-less prokaryotic organism.

*Mmm*SC is a member of the *M. mycoides* cluster (see table 1) which includes *Mycoplasma mycoides* subsp. *mycoides* large colony type (*Mmm*LC), *Mycoplasma capricolum* subsp. *capricolum*, *Mycoplasma capricolum* subsp. *capripneumoniae (Mccp)*, *Mycoplasma mycoides* subsp. *capri* (*M. capri*) and *Mycoplasma* sp. bovine group 7. They share biochemical, immunological and genetic characteristics.

The "*Mycoplasma mycoides* cluster" contains six important mycoplasma of ruminants. Only two of them cause disease in cattle, *Mmm*SC which is the cause of CBPP, and *Mycoplasma* bovine group 7 (Bg 7) which may cause bovine arthritis and bovine mastitis. The four others, two subspecies within *Mycoplasma mycoides* species and two subspecies within *Mycoplasma capricolum* species are responsible for goat respiratory and other diseases.

There is now evidence that some members of "*Mycoplasma mycoides* cluster" may be isolated from both large and small ruminants (*Mccp* and *M. capri* have never been isolated from large ruminants, nor *Bg7* from

sheep/goats). Consequently, animal host specificity is not strict enough to contribute as a valuable criterion for discrimination between *Mycoplasma subspecies*. This presents a real problem for definitive identification.

Species identification may be also complicated by a peculiar, probably ubiquitous feature of mycoplasmas, namely their capability to undergo high-frequency surface antigenic variation. It has generally been anticipated and was first documented for the swine pathogen *M. hyorhinis* (Rosengarten & Wise, 1990). Later, evidence was obtained that this phenomenon also occurs with the bovine pathogens *M. bovis* and more recently *Mycoplasma mycoides subspecies mycoides* (*Mmm*). In the light of the high rate of variability of surface antigens in many mycoplasmas, including the one causing CBPP (*Mmm*SC), diagnostic data obtained with immunobinding assays by using specific monoclonal antibodies as well as polyclonal sera require a more critical evaluation (Rosengarten & Yogev, 1996). The establishment of criteria is needed for identifying mycoplasma strains and for standardising of diagnostic antisera to ensure that reproducible results can be obtained in different laboratories.

3.2. Host specificity

Under natural conditions, CBPP occurs in cattle of the species *Bos* and allied animals including buffalo, yak, bison and even reindeer (Hutyra *et al.,* 1938). These authors reported that goats and sheep were susceptible under experimental conditions. Many of these reports need to be substantiated. Indeed Provost (1988) reviewing the literature could find no evidence to show that the domestic buffalo, *Bubalus bubalis*, was susceptible under natural or experimental conditions. Experimental work in Australia showed that buffaloes could be infected by artificial means but did not spread CBPP to in-contact buffaloes (Newton, 1992). However, Santini *et al.,* (1992) observed pulmonary lesions and isolated *Mmm*SC from seropositive buffaloes which had been in contact with CBPP-affected cattle in Italy. They concluded that buffaloes were susceptible albeit at a low level and that further research was necessary to clarify the role of buffaloes as a reservoir of infection for cattle. Small ruminants, in particular goats, have also been shown to harbour the *Mmm*SC (Hudson *et al.,* 1971). Brandao (1995) isolated *Mmm*SC from the milk of sheep with mastitis, as well as from goats with pneumonia in Portugal outside the endemic region of CBPP. The isolation of *Mmm*SC from these sheep did not result in slaughter of the infected flock or imposition of other CBPP control measures. Experiments with goats in contact with cattle infected by the African strain Afadé – showing clear genetic differences with European strains – suggest a lack of susceptibility in the goat (Belli *et al.,* 1990, Cheng *et al.,* 1995). The pathogenicity of these strains for cattle as well as their epidemiological significance are unknown at present and need to be clarified.

Table 1 Members of the Mycoplasma mycoides cluster

Name	Main disease	Main (and other) hosts
M. mycoides subsp. *mycoides* SC variant	CBPP	Cattle (goats, sheep, buffalo)
M. mycoides subsp. *mycoides* LC variant	Caprine pneumonia, contagious agalactiae[1]	Goats (sheep, cattle)
M. mycoides subsp. *capri*	Caprine pneumonia	Goats (sheep) but rare
M. capricolum subsp. *capricolum*	Caprine pneumonia, contagious agalactiae[1]	Goats (sheep, cattle)
M. capricolum subsp. *capripneumoniae*	Contagious caprine pleuropneumonia[2]	Goats (sheep)
M bovine group 7 (Bg 7)	Arthritis, also mastitis, calf pneumonia	Cattle

[1] = also arthritis [2] = CCPP

3.3. Aspects of CBPP pathogenesis and immunopathology

Contagious Bovine Pleuropneumonia (CBPP) is the only bacterial disease of the List A of the OIE. The disease affects cattle and is caused by *Mycoplasma mycoides subsp. mycoides* Small Colonies (*Mmm*SC). It is often characterised by an inconspicuous and insidious nature that may result in an underestimation of the threat. Under European experimental conditions it cannot be easily reproduced; available vaccines do not confer a satisfactory immunity and little is known about the type of immunity they confer and generally about the immunopathology of the disease.

CBPP is a respiratory disease associated with pneumonia and sero-fibrinous pleurisy. It is transmitted by direct close contact between animals excreting Flugge-type droplets by coughing. In the humid regions of Europe transmission may be facilitated by favourable winds over distances of few kilometres (Abdo *et al*., 2000). The fact that the disease may have a long incubation estimated in months, and that it may occur in a subacute or symptomless form in approximately 20 % of infected animals increases danger of spreading.

No therapeutic treatment is effective; however, farmers resort to heavy antimicrobial treatment in an attempt to reduce disease damage.

The pathological changes, generally characterised by the involvement of only one lung and its gross macroscopic aspects, are well known and may be used for disease surveillance at abattoirs.

However, the pathogenetic mechanism of the disease is not yet fully understood. It has been suggested that auto immune and hypersensitive reactions are essential in the development of lesions. The immunological mechanism involved during infection and ability of the pathogen to evade the immune system, must also be elucidated (Ayling *et al*.,1999a). This lack of knowledge has consequences for *in vitro* diagnostic tests, for assessing immune response and for developing an appropriate vaccine.

It is unclear how the different and often variably severe pathological lesions observed in disease affected cattle are produced; target animal experiments are very expensive, while a valid laboratory animal model is not available. It

is relevant to study the immunopathology of CBPP and to elucidate the pathogenic mechanisms of *Mmm*SC, by identifying the role of the micro-organism and its immunogenic components in eliciting pro-inflammatory and inflammatory reactions.

Cellular elements of inflammatory nature: macrophages, monocytes, lymphocytes, neutrophils that may liberate inflammatory products such as NO_2, myeloperoxidases or cytokines are to be identified (e.g. Tulasne et al., 1996; Nicholas et al., 2000). This approach can identify *Mmm*SC components causing a Th1 response that in all probability contributes to pathological lesions and, at the same time can identify the components causing a Th2 response expected to provide protective response. This approach requires innovative research pathways. These data should, moreover form the scientific basis to the development of a safe vaccine conferring long lasting immune protection.

CBPP can be classified as a re-emerging disease, representing a threat to the livestock industry within the European Community particularly in the context of the extension of the community borders towards the east and the south of Europe.

Little is known about the different immunogenic components of MmmSC upon CBPP infection. Several major proteins of MmmSC appear to be specifically recognised by antibodies from cattle with CBPP, and not by those from cattle infected with other members of the *M. mycoides* cluster. An immunodominant protein of 98 kDa is not common in all *MmmSC* strains, and was lacking from most strains detected in the Italian outbreaks between 1990 and 1993. In recent studies, a surface exposed lipoprotein of 72 kDa (named P 72) was identified and characterised; this lipoprotein was shown to induce an early and persistent humoral immune response in cattle with CBPP.

Some major immunogenic antigens have been identified by Western blot technique for different strains. Bronchial immune responses (IgA) were strong but appear to be directed to fewer antigens than those in serum samples and directed to antigens assumed to be surface exposed lipoproteins (Abdo et al., 1998). Lipoproteins from other mycoplasmas are suggested to play an important role in virulence (Kostyal et al., 1994; Rawadi et al., 1996). Recent studies indicated that non-adjuvanted and ISCOM incorporated *MmmSC* membranes induced pro-inflammatory cytokines (Abusugra et al., 2001).

The role of capsular galactan in inducing pathological lesions has been suggested and the biochemical relationship between capsular galactan and pneumogalactan has been suspected to be the cause of autoimmune reactions. Precipitation of immunocomplexes, observed around lung capillaries, seems to support the theory of autoimmune reactions as components of the pathology of CBPP.

The insidious and inconspicuous nature of CBPP, the variable individual susceptibility of cattle, the pathological changes generally characterised by the involvement of only one lung and its gross macroscopic aspects, the

difficulty to induce protective immunity by traditionally manufactured vaccines, are the problems to be faced in programming disease control.

The apparent but debatable difference in pathogenicity between African and European strains has also been associated with the ability of the former to oxidise glycerol and produce hydrogen peroxide (Nicholas et al., 2000).

3.4. The Disease

Direct contact is the principal mode of transmission. Alternative routes, however, like wind-borne and indirect transmission, cannot be excluded (Masiga *et al.,* 1996; Regalla *et al.,* 1996).

There is considerable variation in severity of signs observed in cattle affected by CBPP, ranging from hyperacute through acute to chronic and subclinical forms. Respiratory distress and coughing, evident on stimulation of resting animals, are the main signs of CBPP. The incubation period of the natural disease may range from 5 to 207 days although Turner & Campbell (1937) reported a range of 29-58 days, and Provost *et al.,* (1987) 20-40 days. In experimental bronchial intubation infections, Regalla *et al.* (1996b) reported disease signs appearing in cattle about 40 days after contact with intubated animals. Serological responses appeared also around 40 days after contact.

(a) Acute forms

The early stages of CBPP are indistinguishable from any severe pneumonia with pleurisy. Animals show dullness, anorexia, irregular rumination with moderate fever and may show signs of respiratory disease. Coughing is usually persistent and is slight or dry. Sometimes fever goes up to 40 – 42 $^{\circ}$C, and the animal prostrates with difficulty of movement. As the typical lung lesions develop, the signs become more pronounced with increased frequency of coughing and the animal becomes prostrate or stands with the back arched, head extended and elbows abducted. While classical respiratory signs may be evident in calves, articular localisation of the causative agent with attendant arthritis usually predominates. Complications in calves may include valvular endocarditis and myocarditis (Martel *et al.,* 1983).

(b) Hyperacute forms

The clinical signs observed in the hyperacute form are much accelerated. The pathological signs are usually characteristic with marked pleural adhesion accompanied by exudative pericarditis (Provost *et al.,* 1987). Affected animals may die within a week exhibiting classical respiratory signs.

(c) Subacute/chronic forms

In the subacute form, signs may be limited to a slight cough only noticeable when the animal is exercised. CBPP in Europe, unlike that caused in Africa where mortality rates are typically 10-70% in epizootics, is characterised by low morbidity and low or non-existant mortality with the majority of infected cattle showing chronic lesions. (Masiga *et al.,* 1996; Regalla, 1996b). These differences may be due to the fact that European cattle are

healthier in general, better fed, subjected to less physical stress, are often permanently housed throughout the year and probably experience strains of lower virulence than in Africa (Abdo *et al.,* 1998). In Italy, during the early 1990s, CBPP forms with mild or without clinical signs were frequently observed in cattle, with lesions of CBPP at slaughter. In infected herds mortality was around 2-3% (Guadagnini *et al.,* 1991). The use of antibiotics and anti-inflammatory drugs may help to mask clinical signs and to accelerate the formation of chronic lesions. In Africa, up to a third of cases that recover from acute disease become potential carriers. This figure is probably higher in Europe where there is far more widespread use of antimicrobials.

3.5. Outbreak definition

The report of the SVC in 1993 recommended that:

"In an area where the disease are unknown, a primary outbreak must be defined by a combination of the following diagnostic factors: a positive serological result, characteristic CBPP lesions, and the isolation and identification of *Mycoplasma mycoides* subsp. *mycoides* variant SC."

The isolation and identification of *Mmm*SC is essential for the confirmation of outbreaks, particularly in new areas. Culture techniques are also required by the OIE for countries wishing to declare freedom from CBPP under the recommended standards for epidemiological surveillance systems for the disease (OIE manual, 1997). The definitive identification of mycoplasmas is usually based on antigenic determination of isolated strains and on serological procedures, but specificity of immunoassays for CBPP is drastically impaired by complex cross-reactions with other mycoplasmas belonging to the "mycoides cluster" as given in Table 1 (Nicolet & Martel, 1996). The use of monoclonal instead of polyclonal antibodies offers a possibility to circumvent this problem.

3.6. Declaration of freedom from disease

The International Animal Health Code 2000 of the OIE (pp 403-412) describes the following requirements for declaration of freedom from CBPP:

- freedom from clinical CBPP for at least 2 years earlier;
- a programme of abattoir surveillance in place for at least 4 years;
- diagnostic procedures for CBPP in use to investigate respiratory diseases;
- surveillance programmes including serological, pathological and microbiological tests, for at least 3 years.

4. DISEASE SITUATION IN THE EU AND OTHER PARTS OF THE WORLD

CBPP is the only bacterial disease of the list A (OIE), widespread in Africa and is recognised to be present in some countries of Asia.

In Africa CBPP is found in an area south of the Sahara, from the Tropic of Cancer to the Tropic of Capricorn and from the Atlantic to the Indian Ocean. Endemic infection extends throughout the pastoral herds of much of western, central and eastern Africa, with Angola and northern Namibia in southern Africa. Newly-infected areas in the 1990s include much of Uganda, parts of Kenya, the Democratic Republic of Congo and most of Tanzania, where recently the disease has spread alarmingly; Rwanda (1994), Burundi (1997), Botswana (1995) and Zambia (1997) were recently reinfected. In Asia CBPP has been reported in recent times from India, Bangladesh and Myanmar.

CBPP largely disappeared from Europe around the end of the 19th century. However, it reappeared in Portugal and Spain in 1951 and 1957, respectively. Seropositive animals have been reported in southern France on a few occasions, between 1980 and 1984. In Italy the disease reappeared in 1990 but was eliminated in 1993 (FAO, 1997). The last case recorded in Spain was in 1994. In Portugal, after a period without cases being diagnosed, the disease reappeared in 1983; Following the implementation of an eradication programme the number of cases has declined rapidly in recent years, from 2818 in 1996, 64 in 1997, 12 in 1998 with a single case in 1999 (OIE, 2001). No cases have been reported in 2000.

5. DIAGNOSIS OF CBPP

5.1. Histology

The detection of specific lesions is an important factor in identifying cattle infected subclinically. The "organising centres" observed in the interlobular septa of lungs with lesions are considered pathognomonic for CBPP (Ferronha *et al.,* 1990; Di Francesco *et al.,* 1998).

5.2. Detection and Identification of *Mmm*SC

5.2.1. Culture

5.2.1.1. Sample collection for culture

From the living animal: nasal swabs and secretions, tracheal and broncho-alveolar washes and pleural fluid and occasionally blood, urine and synovial fluid should be obtained.

From the dead animal: pleural fluid, portions of affected lungs and lung sequestra (scrapings from inside the capsule) and lung-associated lymph nodes, and kidneys should be taken.

Tissues should be transported in insulated containers at temperatures between 0 and +4ºC, to reach the laboratory within 48 hours after collection. For a period of time over 48 hours before dispatching, keep the tissues frozen at –20ºC although some loss of viability of the agent occurs. Swabs should be sent in a suitable medium also refrigerated between 0 and +4ºC, to arrive at the laboratory within 48 hours after collection. These conditions are crucial and often overlooked in the success of mycoplasma isolation particularly in hot climate.

5.2.1.2.Isolation and transport media

Isolation media for *Mmm*SC also traditionally serve as transport media and are based on conventional growth media with the addition of inhibitors to stop the growth of cell-walled bacteria. The maximum concentrations of inhibitors, allowing normal growth of *Mmm*SC (i.e. having no effect on cell yield or growth rate) in PRM medium are: ampicillin, 1.0 gL^{-1}; bacitracin, 0.2 gL^{-1}; penicillin G, 0.05 gL^{-1}; polymyxin B, 0.05 gL^{-1}; and thallium acetate 0.5 gL^{-1}. These inhibitors may be combined to produce a highly selective medium.

5.2.1.3.Growth media

*Mmm*SC is not intrinsically difficult to grow, unlike many other fastidious mycoplasmas such as the one causing contagious caprine pleuropneumonia (CCPP) but requires a fully functioning bacteriological laboratory with access to special mycoplasma media. Many media have been described which enable the growth of *Mmm*SC (e.g., Hudson, 1971; Freundt, 1983; Nicholas and Baker, 1998). A medium (PRM, named after the developers) specifically formulated to give maximum growth rate and yield of SC strains, has recently been described by Nicholas *et al*. (2000) as a result of a recent EU-COST action. Pyruvate is included in the medium as an additional energy source to glucose and increases the growth rate.

5.2.2. Immunobinding

At present, immunobinding assays are the most reliable tests for routine identification of *Mycoplasma* species isolated from clinical material (Poumarat, 1998). All these assays are based on the detection of *Mycoplasma* surface antigens which are believed to be highly specific. However, sensitivity and specificity can be affected in certain circumstances. Since the number of *Mycoplasma* species is increasing, each isolate has to be tested with several sera for complete identification and immunobinding assays involving mycoplasma colonies or imprints of colonies are becoming highly laborious. Immunobinding assays with broth culture bound onto nitrocellulose paper can be affected by a high level of background staining.

5.2.2.1.Establishment of the Dot Immunobinding on Membrane Filtration (MF Dot)

Background staining problems can be reduced by using 0.22 micrometer membrane filter with low protein affinity (Poumarat, 1998). The use of special 96-well microplate whose well bottoms are made of a specific membrane filter offers further advantages over other tests, such as practicality, rapidity, ready standardisation and the possibility of treating many samples against several sera simultaneously. These plates allow the removal of well fluids by vacuum filtration. In this way, mycoplasmas are separated from broth media by trapping them on the filtration membranes and the broth proteins that do not bind to the membrane are easily removed. The membranes are then incubated with specific hyperimmune sera. The unbound immunoglobulins are removed by filtration as above and the bound

antibodies (Ig G) are detected by means of an enzyme-conjugated anti-Ig G (Poumarat, 1998).

5.2.2.2.Performance of Membrane Filtration (MF Dot)

The Dot Immunobinding on Membrane Filtration (MF Dot) is not very sensitive for CBPP. Sensitivity varies from 10^4 to 10^7 mycoplasmas/well depending on the quality of the hyperimmune serum used and mycoplasma species tested. To avoid false-negative reactions, only cultures in which growth turbidity can be visually detected should be used. The blocking of well filters occasionally occurs during the first step of vacuum filtration. A clear broth medium should always be used for M*ycoplasma* cultivation. If necessary, precipitate in the broth medium or cell remains from sample must be eliminated by filtration through a 0,80-micrometer filter before use. Vacuum depression should never exceed 40 to 60 kPa for more than 2 min, otherwise the filtration membrane may be distorted.

Interpretation must always include a reference strain as a positive control. A positive reaction toward two or more hyperimmune sera may occur with field isolates. In most cases, this does not result from technical problems, but from cross-reactions or species mixtures. Mixed cultures of M*ycoplasma* species frequently occur, especially in samples from respiratory tract. Definitive conclusions can only be drawn if immunobinding on these colonies occurs (Rosengarten & Yogev, 1996).

Shared antigens between some species can lead to cross-reactions. In classical cross-reactions between reference strains, the homologous reaction is usually stronger than the heterologous one. In this case, the use of specific monoclonal antibodies can greatly improve specificity.

The MF Dot is an easy and reliable test for the identification of mycoplasmas in general. However, the high rate of cross-reactions between mycoplasmas within the "*M. mycoides* cluster" (Poumarat *et al.*, 1992) can lead to difficulties for CBPP diagnosis.

5.2.3. Immunohistochemistry (IHC)

Immunohistochemistry (IHC) has proved to be a robust assay in the diagnosis of CBPP, particularly where the causative organism, *Mmm*SC, is not recoverable (eg following long transport distances), where the animal has died of acute disease or where serology cannot be performed or is inconclusive (Ferronha *et al.* 1990; Scanziani *et al.*, 1997). The sensitivity of IHC using polyclonal serum can be low and non-specific results occur frequently (Bashiruddin *et al.,* 1999b). Ayling *et al.* (1998) identified a monoclonal antibody, M92/20, for use in IHC confirmation of suspected CBPP cases. This monoclonal shows no background noise, but some cross-reactivity with other *Mycoplasma*s from the "*M. mycoides* cluster" is known; other monoclonals can be evaluated in this test. In an examination of 11 CBPP affected lungs from Portuguese cattle, IHC detected all, while PCR and culture detected 5 and 4 cases respectively. Though the sample size is small, this illustrated that IHC is a sensitive and robust test for CBPP**.** IHC is the test of choice when the diagnostic laboratory is presented with a

carcass in which lung lesions are suggestive of CBPP while serum is not available and mycoplasma culture from lung is unreliable. Samples are taken from lung lymph nodes or lung tissue with suspected macroscopic lesions, fresh or already formalin fixed, and embedded in paraffin wax.

5.2.4. Polymerase Chain Reaction (PCR)

PCR is a rapid and sensitive diagnostic method. It allows detection of *Mmm*SC directly in samples of lungs, bronchial lymph nodes, nasal swabs, pleural fluid and blood. Pre-incubation for 24 h of clinical specimens in growth medium may increase test sensitivity (De Santis *et al.,* 1997). If used for the identification of new isolates it reduces drastically the time required (24-48 h versus 2-3 weeks). The assay has been used as a diagnostic tool to detect *Mmm*SC in the course of CBPP outbreaks that have affected Europe in the recent past (Bashiruddin *et al.,* 1994a, b; 1999a, b).

Detection of the causative agent from bovine samples is one way to confirm a suspect CBPP case. However, isolation and serological or biochemical identification tests are time-consuming leading to significant delays. To overcome this problem, both single and nested PCR systems have been developed for identification of *Mmm*SC (Taylor *et al.,* 1992; Bashiruddin *et al.,* 1994a, b, 1999a, b; Dedieu *et al.,* 1994; Hotzel *et al.,* 1996; Miserez *et al.,* 1997).

Primers based on the sequence of the so-called CAP gene of *M. capri* amplify a 0.5 kb region of *Mmm*SC, *Mmm*LC and *M. capri* but do not amplify DNA from other members of the cluster. Identification is carried out by restriction fragment analysis of PCR product with *Asn*I (Bashiruddin *et al.,* 1994b).

Nested (two step) PCR has improved the sensitivity of detection. Both PCR reactions were highly specific to *Mmm*SC. Two nested pairs of oligonucleotide primers which were designed by evaluation of the most heterologous segments between the P72 gene of *Mmm*SC and the closely related lipoprotein P67 gene of *Mycoplasma* sp. bovine group 7 (PG50) were used (Miserez *et al.*, 1997).

Two PCR detection systems based on the 16S rRNA genes have also been developed for identification of *Mmm*SC with high sensitivity and specificity (Johansson *et al.,* 1998; Persson *et al.,* 1999). Both systems amplify the genes from all members of the *M. mycoides* cluster. In one of the systems, the final identification is performed by restriction enzyme analysis with *Alu*I. The other system (PCR-Laser Induced Fluorescence, PCR-LIF) is based on a sequence length difference between the two 16S rRNA genes of *Mmm*SC and the final identification is performed by fragment analysis of fluorescent labelled amplicons with a DNA sequencer.

Outbreaks of CBPP often require analysis of large numbers of specimens to detect the occurrence of *Mmm*SC. The traditional method for the analysis of PCR products is agarose gel electrophoresis which is useful if a small number of samples is involved.

Colorimetric PCR has overcome the problem: specific dsDNA produced by PCR is captured and detected enzymatically in a 96 well microtitre plate (Bashiruddin *et al.,* 1999a). The results of colorimetric detection of PCR products correlate well with those obtained by gel electrophoresis, which reduces the analysis time by 50 % and allows the use of standard equipment available for ELISA serology.

A comparatively large number of samples can also be handled by PCR-LIF (Persson *et al.,* 1999).

5.2.5. Other methods

Robust and rapid detection systems for the detection and identification of *Mmm*SC are urgently needed, particularly in Africa where PCR is not always practicable and large throughputs are required.

Rodriguez *et al.* (1996) reported a monoclonal antibody-based sandwich ELISA which could detect as little as 10^5 cfu/ml of both *M. mycoides* biotypes within two hours. Sensitivity could be improved significantly by incubating samples for 48 hours. This test does not distinguish between the SC and LC variants, but coupled with pathological and serological information from affected animals, the test could prove useful.

5.3. Detection and Quantification of Antibodies

5.3.1. Conventional tests

Antigenic cross-reactivity among *Mycoplasma* species, especially those of the "*M. mycoides* cluster", has been widely observed, raising practical problems for CBPP serodiagnosis. The first conventional test described is the complement fixation test, followed by the agglutination test and the passive haemagglutination test. The latter ones may be used as screening tests as they detect the IgM response in 2 weeks after infection, but they generally lack sensitivity and specificity (Regalla *et al.,* 1992; ter Laak, 1992).

5.3.1.1.Complement fixation test (CFT)

The CFT, the approved OIE test, although specific, lacks sensitivity. With a positive result being any reaction at 1/10 or higher, the CFT is also not robust. In addition, it requires highly trained staff to perform it accurately and consistently (Regalla, 1995).

In a thorough examination of the CFT in which over 33,000 sera from healthy herds were tested between 1991-1994 in Italy, Bellini *et al.* (1998) reported that the CFT was 98% specific while its sensitivity, based on nearly 600 cattle with specific lesions from 11 infected herds, was only 64%. Isolation of the causative mycoplasma from affected animals was even more insensitive: 54%. It follows that by using the CFT as a screening test, some CBPP affected cattle/herds, in the early or later stages of infection, are likely to be missed.

5.3.2. New tests (LAT; ELISAs; IBT)

5.3.2.1. Latex Agglutination Test (LAT)

A rapid latex agglutination test (LAT), which gives results in less than two minutes, using sera or whole blood, was developed for screening cattle in the field (Ayling *et al.,* 1999a). This test uses a "specific" polysaccharide antigen extracted from the *Mmm*SC capsule, which is then bound to latex beads ("specific"means: found to be specific by empirical means, testing it against different antigens until the one was found that did not cross-react or gave false-positive results). This test has been evaluated using CBPP negative sera from England and CBPP positive sera from Africa, Portugal and Italy. Sensitivity was comparable to the internationally recognised complement fixation test, but is far simpler and more rapid to perform. This test may have great potential in parts of Africa where there are great distances between the outbreaks, usually in nomadic herds, and diagnostic laboratories enabling control measures to be implemented rapidly.

5.3.2.2. Enzyme Linked Immunosorbent Assays (ELISAs)

Indirect ELISAs have been described for CBPP for nearly 20 years using crude antigens (Onoviran and Taylor-Robinson 1979; Le Goff 1986; Poumarat *et al.,* 1989; Nicholas *et al.,* 1996). All appear to be sensitive but may lack specificity.

A blocking ELISA, with a monoclonal antibody which although raised against *Mmm*SC nonetheless recognised all members of the cluster, was compared with the CFT for detecting antibodies in cattle during the Italian CBPP eradication campaign (Mia *et al.,* 1993). There was 96% agreement between the tests on over 300 positive and negative sera. Nevertheless, a small number of sera from apparently healthy herds were positive in the CFT but gave negative results in the ELISA and in both pathological and cultural examinations.

Brocchi *et al.,* (1993) reported monoclonal antibodies which recognized a 70kDa protein from the PG1 strain of *Mmm*SC but did not react with the type strains of other members of the *M. mycoides* cluster. Competitive (c)ELISAs using the monoclonal antibodies were more sensitive than the CFT for detecting antibodies in affected herds and were generally more specific when used in negative herds.

A (c)ELISA has been developed (Le Goff and Thiaucourt, 1998) and validation tests have been performed in Africa (Thiaucourt *et al.,* 1999). This test uses a mouse monoclonal antibody IgG_1 that recognises an epitope localised on a protein with an apparent molecular weight of 80 kDa. Availability of the test seems to be a problem. While this test has considerable advantages in terms of ease of testing and standardisation of results, the cELISA has sensitivity levels similar to the CFT. Clearly more sensitive techniques, such as ELISA using better antigens, enabling mass screening, are necessary backed by highly specific confirmatory tests such as immunoblotting tests.

A Tween 20 treated *Mmm*SC cell fraction shows increased specificity in the indirect ELISA compared to conventional whole cell antigens whilst maintaining sensitivity (Ayling *et al.*, 1999b). In addition, it was shown that incubation times could be significantly reduced allowing a quicker generation of results without affecting the quality of the information. The Tween 20 ELISA was compared to the CFT in an examination of 170 sera from Portuguese herds affected by CBPP. There was 88% agreement between the ELISA and IBT; 81% agreement between the CFT and ELISA; and 79% between the CFT and immunoblotting test (IBT). Furthermore in a study with 88 cattle with CBPP lesions, the ELISA detected antibodies in 92%, IBT detected 91% and CFT only 82% (Ayling *et al.*, 1999b).

Research on the development of tests using antigens that make them more sensitive is clearly needed as small but significant numbers of infected cattle remain seronegative in most tests: about 8% of field sera from CBPP affected cattle are negative, even when using the most sensitive tests like IBT and ELISAs.

It is clear that ELISAs, which are more sensitive in most cases than the CFT, are performed in some European laboratories but doubts about their specificity remain. However as CBPP in Europe is absent or rare, tests of high sensitivity are needed to prevent its re-establishment. Any positive samples can now be confirmed by the immunoblotting tests. A ring trial of available tests is necessary to ensure the selection of the appropriate systems which maintain high sensitivity with acceptable levels of specificity.

5.3.2.3. Immunoblotting test (IBT)

Western (immuno) blotting analysis has been applied with success to the assessment of antibody response in several diseases. This method allows the analysis of the host humoral immune response in relation to the electrophoretic profile of *Mmm*SC antigens, thus overcoming problems related to non-specific binding in other immunoassays.

The study of the immunogenicity of *Mmm*SC led to the development of an immunoblotting test for the detection of the antibody response in sera from cattle infected with CBPP. A common immunological pattern consisting of a combination of 5 immunodominant antigens with apparent molecular weights of 110 (p110), 98 (p98), 95 (p95), 62/60 (p62/60) and 48 (p48) kDa was defined in a preliminary study with sera from both experimentally and naturally infected cattle (Regalla *et al.,* 1996b). The presence of the core profile only in sera from CBPP positive animals confirmed the immunodominance of these five proteins and indicated the longevity of the specific response which was present in these animals regardless of the stage of the disease.

To assess the antigenic homogeneity of that core of 5 bands, 46 isolates of *Mmm*SC from European countries (Portugal [n=24], Spain [n=6], France [n=4] and Italy [n=12]) were studied. The study was also extended to 10 strains from Africa, 3 strains from Australia and to the prototype strain PG1 (Gonçalves *et al.,* 1998). A distinct antigenic difference occurring at the level of 98 kDa was seen. This was constant for all Italian strains with all

sera tested, except in two strains. A Portuguese clone had a similar profile to Italian strains, none exhibiting the 98 kDa. Therefore, the results indicated that the European strains are antigenically different suggesting that the Italian strains are a variant clone with the same geographic origin. In this study it was also verified that the African and Australian strains are different from European strains, but at 71.5 kDa level.

A field evaluation was undertaken for IBT validation as a diagnostic test (Regalla *et al.,* 1999). From 419 cattle tested and originating from herds where *Mmm*SC was isolated, 281 cattle were considered infected, because they were shown to be positive in at least one of the four tests performed: IBT, CFT, bacteriological or histopathological examinations. The distribution of the IBT and CFT test results from the infected animals was the following: 260 serum samples from the above 281 animals were positive in the IBT; 216 serum samples from the 280 animals were positive in the CFT. The relative sensitivities with the respective confidence intervals at 95% were calculated for IBT and CFT. Based on these results the calculated sensitivities were: 92.5% (89.5% to 95.6%) for the IBT and 77.5% (72.2% to 82.1%) for the CFT. The higher sensitivity of the IBT allowed the identification of false-negative cattle. The field evaluation also indicated that this test is important for use in conjunction with CFT or a more sensitive test.

For specificity evaluation, a total of 493 sera from disease free animals coming from regions or countries free from CBPP (including 177 sera with false-positive reactions in CFT), were assayed in IBT. No serum tested showed any positive reaction. As all the sera were negative, the specificity of the test was 100% (Regalla *et al*.1999).

Serial testing, has been carried out since 1998, within the framework of Portuguese eradication programmes. During the last 3 years, there were 2511331 sera (from 429568 herds) from all over the country tested in CFT, from which 12466 (0.5%) were CFT-positive. From these CFT-positive sera 11.715 were tested in IBT, from which only 64 (0.6%) identified the core IgG immunoblot profile of the 5 specific antigenic bands: p110, p98, p95, p62/60 and p48. The total of 64 specific results corresponded to 39 herds, all of them located in the infected areas. However, with the exception of the last outbreak that occurred in February 1999, no *Mmm*SC isolates were obtained from bacteriological examinations performed with the clinical material originating from the slaughtered cattle. All CFT-positive sera originating from disease-free areas gave non-specific profiles in the IBT (see Table 2). During the first 5 months of 2001, no positive results were obtained from a total of 2169 IBT tests, of which 2150 originated from areas identified as being infected.

Therefore, the IBT can be applied to herds of low disease prevalence where both false-negative and false-positive results are often found. The IBT is capable of discriminating false-positive responders which is extremely useful in the final stages of eradication as well as during serological surveillance.

The IBT test protocol is described briefly in Annex I.

The IBT is highly specific and the most sensitive serological test so far described for the detection of cattle affected by CBPP; however, it is not suitable for mass screening.

Table 2. Results of CFT and IBT serial testing performed between 1998 and 2000, within the frameworks of Portuguese eradication programmes (Direcçaõ General de Veterinaria – Ministerio da Agricultura e Pescas, Lisbon, Portugal)

	INFECTED AREAS				FREE AREAS			
SERA	**1998**	**1999**	**2000**	**Total**	**1998**	**1999**	**2000**	**Total**
Total # of samples tested in CFT[1]	**887093**	**863300**	**557229**	**2307622**	**60546**	**79029**	**64134**	**203709**
Total # of CFT positives [2]	**3900 (0.4%)**	**4573 (0.5%)**	**3459 (0.6%)**	**11932 (0.5%)**	**36 (0.6%)**	**344 (0.4%)**	**154 (0.2%)**	**534 (0.3%)**
Total # of samples tested in IBT[3]	**3635**	**4311**	**3316**	**11262**	**26**	**279**	**148**	**453**
Total# of IBT positives[4]	**41 (1.1%)**	**11 (0.3%)**	**12 (0.4%)**	**64 (0.6%)**	**0**	**0**	**0**	**0**
e[5].	**0.005%/CFt**	**0.001%/CFt**	**0.002%/CFt**	**0.003%/CFt**				

CFt – Total number (#) of CFT tested

[1] Total number of samples first tested in CFT
[2] Total number of CFT-positives test results out of (a).
[3] Total number of samples from (b) tested in CBT.
[4] Total number of CBT-positives test results out of (c).
[5] Percentage of IBT positives out of all CFT tested samples (a).

6. STANDARD REFERENCE SERA

The report of the SVC in 1993 recommended that each batch of antigen used, should be titrated using standard sera and selected two sera, sera 840 (low titre) and 845 (high titre). These were obtained from naturally infected cattle in Portugal and prepared by the Laboratorio Nacional de Investigaçao Veterinaria (Lisbon, Portugal), for antigen titration for CFT and CFT harmonisation purposes.

Reference serum 845 is now practically exhausted. However, both sera are necessary for current use in CFT. A third serum (high titre) has been developed in Portugal, but needs to be validated in a ring test among European laboratories.

Selection of sera with the aim of preparation CFT positive standards should be careful and validation should be done within a group constituted by laboratory representatives with high levels of experience in CBPP diagnosis.

7. APPLICATION OF DIAGNOSTIC TESTS

7.1. Test evaluation

Diagnostic tests are commonly used for detection of a disease, for declaring a herd or region to be free from a certain disease, for screening programmes or for eradication programmes. Diagnostic tests are imperfect, the outcomes are not always correct. One should realise that the classification of animals into diseased and non-diseased is also imperfect, resulting in false-positive and false-negative test results (Noordhuizen *et al.,* 1997). Test evaluation is therefore highly important.

The validity of a diagnostic test is represented by characteristics like sensitivity and specificity, and parameters like predictive value positive and negative[6].

Sensitivity and specificity depend on chosen cut-off values; the choice of the cut-off value depends on the purpose of the testing. For example, in case of zoonotic or highly contagious diseases a diagnostic test with high sensitivity is warranted. Sensitivity and specificity are more or less fixed (test feature), while predictive value may alter with the prevalence figures (population feature).

A 2x2 Table (table 3) to calculate sensitivity and specificity of a diagnostic test is reported below.

[6]The public domain software programme (WIN)EPISCOPE for calculating different epidemiological parameters as named above can be downloaded from http://www.zod.wau.nl//qve (Frankena *et al.,* 1990; Thrusfield *et al.,* 2001)

Table 3. Method for calculation of sensitivity, specificity and predictive values of a diagnostic test.

	Reference test ("golden standard")		Totals
Test result	Diseased	Non-diseased	
Diseased	a	b	a+b
Non-diseased	c	d	c+d
Totals	a+c	b+d	N

Sensitivity (%) = a x 100 / (a+c)

Specificity (%) = d x100 / (b+d)

Predictive value (%) of negative result = d x 100/(c+d)

Predictive value (%) of positive result = a x 100/(a+b)

(a) A diagnostic test with high sensitivity is indicated:

- In early phases of the infection;

- When truly diseased animals should not escape testing (no false-negatives desired, e.g. epizootic zoonoses);

- When disease probability is low;

(b) A diagnostic test with high specificity is indicated:

- For confirming a diagnosis which was set earlier; and

- If false-positives would have a large effect (e.g. when test positive animals would be killed).

- If a disease has a low prevalence, the predictive value positive depends largely on the test specificity. Alternative for the parameter "predictive value" is the likelihood ratio, which is not dependent from prevalence (Noordhuizen *et al.,* 1997).

The level of agreement between diagnostic tests can be assessed by calculating the kappa value (see also Noordhuizen *et al.,* 1997).

Parallel testing, where different diagnostic tests are conducted at the same time, is meant to increase sensitivity and predictive value negative. The main target of parallel testing is to rule out a disease.

This procedure is indicated when rapid assessment of the animal health status is necessary, e.g. in case of emergencies and clinical patients. It is also useful for situations where a heavy penalty may be incurred for failing to detect a specific disease (that is in cases where false-negative test results occur).

Serial testing, that is when diagnostic test are conducted sequentially, is meant to increase test specificity and predictive value positive. The main target of serial testing is to correctly identify a disease. This procedure can be indicated for eradication programmes like “test and slaughter”. The element “time” is not as crucial as it is in parallel testing.

7.2. Screening of herds and eradication

Currently available ELISAs in general do not show optimal sensitivity and/or specificity. ELISAs and CFT are both herd tests and not particularly suitable for diagnosis in individual animals. The problems for both tests relate to the standardisation of the antigen, the determination of the cut-off point, and the use of the tests in large populations.

The choice of a test does not only depend on test-specific features but also on aspects like costs, practicality, ease of handling, automation, etc.

As CBPP is absent or rare in EU member states, tests with high sensitivity are needed to prevent its re-establishment.

Positive samples can now be confirmed by the immunoblotting test IBT. PCR has the potential to be used as a routine diagnostic tool for CBPP on different sets of specimens (e.g. lung tissue, lymph nodes, nasal swabs, pleural fluid, blood).

PCR and IHC are tests of choice when the diagnostic laboratory is presented with a carcass in which lung lesions are suggestive of CBPP, as serum will not be available and mycoplasma culture from lung tissue is unreliable.

Abattoir surveillance, searching for lung lesions suggestive of CBPP, is another option. During the Italian outbreaks, abattoir surveillance detected nearly as many outbreaks as serological monitoring, while clinical examination was much less useful (Guadagnini *et al.,* 1991).

8. CONCLUSIONS

- Detection and identification of *Mmm*SC by diagnostic tests cause –in general— problems because of the variation in sensitivity and specificity between the different tests, partly induced by cross-reactivity of mycoplasma variants, features of infection and pathogenicity, and various infection-masking phenomena.

- There is a need for a sensitive screening test such as ELISA to be applied for CBPP diagnosis. However, both standardised antigens and reference sera need urgently to be developed. The CFT European standard reference serum 845 (high titre) is nearly exhausted.

- Although a comparative study of tests among diagnostic laboratories was done in the early 1990s, no proficiency testing needed for test evaluation has been performed in the EU. It is important that a EU reference laboratory under a formal quality assurance system is appointed, to take the lead and co-ordination of diagnostic test development, testing and test evaluation, in order to be adequately prepared for outbreaks of CBPP in the near future, especially in the Mediterranean region.

- For diagnosing CBPP, the CFT and ELISAs can be used in mass screening and eradication programmes but due to their low sensitivity and/or specificity, a confirmatory test is needed. At present the IBT is the most promising for confirmation.

- The IBT is the most sensitive serological test so far described for CBPP, thus leading to an increased detection of infected but clinically silent animals. Therefore it is an ideal test for the assessment of the true (low) prevalence of CBPP in a country. However, IBT is too laborious for mass-screening.

- There is a need for further research on CBPP, especially with regard to the establishment of infection (pathogenicity factors, immunopathology, virulence factors, genomics) and the persistence of infection in chronically affected animals (e.g. reservoirs). The search for new diagnostic tests with high sensitivity and high specificity should be continued.

9. RECOMMENDATIONS

- Standardisation aspects of detection limits and technical procedures

Development of standardised reference antigens and reference sera from different sources for the available diagnostic tests such as CFT, ELISAs and IBT is urgently needed. Because the CFT European standard reference serum 845 (high titre) is now practically exhausted, it urgently needs renewal or replacement. .

A EU-reference laboratory should be appointed to take the lead in development and co-ordination of test evaluations.

- Test comparison and validation

A large panel of new bovine sera should be tested comparatively in different test systems among EU laboratories, in order to evaluate and validate these tests for their different purposes, i.e. detection, screening, eradication. Sensitivity and specificity are major issues.

- Test application

For CBPP serological diagnosis the following tests should be applied for screening or eradication purposes: CFT and ELISAs. Since these two tests show limitations with regard to sensitivity or specificity, the IBT should be evaluated as confirmatory test.

When detection and identification is needed (e.g. in case of primary outbreak), the conventional bacteriological examinations (culture, isolation and identification) and the MF DOT and PCR should be applied.

If serum samples are not available and when culturing from lung tissue would be unreliable, then IHC could be the test of choice (e.g. in individual carcasses).

- Future research

In addition to the need to develop reference antigens and sera, further research is required in the area of the CBPP pathogenesis and the immunopathology of infection, in order to provide the rational basis for applying a diagnostic test and to implement control measures. The mechanisms involved in the invading process after the first contact of mycoplasmas with host cells are unknown. In depth study of immunopathology will not only facilitate the development of new diagnostic tests, but at the same time will yield useful information for vaccine development. Factors responsible for chronicity of the infection or in the so far serologically undetected phase of the chronic disease can be attributed to a variability in surface antigens which interact with the host during the course of infection. Strains from different geographical regions may be distinguished by a 71.5 kDa protein present in both African and Australian isolates and absent in European isolates. Other pathogenicity features, possibly related to biochemical characteristics of certain strains (e.g. glycerol oxidation) could be detected by this type of research and through genomics research. The latter may also be helpful in developing new diagnostic tests, such as for milk.

10. ANNEX I: SHORT DESCRIPTION OF THE IMMUNOBLOTTING TEST, THE IBT PROTOCOL

(Goncalves *et al.,* 1998; Laemmli, 1970; Lowry *et al.,* 1951; Towbin *et al.,* 1979)

10.1. Strain, growth conditions and antigen preparation

A log phase culture of an antigenically characterised *Mmm*SC Portuguese isolate B103 or an other antigenically well characterized *Mmm*SC strain, growing in modified Gourlay-medium at 37°C, is harvested by centrifugation at 14.600 g for 1 hour at 4°C, followed by three washes in phosphate buffered saline solution (PBS, 0.1M Na_2HPO_4, 0.1M NaH_2PO_4, 0.15M NaCl, pH 7.2). Washed cells are resuspended in the same buffer and stored at –20°C (Goncalves *et al.,* 1998)

10.2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

Estimate the protein content of thawed mycoplasma cells by the method of Lowry et al. (1951). Mix the samples with lysis buffer (500 mM Tris/HCl pH 6.8; 4.6% (w/v) SDS; 20% (v/v) glycerol; 10% (v/v) 2-mercaptoethanol and 0.004% bromophenol blue) and boil for 5 min. Separate equal amounts of lysates (50 µg of protein per each 10 mm of well) by SDS-PAGE in a 1.5 mm thick, 4% stacking/5-15% gradient resolving gel and the electrophoresis is run at a constant current of 40 mA (Laemmli, 1970).

Transfer the separated proteins to 0.45 µm nitrocellulose membranes (NC) at 70 V constant voltage in transfer buffer (20% methanol in 192 mM glycine, 25 mM Tris-HCL, ph 8.3) for 1.5 hour (Towbin *et al.,* 1979).

10.3. Preparation of antigen strips

Dry the membrane, labelling the side on which the proteins were western-blotted. Incubate the NC membrane in blocking buffer (PBS containing 5% skim-milk, 1M glycine and 1% egg albumin) for 2 h at room temperature (22 +- 2^0C). After washing at room temperature for three times 15 min. washes in 0.1% (v/v) Tween 20 in PBS and again for 10 min in PBS alone. Dry the NC sheet and cut strips with 3 mm wide serial labelling each strip. These strips constitute the antigen used for immunoblotting and should be kept at – 20 ^{0}C until used.

10.4. Quality control of antigen strips.

After performing the Western blot, cut and stain with Indian ink, one strip with 20 mm wide from each side of the membrane, to verify the entire range of the transferred cellular proteins and molecular weight standards.

After membrane blocking (see above, point 10.3, lines 26 and 27), test against the positive control serum, one strip from each edge of the

membrane, to identify the specific bands of 110, 98, 95, 62/60 and 48 kDa (see also the Figure below).

10.5. Test procedure

Serum samples to be tested are not heat treated. The strips must be kept with the antigen side up during the procedure.

(1) Prepare test serum samples diluted to 1:3 and positive control sera and negative control sera in dilution buffer (PBS containing 0.1% skim milk and 0.1% egg albumin).

(2) Incubate the antigen strips in the mentioned test samples (controls included) at 37ºC for 2 h with continuous agitation. Washing as above.

(3) Incubate for 1 h at room temperature (see above) with appropriate dilution of peroxidase-conjugated anti-bovine-IgG (H+L chains) in dilution buffer with continuous agitation.

(4) After washing as above, the substrate comprising 30 mg 4-chloro-1-naphtol dissolved in 10 ml methanol jointed to 50 ml PBS and 30 µl H_2 O_2 was added and left in the dark with continuous agitation until the protein bands are suitably dark. Stop the reaction with distilled water.

(5) Reading the results: After drying the strips the reading is carried out. A positive result is based on the presence of the core IgG immunoblot profile of five specific antigenic bands of 110, 98, 95, 62/60 and 48 kDa. Sera giving a similar immunological profile are considered positive. No common patterns with the forenamed five bands are seen in negative results. Due to the variability of bands that could be found in negative sera, it is not possible to give values of intermediate results.

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12. ACKNOWLEDGEMENTS

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