Contagious bovine pleuropneumonia *

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Summary: Contagious bovine pleuropneumonia (CBPP), which only affects species of the genus *Bos* (cattle and zebu), is caused by *Mycoplasma mycoides* subsp. *mycoides* (SC).

The originality of CBPP and its importance in the history of medicine, biology and veterinary medicine are highlighted in Chapter I of this review article.

Chapter II presents the clinical and lesional aspects. The usual form of the disease is acute while the hyperacute form is less frequent; the subacute and chronic forms are common. The essential lesions, located in the lungs and pleura, are almost invariably unilateral. Hypertrophy of the bronchial lymph nodes is a constant feature of the disease. Characteristic microscopic lesions accompany each stage of its development.

Chapter III deals with epidemiology. Once widespread, CBPP has been reduced considerably and even eradicated in many countries. It still occurs sporadically or in enzootic form in a large part of Africa as well as certain Asian countries. The virulence of the pathogenic agent, its resistance to physical and chemical conditions, the modes of transmission, receptivity and factors favouring infection are also discussed.

The diagnosis of CBPP is the subject of Chapter IV. After a short presentation of clinical, post-mortem and differential approaches, experimental diagnosis is discussed in detail. There are two types of procedure here: isolation followed by identification of the pathogen by examining its biochemical properties, with serological confirmation by the growth-inhibition and/or immunofluorescence tests; and tests for circulating antibodies in serum, or circulating antigen (galactan) in serum, pleuropneumonic lymph or organ homogenate. Formulae for appropriate media are included in an Appendix.

Finally, in Chapter V, different methods of prophylaxis are described. Under European conditions, only the disease control procedure of eradication is applicable. In Africa, control measures must include annual vaccination of cattle. The author describes types of vaccine available and the practice of vaccination against pleuropneumonia. In Australia, CBPP has been eradicated by combining disease control and vaccination procedures.

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I. HISTORY AND IMPORTANCE

Contagious bovine pleuropneumonia (CBPP) is a disease remarkable for its originality and complex paradoxes, and also for a history marked by the slow evolution of modern veterinary medicine and microbiology.

HISTORICAL IMPORTANCE FOR MEDICINE AND BIOLOGY

The rise of the concept of contagion

If the writings of Claudius Aelianus and Cassius Dionyssus of Utica are to be believed, the disease was already known to the ancient world. Until the middle of the 16th century, CBPP remained confined to the western Alpine area (Bavaria, Württemberg, Switzerland, Northern Italy and Tyrol). It was the subject of one of the first works to be written on animal disease control. Published in Bern in 1773, this work by Albert de Haller recommends mass slaughter of affected and suspect animals as the best way of controlling the disease.

In an account of "diseases of the chest", Bourgelat (1765) distinguished pleuropneumonia from other "putrid fevers" of the lungs, and described the unique aspect of exudative pleuropneumonia lesions. The contagiousness of CBPP was suspected by Chabert in 1792, and a lengthy controversy followed between the proponents of spontaneity (who founded their arguments on experiments in which healthy cattle placed in contact with affected cattle failed to develop the disease, for the concept of immunity following infection was as yet unknown), and those of contagion, supported by a number of French practitioners. The latter triumphed towards the middle of the 19th century, well before the Pasteur era, thanks to the keen observations of Delafond (a Normandy veterinarian) and Yvart, and above all to the work of a French Ministerial Committee created in 1850 under the direction of H. Bouley. The dogma of spontaneity foundered, and that of contagion was firmly established, along with the concept of immunity following both severe and indistinct forms of illness.

Without knowledge of an aetiological agent, scientific reasoning had laid the foundations for the study of contagious diseases, with which CBPP was intimately associated.

The first attempt to vaccinate in veterinary medicine

During the same period (1852), a procedure for vaccination, then referred to as inoculation, was discovered. With some minor modifications, it has survived to this day. A Dutch doctor, Louis Willems of Hasselt, was responsible for the discovery of the second procedure for preventing a contagious disease, the first being Jenner's
treatment of smallpox. Willems's efforts were directed not so much towards vaccination as at the reproduction of the disease in different animal species (including cattle) by using fluid ('lymph') from pleuritic exudate. His observations demonstrated the strict specificity of the disease for cattle. The uniqueness of inflammatory lesions of connective tissue and muscle prompted him to inoculate the 'lymph' subcutaneously into the trunk or the limbs, giving rise to a voluminous oedematous reaction ('Willems's reaction') without causing the disease. This reaction was fatal, and so the inoculation sites were described as 'forbidden regions'. His most important observation was that the oedema was absent, or less pronounced, when inoculation was made into the tail tip, or into other dense connective tissue, such as the bridge of the nose. Such inoculation protected the animals when they were later placed in contact with infected cattle. This relatively safe 'preventive inoculation' led the Bouley Committee to repeat the experiments, confirming the results in 1854. It is one of the paradoxes of CBPP that scientific control became possible even before the causal agent was identified.

**First culture of a ‘filtrable virus’**

This was not the only unusual feature. From the start the Pasteur era witnessed a flourishing of research on the disease, and descriptions of numerous micro-organisms which were supposed to be responsible. One of the best studied was *Pneumobacillus liquefaciens bovis* of Arloing (between 1885 and 1896), and there were lively arguments between this professor of Lyons and Nocard, who challenged the notion of its specificity. Nocard would later culture the aetiological agent in 1898.

Nocard and Roux, with Borrel, Salimbeni and Dujardin-Beaumetz, adapted the technique which two of them had developed with Metchnikoff for preparing cholera toxin, and produced cultures of CBPP serous fluid within collodion sacs implanted into animals. These sacs, filled with broth medium containing a trace of serous fluid, then sealed carefully and inserted within the peritoneal cavity of a rabbit, were found after 15-20 days to contain a fluid which was opalescent, slightly cloudy and albuminous. The fluid contained neither cells nor bacteria which could be cultured on the usual media. Microscopic examination under the highest magnification (× 2000) revealed a great number of small, refringent and mobile bodies so fine that it was hard to determine their shape, even after staining. These changes were definitely due to the growth of the micro-organism, because the contents of a second sac filled only with broth medium, inserted into the peritoneal cavity of the same rabbit, remained transparent and clear. These workers were able to passage cultures within collodion sacs from rabbit to rabbit.

No microbial development took place when the cloudy serous fluid was added to ordinary culture media incubated *in vitro*. However, the CBPP organism could be cultured readily by inoculating Martin’s peptone broth containing cow or rabbit serum.

These workers stated that the discovery of the CBPP agent was important not only because of difficulties overcome, but also because it offered a means for studying other agents, about which little was known at that time.

In 1900 Dujardin-Beaumetz succeeded in culturing the agent on agar, and observed minute colonies looking like fried eggs.
Study of the morphology of the agent continued. In 1910 Borrel, Dujardin-Beaumetz, Jeanet and Jouan examined it in the live state, also after staining by Giemsa's method, by ultra-microscopy, and by overstaining after application of a mordant. These procedures disclosed other, highly polymorphic forms, some of which were enclosed within a mucoid matrix. The authors proposed the name *Asterococcus mycoides* for the CBPP agent in order to embody its principal characteristics of a mucoid sheath, pseudo-mycelial filaments and multiple polarity.

Thus the technique of overstaining made it possible to describe the various aspects of a filtrable micro-organism which, before the work of Bordet and their own findings, had been considered to be invisible.

Although this was a major development, the concept of filtrable viruses which could be cultured *in vitro* set microbiologists onto a false trail for the next 20 years. The agent of contagious agalactia was placed in this category by Bridre and Donatien, who discovered and cultured it in 1925. Even in 1927 Orskov still described the agent as ‘pleuropneumonia virus’.

The confusion increased when several bacteriologists (Frosch, Nowak, Wroblewski) interpreted the kinetic behaviour of cultures of these agents as resembling the fungi imperfecti. Thus Nowak gave the CBPP agent the name it has today, *Mycoplasma mycoides*, because of its fungal morphology.

**Extension of the new group**

The number of micro-organisms of this type continued to increase. In 1934 Shoetensack described CBPP-like organisms in Japan, obtained from secretions and tissues of dogs with distemper. In 1936 Laidlaw and Elford isolated two apparently saprophytic strains from sewage filtrate. These strains did not require serum in the culture medium. In 1937 Seifert found similar organisms in soil and decomposing matter.

Between 1937 and 1940 Lieneberger and Findlay, Sabin, and Edward used rats and mice to isolate a number of these micro-organisms which they began to call, with Sabin, pleuropneumonia-like organisms or PPLO.

Not until the 1960's did the pioneering work of Edward, Freundt and others characterise the order *Mycoplasmatales*, derived from the name *Mycoplasma*, which was a taxonomic error made by Nowak.

**IMPORTANCE IN THE HISTORY OF VETERINARY MEDICINE**

Some important dates in the history of CBPP are given below:

1550: clinical description by Gallo in Italy under the name ‘polmonera’
1554: clinical description in France by Charles Testienne in his “Traité d’agriculture” (Treatise on Agriculture)
1732: clinical description by Jacob Schenchzer in Switzerland
1765: detailed clinical description by Bourgelat
1773: regulations for control by slaughter, signed by Albert de Haller of Bern
1792: Chabert’s instructions concerning pleuropneumonia, which affirm its contagiousness.
At the beginning of the 19th century, convoys of cattle following the Napoleonic armies spread the disease in Western Europe, first in Flanders and then in Holland.

England was infected from Holland in 1840, with 200,000 deaths in 1860 and one million six years later.

The United States became infected in 1843, but the disease disappeared in 1892. Spain became infected in 1846 and Scandinavia in 1847.

The disease has never occurred in South America. A brief episode occurred in Guadeloupe towards the middle of the century, following imports of cattle from Senegal.

In 1854 South Africa became infected through a bull imported from Holland. In 1858 cattle from Britain infected Australia and then Asia (Shanghai in 1919, Hong Kong in 1920). Japan became infected in 1924 and the disease did not disappear until 1932.

As far as the African continent (excluding South Africa) is concerned, North Africa seems to have escaped the disease. It has occurred in Central and Western Africa from time immemorial, evolving at present along with vaccination procedures and other factors. The same applies to East Africa.

Economic losses in livestock farming were considerable during the 18th century. Epidemics became less frequent from 1890 as a result of strict application of control measures (prohibition of movement and slaughtering).

Major campaigns resulted in Britain eradicating the disease in 1898, USA in 1892, South Africa in 1916 and Japan in 1932. By the end of the 19th century the disease had disappeared from most of Europe, with the exception of Spain, Poland and Russia.

Infection broke out again in Germany and Austria in 1916, and it persisted until 1923. The USSR became free from the disease in 1935, and Poland in 1936.

The geographical distribution of CBPP in 1987 may be summarised as follows:

1. The disease was eradicated from Australia in 1973, following a large-scale campaign which commenced in 1960.

2. In Europe, a few outbreaks were declared in France, Spain and Portugal between 1980 and 1984.

3. There is little information concerning Asia. It seems that CBPP is still present in India (Assam), as well as in Kuwait and Bahrain. The last two countries became infected recently following importation of beef cattle from Africa.

4. In Africa the remaining major foci of infection tend to be located in the dry tropics (particularly north of the Equator), and many countries are affected.

Vaccination has ensured that major epizootics do not last long, but CBPP is still endemic, and remains more or less concealed.

African countries bordering the Mediterranean and those situated on the Equator are free from the disease, as are also Zambia, Malawi, Zimbabwe, Swaziland, Mozambique, Botswana, South Africa and Madagascar.
II. CLINICAL PICTURE AND LESIONS

CLINICAL PICTURE

The incubation period is poorly defined. In healthy cattle placed in contact with infected cattle, it ranges from 20 to 123 days (20-30 days in 46% of animals, 31-40 days in 23%, 41-60 days in 11%, and over 2 months for the remainder).

Its usual form is acute. The hyperacute form is rare, but subacute and slow forms are common.

Chronic infections associated with sequestered lung lesions are a special problem in CBPP, and their detection is a major obstacle to disease control.

Acute form

a) The period of invasion corresponds to the congestive phase, and lasts for no more than 5 days. There are the usual general signs of an infectious disease — dullness, anorexia, irregular rumination, fall in milk yield. High fever does not always occur, and the intensity of fever varies to the same extent as the rapidity of the course of the disease; in most cases it does not exceed 40°C.

Respiratory signs which attract the attention of the clinician are still slight, but significant:

- polypnea (up to 30 inspirations a minute)
- stiff posture and arched back
- lowing when the animal rises, or is jostled by others, and during percussion of the chest wall
- under the same circumstances, cough may also be present, but it is infrequent, slight, dry and fitful, without being repeated.

b) The clinical stage corresponds to the stage of hepatisation in the lungs, which soon leads on to severe pleurisy.

The general signs become more pronounced and the hyperthermia reaches a plateau at 40-42°C. Rumination is almost completely suspended and anorexia is practically complete; the animal is prostrate and any movement is painful.

Moaning upon expiration is more pronounced, and is heard during standing up and sometimes at even the slightest movement. An orthopneic posture is often adopted, because it is a considerable effort to breathe.

Signs of pleuropneumonia soon become evident:

- short and rapid breathing (50-55 movements a minute)
- appearance of interrupted or discordant breathing
- a moist cough accompanied by a little foamy sputum
- pronounced chest pain, sometimes restricted to certain regions of the ribs
- extensive zone of dullness upon percussion, irregular in outline (lobar hepatisation) or partly sloping and at the upper horizontal level (formation of pleural transudate)
at a severe stage of the acute form, the mouth is held open with the tongue protruding, and there is much mucus around the nostrils, as well as foam around the mouth (38).

Auscultation provides a varied picture:

- silence in zones of dullness
- exaggerated murmur in still healthy areas of the lung
- moist, crepitant rales in pneumonic foci
- tubular respiration sounds, sometimes very clear
- droplet noise.

All these symptoms constitute a complete clinical picture, which is rarely seen in its entirety. Nevertheless, the clinician will observe enough signs to reach a judgement of severe pleuropneumonia.

Complications which may occur at the end of this clinical stage are arthritis, pericarditis, peritonitis and abortion.

An articular localisation, seen particularly in calves, may be the only manifestation of \textit{M. mycoides} infection in these animals. In such cases pulmonary involvement is rare, but there may be cardiac complications in the form of valvular endocarditis and myocarditis (53).

c) The \textit{terminal period}: General illness is always very severe during the clinical stage, but recovery is far from exceptional. It is a slow process (at least several weeks), characterised by regression of the lesions and the clinical signs derived from them. Resorption of lesions is never complete, and sequestered lesions or pleural adhesions remain.

\textbf{Hyperacute form}

This is characterised by accelerated evolution of the symptoms described above. Death takes place after a week at the most, exceptionally after 1-3 days (81), and seems to be due to asphyxia, as pleural exudation is rapid and severe; there may also be heart failure from exudative pericarditis.

\textbf{Subacute and chronic forms}

These are very common, with various intermediate degrees.

The subacute form develops unobtrusively and is not accompanied by spectacular signs; the lesions are often not extensive.

Attention is drawn to the affected animal by its sick appearance and the presence of a cough. Fever is intermittent and never very high.

In most cases the outcome of this syndrome is the development of chronic lung lesions, with a return to a healthy appearance which simulates recovery.

A diagnosis of chronic pleuropneumonia is often made only after slaughter in animals which showed no sign of illness, when \textit{M. mycoides} is isolated from the lung lesions.
Macroscopic lesions (53, 77)

The essential lesions are located in the lungs and pleura. They are almost invariably unilateral, without predilection for one side or the other, and are often in the posterior part of the chest (53).

a) Pleura

Dry pleurisy: the pleura is much thickened, with adhesions joining the parietal membrane to the pulmonary membrane. There is no exudate in the thoracic cavity. This form is most often chronic.

Exudative pleurisy: acute inflammation with sero-fibrinous exudate, often abundant (up to 30 litres) affects the pleural membranes. Its colour is amber and it is often cloudy and tinged with blood, coagulating readily upon contact with air; the fluid occupies all of the floor of the pleural cavity.

'Omelettes' of fibrin are often found floating in this fluid, or attached to the parietal pleura and the lung surface (a pathognomonic sign).

b) Lungs

The framework of perilobular connective tissue is infiltrated and distended by amber serous fluid arising from lymphatics, so that every lobule seems to be within a 'frame'.

Hepatisation of lobules commences at the periphery and proceeds towards the centre of the lobes. The lobes become dark red and often cover an extensive area of lung uniformly, giving it a marbled appearance (yellow marbling on a red background). Areas of old lesions resemble brawn, with lobules of various colours (red, grey or yellow), indicating different stages of hepatisation. The lung parenchyma is friable and very dense.

The next stage is that of necrosis, the lungs becoming grey or yellowish-grey (38). Such an area is surrounded by a double line of yellowish tissue, one outside the lobule and the other in its centre (around the broncho-vascular axis) (53). If the lesion is not too large, its becomes surrounded by a fibrous capsule.

In animals which have recovered, or are at the chronic stage, sequestra are frequently present, composed of islets of necrotic and liquefied parenchyma, sometimes caseous, enclosed within a fibrous matrix which prevents any further extension of the lesion. Their size and location are variable. In deep locations they can be detected only by palpation of the lung tissue. Under the pleura they often coincide with a pleural adhesion. A sequestrum may be drained by a bronchus, and this results in discharge of the causal agent. Sequestra vary in size from 1 to 30 cm (5, 38).

To these lesions of lungs and pleura is always added hypertrophy of the bronchial lymph nodes. The bronchial mucous membrane is often congested, and the lumen of bronchi is usually obstructed by false membranes and fibrous plugs suspended in a foamy exudate.

Additional lesions may be seen on other serous membranes — exudative pericarditis, exudative peritonitis and synovitis.
In the most severe cases of CBPP there are also renal lesions, with well-defined yellowish infarctions, easy to see once the capsule is removed. They penetrate more or less extensively into the kidney tissue (38).

The frequency of distribution of acute, subacute and chronic lesions found when an affected herd is slaughtered will vary according to duration of the disease within the herd. At the onset and during the first two months, acute lesions are dominant, while as time passes the chronic lesions become commoner, compatible with a healthy appearance of the animals.

**Microscopic lesions** (77)

The *primary lesion*, occurring at the moment of dilatation of the perilobular spaces, is an influx of polymorphs into the septa. These then become organised by forming a border at the edge of the interlobular spaces.

At the same time there are changes in the lymph nodes, with the afferent canalicules filled by lymph, and necrotic foci surrounded by reticular cells, centred on a thrombotic arteriole.

The *secondary lesion* is characterised by alveolar involvement, with development of intra-alveolar oedema which coagulates and then becomes invaded from the septa by neutrophils and macrophages, many of which die.

The most striking feature is the difference in the degree of evolution between adjacent lobules, a characteristic pathological feature of CBPP.

The neutrophils and macrophages then form a well-defined inflammatory zone, separating the lobule from its septum.

Vascular lesions (thrombosis, perivascular organisation, necrosis) are pathognomonic for CBPP.

The *tertiary lesion* is inflammation of bronchioles and then bronchi, by an ascending route commencing at alveoli. There is a catarrhal or fibrinous exudate, rich in neutrophils and macrophages, containing large numbers of mycoplasmas.

### III. EPIDEMIOLOGY

**DESCRIPTIVE EPIDEMIOLOGY**

**Geographical distribution**

CBPP was widespread until the middle of the 19th century; its area of distribution has since been reduced considerably by the successful application of control measures.

The only areas never to have been affected are South America and Madagascar. Numerous countries have been able to eradicate the disease, such as the USA in 1892 and Australia in 1973. Europe had been free from the disease since the beginning of the century, but it has returned in the form of persistent outbreaks in Spain and Portugal where infection has apparently occurred relatively recently. The disease is still serious in African countries and in certain regions of Asia (Table I).
TABLE I

List of countries which declared outbreaks of CBPP in 1985

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<th>Africa</th>
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+ = low sporadic incidence  ? = suspected
++ = moderate enzootic occurrence  () = confined to certain regions
+++ = high occurrence  V = vaccination

Evolution

In countries or regions infected for some years, CBPP takes an enzootic form with sporadic cases, usually subacute.

When introduced into a country for the first time, the disease tends to spread rapidly like an oil spot on water, resulting in numerous foci in which many animals become infected and develop the acute clinical form.

Symptoms are observed to decline in severity in proportion to the time that the disease has been present in a herd or a region.

Strains of the causal agent vary in pathogenicity and infectivity in different epidemics, and in the same epidemic depending on the moment of isolation.

- Variation during the same epidemic:

Within a given herd, the disease has a more explosive character at its onset than at its end, and the decrease in pathogenicity can be reproduced experimentally (5, 14).

Within a given region, all animals in infected herds die at the onset of infection but many recover at the end of the epidemic. The last strains to be isolated are of lower virulence than the first strains.
Variation from epidemic to epidemic:

Tests conducted during different epidemics give results which agree with clinical findings. It is possible to distinguish very virulent, velogenic strains, such as the Gladysdale strain, from hypovirulent, mesogenic or lentogenic strains, such as the Mara, Oremit and V5 strains (4).

ANALYTICAL EPIDEMIOLOGY

Aetiology

Pathogenicity

The pathogenicity of strains of *M. mycoides mycoides*, a pneumotropic micro-organism strictly adapted to bovines, can be measured accurately only by administration in aerosol form into the bronchi of animals previously unexposed, under well-controlled conditions. In this way the strains can be classified as hyper- or hypovirulent, requiring inocula of $10^2$ and $10^9$ mycoplasmas per ml of culture, respectively.

In addition, a test for mycoplasmaemia in mice may be a guide, particularly in identifying vaccine strains (90).

However, inoculation experiments have to take into account a certain number of virulence factors inherent in the organism itself.

These concern the strain, colonial morphology, number of subcultures and age of the culture. Animal experiments enable large-scale verification of the slight but well-attested nuances associated with differing degrees of virulence in strains responsible for the naturally-occurring disease. Certain severe and rapidly-spreading epidemics are caused by hypervirulent, velogenic strains, while other epidemics are caused by hypovirulent, lentogenic strains. Finally, it seems that virulence declines as the disease evolves. In contrast to exaltation of virulence by passage, this is an instance of spontaneous attenuation, perhaps engendered by the acquisition of spontaneous immunity formed during indistinct and inapparent infections.

Strains thus show different degrees of virulence from the moment of isolation.

Filamentous ‘comet-shaped’ forms seem to have been wrongly considered the only virulent forms, because corpuscular forms behave in an identical manner at the same stage of subculture. Furthermore, contrary to the general rule of bacteriology, S-forms do not appear to be hypervirulent in comparison with the R-forms of dissociation.

Broth cultures of a freshly-isolated strain are undeniably the most virulent, while during the various passages, subcultures undergo a degradation of their pathogenicity when administered into bronchi or subcutaneously. Application of this rule in vaccine preparation verifies its authenticity.

Nevertheless, it is difficult to state, even for a given strain cultured under the same conditions, that virulence will be attenuated to the same degree after the same number of passages.
Resistance of the pathogenic agent

The resistance of *M. mycoides mycoides* SC to environmental factors is low, and it is capable of surviving for only 2-3 days in tropical regions, and rather longer (1-2 weeks) in temperate regions.

There have been numerous studies of its resistance to physical and chemical factors, as follows:

*Heat and cold*

Being unsporulated and without a cell wall for protection, *M. mycoides* has only slight resistance to high temperatures, but in this case as well, the figures obtained vary according to the medium in which it is suspended and the experimental conditions (22, 66, 91, 93).

Within 'lymph' it is inactivated within: 240 minutes at 45°C; 60 minutes at 50°C; 5 minutes at 55°C; under 2 minutes at 60°C.

The organisms suspended in normal saline are inactivated in two hours at 45°C, and practically immediately at 47°C (58). However, magnesium sulphate in molar concentration has a thermoprotective effect, by a mechanism not yet understood (79).

The relative sensitivity of fluid cultures to heat is important in the case of live CBPP vaccines in fluid form, for they must not be exposed to excessive heat, particularly in tropical countries (37).

By contrast, cold is an excellent preservative, and the organism can survive for at least a year in frozen, infected lungs. Preservation in liquid medium is difficult to quantify, because it depends on pH, as a result of the harmfulness of acid for mycoplasmas. As a rule, broth cultures can be stored for 2-4 months at 4°C, and this affords a new approach which can be applied to vaccines (25).

The freezing of cultures has no immediate harmful effect. Paradoxically, it may even enhance the titre of cultures, no doubt as a consequence of rupture of pseudomycelia. A temperature of −20°C, or better still −30 or −70°C enables the organism to be kept for several years, although the titres fall by 1 or 2 log units.

It is also worth noting that repeated freezing and thawing destroys mycoplasmas suspended in water, but it has no effect on mycoplasmas suspended in normal saline or another isotonic or hypertonic solution (31).

*Lyophilisation*

This can be used to preserve the organism, in preference to slow desiccation, which was once widely used in the preparation of 'virulent setons' impregnated with 'lymph' air-dried on the strips. The success of lyophilisation rests on the incorporation of protective substances, for it completely inactivates mycoplasmas suspended in distilled water. Numerous protective diluents have been tested both for preserving strains and for vaccine production, and the best preparations contain bovine albumin or serum. In practice, however, peptones are widely used; skimmed milk is not recommended for prolonged preservation. Whatever the protective substance, there is always a considerable loss of titre, at least 90%, though the lyophilised product is remarkably stable, surviving for many years when stored at 4°C, 18-24 months at 37°C, and several minutes in boiling water – an impressive result indeed.
Light and radiation

Ultraviolet radiation inactivates cultures within a few minutes, with a loss of $10^6 \log_{10}$ in 15 min. Sunlight has little effect, providing the container cuts out ultraviolet light and the temperature within the container does not rise above 45°C (36).

Ultrasound

The organism is inactivated in a few minutes by exposure to ultrasonic oscillations of 9 Kc. Composition of the medium has no effect in this instance (31).

Osmotic shock

The osmotic pressure of the cytoplasm of *M. mycoides mycoides* SC at 37°C is 11.6 atm. Despite the lack of a rigid wall, placing the organisms in a hypotonic solution or even distilled water is not followed by immediate lysis at freezing point, although it does occur at 37°C. Inversely, the organism can tolerate an osmotic pressure six times its own, with only slight loss in viability of the culture. The presence of bivalent and trivalent cations has a protective effect (31, 79).

This relative resistance to osmotic shock is attributable to the high cholesterol content of the cytoplasmic membrane, which forms bridges between phospholipid molecules, facilitating deformation of the membrane. In fact, changes in shape and volume of the CBPP organism are synchronous with changes in the tonicity of the medium (83, 84).

Wetting agents

Saponins and digitonin lyse *M. mycoides mycoides*, as do bile and bile salts (including sodium desoxycholate, at a concentration of $3 \times 10^{-5}$ M).

Action of antiseptics

All the ordinary antiseptics are active (93):

- 1% phenol solution in 3 minutes
- 0.5% formaldehyde solution in 30 seconds
- 0.01% mercuric chloride solution in 1 minute
- calcium hydroxide solution in less than 5 minutes
- ether in less than 5 minutes
- 0.004% mercurochrome in 1 hour.

Apart from its activity as a wetting agent, alcohol has no effect, and the same is true of boric acid.

Modes of transmission

Virulent material

The chief type of infective material is the pleuropneumonia 'lymph', which exudes from the cut lung or accumulates by exudation within the pleural cavity.

In acute forms of the disease, the excretion of infective material is considerable and continuous; in subacute and chronic forms, excretion is less abundant, irregular and sometimes absent.
While respiratory excretion is the prime source, there is a certain degree of mycoplasmaemia during the early febrile stage, which renders other organs infective, such as the brain, liver, kidney, lymph nodes, uterus, fetus and fetal membranes.

In this respect urine may also be important, for considerable numbers of *M. mycoides mycoides* have been recovered from urine (55).

Invasion of the blood suggests the possibility of transmission by tick vectors, with the creation of a natural reservoir of infection (56) and the possibility of repeated initial sensitisation of cattle, the disease then being elicited by direct contact.

**Chronic carrier state**

The frequently occurring clinical recovery is generally accompanied by the sequestration of lung and pleural lesions, with perivascular cellular organisation and formation of a fibro-sclerotic external barrier. The lesion is still active immunologically, providing the immunity of superinfection, together with tuberculin-type delayed hypersensitivity. Sequestra are usually closed, but an occasional pulmonary sequestrum may open into a draining bronchus, with the dangerous epidemiological consequence of carriage and excretion of the causal agent. The cycle of infection is completed when the carrier emits aerosols of the causal agent, exposing disease-free animals in contact to repeated possibilities of infection.

**Modes of infection**

The disease is transmitted by direct contact, in the short term and over small distances by the coughing animal, emitting Flugge-type droplets from the mucous membrane of pharynx, trachea and bronchi, and from saliva, or even micro-droplets of urine transported by an air current (55).

Three examples of anecdotal evidence demonstrate some minor departures from this rule which, it should also be mentioned, excludes risk of infection to meat: experimental transmission by infective aerosols over a distance of 55 m, another case of transmission across a double barbed-wire fence (but with a favourable wind) and an example of infection between shipments of cattle by truck.

**Receptivity**

*Species* of animal is an important consideration, for under natural conditions CBPP affects species of the genus *Bos: Bos taurus* (cattle) and *Bos indicus* (zebu) are equally susceptible. In Africa the more frequent involvement of zebu is an epidemiological rather than an aetiological factor, for the disease is more serious among large zebu herds in the interior of the continent than among small cattle herds near the coast.

It is exceptional to detect the infection in bison (*Bison bonasus*) and yak (*Bos [Poephagus] grunniens*) in zoos.

It has been reported that the domestic buffalo (*Bubalus bubalis*) is susceptible, and that outbreaks still occur among this species in Assam. Experiments on the feral buffaloes of Northern Australia have shown that introduction of infective material directly into the bronchi is followed by a respiratory disease, but the subsequent course of the disease is abortive, and the animals are not infective for other buffaloes. The African wild buffalo (*Syncerus caffer*) is not susceptible (89).
A unique case, and thus of doubtful validity, was reproduction of Willems’s phenomenon in a young roan antelope (*Hippotragus equinus*). Despite the detection of antibodies to *M. mycoides* in the gnu (*Gorgon taurinus*), subcutaneous infection of these antelopes was not followed by any reaction.

Finally, pleuropneumonia has not been recorded in camels.

**Breed** is also a factor in both natural infection and the response to inoculation of attenuated vaccine strains, as follows:

**Breed factors in naturally occurring disease**

Among zebu, some breeds are remarkably resistant to CBPP, which has little effect on them, and these include Somba, the breed of coastal lagoons of Benin, and the small Côte d’Ivoire breed.

The Masai breed of Tanganyika is equally resistant, and 80-85% of them recover without treatment, whereas the European breeds and their crosses are more receptive.

In Zambia there is a high degree of infection among Barotse and Mashululumbive breeds, while in Sudan, by contrast, almost 40% of zebus are resistant to experimental infection.

Among cattle, it was found during the previous century that the Dutch and Flemish cattle of Europe were more susceptible than Swiss, while in present-day Kenya, Jersey cattle are affected more often than Friesians.

N’Dama cattle of Guinea seem to be more susceptible than the zebu and crossbred N’Dama of Senegal.

In Australia, dairy cows of Anglo-Normandy breeds were more susceptible to experimental infection than Hereford beef cattle.

**Breed factors following vaccination and experimental infection**

In these cases, similar variations are observed, but it is not legitimate to separate one from the other, since in both the only effect is a Willems’s reaction.

As a rule, cattle are more susceptible than zebu, at least in Africa. Thus breeds of the Benin coast and the Côte d’Ivoire, reported to be resistant to the disease, develop particularly severe reactions to the inoculation of infective ‘lymph’, or strains of *M. mycoides* which are still pathogenic. This finding has led some laboratories to prepare separate vaccines, differing in residual virulence, for the two species.

Nevertheless, Curasson (14) expressed the opposite opinion: application of Willems’s method in both West Africa and Europe at the end of the last century was followed by high post-vaccinal losses among zebu, up to 5%, compared with barely 1% in Europe. Kouri cattle of Lake Chad proved to be no more sensitive than the zebus of the same environment, while vaccination in Central Africa showed that N’Dama and Baoul cattle did not develop greater reactions than zebu inoculated with a relatively pathogenic egg-culture vaccine, provided that the inoculation was performed correctly.

In fact, it is inadvisable to draw up strict rules. The apparently high resistance of zebu has been ascribed to contamination of the environment, which confers on progeny a certain acquired resistance, sustained by successive reinfections. No
immunogenetic proof has been offered to confirm this supposition. The concept of an average level of breed susceptibility remains useful, with important consequences for vaccination campaigns (36).

Age of the animal also has various repercussions in the naturally occurring disease and the response to vaccination.

In the natural disease, the susceptibility curve is sigmoid in shape with three phases:

- an initial phase of low susceptibility in unweaned animals, which develop only minor lesions of tendons and joints, and not the severe pulmonary form;
- a subsequent phase of moderate susceptibility, gradually increasing until 12-18 months of age;
- a final phase of full susceptibility, which explains the choice of cattle over two years of age for experiments on the virulence of *M. mycoides*, in order to eliminate aberrant responses.

The mechanism of these variations is unknown, and serological testing does not provide a satisfactory explanation of the acquisition of spontaneous, occult immunity by animals in contact with infected animals. Here too, an average level of susceptibility according to age remains the only valid generalisation.

Similar variations are found in both experimental infection and vaccination. If it is true, as stated by Willems, that calves up to six months of age respond poorly to the inoculation of virulent 'lymph', developing no more than slight, transient oedema, it is a fact that numerous cases of polyarthritis develop as a consequence. To this may be added cases of coronary valvular disease and myocarditis, encountered among calves less than three months old.

At a later age, heifers respond weakly to vaccination, but nevertheless become more resistant to experimental infection than adult cows.

Individual factors complicate further the multiple aspects of susceptibility.

To start with, individual factors in a group of animals of the same species, breed or age cannot be predicted, to the dismay of epidemiologists and hygienists.

Thus, in South Australia, it has been found that aged dairy cows are more sensitive to both vaccination and challenge infection than steers from central Australia (36). However, this rule includes numerous exceptions from group to group in each type of cattle. Thus some adult dairy cows were as sensitive as steers, while, inversely, certain steers were as sensitive as those cows classified as being least sensitive. Such variation in the sensitivity of a group is far from remaining constant, and seems to follow unpredictable cycles.

According to Turner, 17 cattle in Australia which resisted an initial challenge contracted the disease after a second challenge. In general, some groups of animals show an evolutive receptivity in both directions — according to the region and type of husbandry — and may not contract a disease which decimates other herds at the same time, e.g. 30% in Tanganyika, 58% in Kenya, 10-30% in West Africa. In addition, the kinetics of the disease in a herd extends over about 20 weeks.

Aerosol infection, as studied by Turner and Campbell in Australia, gave rise to the following divergent pattern: 8% developed the hyperacute form, 14% acute, 30%
mild, 23% inapparent infection with serological conversion and 25% were fully resistant; 10% of survivors developed sequestered lesions. The mortality rate generally varied from 50 to 90%.

This demonstrates the difficulty in interpreting the results of tests of vaccines, as challenge infection may provide a fairly small proportion of positive results, with animals incorrectly classed as protected.

Such group variation has repercussions for vaccination, to the prejudice of statistical interpretation of results, because the same batch of vaccine inoculated into apparently similar cattle, might provoke alarming, unforeseen reactions in a given group, with a temporal evolution of vaccine reactions similar to that of responses to infection. Similarly, a group of cattle refractory to inoculation of vaccine might contract the disease a few weeks later. We have to admit an ignorance of the immune mechanism, and this affects vaccination programmes. Instability in receptivity is the major obstacle to vaccination against CBPP, which is based on an average receptivity of species, breed, age group and individual. But the present difficulties do not rule out the hypothesis of a pathogenesis directed by a variable allergy depending on past and present pathological and immunological events.

The strictly individual factors are not excluded, and they are usually regarded as non-specific. But when applied to a whole herd, they may determine group factors.

An intercurrent disease, such as rinderpest in Africa, frequently brings about the same type of synergic-pathogenic combination.

Subsequent vaccination runs the risk of producing a similar situation. Thus the use of caprinised rinderpest virus vaccine considerably increased the reactions to CBPP. Similarly, anthrax vaccine can ‘reactivate’ a weak Willems’s reaction induced by an attenuated strain of \textit{M. mycoides}, with the development of a fatal Willems’s reaction at the site of the first inoculation, despite the presumed existence of immunity (58).

The difficult and elusive study of the pathogenicity of \textit{M. mycoides} enters into the practical application of systematic vaccination. The approach to immunisation against CBPP is founded on the following concepts:

- The similarity of sensitivity of cattle and zebu must not occlude the variability and changing patterns of sensitivity in a given breed or group of individuals. The result has been the arbitrary definition of an average level of herd sensitivity, in herds calling for vaccination, but it too entails some surprises and deceptions. Vaccine testing must include a sufficient safety margin to cater for the most sensitive cattle.

- The relative permanence of resistance in very young animals and the full susceptibility of adults has led to vaccination of calves at three months, without an immune hiatus unfavourable for a statistical immunological cover.

- Relationships between sensitivity and regional conditions of cattle husbandry have led to the preparation and testing of vaccines for use on a regional basis. Similarly, when a new vaccine is developed, it has to be tested on the animals on which it will be used. Accidents which have occurred despite all precautions and predictions should encourage the use of strains of relatively low virulence. These cannot provide total immunity, but they may somewhat enhance and regularise the average herd resistance in a region.
One may agree with Curasson that in regard to investigation and control, no disease is as misleading as CBPP.

Favouring factors

These concern the type of husbandry, climate or season, and feeding.

*Type of husbandry.* This affects both epidemiology and aetiology, and may well be the crucial factor, since CBPP is essentially related to the movement of animals. Areas completely free from infection can adjoin endemic areas if there is no movement of cattle between them. A good example is the disease-free plateau of Adamaoua in Cameroon, separated by a 1,000 m drop from the infected plain of Benoue. In central Australia, experience has shown that in a dry climate, CBPP becomes extinguished spontaneously, provided the infected herds are kept in strict isolation, without introducing new animals.

The inherent virulence of strains, host susceptibility and the occult immunity of carriers are intimately mixed with strictly immunological factors governing infection.

It is both true and false that CBPP is a disease of nomadic, or at least transhumant cattle. In fact, in intertropical Africa the disease occurs especially in that segment of farming populations and breeders living within the 500 isohyet, who have little experience of livestock husbandry. They thus contract to send their cattle off to join the large transhumant herds of the Sahel, to seek water and fresh grazing. These newly-introduced cattle are likely to pay a heavy toll to disease, revealing the existence of carriers in the nomadic cattle, and thus sparking off the disease in the reception herd. Further commercial transactions involving Sahelian cattle, whether for slaughter, breeding or use for traction, are then responsible for fresh outbreaks.

This establishes a surprising contrast between the apparent mild course and low incidence of the disease among nomadic cattle, on the one hand, and the severity and catastrophic nature of the disease in areas populated by farmed cattle, on the other. Interventions to eliminate the source of infection should be conducted among the latter, but even moreso among the former.

The African example is exactly mirrored in the former epidemiological status of Australia, when large herds of slaughter cattle were driven from North Queensland to the south-eastern coast, spreading the infection as they travelled.

Few remedies can be applied to this ecological factor, for to prohibit transhumance in Sahelian countries would destroy cattle breeding under the present conditions of social life and economic infrastructure. In addition, there are other ancillary factors which complicate the effect of the husbandry system on the disease, as follows:

- Local husbandry practices in Sahelian Africa involve compact grouping of herds during grazing, mixing with other herds at watering points, and confinement at night within small enclosures — such conditions are eminently favourable for infection.

- The intensity of infection is low in Sahelian regions, where herds are spread out, the air is dry, and the sexes are kept separate. Under these conditions the disease may persist for a long time, only becoming evident when the animals are moved, new stock introduced or animals exchanged between tribes or traded (dowry payments for example).

- The difference in general susceptibility between herds is relatively pronounced in the Sahel, and low within the Sudano-Guinean zone. There is a natural tendency
for CBPP expression to wind down at the end of an epidemic, and to show a sinusoidal course with time, each cycle being completed by the appearance of a sufficiently large unexposed population.

*Climate and season* are important more for the way in which they affect the type of husbandry than for any direct effect on the disease.

According to Turner, a dry climate diminishes the risk of spread, because infective aerosols from contaminated cattle evaporate rapidly, and the pathogen is inactivated by ultraviolet rays. In regard to the disease itself, once established and regardless of racial susceptibility, its severity is identical in dry or humid climates.

Season seems to play a role in stimulating infection, particularly the rainy season, when the animals are exposed to cool downpours.

The role of *feeding* has not been examined experimentally as an aetiological factor. At one time the disease was a scourge of premises housing young animals, but in general nutrition seems to have only an ancillary role.

### IV. CLINICAL, DIFFERENTIAL AND EXPERIMENTAL DIAGNOSIS

#### CLINICAL DIAGNOSIS

*Species concerned:* large ruminants — cattle, zebu, bison, yak and (with less certainty) domestic buffaloes. The naturally occurring disease is not transmissible to sheep and goats. The disease has never been verified in camels, wild buffalo, giraffe nor the larger African antelopes (80).

CBPP is to be suspected in an endemic zone whenever general illness is coupled with pleuropneumonia, and the suspicion is strengthened when the contagious nature of the disease becomes clear (73).

As a rule, the complete syndrome is never observed in any one animal. It is advisable to examine many sick animals in the same outbreak in order to obtain a full idea of the syndrome. Sometimes (e.g. in zebu of the African Sahel) the symptoms are relatively scarce, and only statements of the owners serve to identify the sick animals.

#### POST-MORTEM DIAGNOSIS

The pathognomonic lesion is the characteristic type of lung hepatisation found in this disease. Its significance is always supported by finding extensive pleural lesions and abundant pleuritic fluid (38). This hepatisation of lung lobules (with centripetal extension) first gives the lung a marbled appearance, followed by a brawn-like appearance when different stages are present simultaneously.
In most cases the respiratory tract lesions are dissymmetrical (53), affecting the more posterior parts of the lungs.

Another pathognomonic finding is exudative pleurisy (with an exudate amounting to 30 litres in some cases), and ‘omelettes’ of fibrin which coagulate when the chest is opened. The ‘omelettes’ float in the pleural fluid and may cover the parietal wall as well as the lung surface (53).

Under the microscope the vascular lesions of thrombosis, perivascular cell infiltration and necrosis are also pathognomonic.

Features of the chronic form are as follows:
- Sequestra are present when the cut surface of the lung is inspected, although they may be very small and difficult to find.
- In place of sero-fibrinous exudation, a fibrous tissue is present on the pleura, leading to adhesions between its parietal and pulmonary surfaces. These adhesions can be quite strong, making it difficult to extract the lungs without cutting or without leaving behind some lung tissue attached to the chest wall (53, 73).

**DIFFERENTIAL DIAGNOSIS**

It is hard to make a clinical distinction between CBPP and bronchopneumonia and pleuroneumonia resulting from mixed infections (lung disease caused by both viruses and bacteria, such as *Pasteurella*) (53).

If it is not possible to slaughter an affected animal, attempts can be made to isolate mycoplasmas from the blood, or by making cultures from nasal swabs in culture medium containing bacterial inhibitors. It is also possible to conduct serological tests (38).

At post-mortem examination it is important to distinguish CBPP from:
- East Coast fever, in which an oedematous fluid may dilate the septa and infiltrate the parenchyma. However, this is neither true pneumonia nor pleurisy (38).
- Acute bovine pasteurellosis, the disease closest to CBPP, produces a similar marbling of the lungs, although usually the entire tissue of both lungs is affected (in contrast to CBPP) (53). In addition, staining a lung section with methylene blue will reveal the coccobacilli with bipolar staining.
- A sequestrum of CBPP may be confused by an inexperienced person with an old *Echinococcus* (or hydatid) cyst. The precipitation test will distinguish the two conditions, should the cyst have become infected secondarily by bacteria.
- Actinobacillosis lesions may resemble old sequestra, and in such cases microscopic examination and the preparation of cultures is required.

In every case, experimental diagnostic procedures are advisable.

**LABORATORY DIAGNOSIS**

There are two types of procedure:
- Isolation followed by identification of the pathogen by examining its biochemical properties, with serological confirmation by the growth-inhibition and/or immunofluorescence tests.
b) Tests for circulating antibodies in serum, or circulating antigen (galactan) in serum, pleuropneumonic ‘lymph’ or organ homogenate.

**Collection of samples**

A number of procedures have to be followed in order to isolate mycoplasmas (39).

- **In the live animal**, swabs of the nasal mucosa should be taken or nasal discharge collected from affected cattle.

The pathogen may be either scarce or very numerous, depending on the stage of the lesions, and failure to isolate is not conclusive. The mucus or sero-fibrinous exudate (sometimes blood-tinged) can be collected on a sterile swab, which must not be allowed to dry out. This can be avoided by plunging the swab into a tube of semi-agar transport medium, containing substances which inhibit the growth of other bacteria (formula in the Appendix).

It is also possible to take a few ml of pleural fluid, collected under aseptic conditions by puncture of the thoracic cavity in its sloping part, between the 7th and 8th ribs. This procedure is practically painless and without risk. For a sample to be dispatched to a laboratory, 100-1,000 IU benzylpenicillin or 0.15 mg ampicillin should be added to each ml, as well as thallium acetate to give a final concentration of about 1:8,000 (between 1:5,000 and 1:10,000).

For serological tests, 10 ml of whole blood are needed, preferably in a glass tube to avoid haemolysis. The laboratory will require 5 ml of serum (decanted after centrifugation). The latter may be stored in the frozen state. If it is sterile, it may be kept for a few days at 4°C.

- **Carcass sampling**: Portions should be collected of hepatised lung, thoracic ‘lymph’, bronchial exudate and lymph nodes draining the broncho-pulmonary tract.

A post-mortem examination conducted in the field provides excellent samples, using a minimum of precautions.

Once the chest is opened, a sample of pleural fluid can be taken by pipette or syringe under practically aseptic conditions. It is useful to have a small, portable gas burner to sterilise surfaces.

Once lung lobes bearing lesions have been located, fragments of lesions in the stage of red hepatisation are taken, together with the sero-fibrinous contents of major bronchi supplying the infected lobes. Tracheo-bronchial and/or mediastinal lymph nodes are removed intact, within their capsule, to avoid contamination.

In cases of the chronic form with old lesions, isolation of the pathogen may prove difficult. In general, the pleural cavity no longer contains inflammatory fluid and the lung lesions are at the stage of grey hepatisation or even fibrosis. The only possibilities are hypertrophied lymph nodes, mucus from bronchi supplying the affected lung tissue, and perhaps the contents of a sequestrum (or at least a scraping from the internal surface of the fibrous capsule).

If serological tests are required, it is necessary to collect the blood clot from within the heart in order to separate serum by exudation, preferably using sterile glass containers. Serum samples must not be frozen until the clot has been separated and the sample centrifuged in order to remove any remaining cells.
All samples should be dispatched in an insulated container kept between freezing point and 4°C to a competent laboratory (see Appendix). Fluid samples ('lymph' or exudate) may also be inoculated deeply into a transport medium suitable for mycoplasmas (see Appendix).

If dispatch has to be delayed, the samples can be stored under the following conditions:

- at +4°C for a few days
- at -20-25°C for several weeks or months (apart from samples in transport medium)
- freeze-dried (organ homogenates, exudate, 'lymph'), followed by storage at +4°C (or better at -20°C), in which case the samples can be kept for years.

Obviously samples required for pathological examination must not be frozen, but placed as rapidly as possible (in order to fix them) in 10% formaldehyde solution, using a volume 20-40 times the volume of the sample. An alternative fixing agent is Bouin's fluid.

MICROBIOLOGICAL DIAGNOSIS

Culture media

The isolation of *M. mycoides* subsp. *mycoides* (SC, bovine biotype) poses no major problems for a suitably equipped laboratory.

All culture media should contain:

- a basic medium composed of meat extract (such as beef heart infusion) or peptone (Tryptose Difco, Tryptone Difco/Oxoid) or both;
- extract or autolysate of beer yeast (fresh or commercially supplied) which provides the growth factors (group B vitamins and nucleic acid precursors);
- serum in the proportion of 10-20%. The 10% proportion has been recommended for favouring the frequent (but not invariable) appearance of 'comet' forms (93). Complement in the serum is inactivated by heating at 56°C for 30 min. The species of animal is not important, but the serum has to be tested beforehand (e.g. by the growth-inhibition test) to make sure that it is free from antibodies or other substances which could inhibit the growth of mycoplasmas. Preference may be given to horse serum because it rarely contains inhibitors of mycoplasmal growth. Serum from aged horses is better than that from foals, because it contains more growth substances (particularly sterols).

Sterol is a vital component of the membranes of mycoplasmas, which are incapable of synthesising it themselves (hence the need to incorporate it in culture media).

To promote the growth of *M. mycoides*, glucose, a buffer system, DNA and possibly glycerol may be added.

It is essential to add inhibitors of bacteria at the moment of isolation, such as thallium acetate at a final concentration of 1:5,000 to 1:10,000, and benzylpenicillin at 250 or 1,000 IU per ml of medium. The concentration must never exceed 1,000 IU/ml, because higher concentrations favour the appearance of L-forms.
Benzylpenicillin has never shown to have an inhibitory effect on *M. mycoides* subsp. *mycoides* (SC, bovine biotype). 'Fungizone' (amphotericin) may also be added at 5 µg/ml if fungal contamination is suspected.

Culture media may be liquid or solid (prepared by adding agar at about 15 g/l, or agarose). Liquid media are best sterilised by filtration. Agar media are autoclaved, after which serum, yeast extract and inhibitors are added when the medium has cooled to 55-60°C. Formulae for the media are provided in the Appendix.

Isolation

There are many techniques, preferably starting with copious sown cultures.

**Isolation on solid media** (containing bacterial inhibitors)

A few drops of lung ‘lymph’, pleural fluid or lung homogenate are deposited and spread. It is also possible to make a direct impression on agar with the cut surface of a lung lesion or lymph node, without spreading it.

Certain authors (2, 27) report that mycoplasmacidal substances (antibodies, complement, lysolecithin, lecithin) may be present in pathological specimens, so that it is best to sow first in mycoplasma broth by a system of Pasteur dilutions, and then to sow agar media from these dilutions. Contrariwise, it is plausible that organ homogenate (or a smear) sown directly onto an agar plate preserves the original habitat of the organism and aids adaptation to artificial media. Such problems have not been encountered in the case of *M. mycoides* subsp. *mycoides* (SC, bovine biotype), but it is still true that isolation should be performed as rapidly as possible.

**In liquid media** (with bacterial inhibitors)

The best method is still that of Pasteur dilutions, which makes it possible to eliminate bacterial contamination from the 2nd or 3rd tube, with mycoplasmas obtained in a pure state from the 4th tube. This is an example of the joint effect of two systems, dilution and inhibitors, in eliminating contamination.

The procedure is to take up 1 ml of the sample in a Pasteur pipette and inoculate it into 9 ml of medium in the first tube. After mixing the contents, 1 ml is transferred to 9 ml of medium in the second tube, and so on down the dilutions, changing the pipette for each tube.

In conclusion, it is fairly easy to culture *M. mycoides* and the following procedure is recommended:

- sowing the specimen on agar by the standard technique;
- sowing a series of 5 tubes by the Pasteur dilution technique.

Cultures of mycoplasmas grow relatively slowly, and the cultures have to be incubated for several days at 37°C in hermetically-sealed tubes, while agar plates are placed in an oven containing water (to provide 80% humidity) so that they do not dry up.

An atmosphere of CO₂ is unnecessary; ordinary air is adequate for *M. mycoides*.

**DIRECT DIAGNOSIS**

Little information will be provided by this procedure if the observer is not well experienced.
Examined in the fresh state by phase-contrast microscopy (with or without a dark background), 'lymph' or bronchial exudate may be seen to contain fine particles, neither cocci nor bacilli, in the form of small detached elements or filaments of varying length, subject to brownian movement, in the midst of cellular debris, fibrin filaments and contaminating bacteria — hence the difficulty for the inexperienced eye.

Examination after staining (May-Grünwald-Giemsa) reveals small pinkish-violet grains mixed up with cells, fibrin and various bacteria, more or less numerous. The presence of such particles is a good indication of mycoplasmas, but again such observation requires trained personnel. This staining procedure has the advantage of permitting a cytological study, which cannot be done when Gram stain (or some other stain) is used.

**Immunofluorescence** (71) is another direct technique applicable to diseased material, and its specificity is excellent.

Smears are prepared with a suitable fluid ('lymph', exudate, oedematous fluid) and fixed in methyl alcohol for 3 minutes. Portions of lung lesions (incipient or in the stage of red hepatisation) are fixed in Bouin's fluid (or 10% formaldehyde), then treated in the same way as specimens for pathological examination.

The immunofluorescence technique is direct or indirect. The direct method is quickest, but less specific than the indirect method, which takes longer but is more specific. Preference should be given to the latter. In either case it is necessary to test negative serum as a control.

The immune serum employed must contain antibodies to *M. mycoides* subsp. *mycoides* (SC, bovine biotype) in high titre, preferably assayed by the growth-inhibition test, because the type of antibody involved in each test is the same (external, membrane antigen). Alternatively, complement fixation and passive haemagglutination can be used.

The conventional technique for fluorescein labelling is employed for both types of immunofluorescence. In parallel to reference sera, an additional control serum may be obtained from a known case of the disease, having been tested previously by the standard serological techniques.

After absorption, the sera are titrated by immunofluorescence in high dilutions, in order to avoid cross-reactions with other species and non-specific fluorescence.

As concerns the results obtained for pathological fluids, so long as smears have been prepared and fixed immediately after collection (which preserves the morphology of the mycoplasmas), this test is very accurate, although there may be some non-specific fluorescence (from degenerated cells, nuclei, fibrin fragments, etc.). When applied to histological sections, the results are more difficult to interpret by the inexperienced eye (non-specific fluorescence). Nevertheless, mycoplasmas are easily identifiable in the perivascular lymphatic fissures, the perilobular lymphatic spaces, and also in the images of phagocytosis.

Mycoplasmas are very abundant in early or acute lesions, and become scarce in subacute and chronic forms.

In conclusion, direct examination by immunofluorescence of a fresh specimen after staining should be entrusted to a very competent laboratory.
Culture and staining

Culture

Within 3-5 days a slight, homogeneous cloudiness appears in the culture tubes. Microscopic examination of cultures, preferably low in serum (e.g. containing 10%), may disclose silky, fragile filaments called 'comets' (38), extending from the surface of the liquid into its interior, though they are not always present, and they disappear when the tube is shaken. Next, the culture takes on a uniform opacity, and whirls appear when the tube is shaken in a circular movement. This is the mucoid stage of mycoplasmal growth and is due mainly to a matrix of galactan which surrounds colonies (26, 80). This observation is valuable, because it is manifested solely by strains of the bovine SC biotype of *M. mycoides* subsp. *mycoides* and strains of the CBPP agent.

Microscopic examination of cultures in the fresh state (preferably by phase contrast) provides evidence of the presence or absence of contaminants, the density of the culture, and the probability that the cloudiness is due to mycoplasma. The particles are neither cocci nor bacilli; much smaller than typical bacteria, they are arranged in small points, bunches are long slender filaments (in young cultures). There is pronounced brownian movement in the culture, but this is not true motility. In aged cultures the only elements present are small and coccoid (0.2 µm on the average).

On agar media the colonies which form are characteristic of the order *Mycoplasmatales*, having an average diameter of 1 mm with a dense centre. The centre of the colony is incrusted in the agar (perhaps due to a slight proteolytic activity). The colonies can be seen readily by using a stereoscopic microscope under transillumination and at a magnification of 25.

Staining

In a doubtful case the entire colony can be stained with one of two types of stain.

1. May-Grünwald-Giemsa staining

A block of agar supporting colonies is placed face down on a microscope slide, which is then carefully immersed in Bouin's fluid. After 15 minutes the colonies have become attached to the slide and the agar block is detached. The slide is washed thoroughly with distilled water buffered to pH 7, then stained by the standard May-Grünwald-Giemsa procedure. The slide is examined dry at a magnification of 16 (or 40), or for fine detail under oil immersion at × 100.

2. Dienes's staining

A block of agar supporting colonies is placed on a slide with the colonies uppermost. A cover slip is coated with Dienes's stain (see Appendix), which is allowed to dry. The cover slip is applied face down to the colonies. After 15 min the colonies can be examined dry at a magnification of 16 or 40.

In either procedure, the typical structure of mycoplasma (or acholeplasma) colonies can be recognised: a dense, intensely stained centre, a granular structure becoming thinner towards the edge, and a finely serrated border.

The applications of staining are:
Identification of colony structure: it is possible to distinguish a mycoplasma from a very small colony of other bacteria, which have a well-differentiated structure, often intensely and uniformly stained; vacuoles may or may not be present.

Exclusion of false colonies, such as debris from the inoculum, or ‘St. Catherine’s wheels’ (crystals which disappear under the action of the phenol contained in Dienes’s stain), etc.

Because it is more rapid and more reliable than the May-Grünwald-Giemsa method, the use of Dienes’s method is to be preferred.

Species identification

Purification

The colony observed is cloned by cutting the agar block which supports it, and then transferring it to a tube of broth. If a broth culture should become contaminated, it is possible to filter it through disks of pore size 0.45 or 0.22 µm (such as Schleicher or Millipore), because only mycoplasmas can pass through, and other bacteria are retained.

The two principal tests are growth inhibition and immunofluorescence. Other tests, used less often, include galactan assay and the slide agglutination test. The same type of serum is used for all these tests.

1. Growth inhibition

The technique, as described by Clyde (11), consists of sowing agar, in a sheet, with different dilutions (by the same procedure used for testing sensitivity to antibiotics). Once the dishes have dried, wells 3 mm diameter are punched into the 4.5 mm-thick agar. The wells are filled with immune serum. The dishes are kept at room temperature for 5-6 hours, and then placed in a standard incubator. The result is read after 48 and 72 h. A zone of inhibition of at least 2 mm, measured from the edge of a well to the edge of the colony, is a positive result.

It is necessary to distinguish between the SC type of CBPP agent and the LC type of *M. mycoides* subsp. *mycoides* of caprine origin. The latter is pathogenic for cattle only under exceptional circumstances (38). They can be distinguished by their metabolic activity, as follows.

Strains of caprine origin (LC type) have a fast rate of growth. Average colony size is greater than 2 mm, and they appear on agar within 24 h. Their proteolytic activity (against coagulated serum or thin paper strips) is considerable. In addition, they resist heating to 45°C for 48 h, which is not the case with biotype SC (13). Comet forms are never observed.

Of course, these are only relative differences, but they are still important.

Many colonies can be purified simultaneously, e.g. when colonies of *M. arginini* are also present. The different colonies can be observed on the same plate. The best procedure is to perform three successive purifications.

2. Elimination of L-forms

After 2 or 3 subcultures, inhibitors of bacteria should be omitted. This will eliminate L-forms (spheroplasts and protoplasts). In addition, omission of penicillin allows the original bacteria to regain their shape, being more readily detectable (74).
3. The digitonin test (23)

This simple test distinguishes mycoplasmas, which are sensitive to digitonin, from acholeplasmas, which are not.

4. Biochemical characteristics

The characteristics of *M. mycoides* subsp. *mycoides* (SC, bovine biotype) are listed below. (See the Appendix for particulars of the biochemical tests).

- films and spots : –
- glucose fermentation : +
- reduction of tetrazolium salts : + (under aerobiosis and anaerobiosis)
- arginine hydrolysis : –
- phosphatase hydrolysis : –
- proteolytic activity : – or weak

The bovine SC biotype is not the only mycoplasma possessing these metabolic properties. Consequently, to make a definitive identification it is necessary to perform serological tests, using growth inhibition (or glucose metabolism inhibition) by a specific reference immune serum, or direct or indirect immunofluorescence.

It is impossible to separate the two biotypes by growth inhibition with reference immune serum. Nevertheless, Perreau (73) demonstrated that it was possible to distinguish SC from LC by using serum from naturally infected cattle. This had a selective action and enabled the bovine and caprine SC types to be distinguished from the LC type.

**TABLE II**

*Growth-inhibition test*

<table>
<thead>
<tr>
<th>Strains of <em>M. mycoides</em></th>
<th>Serum against Pg1 (SC)</th>
<th>Serum against 7302 (LC)</th>
<th>Serum against D.III (natural cases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bovine SC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KH1J</td>
<td>3</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>T1</td>
<td>2</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Fatick</td>
<td>1.5</td>
<td>5.5</td>
<td>4</td>
</tr>
<tr>
<td>Afade</td>
<td>1</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Gemu-Goffa</td>
<td>4.5</td>
<td>5.5</td>
<td>4.5</td>
</tr>
<tr>
<td>caprine SC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vom</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>C11</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Pillai G</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>caprine LC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7302</td>
<td>1</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>S1</td>
<td>2</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>7937</td>
<td>2</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>7957</td>
<td>1</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>7958B</td>
<td>3.5</td>
<td>5.5</td>
<td>–</td>
</tr>
<tr>
<td>7958C</td>
<td>2</td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td>7959</td>
<td>1.5</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td>8062</td>
<td>1</td>
<td>3.5</td>
<td>–</td>
</tr>
</tbody>
</table>
Serum sensitivity assay

This test, still in the developmental stage (86), distinguishes the two biotypes by the presence of guinea-pig complement, which has a greater slowing or inhibitory effect on the growth of the bovine SC biotype than on the caprine LC biotype.

5. Immunofluorescence

This test serves as a complement to the growth-inhibition test. Various techniques have been described (85). It is best to use entire, unfixed colonies on agar blocks. The longer indirect method is the most specific, though the quicker direct method still gives good results. In either case it is necessary to use a negative serum as a control. Sera have to be assayed before use, and a dilution is chosen which eliminates interspecies and non-specific cross-reactions. To the experienced observer, the intensity of fluorescence (from negative to ++++) enables SC to be distinguished from LC. As in the growth-inhibition test, however, the problem of distinguishing LC from SC remains. In this test as well, serum from a natural case of CBPP may be used to resolve the problem.

CONCLUSION

Isolation and microbiological diagnosis of the bovine SC biotype of *M. mycoides* subsp. *mycoides*, supported by clinical signs and post-mortem findings in cattle suspected of having CBPP, should create no major problems for well-trained laboratory personnel, provided that a rigorous methodology is adhered to, and that the samples are collected and processed as rapidly as possible.

There is the possibility of contamination by other mycoplasmas, but successive purifications should eliminate them. Biotype LC can be distinguished from biotype SC by observing differences in metabolism (LC more active than SC) and by applying the growth-inhibition and immunofluorescence tests, with the aid of serum from infected cattle.

SEROLOGICAL DIAGNOSIS

Among the diseases investigated in veterinary medicine, CBPP is one of the best understood from the serological aspect (28, 72).

Here we shall outline the numerous diagnostic tests available, paying special attention to the advantages and limitations of each one.

Detection of circulating antigen

The specific antigen (galactan) which occurs on the surface of *M. mycoides* can be detected readily during the clinical stage of the disease, for it is distributed widely throughout the body, including blood serum, lymph, lung fluid, pericardial fluid and also urine.

Such an excess of antigen saturates the various antibodies, leading to the formation of circulating immune complexes. These cannot be detected by conventional serological techniques, hence the value of testing for galactan in the circulation.
For this purpose the **double agar immunodiffusion test** is without doubt the test used most often (30, 88, 98). The result is read after about 24 h of diffusion.

For field use there is a simplified technique, using thick disks of filter paper saturated with serum, which are placed on a thin layer of agar. The line of precipitation appears sooner and it is easier to see (81).

The gel medium acts as a filter and only passes soluble antigen. It is also possible to test organ homogenates.

**Interfacial precipitation in liquid medium** (94) provides results within 2-10 minutes, but it requires completely translucent samples if the precipitation ring is to be seen.

These two tests are easy to perform and very reliable, and the sole requisite is a good precipitating serum. A positive reaction provides proof of infection.

Circulating antigen is most likely to be detected 6-10 weeks after the onset of the disease. Beyond this period, and particularly in chronic carriers, the frequency of detection diminishes sharply (24).

**Tests for antibodies**

**Slide agglutination test:** This is the quickest way of detecting antibodies when performed with stained antigen (60, 61). A result, of qualitative or semi-quantitative status, is obtained within 2 minutes or less, and is expressed by a number of plus signs (78, 96).

Interpretation is easiest when serum is used. But since the antigen contains anticoagulant, whole blood may also be used, and so this test is eminently suitable for field use (47, 78).

The reliability of the results is excellent during the acute stage of the disease, but it declines rapidly when the disease enters a chronic stage (24), for large amounts of circulating immune complexes are present during this stage (95).

Nevertheless, the test retains its value when applied on the herd scale, for an infected herd contains animals at all stages of the disease; it should not be used to test individual animals.

**Complement fixation (CF) test:** Numerous techniques have been described and are still more or less in use, while the technique of Campbell and Turner (8) is a favourite among users, and is the procedure recommended by the OIE. It is not very sensitive, and so results that are either doubtful or difficult to interpret are rarely obtained. It is also very specific, so that false-positive results are extremely rare (19).

Antibodies are detectable by using the standard technique, from about 10 days after the onset of the disease and during the subsequent few months. During the clinical stage of the disease the rate of detection is reliable, and practically no sick animal will give a negative result. However, for animals entering the chronic stage the percentage of false-negative results increases (24, 96).

In countries where vaccination is practised, a presumptive diagnosis of CBPP cannot be made, because the CF test can give positive results for 3-6 months after vaccination, owing to the presence of vaccinal antibodies (38). CBPP occurs often in countries where traditional husbandry takes the form of nomadic herds, which
are difficult to control. This created an incentive to develop a technique for use under field conditions (28, 81). However, the best working conditions can be fulfilled only in a laboratory, for the CF test is still difficult to perform, requiring well-trained personnel.

Certain workers have tried to make the test more sensitive (48, 82), but this has the disadvantage of increasing the false-positive reactions.

**Immuno-enzyme techniques (including ELISA)** are inherently very sensitive and are already being used for the diagnosis of CBPP (65). The automation of each step, including reading the result, makes them readily reproducible, provided that all the components are strictly defined and tested.

Whereas the CF test detects mainly the anti-galactan antibodies, enzyme immunoassay has the advantage of detecting all antibodies to CBPP when the antigen consists of a lysate of *M. mycoides*. Despite this difference, there is good correlation between the results of the two techniques (46).

Another potential advantage, which has not yet been demonstrated, would be to improve the detection of chronic cases, thanks to the high sensitivity of the method, capable of detecting traces of antibody.

The results obtained with negative serum samples vary according to whether the samples come from an endemic zone or not. Consequently, the threshold for positivity has to be determined carefully and adjusted if necessary. This represents an obstacle to standardisation.

**Passive haemagglutination** (9, 12, 70) and the *latex particle agglutination test* (69) have also been applied to the detection of antibodies in CBPP. When the sensitising antigen is a lysate of whole organisms, the erythrocytes have to be pretreated with formaldehyde or glutaraldehyde. On the other hand, when galactan antigen is used, this becomes adsorbed to the surface of erythrocytes spontaneously.

Use of the last-named antigen provides good correlation with CF titres. Nevertheless, the sensitivity threshold is always difficult to establish, because non-specific cross-reactions occur with low dilutions of serum. The technique is therefore not recommended for diagnosis, and is useful solely in the study of particular serum samples.

No test is capable of detecting infected animals at every stage of the disease. However, the CF test has already proved valuable in the eradication and control of CBPP. It is still the reference method, because the materials and methods are fully standardised (15).

If, after applying various techniques, one tests the remaining negative sera for circulating antigen and then by an immuno-enzyme assay, very few infected animals will escape detection.

* * *
APPENDIX
Chapter IV – Diagnosis

Staining colonies of mycoplasma

Dienes's method

Composition of the stain:

- Methylene blue: 2.5 g
- Azur II: 1.25 g
- Maltose: 10.00 g
- Sodium carbonate: 0.25 g
- Benzoic acid: 0.2 g
- Distilled water to 100 ml

1. The stain is spread on cover slips measuring 24 × 24 mm, which are then allowed to dry.
2. The slips are each cut into 4 squares by using a diamond knife.
3. Blocks of agar (bearing colonies) are cut out and placed on slides, with the colonies uppermost.
4. A portion of stain-coated glass slip is applied to each agar block, with the stain face down. The glass should overlap the edge of the agar.
5. The space between slide and cover slip is filled with molten paraffin wax containing 10% yellow soft paraffin. Upon cooling, the paraffin seals the preparation and makes it possible to examine under oil immersion lens without displacing the cover slip.
6. Staining is normally completed within a few minutes, but it is advisable to wait for 10-15 min.
7. If the agar is too thick or too opaque, it should first be pared by using a razor blade.
8. The stain may be diluted if the agar takes up too much stain.

Culture Medium Bacto-Tryptose ‘Difco’

- Beef heart infusion: 850 ml
- Bacto-Tryptose ‘Difco’: 20 g
- Bacto-yeast extract ‘Difco’: 6 g
- Glucose: 2 g
- Sodium chloride: 5 g
- Anhydrous disodium phosphate: 2.5 g

The above constituents are sterilised by heat.

- Horse serum: 150 ml

Optional additives:

- Thallium acetate 10% soln: 1.5 ml
- Penicillin: 200,000 IU
Notes:
The pH must be adjusted to 7.6-7.8.
Heart infusion is prepared in a glass vessel, with demineralised water as used for cell culture.
Solid medium is prepared by incorporating 10 g of agar ('Ionagar' Oxoid) into the sterilisable fraction.
Liquid media are best sterilised by passing the complete medium through a filter.
Such medium can be enriched by increasing the proportion of horse serum (up to 20%) and adding fresh yeast extract (5-8%).

Mode of use
For bovine, caprine and ovine mycoplasmas, including both subspecies of *M. mycoides*.

**DE Medium**
(from the Institut d'Elevage et de Médecine vétérinaire des Pays tropicaux, Maisons-Alfort)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto PPLO broth 'Difco'</td>
<td>21 g</td>
</tr>
<tr>
<td>Bacto-Tryptose 'Difco'</td>
<td>10 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>1 g</td>
</tr>
<tr>
<td>Bacto yeast extract 'Difco'</td>
<td>5 g</td>
</tr>
<tr>
<td>Demineralised water</td>
<td>700 ml</td>
</tr>
</tbody>
</table>

The components are dissolved by warming, preferably not above 80°C. This fraction is sterilised by heat. The following are added to it:

- 25% extract of fresh yeast 100 ml
- Horse serum 200 ml
- Penicillin 200,000 IU
- 10% thallium acetate solution 1.25 ml

Adjust the pH to 7.6-7.8 and sterilise by filtration.

*Note:* Solid medium is prepared by incorporating 10 g agar ('Noble' Difco or 'Ionagar' No. 2 Oxoid) into the sterilisable fraction (700 ml).

**HIB (Heart Infusion Broth) Medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart infusion broth 'Difco'</td>
<td>25 g</td>
</tr>
<tr>
<td>Neopeptone 'Difco'</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Bacto-Casitone 'Difco'</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>2 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>700 ml</td>
</tr>
</tbody>
</table>

The constituents are dissolved by heating to 80°C. Add:

- 25% extract of fresh yeast 100 ml
- Horse serum 200 ml
- Penicillin 200,000 IU
- 10% thallium acetate solution 1.25 ml
The pH is adjusted to 7.6 and the medium is sterilised by filtration.

Solid medium is prepared by incorporating agar into the sterilisable constituents, as above, or 'Difco' heart infusion agar may be used.

The two preceding media are used for culturing various species of mycoplasmas. In the case of *M. mycoides* or other mycoplasmas which are easy to grow, the proportion of serum can be reduced to 15 or 10%.

**Media for biochemical tests and inhibition of metabolism**

(From details supplied by the Animal Mycoplasmas Reference Centre, University of Aarhus, DK-8000 Aarhus C, Denmark)

**Glucose hydrolysis**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart infusion broth 'Difco'</td>
<td>180 ml</td>
</tr>
<tr>
<td>Horse serum</td>
<td>40 ml</td>
</tr>
<tr>
<td>25% fresh extract of yeast</td>
<td>20 ml</td>
</tr>
<tr>
<td>0.2% DNA solution</td>
<td>2.6 ml</td>
</tr>
<tr>
<td>50% glucose solution</td>
<td>1 g (dry) or 2 ml</td>
</tr>
<tr>
<td>Phenol red (Int. Pharm.)</td>
<td>5 ml</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>7.8</td>
</tr>
</tbody>
</table>

**Arginine hydrolysis**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart infusion broth 'Difco'</td>
<td>180 ml</td>
</tr>
<tr>
<td>Horse serum</td>
<td>40 ml</td>
</tr>
<tr>
<td>25% fresh extract of yeast</td>
<td>20 ml</td>
</tr>
<tr>
<td>0.2% DNA solution</td>
<td>2.6 ml</td>
</tr>
<tr>
<td>38% L-arginine HCl solution</td>
<td>2.5 g (dry) or 8.5 ml</td>
</tr>
<tr>
<td>Phenol red (Int. Pharm.)</td>
<td>5 ml</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>7.3</td>
</tr>
</tbody>
</table>

**Tetrazolium reduction**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart infusion broth 'Difco'</td>
<td>180 ml</td>
</tr>
<tr>
<td>Horse serum</td>
<td>40 ml</td>
</tr>
<tr>
<td>25% fresh extract of yeast</td>
<td>20 ml</td>
</tr>
<tr>
<td>0.2% DNA solution</td>
<td>2.6 ml</td>
</tr>
<tr>
<td>2% triphenyltetrazolium chloride</td>
<td>5 ml</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>7.5</td>
</tr>
</tbody>
</table>

Note: All these media are sterilised by filtration.

The usual inhibitors of bacteria (penicillin, thallium acetate, etc.) may be added.

Addition of DNA is not indispensable for mycoplasmas which are easy to grow or are well adapted to artificial media.

**Testing the proteolytic activity of mycoplasmas**

The standard procedure uses a medium containing coagulated horse serum.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic medium ('Difco' PPLO broth)</td>
<td>20 ml</td>
</tr>
<tr>
<td>Horse serum</td>
<td>75 ml</td>
</tr>
<tr>
<td>Fresh extract of yeast</td>
<td>2 ml</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>3 ml</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>7.8</td>
</tr>
</tbody>
</table>
Distribute 5 ml portions into 16-18 screw-capped tubes. Place at a slope and coagulate by heating to 90°C for 45 min.

Leave overnight at room temperature or in a refrigerator.

Pour off the water of condensation, screw down the stoppers and store at 4°C.

A copious sowing is made onto the surface of the medium, preferably by streaking with a young culture (24-48 h).

Proteolysis is manifested by the formation of a gutter along the streak, the medium becoming soft and transparent. Eventually fluid resulting from digestion accumulates at the bottom of the tube.

The phenomenon may appear after a week, but it is advisable to keep the tubes in the incubator for a month.

**Test for phosphatase activity of mycoplasmas**

This is a very important biochemical test.

The enzyme activity may be examined in solid or liquid media.

- Basic agar medium (may be omitted) 74 ml
- Heated horse serum 20 ml
- Extract of fresh yeast 5 ml
- Penicillin at 200,000 IU/ml 0.2 ml
- 1% thallium acetate solution 1 ml
  (final dilution 1:10,000)
- 1% Na phenolphthaleine diphosphate 1 ml
  (final dilution 1:10,000)
- pH – adjusted to 7.8

After the culture has grown (3-4 days), phosphatase is revealed in solid medium by the immediate appearance of a pink to dark red colour of the colonies when a few drops of an alkaline solution (38-40% NaOH or KOH) are placed onto the colonies.

In liquid medium the same colour develops under the same conditions, but it disappears rapidly, so that the result has to be read immediately.

*Notes:* The horse serum must be depleted of complement by heating to 60°C in order to remove serum phosphatases.

Inhibitors of bacteria are not required if the preparation is reasonably free from other bacteria.

The colour indicator is available commercially as ‘Sigma’ (distributed by OSI).

**Preparation of yeast extract**

1. Prepare a homogeneous suspension of 450 g fresh yeast (obtained from a baker) in 1,800 ml of demineralised water.

2. Adjust the pH to 4.5 with hydrochloric acid and heat to 80°C for 38 min, shaking frequently.
3. Centrifuge, clarify and sterilise by filtration through an EKS disk, after readjusting the pH to 7.6-7.8. Freeze at −20°C if not required immediately.

4. Add to media at 100 ml per litre.

**Transport Medium**

This is composed of heart infusion broth without peptone and without glucose, to which is added 10% yeast extract, 20% serum and 3% agar. It is poured into screw-capped tubes 100 x 10, with penicillin (500 IU/ml) and 1:7,000 thallium acetate.

The swabs are inserted in as sterile a manner as possible, and their ends are cut off. Samples can be kept in this way for several days.

The tubes are placed in an incubator for 24-48 h before taking the first sample from them.

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**V. PROPHYLAXIS**

**EUROPE**

**INTRODUCTION**

Feared during antiquity, CBPP was a scourge of European cattle during the 18th and 19th centuries.

The breeding-ground of infection in Europe was the western part of the Alpine range (Bavaria, Württemberg, Switzerland, Tyrol and Northern Italy). It seems to have appeared around 1713, extending during the 18th century through most of Europe, where it occasioned considerable losses, and also led to the establishment of the first public, scientific establishment created for the control of an infectious disease – the Lyons Veterinary School, founded by Bourgelat in 1762.

The first regulations for eradication by slaughter date back to 1773, under the signature of Albert de Haller of Berne. Irrefutable proof of the transmissible nature of the disease was not obtained until 1854 by a French Ministerial Committee which started work in 1850 with Bouley as Rapporteur (63).

By the end of the 19th century, most of the foci had been extinguished. These first major successes were attained by systematic application of disease control measures and the slaughter of affected and exposed animals, implemented on this occasion by means of special laws.

The outbreaks of CBPP detected in the Pyrenean area in 1967 and then in 1980, 1982 and 1984 showed that the disease had not been completely eliminated from Europe, and particularly from the Iberian Peninsula. An extension of these outbreaks is still to be feared, as demonstrated by the development of the infection in northern Portugal since January 1983. A Working Group has been formed by the Commission of the European Communities, and its first meeting was held in Brussels on 16-17 June 1983, to examine ways of protecting the European cattle population and to initiate epidemiological surveillance.
THE THEORETICAL PRINCIPLES OF DISEASE PREVENTION

Under European conditions, treatment and vaccination are completely contra-indicated, and only the disease control procedure of eradication is applicable. This conclusion is based on three essential considerations:

*Mycoplasma mycoides* subsp. *mycoides*, the agent of CBPP, is strictly a parasite of domesticated bovines, which constitute the only known reservoir of infection.

Its contagiousness is neither high nor subtle. Direct infection by the airborne route precludes any indirect or distant transfer. Close contact, repeated and prolonged, is needed for transmission to occur under natural conditions.

In Europe the disease is seldom fatal. A certain proportion of animals which have apparently recovered become chronic carriers through encapsulated lung lesions, which ensure perpetuation of the infection. Chemotherapy, including antibiotic therapy, may give excellent results from the clinical standpoint, but it does not sterilise the infection and favours the creation of chronic carriers. A lung sequestrum may open into a bronchus, and then a carrier becomes an excretor and disseminator of infection.

One of the most important protective measures against CBPP is the use of laboratory tests to detect asymptomatic carriers.

PRACTICAL MEASURES IMPLEMENTED

In a disease-free area, protective measures must be taken to avoid establishing sources of infection, with prohibition of imports of live cattle from countries known or suspected of being infected (intertropical Africa, Middle East, Asia).

Any permitted derogation must be accompanied by rigorous surveillance, with certification that the animals have come from a disease-free area, and that they are in good health, together with quarantine and two serological (CF) tests conducted a month apart (method of Campbell and Turner).

In a region at risk of infection (adjoining an infected region), these protective measures must be reinforced by controlling all movements of cattle, in particular clandestine crossings of frontiers. Investigation of the new cases of CBPP which appeared in northern Portugal (between the Douro and Minho rivers) in January 1983 revealed that the source of infection was a cow introduced illegally from Spain.

Transhumance of cattle must be controlled rigorously, and common grazing land closely surveyed. Outbreaks which occurred in the Pyrenean zone between 1980 and 1984 were all associated with mixing of herds at mountain pastures during the summer. A Franco-Spanish Veterinary Committee met at Perpignan on 12 July 1982 in order to exchange information on the general disease situation on each side of the frontier, and resolved to strengthen the health guarantees required for cattle entering international grazing land. Such cattle can only come from herds free from CBPP, and have to be negative to the complement fixation test for CBPP. Serological testing must be repeated upon return from summer grazing.

In a region infected accidentally, immediate steps have to be taken to detect and neutralise new foci.
Epidemiological vigilance has thus been increased in southwestern France. All herds using international summer grazing undergo two serological tests before departure to the mountains at the beginning of May, and after their return in October. Practising veterinarians have been asked not to treat animals with suspicious clinical signs until the possibility of CBPP has been eliminated by laboratory tests. Finally, the meat inspection service has been asked to pay special attention to possible CBPP lesions, particularly chronic lesions, with recourse to laboratory testing in doubtful cases to establish their aetiology.

Immediate slaughter of animals found to be infected and those exposed to infection is an essential step, which implies complete understanding on the part of the owners, assisted by adequate compensation.

The premises concerned are placed under an infected premises order, and an observation zone is specified, taking into account local geography and the risk of contamination from the focus. Prohibition of movement from the observation zone is maintained until there have been three consecutive negative serological tests, carried out at monthly intervals.

The measures have proved to be very effective on each occasion when they have been applied rigorously and rapidly within a country provided with a well-structured animal health service and a system of cattle farming which is easy to monitor. Difficulties encountered in overcoming the last endemic in northern Portugal have been ascribed to a collective system for the machine milking of dairy cows.

Among the investigation techniques, the complement fixation test plays an important part, particularly for detecting asymptomatic carriers.

The European Working Group, meeting in Brussels in 1983 (28), recommended that priority be given to harmonisation within the European Communities of the Campbell and Turner test (8), modified in order to provide a simpler micro-method.

The quality of results obtained in twelve laboratories representing nine European countries (15) was very satisfactory. The results could be interpreted by the criteria established by Campbell and Turner (8). A very small proportion of false-positive results does not diminish the reliability of the test, but illustrates a serological relationship between \( M. \text{mycoides} \) subsp. \( mycoides \) and other mycoplasmas which can infect cattle (18).

A precise response to this problem would help explain the rare instances when a positive test is given by cattle known to be free from CBPP.

AFRICA

The control measures which have led to eradication of CBPP from numerous countries, and which are still being applied in Europe, are impracticable in Africa for the following reasons.

Systematic serological detection of infected cattle and cattle harbouring lung sequestra is impossible in regions where extensive husbandry is the main type of cattle keeping.
There is no question of being able to prohibit the movement of herds, or to slaughter infected animals.

Consequently, recourse must be made to an alternative policy of prophylaxis. In Africa this entails the annual vaccination of cattle.

THE BASIS OF VACCINE PROPHYLAXIS

Animals which have recovered possess a reliable immunity to reinfection. The immune processes during and after infection with *M. mycoides* subsp. *mycoides* are only partly understood. It is practically certain that humoral and cellular immunity are both involved, but little is known of the true nature of antibodies responsible for humoral immunity. It is probable that a serum antibody, detectable by passive protection of mice, plays a certain role (90). It is known that protection coincides with the presence of mycoplasmas in ganglionic sites (nerve ganglion and organic lymph nodes) (54).

For vaccination to be effective, it is imperative that mycoplasmas multiply within the body, probably bringing about a type of premunity.

For reasons of convenience, the route of inoculation used most often is subcutaneous, into the loose connective tissue posterior to the scapula, and this means that the residual pathogenicity of vaccine strains must be as low as possible in order to avoid serious post-vaccinal reactions (Willems's reaction).

Since there is no reservoir host among wild animals, the only species involved in vaccination are cattle and zebu.

VACCINES AGAINST PLEUROPNEUMONIA

Numerous types of vaccine (inactivated, live, egg-culture, etc.) have been used, together with many attenuated strains. At present only live vaccines are used, consisting of fluid cultures of two strains, strain T₁ and strain KH₃J.

Strain T₁ is the most virulent, particularly for breeds of cattle, but also for certain zebras (determined solely by local experience). Its immunogenicity is excellent, however, and resistance persists for at least a year, though it will not be absolute for all members of a vaccinated group.

**Strain T₁ pleuropneumonia vaccine**

*History of the strain*

This strain was isolated in 1951 by Sheriff and Piercy from a clinical case of CBPP in Tanzania. It has remained only moderately virulent since its isolation.

Vaccines are prepared from broth cultures, using the 44th egg passage of the strain.

Tens of millions of doses of this vaccine, labelled T₁/44, have been prepared. However, it is well known that vaccines in liquid form are difficult to use because they are bulky to store and distribute. They have to be kept refrigerated constantly, including during transport to the place of vaccination. Despite such precautions, their storage life is limited to one month after preparation.
For this reason, the possibility of freeze-drying was investigated and adopted by the veterinary laboratories at Farcha and Dakar, and subsequently implemented at Debre-Zeit, Bamako and Niamey.

At the same time research was undertaken into the possibility of combining strain T₁ with cell-culture rinderpest vaccine, to produce a combined vaccine. Since streptomycin is present in rinderpest virus culture fluid, strains of T₁/44 resistant to streptomycin were produced by the IEMVT Laboratory at Maisons-Alfort, and also at Farcha. The Maisons-Alfort mutant (strain T₁-SR), obtained after 3 passages in the presence of increasing concentrations of streptomycin, was investigated for its immunogenicity at the Dakar Laboratory.

Thus two strains of T₁ became available, the original T₁/44 and strain T₁-SR, which had undergone additional passages in broth. Their immunogenic properties were identical, but T₁-SR seemed to result in fewer local, post-vaccinal complications among cattle breeds. There was a slight difference between them when protoplasmic proteins were examined by electrophoresis in polyacrylamide gel (disparity of bands in the central part of the pattern). Strain T₁-SR was considerably dependent upon streptomycin, in the sense that the yields from cultures containing this antibiotic were 10 to 1,000 times greater in terms of viable units than from cultures without streptomycin.

**Production techniques**

These differ according to whether the vaccine is to be delivered in liquid or freeze-dried form.

**a) Liquid vaccine (3)**

A number of cultures per litre are sown with strain T₁/44, available in freeze-dried form from egg culture, after two passages in serum broth. The material for sowing is provided by two further subcultures, one of them done in terminal dilutions, followed by sowing into flasks.

10 ml of culture per litre are sown into 20 litres of serum broth filtered through disk (Gourlay's medium), then the mixture is distributed into flat bottles of 200 ml capacity (medicine bottles). The bottles are plugged with cotton wool and incubated for 3 days at 37°C. During incubation the rate of growth is monitored by biochemical assay of lactate dehydrogenase in samples of culture fluid.

When the growth phase is completed, the bottles are sealed with rubber stoppers, labelled and stored at 4°C. The expiry date is 38 days after stoppering. During the first month it has been calculated that there is a fall in titre of only 1 log₁₀ viable units, provided that the culture is always kept cold.

**Testing**

Two days after sealing the following tests are performed on a sample of the batch:

- bacterioscopic examination against a dark background, and after Gram staining;
- sowing onto ordinary broth, thioglycollate broth (incubated anaerobically) and blood agar;
- 0.25 ml is inoculated into the peritoneal cavity of each of 3 mice.
These tests for bacterial contamination and pathogenic organisms must be negative. The density of viable units is estimated by culturing decimal dilutions of the vaccine, either in tubes of serum broth or on serum agar. Statistical calculation gives the number of viable units per ml.

The minimum titre is $5 \times 10^8$ viable units per ml of vaccine, which means that during its period of validity, a vaccine dose of 0.5 ml contains a minimum of $10^7$ viable units.

Efficacy testing cannot be performed for each batch in view of the time required (3 months, which is 2 months longer than the period of validity) and the cost (36 three-year-old cattle). It is done only occasionally, mostly for research purposes.

**b) Freeze-dried vaccine**

The same starting strain ($T_1/44$) is used, and culture flasks are sown as before. For production of a combined vaccine, strain $T_1$-SR is used.

Two passages in fluid medium (containing 200 µg streptomycin per ml if strain $T_1$-SR is used) provide the conditions for logarithmic growth. Five litres of F-66 medium (78) are placed in a 10-litre flask provided with a magnetic stirrer. The medium is not stirred for the first 24 h at 37°C, then stirring commences. The culture is harvested after 72 h. There is not advantage in prolonging culture, even by a period of refrigeration, for subsequent processing.

When strain $T_1$-SR is used, streptomycin is added to the culture medium at 200 mg/l. This improves the growth of the strain both in rapidity (peak growth after 65 h) and in viable units, a density of $10^{10}$/ml being obtained frequently.

At harvesting, dried skimmed milk is added at 45 g/l. 20 ml bottles are each filled with 5 ml of vaccine and it is freeze-dried. Certain aspects of freeze-drying will be mentioned later. The bottles are stoppered and sealed under vacuum or under nitrogen.

Each bottle contains 20 doses. For use the vaccine is reconstituted with cold distilled water or a molar solution of magnesium sulphate (248 g/l) at room temperature – this molar solution is a remarkable protectant for mycoplasmas against inactivation by heat.

Freeze-dried vaccine is stored at $-20^\circ$C, and its storage life is at least a year. It is usually dispatched in insulated containers with ice cubes. Storage at the secondary point of distribution has to be at $-18^\circ$C. From there vaccine may be transported in an insulated box with ice cubes, or more simply wrapped in a moist cloth, suitable for a few days.

**Testing**

This is the same as for liquid vaccine. The minimum titre is $10^7$ viable units per dose of vaccine at the moment of inoculation, which demands a much higher titre at the time of testing during production. In practice the vaccine contains at least $10^{9.7}$ units.

**Strain $\text{KH}_3J$ pleuropneumonia vaccine**

**History of the strain**

This is obscure. It is known to have originated from Juba (Sudan), but its original virulence is unknown. It was subjected to frequent subculturing at the Khartoum
Laboratory until, in 1948, it was imported by the Vom Laboratory in Nigeria, together
with other Sudanese strains.

The strain has some special cultural characteristics. When grown in broth it forms
the smooth type without the filamentous form. On agar it produces two types of
colonies, one of which is a large colony with the central raised area characteristic
of \textit{M. mycoides} colonies and surrounded by a wide, clear peripheral zone. The colony
type is unstable, and either type can give rise to one or other colony type in the next
generation. They are of equal antigenicity.

Vaccine is currently prepared by fluid culture of the 88th generation of the strain
in egg culture.

As in the case of strain \textit{T}_1, a streptomycin-resistant variant has been developed
(variant \textit{KH}_3\textit{J-SR}) at the Farcha Laboratory for the preparation of the first mixed
vaccine against rinderpest and CBPP.

There is also an 'Australian' variant of this strain, obtained from the 85th
generation, followed by four consecutive passages in cattle (being recovered from
lymph nodes), with a minimum of subculturing between passages. The fourth passage
represents approximately the 90th generation.

\textit{Production techniques}

\textit{a) Liquid vaccine}

The production technique is identical with that of strain \textit{T}_1. Several years ago
the Australian variant was being used.

The cultures are very rich, for the titre may reach $10^{12}$ viable units per ml (13).
Preservation is the same as for strain \textit{T}_1, except that it is recommended for use
within a few days after production, because it does not remain stable for long.

\textit{b) Freeze-dried vaccine}

The procedure is the same as that used for strain \textit{T}_1, with or without
incorporation of streptomycin in the medium, according to the strain used. Dried
skimmed milk is added before freeze-drying.

The minimum titre required after freeze-drying is $10^8$ per vaccine dose of 1 ml
at the time of its use, though owing to the richness of the cultures, the actual titre
is usually $10^9$ or even $10^{10}$.

\textit{General techniques of inoculation}

Problems associated with storage and dilution of freeze-dried vaccines will be
discussed later. This section relates to the general rules for inoculating the vaccines.

\textit{Strain} \textit{T}_1: In English-speaking countries of Africa, the dose of these vaccines is
0.5 ml, injected under the skin of the tail tip.

In French-speaking countries, zebu are injected subcutaneously in the upper part
of the posterior scapular region with 1 ml of vaccine. Vaccination policy for cattle
breeds is not entirely standardised, but most users prefer the subcutaneous costal site
(50, 68).

\textit{Strain} \textit{KH}_3\textit{J}: 1 ml subcutaneously, regardless of breed.
General considerations on post-vaccinal reactions

*Strain KH.* No local reaction of the Willems type has been recorded, even after quite large amounts have been injected beneath the skin. Serological conversion is rare - it is neither early, high nor prolonged.

Nevertheless, mycoplasmas do appear in the blood for a brief period after vaccination, and can be recovered during two months from lymph nodes and from lung tissue (49).

*Strain T.* Mycoplasmas also appear briefly in the blood, and subsequently the organism or its antigens can be demonstrated in various tissues (lymph nodes, lung, spleen, kidney) for up to 204 days after vaccination (54).

Serological conversion (development of CF antibody) takes place in a considerable proportion of animals, varying from 30 to 100% according to different authors, commencing at about 8 days and culminating at 21 days; the antibody disappears 2 months after vaccination. Upon revaccination 12% of animals may show a fresh upsurge of antibody.

Local reactions vary in incidence, intensity, species (cattle or zebu) and vaccination site. They are entirely unpredictable when cattle are vaccinated for the first time, though they are usually most severe in infected herds.

Reactions in zebu. After vaccination into the tail, as practised in East Africa, 1% of the animals develops a local reaction characterised by erythema and swelling (of variable intensity) extending along some 12 cm of the tail. In some animals the oedematous reaction extended along the entire tail and into the gluteal and perineal regions, ending fatally. Some cases of oedema terminated in necrosis with loss of the tail tip.

After subcutaneous retroscapular vaccination, 3 or 4% of animals develop a transient local reaction between 3 and 30 days afterwards. The reaction is oedematous at first, then the swelling becomes firmer, and may undergo necrosis in some cases. Fatal generalisation is rare.

The occurrence of local reactions should be acknowledged, but it should not be an obstacle to vaccination. However, when vaccinating a given bovine population, it is wise to test their general sensitivity first by vaccinating sample groups (10, then 100, then 1,000) before undertaking large-scale vaccination (20).

Reactions in cattle. The reactions are more severe than in zebu in both number and intensity. After caudal vaccination about 25% develop a reaction of variable intensity, sometimes with ulceration and necrosis, leading in severe cases to death or loss of the tail. In the Guinean zone, loss of the tail can have serious consequences because it helps to protect the animal against the numerous blood-sucking flies.

Reactions over the ribs are just as frequent, if not more so, and the consequences may be severe: necrosis, ulceration, secondary infection and myiasis.

Under such circumstances it is necessary to treat cattle which develop reactions to the vaccine. Such treatment may be given during the visit which is usually made about 20 days after a herd of cattle has been vaccinated with strain T, using tylosin or spiramycin. Finally, there is a risk that vaccination may reactivate a latent case of pleuropneumonia.
THE PRACTICE OF VACCINATION AGAINST PLEUROPNEUMONIA

The research worker developing highly active vaccines against CBPP must remain attuned to the requirements of the users of the vaccine, and to implementation of vaccination campaigns and analysis of the results. In the end, it is the result which counts.

Comments on the choice, storage, distribution and use of vaccines

Choice of vaccine strain

The need for harmonisation and standardisation has led to the exclusive choice of strains T_1 and KH_3J for vaccination campaigns. There is no doubt that the immunogenicity of the former is better than that of the latter strain, and it is the most extensively used. It appears, however, to elicit local complications, more so among cattle than zebu, an opinion supported by field evidence rather than by laboratory testing. Moreover, as already mentioned, vaccination complications are unpredictable, sometimes affecting one group of zebu while their neighbours are unaffected. Consequently the planning of a vaccination campaign against CBPP must include, in cooperation with the vaccine producer, a test of the sensitivity of the group or groups of animals to be vaccinated, performed under different ecological surroundings in order to detect differences which might exist between herds of the same species or even of the same breed, but kept under different conditions.

In the case of zebu, sufficient experience has now accumulated to show that strain T_1 is of universal application, and any post-vaccinal reaction which does develop can be treated by antibiotics 20-25 days after vaccination.

A similar policy is tempting for cattle, and it has been systematically practised in Côte d'Ivoire and Togo (50, 68). Nevertheless, recent research (99) has shown that when cattle are treated with tylosin a month after vaccination, 25% are incapable of resisting a subsequent challenge infection. This observation is readily explained by the necessity for mycoplasmas to survive in lung tissue or lymph nodes (54) in order to elicit resistance to infection.

In view of this problem, the procedure cannot be recommended for routine use in vaccination campaigns. It would seem to be preferable to inoculate strain KH_3J twice a year in the case of cattle, at least those of African breeds, for little is known about the responses of cattle of European breeds. Obviously the problem of immunising African cattle against CBPP has not yet been solved in a completely satisfactory manner.

Preservation of vaccines

Fluid vaccines

It is agreed that fluid CBPP vaccines are fragile. Exposure to a temperature above 45°C inactivates them (37). Their half-life is about 10 days at 4°C, which means that they have to be stored at this temperature (and shielded from sunlight) until use. They can be used up to 30 days after preparation, provided they have been kept cool all the time.

Consequently, the countries which have adopted this type of vaccine have had to arrange for a 'cold chain' between the laboratory and the vaccination site, by using
insulated containers capable of holding 14 bottles (5,600 doses of 0.5 ml) and intermediate refrigeration. Vaccine is dispatched from the laboratory 5 days after sealing, often before the results of microbiological testing become available. Despite these handicaps, the users of this method prefer fluid vaccine because of difficulties encountered in freeze-drying.

Freeze-dried vaccines

There is no scientific obstacle to the correct freeze-drying of mycoplasmas. The failures experienced by some workers (40, 67) might be due to an imperfect knowledge of the production technique, which requires prolonged lyophilisation during which the vaccine is not exposed to temperatures above freezing point until the residual moisture content falls to 3%. This operation can be performed only with the plate type of freeze-drier, and not with the centrifuge system. Under these conditions, and with a good stabiliser, the loss in viable units does not exceed $1 \log_{10}$ — in other words, with a harvest of up to $10^{10}$ viable units per ml, it is possible to prepare a very potent freeze-dried vaccine which is thus much easier to store.

When freeze-drying is conducted in such a way that the residual moisture amounts to 1.5%, the half-life of the vaccine is 4 days at 45°C, 1.6-2.8 weeks at 37°C, 2.8-4.6 weeks at 28°C and 30-40 weeks at 4°C.

These figures mean that a vaccine which contains, when tested during production, a concentration of $10^8$ viable units per dose (which is on the low side, encountered in batches of only moderate quality) can be kept for 2-4 months at 28°C, 1.5-3 months at 37°C and 12-14 days at 45°C (16). As a safety measure, to take account of the cumulative effect of thermal inactivation under different temperature conditions to which the vaccine may be exposed during storage, these times may be halved, yet the benefits of freeze-drying for the vaccinator and the cattle vaccinated remain convincing.

By continuing freeze-drying beyond the 1.4% moisture limit (at the cost of additional expenditure of electrical energy), resistance to heat is enhanced, and the storage life at 37°C is practically doubled (16).

Attempts have been made to improve this heat resistance further by refinements in stabiliser and freeze-drying procedure, and by phenotypic selection of variant vaccine strains which are more resistant to inactivation at 45°C. At present it is clear that a combination of techniques for producing high culture yields of *M. mycoides* (by adding streptomycin to the culture medium) and careful freeze-drying technique have resulted in the practical solution which the user has been looking for: a CBPP vaccine which can be stored without taking special precautions.

It remains necessary to ensure that bottles of vaccine are never exposed to direct sunlight. An elementary and easy precaution is to keep the bottles packed in wood shavings or wrapped in moist cloth; evaporation has a cooling effect and the necessary protection from the sun’s rays is achieved.

Use in the field and reconstitution of freeze-dried vaccines

Certain rules applicable to biological materials need to be reiterated because they are sometimes overlooked.

- Only use injection equipment (syringes and needles) which has been sterilised by boiling, and not by antiseptics (which are harmful to *M. mycoides*).
- Only use cooled injectable fluids, particularly in the case of fluid vaccines.
- Use a syringe no larger than that required to inoculate about ten animals.
- As an additional precaution, especially under high ambient temperatures, surround the syringe with moistened cotton or cloth in order to maintain its coolness.
- Use up a bottle of vaccine within half an hour. It is inadvisable to try to economise by replacing left-over vaccine in the cold container, to be used later.

It is vital to observe the manufacturer’s recommendations for dose and inoculation site.

There is still the problem of correct reconstitution of freeze-dried vaccines, and two examples have been selected.

(1) The user possesses mobile refrigeration equipment (insulated container filled with ice cubes, or a portable refrigerator) which enables the diluent (either distilled water or saline solution, according to the manufacturer’s instructions) to be cooled before reconstitution. Thus no special precautions have to be taken, provided that the reconstituted vaccine is diluted so as to yield the adequate number of organisms per vaccine dose, and that it can be kept cold in between the loading of syringes. Any increase in temperature of the vaccine could be particularly harmful.

One must never succumb to the temptation of using surface water, even after filtration, boiling and cooling, to dilute the vaccine. Such water is likely to have a pH adverse to the good survival of mycoplasmas. Similarly, tap water should never be used, because in towns it may be strongly chlorinated and therefore harmful to mycoplasmas.

(2) The user does not possess mobile refrigeration equipment. This is a common occurrence in Africa and it is precisely for this reason that a refined but simple technique has been developed for the field use of freeze-dried vaccines. In most cases the vaccines are transported with no special precaution other than packing in shavings or wrapped in damp cloth. This means that the vaccine diluent is at ambient temperature or thereabouts. Under such conditions, assay of viable units would reveal sudden inactivation when the vaccine is reconstituted with distilled water or normal saline, followed by thermal degradation depending on the diluent (being most pronounced with water) and temperature. By contrast, reconstitution and dilution of freeze-dried vaccine by a strongly hypertonic fluid medium provides remarkable thermal stabilisation (79). Many electrolytes have been tested (sodium chloride, sodium sulphate, magnesium chloride, magnesium sulphate). The best results were obtained with molar concentrations of magnesium sulphate, since it also has a thermoprotective effect for rinderpest live vaccine, which is widely employed in the mixed vaccine against CBPP and rinderpest.

The procedure is very simple. The user prepares in advance 25 g sachets of magnesium sulphate (MgSO₄·7H₂O), a stock of distilled water (not water straight from the tap) and some 100 ml bottles. The water can be transported to the vaccination site, like the vaccine, without special precautions. At the site the user makes up a molar solution by adding a 25 g sachet to an empty 100 ml bottle, followed by water to make up 100 ml of solution. This is a 1 M solution of MgSO₄ which can be used immediately to reconstitute the vaccine. Reconstituted vaccine may be kept for 2 h at 45°C, but in practice it is best to discard any remaining at 30 min after reconstitution.
Magnesium sulphate is cheap, and there are no safety hazards in handling it. Thus the procedure is economical in comparison with the manufacture and transport of ice needed to refrigerate the other type of diluent. It can thus be recommended even for those who already have refrigeration equipment, because it provides the best thermal protection of the vaccine.

Application of vaccination

Within the context of full eradication of CBPP, it would be ridiculous to suppose that vaccination was of major value to protect individual animals, as is the case with tetanus in horses. Even if the disease is present in a small herd of only a few cattle, epidemiology shows that CBPP is a disease of medium and large herds, and it is to these that attention should be directed.

METHODS OF PROPHYLAXIS

Without going into abstruse epidemiology, it can be stated that the epidemiological pattern of CBPP is simple: direct transmission by contact of susceptible bovines with an infected bovine (in the acute or chronic stage), without any vector or telluric reservoir being implicated.

Consequently, it is apparent that the standard methods of disease control are applicable. The examples of Europe and the USA illustrate how well-founded this concept is. However, when we turn to cases such as that of the African continent, where ecological and/or sociological situations do not permit the full application of such measures, the emphasis changes towards vaccine prophylaxis. An exceptional case is Australia, where the disease has been eradicated by judicious combination of both techniques.

AUSTRALIA

The information on which this section is based has been drawn largely from publications up to 1973. In that year, Australia was officially declared free of CBPP, the last known case having occurred in 1967 (10). The control measures to be described are applicable to other countries but, just as their application within the various States in Australia were varied to suit the local conditions, so may the measures have to be adjusted to suit each country. This short review is necessarily a simplification of the detailed treatments given by others cited in the text.

History

CBPP was introduced to Australia in 1858 with a group of five cattle brought by ship from England and landed at the port of Melbourne in the south-east of the continent (87, 92). Despite attempts at prevention, it spread rapidly in a northerly direction to the adjoining State of New South Wales and further north to Queensland, reaching the north of that State by 1864. Further spread westward to the Northern Territory and Western Australia was slower. Much of this spread has been attributed to movements of working oxen (87). Spread to the south of Western Australia resulted from the landing by ship of infected cattle from Adelaide. By the early 1900's, the
disease was present in most of the areas where cattle were run in Australia, except for the island State of Tasmania (87), which has remained free.

The cattle industry

There are two major components of the cattle industry. The intensive dairying industry is concentrated in the coastal areas of the east, south and south-west of the continent, or in those inland areas that can sustain the high stocking rates required.

Breeding of beef cattle is largely confined to the northern inland areas of Western Australia, the Northern Territory and Queensland, where it is hot and dry from March to November and where cattle properties are usually unfenced and measure thousands of square kilometres in area, with stocking rates of only 3 to 15 animals per square kilometre (92). To provide fattened for the market, 'store' cattle were gathered annually into mobs of about 1,200 head from properties in these northern areas and were walked east to rail heads or southern areas to better grazing in the eastern States and South Australia (87). This involved journeys of many hundreds of kilometres over many months for up to 1 million cattle annually (97). In recent times, cattle have been moved from these remote areas by motor transport.

The pattern of CBPP in Australia

The spread of CBPP throughout Australia occurred despite attempts at control by slaughter when clinical cases were recognised. Lack of knowledge of the often long incubation period and of the potential for spread by apparently healthy carriers resulted in failure of many attempts to stem the spread.

Only a few years after the initial southward spread, it became recognised that some cattle in their southward journey were bringing the disease back with them from the north, and reinfecting southern cattle (92). Restrictions were placed on the movements of cattle between States as early as 1861. These lapsed when it became apparent that the disease was widespread, but were reimposed later (87).

Seddon (87) has described in detail many of the attempts at control. These included compulsory notification of the presence of the disease, destruction of affected animals, quarantine of infected herds and vaccination of all in-contact animals. As outbreaks were controlled in particular areas, such as the closely settled dairying areas, restrictions on cattle movements into these were imposed to maintain that freedom. Details of the regulations for each State were described (87).

Methods of control of the disease gradually improved with the increase in knowledge gained from field experience and from the results of research.

In the 1930's, the team led by Dr A.W. Turner of CSIR (later CSIRO) profoundly improved the methods for diagnosis and prevention of CBPP.

The efficient complement-fixation (CF) test devised by Campbell and Turner (7, 8) was to play an important role in field control and in monitoring research on preventive vaccination (inoculation).

The culture vaccine (6) prepared from the V5 strain of *Mycoplasma mycoides* subsp. *mycoides* was an important factor in the control of CBPP.

Experimental work using aerosol or natural challenge showed that animals vaccinated three years previously were still immune to challenge, that immunity
developed early after vaccination (6), and that calves should not be vaccinated under the age of two months as they tended to develop joint and heart lesions with some deaths (92); much data was obtained on the incubation period of the disease, the persistence on the organism in lesions, and persistence of antibody after vaccination. All of this was basic to the development of control measures.

South Australian herds have been free of CBPP since 1952 (35) as have those in the Central Australian Protected Area since 1956 (35). CBPP ceased to be enzootic in the south of Western Australia in 1906 (87), in Victoria in 1929 (29) and in New South Wales in 1941 (35), the sporadic outbreaks which occurred in these areas having arisen from contact of susceptible cattle with infected cattle introduced from the enzootic areas in the north of the country. Control of such outbreaks required a particular strategy while other strategies were required for cattle travelling from the enzootic areas and for those resident in the enzootic areas.

Control of outbreaks

While control measures varied in details from State to State, they were based on the same principles – slaughter of clinical cases, quarantine, CF testing and vaccination of the herd and slaughter of positive reactors to the test. All surrounding herds that could have contact with the infected herd were placed in quarantine, bled for CF testing and vaccinated. Regular inspections to detect clinical cases were carried out, and in Victoria temperatures were recorded as they were a useful guide in detecting infected animals in the absence of other clinical signs (29). CF testing was repeated after waiting for the antibody levels provoked by vaccination to subside. This usually occurred within 7 weeks (92) but could take up to 5 months (29).

Where appropriate legislation was in place, as in Victoria, and when it was considered advisable, whole herds were slaughtered and the owner compensated.

Application of these control measures was effective in bringing outbreaks under control, and the main aim in the southern States became prevention of the introduction of the disease.

Control in the enzootic areas

The enzootic areas were composed of large, unfenced properties where it was impossible to round up all the cattle for vaccination or serological testing. Two strategies were used.

a) Travelling cattle

The annual turn-off of 'store' cattle destined to walk east and south to fattening areas posed a major threat to susceptible cattle en route and at their destinations. To minimise spread of the disease, such cattle were required to be vaccinated before setting off. Also, such cattle could move only when the drover in charge held a permit issued by the appropriate veterinary authority containing details of their origin, of the animals in the mob and of their destination.

The efficiency of vaccination was high: laboratory tests showed that 3% (92), and field experience that fewer than 1% (51) of vaccinated animals failed to develop immunity.
Vaccination was usually carried out without veterinary supervision and problems arose when the procedures used were faulty. Mahoney (52) observed that up to 10% of animals may at times have been left unprotected, and this position was aggravated when, to offset possible losses on the long walk, other cattle that may not have been vaccinated were illegally incorporated into the mob. As a consequence of these studies, supervision of vaccination by trained personnel was increased. When vaccination was properly carried out, the animals were protected from the risk of disease from contact with infected cattle encountered en route and posed no risk to susceptible cattle, unless any animals in the mob were incubating the disease at the time of vaccination.

b) Cattle in the enzootic area

The second strategy was to vaccinate as many animals as possible in the enzootic area. While owners complied with the requirement to vaccinate travelling cattle, as outbreaks en route would invoke quarantine and costly delays, they were reluctant to attempt to vaccinate cattle resident on the property. At first encouragement and later enforcement resulted in the annual crop of young cattle being vaccinated at branding or weaning, with the aim of building up a resistant herd over several years. In such a situation, the disease often died out for want of susceptible animals (10).

One of the difficulties in dealing with these large areas was to define which properties were infected. This was solved largely by 'trace back' from abattoirs. As lesions were recognised in abattoirs and proven in the laboratory, efforts were made to trace the property of origin of such animals. This proved to be a very effective step in defining the infected properties (59).

These strategies for dealing with animals in, and leaving from, the enzootic areas were successful.

Vaccination

Under Australian conditions tail tip vaccination with the live culture vaccine made from the V5 strain was very efficient, at least 97% of cattle being protected (92). Severe reactions sometimes occurred, resulting in the loss of a portion of the tail (about 1% of vaccinates), or in extreme cases, in severe swelling in the gluteal region with occasional deaths, but the numbers so affected were low enough to be acceptable under the prevailing conditions (92).

Another disadvantage was the interference with serological diagnosis. Although the CF antibody detected in response to vaccination was usually below a positive titre seven weeks later (92), in some animals it remained at high levels for 5 months (29) and even longer (92). This had to be taken into account when a vaccinated herd was being monitored for freedom from CBPP. In an attempt to obviate this problem, Hudson (34) introduced the KH₃J strain from Africa and prepared experimental vaccines with this strain, alone and together with egg fluids or extracts of brain. He was able to show that effective immunity was produced with these vaccines and that there were no severe local reactions nor serological response as measured by the CF test. Despite these apparent advantages, the KH₃J vaccines did not replace the V5 vaccine in the eradication campaign (76).

Eradication of CBPP from Australia

An upsurge in the number of outbreaks in the early 1950's was followed in the next two years by outbreaks in the southern States. In turn, these occurrences led
to recommendations for a combined approach toward eradication of the disease from the whole country (59). Extensive vaccination programmes in place in Queensland resulted in a dramatic lessening of the prevalence there of CBPP (59). In 1959, a national eradication campaign was planned and in 1961, it was implemented.

Efforts were now directed towards defining the prevalence of the disease on the large properties in the enzootic areas by close inspection and by tracing back to the properties of origin any CBPP lesions observed at the abattoirs and proven in the laboratories. Vaccination of young cattle in the enzootic areas and even whole herd vaccination were encouraged and later enforced to gradually build up resistant herds (10). The standard (Campbell and Turner) CF test and the field test of Huddart (43, 44) were applied to large numbers of cattle in some areas to allow assessment of the disease status of these areas. The control measures in travelling cattle were rigorously enforced. During this period, a contribution to the eradication campaign came from alterations to methods of moving cattle. The growth of vehicular transport of cattle in ‘road trains’ along improved ‘beef roads’ to rail heads minimised the number of cattle moving on foot, removing the most important factor in the continual spread of the disease.

Control of cattle movements was vital to the success of the campaign. Areas within the country were designated infected (quarantined), protected or free. Protected areas were those, usually next to infected areas, in which no cases had occurred over several years as shown by serological testing and absence of lesions on slaughter of animals from the area, and thus were probably free (76). Such areas acted as buffers between free and infected areas. Movements into and out of all three areas were strictly controlled by regulations agreed to by representatives of all States and the Commonwealth (76). Pierce (76) described in some detail the intense vaccination and inspection activity at abattoirs between 1964 and 1969, and the large reduction in the size of the areas classed as infected. In 1968, examination of large numbers of lungs at abattoirs had shown no evidence of CBPP, and in successive years, the findings were the same. Vaccination was scaled down (10) and by 1971 was prohibited, more use being made of CF testing. In August 1973, the whole country was declared free of CBPP, the last known case having occurred in 1967 (10).

In retrospect, the important features of this extensive and successful eradication campaign were the definition of the disease status of all areas, rigorous control of movements of cattle between areas, vaccination of travelling cattle and of young cattle on properties, examination of lungs at abattoirs and tracing the property of origin of any lesions. CF tests were useful, particularly when vaccination was stopped, in surveying properties for evidence of infection and in determining the status of animals slaughtered at abattoirs.

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REFERENCES


