CHAPTER 2.1.15.

RINDERPEST

SUMMARY

Classical rinderpest is an acute, viral disease of domestic cattle, buffaloes and yaks characterised by high morbidity and mortality rates. Sheep, goats, pigs and wild ungulates may also be affected. Clinically this form of the disease is characterised by pyrexia, the progressive development of shallow erosions on the gums, tongue, cheeks and hard palate, together with serous or mucopurulent ocular and nasal discharges. Alimentary tract involvement is marked by the development of diarrhoea or dysentery, leading to severe dehydration and depression. Rinderpest conforming to this description has not been seen since 2001 (Pibor, Southern Sudan). A milder form of the disease, with the potential to regain classical characteristics, used to occur in association with endemic situations, latterly in East Africa; it has not been positively diagnosed since 1997 (Tanzania) and could have died out, in which case wild-type rinderpest virus may no longer exist. Based on historical virus collections, three genetically distinct lineages of the virus have been recognised as causal agents of rinderpest disease in Africa and Asia. The Food and Agriculture Organisation of the United Nations (FAO) launched a Global Rinderpest Eradication Programme (GREP) in 1992, calling for eradication of the virus by the year 2010. The success of this programme may be judged by the fact that two of the three rinderpest lineages have now assuredly been eradicated and the third might well have joined them.

Identification of the agent: Clinical confirmation of classical rinderpest is based on the finding of individual or small groups of animals showing pyrexia, inappetence, depression, shallow erosions of the upper and lower lip and gum, erosions or blunting of the cheek papillae, serous or mucopurulent ocular discharges and/or nasal discharges, diarrhoea, recumbency and possibly death. Laboratory confirmation is based on demonstrating the presence of the virus, virus-specific RNA or precipitating antigens in samples from the spleen, lymph nodes, or ocular or nasal secretions of acutely infected animals. It is particularly important to isolate the virus if a geographical extension or significant animal health deterioration has occurred. Following the successes of global eradication, rinderpest-free countries may now confirm the presence of peste des petits ruminants (PPR) in sheep or goats based on the clinical appearance of affected animals and the presence of precipitating antigens, even though both the clinical signs and the virus-induced antigens are common to both viruses.

In cases where rinderpest is suspected, post-mortem examinations should pay particular attention to the abomasum, which may be highly engorged or show a grey discoloration; to the Peyer’s patches, which may show lymphoid necrosis; and to the development of linear engorgement and blackening of the crests of the folds of the caecum, colon and rectum. The principal differential diagnoses are PPR in sheep and goats, and bovine viral diarrhoea/mucosal disease and malignant catarrhal fever in cattle; differentiation of these diseases requires the use of appropriate laboratory methods.

Serological tests: The OIE has developed a set of Recommended Standards for Epidemiological Surveillance for Rinderpest (the ‘OIE Pathway’) that governs the actions of Member Countries wishing to demonstrate that they have achieved freedom from infection. To this end, a competitive enzyme-linked immunosorbent assay (ELISA) has been described to determine the presence of rinderpest antibodies in animals that have been infected with field virus or received rinderpest vaccine. An indirect ELISA has also been described. Whatever test is used it should be sensitive with respect to the lineage of virus likely to be present and be highly specific. Neutralising antibody estimations may be used for the same purpose. Member Countries may wish to seek expert advice from an OIE Reference Laboratory or the GREP Secretariat with regard to the selection of the test most appropriate for their purpose.
**A. INTRODUCTION**

In recent years the Global Rinderpest Eradication Programme (GREP) of the Food and Agriculture Organisation of the United Nations (FAO) has made enormous progress in organising and documenting the decline of rinderpest (13). Historically, the virus was widely distributed throughout Europe, Africa, and Asia; recently however, it has only occurred in Africa and Asia. Gene sequence analysis has shown that all known rinderpest isolates fall into one of three non-overlapping phylogenetic lineages, and in recent years it has been possible to describe the virus' distribution in lineage-specific terms. Thus, the so-called Asian lineage (lineage 3) was only ever recorded in Afghanistan, India, Iran, Iraq, Kuwait, Oman, Pakistan, Russia, Saudi Arabia, Turkey, Sri Lanka and Yemen. As a result of concerted and coordinated vaccination and surveillance campaigns, this virus lineage has failed to resurface since September 2000 (Pakistan). Although evaluations are not yet complete, it is almost certain that this virus has been successfully eradicated.

Rinderpest virus lineages 1 and 2 have only been recorded from Africa. Lineage 1 appears to have been distributed from Egypt to southern Sudan and eastwards into Ethiopia and into northern and western Kenya. On the other hand, lineage 2 has been recorded from both East and West Africa and at one time may have been distributed in a sub-Saharan belt running across the whole of the continent (12). Now however, as the result of further coordinated vaccination and surveillance programmes (Pan African Rinderpest Campaign in particular), neither West nor Central Africa have reported rinderpest since 1988 (Ghana/Burkina Faso). Until recently both lineages were being reported from eastern Africa, but it is now clear that lineage 1 was eliminated from southern Sudan in 2001 by intensive vaccination.

Reappearing in 1994, 1996 and 2001 in wildlife, Lineage 2 has been transmitting within the Somali pastoral ecosystem (9) where its continued presence caused considerable concern (10). In 1994, this virus reappeared in south-east Kenya where its effects were expressed most dramatically in buffaloes in Tsavo National Park (7) thereby illustrating its ability to engage in cryptic persistence for a period of at least 30 years, during which time it is likely to have been transmitted with a low level of virulence among susceptible cattle. Although this virus is now seen as having evolved to the point where it has been possible for it to escape veterinary attention in remote areas, its presence did not go unnoticed by the nomadic pastoralists whose cattle it infected. Nevertheless, it is clear that this virus has not been confirmed within this last reservoir since 2001 and although not yet an accredited achievement, it is more than likely that sporadic vaccination has broken the transmission chain of Lineage 2.

Rinderpest is caused by a negative-strand RNA virus of the Morbillivirus genus within the family Paramyxoviridae. Classic descriptions of rinderpest refer to it as a highly fatal disease of domestic cattle, buffaloes and yaks. The virus also affects some breeds of pigs and a very large variety of wildlife species within the order Artiodactyla, although not always in a clinically apparent form; a recent review views sheep and goats as susceptible but largely epidemiologically unimportant hosts of rinderpest (14).

Although in its final stages of eradication, some strains of rinderpest had evolved into a mild, nonfatal, infectious disease of cattle, all strains retained two very dangerous attributes. The first was an almost certain ability to undergo virulence modulations. The second was an ability to infect game animal species and, in buffaloes, eland, giraffe, lesser kudu and warthog, to cause an acute infection associated with high levels of mortality.

Classical rinderpest has an incubation period of between 1 and 2 weeks, the clinical disease is characterised by an acute febrile attack within which prodromal and erosive phases can be distinguished. The prodromal period lasts approximately 3 days, during which affected animals develop a pyrexia of between 40 and 41.5°C together with partial anorexia, constipation, congestion of visible mucosae, serous ocular and nasal discharges, depression and drying of the muzzle. However, it is not until the onset of the erosive phase, and the development of necrotic mouth lesions, that a tentative clinical diagnosis of rinderpest can be made. At the height of fever, flecks of necrotic epithelium appear on the lower lip and gum and in rapid succession may appear on the upper gum and dental pad; on the underside of the tongue, on the cheeks and cheek papillae and on the hard palate. Through the enlargement of existing lesions and the development of new foci, the extent of the oral necrosis can increase dramatically over the following 2–3 days. Much of the necrotic material works loose giving rise to shallow, non-haemorrhagic mucosal erosions.
Diarrhoea is another characteristic feature of rinderpest and develops 1–2 days after the onset of mouth lesions. The diarrhoea is usually copious and watery at first, but later on may contain mucus, blood and shreds of epithelium and it may be accompanied, in severe cases, by tenesmus. During the erosive phase, necrosis may be observed in the nares, in the vulva and vagina, and on the preputial sheath. Anorexia develops, the muzzle dries out completely, the animal is depressed, the breath is fetid and mucopurulent ocular and nasal discharges develop.

Deaths will occur but the mortality rate will be variable and may be expected to rise as the virus gains progressive access to large numbers of susceptible animals. Initial mortality rates will probably be in the order of 10–20% and, in the terminal stages of the illness, animals may become recumbent for 24–48 hours prior to death. Some animals die while showing severe necrotic lesions, high fever and diarrhoea, others after a sharp fall in body temperature, often to subnormal values. Alternatively, the pyrexia may remit slightly in the middle of the erosive period and then, 2–3 days later, return rapidly to normal accompanied by a quick resolution of the mouth lesions, a halt to the diarrhoea and an uncomplicated convalescence.

Typically the carcass of the dead animal is dehydrated, emaciated and soiled. The nose and cheeks bear evidence of mucopurulent discharges, the eye is sunken and the conjunctiva congested. In the oral cavity, there is often extensive desquamation of necrotic epithelium, which always appears sharply demarcated from adjacent areas of healthy mucosa. The lesions frequently extend to the soft palate and may also involve the pharynx and the upper portion of the oesophagus; the rumen, reticulum and omasum are usually unaffected, although necrotic plaques are occasionally encountered on the pillars of the rumen. The abomasum, especially the pyloric region, is severely affected and shows congestion, petaechiation and oedema of the submucosa. Epithelial necrosis gives the mucous membrane a grey colour. The small intestine is not commonly involved except for striking changes to the Peyer’s patches where lymphoid necrosis and sloughing leaves the supporting architecture engorged or darkly discoloured in long-standing cases; in either event the lesions are referred to as ‘zebra striping’.

Taking the lineage 2-associated form of rinderpest as an example of the mild form adopted by the virus within endemic situations, the incubation period is between 1 and 2 weeks and the ensuing clinical disease is little more than a subacute febrile attack in cattle. The fever is not invariable; it is short-lived (3–4 days) and not very high (38–40°C). The depression that characterises more acute forms of rinderpest is absent from mildly affected animals and, as a result they might not lose their appetite, they will probably continue to graze, water and trek as well as unaffected animals. These animals do not usually develop diarrhoea. On close examination there may be some slight congestion of the visible mucous membranes and small, focal areas of raised, whitish epithelial necrosis may be found on the lower gum – sometimes no larger than a pin head – along with a few eroded cheek papillae. Some animals may escape the development of such erosions, the appearance of which is fleeting. Other animals may show a slight, serous, ocular or nasal secretion but, in contrast to the more severe forms of the disease, these do not progress to become mucopurulent.

Even though infections with lineage 2 may pass unnoticed in cattle, the virus is highly infectious for wildlife species, and among those generally regarded as highly susceptible (buffalo, giraffe, eland, and lesser kudu) it causes fever, a nasal discharge, typical erosive stomatitis, gastroenteritis, and death. Kock (7) observed that in addition, buffaloes infected with lineage 2 showed enlarged peripheral lymph nodes, plaque-like keratinised skin lesions and keratoconjunctivitis. Lesser kudus were similarly affected, but whereas blindness – caused by a severe keratoconjunctivitis – was common, diarrhoea was unusual. Eland also showed necrosis and erosions of the buccal mucosa together with dehydration and emaciation. Therefore, under the present circumstances, a diagnosis of rinderpest in any of these species points to the likelihood of the simultaneous transmission of the virus, even at a subclinical level, in neighbouring cattle.

### B. DIAGNOSTIC TECHNIQUES

#### 1. Identification of the agent

In view of the high level of expectation surrounding the Global Rinderpest Eradication Campaign, any recent outbreak of rinderpest would be of immense epidemiological significance, not only as a threat of pandemic eruption but as a indicator of a lacuna in global surveillance. Consequently, until a country has reached an accredited rinderpest infection free status (based on serosurveillance), samples from all outbreaks considered rinderpest-like on clinical or pathological grounds must be routinely submitted for laboratory examination. For rinderpest, a variety of suitable laboratory tests is available, but under the circumstances outlined above it is of paramount importance to isolate the virus, identify its lineage and assess its virulence in experimental cattle (1). Blood in anticoagulant is the preferred sample wherever possible. On average, the onset of viraemia slightly precedes the onset of pyrexia, and may continue for 1–2 days after pyrexia begins to wane. Consequently, animals showing a pyrexia are probably viraemic and therefore the best source of blood with which to attempt
virus isolation. However, as occasional febrile animals may no longer be viraemic, samples from several febrile animals should be collected for submission. It is important to ensure that there is adequate tissue available for at least two virus isolation attempts from the initial submission of a suspected outbreak. The other procedures described should only be attempted if there is extra tissue available.

a) Virus isolation

Rinderpest virus can be cultured from the leukocyte fraction of whole blood that has been collected into heparin or EDTA (ethylene diamine tetra-acetic acid) at final concentrations of 10 international units (IU)/ml and 0.5 mg/ml, respectively. Samples should be thoroughly mixed and transferred to the laboratory on ice, but never frozen. Virus can also be isolated from samples of the spleen, prescapular or mesenteric lymph nodes of dead animals; these samples may be frozen for transportation.

To isolate the virus from blood, uncoagulated blood is centrifuged at 2500 g for 15 minutes to produce auffy coat layer at the boundary between the plasma and erythrocytes. This is removed as cleanly as possible, mixed in 20 ml physiological saline and recentrifuged in a washing procedure designed to remove any neutralising antibody present in the plasma. The resulting cell pellet is suspended in cell culture maintenance medium and 2 ml aliquots are distributed on to established roller tube monolayers of primary calf kidney, B95a marmoset lymphoblastoid or African green monkey kidney (Vero) cells. The culture maintenance medium should be decanted and replaced every 2 or 3 days and the monolayer observed microscopically for the development of cytopathic effects (CPE). These are characterised by refractility, cell rounding, cell retraction with elongated cytoplasmic bridges (stellate cells) and/or syncytial formation. The speed with which the CPE develops varies by substrate and probably by strain of virus also. Up to 12 days should be allowed in primary cells, a week in Vero and 2–4 days in B95a cells. Blind passages may be attempted before declaring an important sample negative, but a preferable technique would be to inoculate the cell suspension, and any residue of the original sample, intravenously into a rinderpest-susceptible ox and attempt to re-isolate the virus from its blood. Isolates of virus can be partially identified by the demonstration of morbillivirus-specific precipitogens in infected cell debris, or completely identified by the demonstration of specific immunofluorescence using a conjugated monoclonal antibody (MAb).

Alternatively, 20% suspensions (w/v) of lymph node or spleen may be used. These should be made by macerating the solid tissues in serum-free culture maintenance medium using standard grinding or shearing techniques and inoculating monolayers as before. The release of virus from solid tissue can be achieved in several ways. Perhaps the easiest is with a pestle and mortar, but this technique requires the use of sterile sand as an abrasive. Alternatively, tissue may be ground without an abrasive using all-glass grinders, for example, a Ten Broeck grinder. Shearing techniques are equally applicable using, for example, Silverson or Waring blenders. Virus-containing suspensions are clarified by low-speed centrifugation. The volume of the inoculum is not critical; a working volume is between 1 and 2 ml. Commonly used antibiotics are penicillin and streptomycin in combination, each at a concentration of 100 IU/ml. A similar broad-spectrum cover can be obtained using neomycin at 50 µl/ml. Fungizone should be included at 2.5 µg/ml.

b) Antigen detection by agar gel immunodiffusion

The agar gel immunodiffusion (AGID) tests may be conducted in Petri dishes or on glass microscope slides (5). In either instance the surface should be covered with agar to a depth of about 4 mm using a 1% aqueous solution of any high quality agar or agarose. Wells are usually cut in a hexagonal pattern of six peripheral wells around a single central well. For slides, wells should be 3 mm in diameter and 2 mm apart. For Petri dishes, the wells can be increased to 4 mm in diameter and the distance between wells to 3 mm. The closer the wells are placed from each other, the shorter the reaction time.

Using a small volume pipette, rinderpest hyperimmune rabbit serum should be placed in the central well. Similarly, control positive antigen, prepared from the macerated lymph nodes of rabbits infected with the Nakamura III lapinised strain of rinderpest, should be placed in alternate peripheral wells (i.e. one, three and five). Negative control antigen is placed in well four. Test antigens are obtained as exudates from the cut surface of spleen or lymph nodes submitted for testing; if no exudate can be obtained a small portion of the sample should be ground with a minimum of saline. Ocular exudates may be squeezed directly from the swabs or, alternatively, by compression in a microtip (the cotton wool should be cut off the swab and placed into the wide end of a plastic 50–250 µl pipette tip; the stem of the swab may then be used to compress the cotton wool and force a small volume of exudate out of the narrow end of the tip). Test samples are added to wells two and six. Tests are best developed at 4°C or low ambient temperatures. The reaction area should be inspected from 2 hours onwards for the appearance of clean, sharp lines of precipitation between the wells forming a line of identity with the controls. Tests should be discarded after 24 hours if no result has been obtained. The result is not acceptable unless precipitation reactions are also obtained giving a line of identity with the control positive antigen preparation.

Although the test is neither highly sensitive nor highly specific, it is robust and adaptable to field conditions. A positive reaction from a large domestic ruminant should be treated as if it were rinderpest. From a small
ruminant, a positive result should be treated as having been derived from a case of rinderpest or peste des petits ruminants (PPR) and requiring further differentiation.

c) **Histopathology and immunohistochemistry**

At post-mortem examination, tissues should be collected and placed in 10% neutral buffered formalin for histopathology and immunohistochemistry; the base of the tongue, retropharyngeal lymph node and third eyelid are suitable tissues. Sections stained with haematoxylin and eosin should be examined for the presence of syncytial cell formation, and cells with intranuclear viral inclusion bodies. The presence of rinderpest antigens can be demonstrated in the same formalin-fixed tissues by immunoperoxidase staining following the quenching of endogenous peroxidase activity. If a polyclonal antiserum is used, this test will fail to differentiate between rinderpest and PPR. However, this problem can be circumvented by using monoclonal antibodies specific for rinderpest and PPR in duplicate tests (3).

d) **Lineage identification using the reverse-transcription polymerase chain reaction**

The reverse-transcription polymerase chain reaction (RT-PCR) (6) produces DNA suitable for gene sequence analysis. Viral RNA can be purified from spleen (not ideal due to its high blood content), lymph node and tonsil (ideal), peripheral blood lymphocytes (PBLs), or swabs from eyes or mouth lesions (contingent). Solid tissues (0.5–1.0 g) are minced and homogenised with 4.0 ml denaturing solution, eye and mouth swabs are treated with 1.0 ml, and purified PBLs (from 5 to 10 ml whole blood) are treated with 0.4 ml according to the published procedure. Solution D (disruption solution): the procedure is that recommended to minimise the hazard of handling poisonous guanidium thiocyanate. It should be carried out in a chemical safety hood. The following are the amounts of guanidium thiocyanate for a 250 g bottle, but the volumes can be adjusted for other quantities. Do not attempt to weigh out the guanidium thiocyanate, but dissolve it in the manufacturer’s bottle by adding 253 ml sterile distilled water, 17.6 ml 0.75 M sodium citrate, pH 7.0, and 26.4 ml 10% sarcosyl, then heat to 65°C in a water bath to dissolve. This solution can be kept for several months in the dark at room temperature in a chemical safety cabinet. The final solution D is made by the addition of 0.36 ml 2-mercaptoethanol to 50 ml of the stock solution. This solution should not be kept for more than 1 month. In the past few years, RNA extraction spin columns have become widely used for fast purification of high quality RNA (RNeasy kit, Qiagen) The resulting RNA is precipitated with 2.5 volumes of ethanol, washed in 70% ethanol, dissolved in sterile water, or TE buffer (Tris/EDTA, 10 mM, pH 7.5, 1 mM EDTA) and stored at −70°C or −20°C until required. The cDNA synthesis is carried out using random hexanucleotide primers to enable several different specific primer sets to be used in the PCR amplification step. Aliquots of the resulting cDNA are amplified using at least three primer sets that can detect and differentiate between the two morbilliviruses. These primer sets include two ‘universal’ sets based on highly conserved regions in the phosphoprotein and nucleoprotein genes that should detect all morbilliviruses, and rinderpest virus-specific sets based on sequences in the fusion protein genes of the virus. The PCR products are analysed on a 1.5% (w/v) agarose gel along with a suitable DNA marker to identify the specific DNA product. A positive control such as measles or canine distemper virus RNA, and a negative control using sterile distilled water instead of RNA, must be included in each RT-PCR. Positive reactions should be confirmed either by using ‘nested’ primer sets based on the F gene sequences or by sequence analysis of the DNA product. It is important to use more than one set of primers for the PCR step when testing for the presence of RNA viruses, as their nucleotide sequences can vary significantly and one change at the 3'-end of the primer sequence may result in failure of the primers to amplify the DNA. The World Reference Laboratory in the United Kingdom (UK), which is also an OIE Reference Laboratory for rinderpest, and the OIE Reference Laboratory in France (see Table given in Part 3 of this Terrestrial Manual), can advise on use of the technique for field sample analysis.

Most recently, a simple Taqman real-time RT-PCR assay for RPV diagnostic has been described. This real-time RT-PCR assay for rinderpest virus has been validated to be highly sensitive in infected tissue culture supernatant and clinical samples from experimentally infected cattle. The assay has proved to be able to detect isolates representative of all known phylogenetic lineages of the virus and clearly differentiate from PPR virus and other look-alike diseases (foot and mouth disease virus, bovine viral diarrhoea virus, bovine herpesvirus, vesicular stomatitis virus). The analytical sensitivity of the L10 primer-probe system exceeded 1–100 TCID$_{50}$ (50% tissue culture infective dose)/ml, depending on the rinderpest virus strain. Comparison of samples from experimentally infected animals showed that white blood cells and conjunctival swabs are the sample of choice for epidemiological surveillance of the disease, allowing the preclinical detection of the disease by 2–4 days. In the event of a rinderpest virus outbreak, this portable, single-tube format, real-time RT-PCR has the capability of preclinical diagnosis, thus aiding efforts to prevent further transmission of disease.

e) **Differential immunocapture ELISA**

Neither clinical observations nor AGID tests can differentiate between rinderpest and PPR; consequently, if either disease is suspected in sheep or goats in countries where both diseases occur, other tests like the real-time PCR must be used. Rapid differentiation can be achieved using a differential immunocapture
ELISA test (8). This test employs MAbs directed against the N protein of the two viruses. One MAb, with a reactivity against both viruses, is used as a capture antibody, while a second biotinylated MAb specific for a nonoverlapping antigenic N protein site, and specific against either rinderpest or PPR, is used to determine which N protein has been captured.

High protein-binding ELISA plates (or strips) are coated with 100 µl/well of capture antibody. After three washes, the wells are loaded with 50 µl of test sample diluted 1/10 in a lysis buffer, 25 µl of the manufacturer’s recommended dilution of the virus-specific MAb and 25 µl of streptavidin peroxidase at a final dilution of 1/3000. The wells are then placed on an orbital shaker for 1 hour at 37°C, after which time they are again washed; following the addition of 100 µl of ortho-phenylenediamine (OPD), the wells are re-incubated at room temperature for 10 minutes. Reactions are halted by the addition of 100 µl of 1 N sulphuric acid, and the results, measured at 452 nm with an automated ELISA reader, are expressed as absorbance values.

f) Chromatographic strip test

While not a definitive diagnostic test, a rapid chromatographic strip test (penside test; ref. 4) has proved a useful tool for assisting field personnel in investigating suspected outbreaks of rinderpest.

2. Serological tests

a) The competitive enzyme-linked immunosorbent assay (the prescribed test for international trade)

A competitive ELISA is available for the detection of rinderpest antibodies in the serum of animals of any species previously exposed to the virus. The test is based on the ability of positive test sera to compete with a rinderpest anti-H protein MAb for binding to rinderpest antigen. The presence of such antibodies in the test sample will block binding of the MAb, producing a reduction in the expected colour reaction following the addition of enzyme-labelled anti-mouse IgG conjugate and a substrate/chromogen solution. As this is a solid-phase assay, wash steps are required to ensure the removal of unbound reagents.

The rinderpest antigen is prepared from Madin–Darby bovine kidney cell cultures infected with the attenuated Kabete ’O’ strain of rinderpest virus. The viral antigen is extracted from the infected cells by repeated cycles of sonication and centrifugation. The MAb was obtained by fusing the splenocytes of hyperimmunised mice with the NSO myeloma cell line, and then shown to be rinderpest H protein specific (2); this MAb has now been designated as C1. Both C1 and standardised rinderpest antigen are directly available from the OIE Reference Laboratory for Rinderpest in the UK (see Table given in Part 3 of this Terrestrial Manual). Kits are available commercially.

o Test procedure

i) Reconstitute the freeze dried rinderpest antigen with 1 ml of sterile water and further dilute it to the manufacturer’s recommended working dilution using 0.01 M phosphate buffered saline (PBS), pH 7.4.

ii) Immediately dispense 50 µl volumes of the diluted antigen into an appropriate number of wells of a flat-bottomed, high protein-binding ELISA microplate using two wells per test serum. Tap the sides of the microplate to ensure that the antigen is evenly distributed over the bottom of each well and, having sealed the plate, incubate it on an orbital shaker for 1 hour at 37°C. Wash the wells three times with 0.002 M PBS, pH 7.4.

iii) Add 40 µl of blocking buffer (0.01 M PBS, 0.1% [v/v] Tween 20 and 0.3% [v/v] normal bovine serum) to each test well followed by 10 µl volumes of all test sera.

iv) Follow the manufacturer’s recommendations to prepare a working dilution of the MAb in blocking buffer, and add 50 µl of this to each test well. Seal the plates and re-incubate on an orbital shaker for 1 hour at 37°C.

v) Follow the manufacturer’s recommendations to prepare a working dilution of rabbit anti-mouse immunoglobulin horseradish peroxidase conjugate in blocking buffer and add 50 µl to each test well. Seal the plates and re-incubate on an orbital shaker for 1 hour at 37°C.

vi) At the end of this period the plates are washed as before and immediately refilled with 50 µl volumes of substrate/chromogen mixture (1 part 3% H₂O₂ to 250 parts OPD), and incubate at room temperature for 10 minutes without shaking. Then add 50 µl of a stopping solution consisting of 1 M sulphuric acid.

vii) The test system must include known rinderpest positive and negative serum samples, a MAb control and a conjugate control.

viii) Measure the resulting absorbance values on an ELISA reader with a 492 nm interference filter and express the test results as percentage inhibition values compared with the value obtained using the
MAb control. Inhibition values of 50% or more are considered to be positive and values below 50% are considered to be negative.

Lowering the positive/negative threshold to 40% or less increases the sensitivity of the test, but inevitably affects specificity by increasing the proportion of false-positive test results encountered. In practice, the 50% value is recommended by GREP at which level sensitivity is at least 70% and specificity exceeds 99%. The sensitivity needs to be taken into account when designing sampling frames for serosurveillance.

An indirect ELISA method has been developed and might be useful for rinderpest surveillance programmes, especially in areas in which lineage 2 rinderpest virus could be present (17). However, the performance characteristics of the test indicate a problem with specificity and therefore its use will require confirmatory testing.

b) Virus neutralisation

The ‘gold standard’ virus neutralisation (VN) test is performed in roller-tube cultures of primary calf kidney cells following the method of Plowright & Ferris (11); the test has been validated in experimentally infected cattle. In the roller tube procedure, sera, that has not been inactivated, are diluted at intervals of 1 in 10 and then, starting with undiluted serum, mixed with an equal volume of $10^{3.5}$ TCID$_{50}$ per ml of the attenuated Kabete ‘O’ vaccine strain virus. Mixtures are held overnight at 4°C, after which 0.2 ml volumes are inoculated into each of five roller tubes, immediately followed by 1 ml of dispersed indicator cells suspended in growth medium at a rate of $2 \times 10^5$ cells per ml. Tubes are incubated at 37°C, sloped for the first 3 days, after which they are replenished with maintenance medium and placed on a roller apparatus. They are examined regularly for virus-specific cytopathology and positive tubes recorded and discarded; the final examination takes place on day 10.

For calculating end-points, the virus dose is regarded as satisfactory if the final dilution falls within the range $10^{1.8}$ to $10^{2.8}$ TCID$_{50}$/tube. This test should be used to examine the sera of ELISA reactors during national serosurveillance programmes designed to demonstrate freedom from infection, or to qualify susceptible cattle for vaccine testing. Under these circumstances, the presence of any detectable antibody in the 1/2 final serum dilution is considered to indicate previous infection with rinderpest virus. The VN test is the test of choice for the examination of wildlife serum samples.

A microplate method may be used as a screening test. In this procedure, an initial serum dilution of 1/5 is further diluted at twofold intervals. Thereafter, 50 µl volumes of serum are incubated with 50 µl volumes of virus diluted to contain between $10^{1.8}$ and $10^{2.8}$ TCID$_{50}$ (15). Following a 45-minute or an overnight incubation period, between 1 and $2 \times 10^5$ calf kidney, lamb kidney or Vero cells are added as indicators. Tests are terminated after 6 or 7 days. Such tests may give indications of non-specific neutralisation at high serum concentrations. There appear to be factors in some normal (with respect to prior rinderpest exposure) sera that bring about the failure of the virus to penetrate and replicate in indicator cells. In the tube test, these factors were probably removed during changes in maintenance medium; in the microplate method, they remain present the whole time. If the most concentrated final serum dilution is limited to 1/10, the effect disappears.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Many countries have used rinderpest vaccine to reduce the incidence of rinderpest to zero, and then followed the OIE Pathway in order to have their rinderpest-free status internationally recognised. To obtain this status, the process of annual rinderpest vaccination has largely been replaced by active and passive clinical and serological surveillance. Intensive focal vaccination with homologous vaccine (immunosterilisation) was retained for dealing with emergency management (16).

The live attenuated tissue culture rinderpest vaccine (TCRV) described in previous editions of this Terrestrial Manual was developed by Plowright by the serial passage of the virulent bovine rinderpest strain Kabete ‘O’ (RBOK) in primary bovine calf kidney cells. Due to the success of the Global Rinderpest Eradication Programme, it is believed that few vaccine manufacturers continue to make this product, although a number of them may be storing considerable stocks. However, the description published in the previous edition of the Terrestrial Manual will be repeated here so that it is available if conditions change.

1. Seed management

a) Characteristics of the seed

Seed lots used in the manufacture of TCRV must produce a cell-culture vaccine that is safe, that confers an immunity in cattle lasting at least 5 years, that retains its attenuated characteristics during at least five back passages in cattle, and that lacks the ability to spread by contact. Substrains of RBOK used in the
manufacture of TCRV must be identifiable by written historical records, which must include information on the origin of the strain and of its subsequent manipulations.

b) **Method of culture**

Vaccine seed must be maintained in a seed-lot system between passage levels 90 and 120. Seed-lot virus must be preserved in a freeze-dried state at a temperature of –20°C or lower. The virus must be cultured in Vero cells or primary or serially cultivated kidney cells derived from a normal bovine foetus or a very young calf. Serially cultivated cells may not be more than ten passages removed from the primary cultivation.

c) **Validation as a vaccine**

Seed lots must be shown to be:

i) **Pure**: Free from contamination with viruses, bacteria, fungi or mycoplasmas.

ii) **Safe**: Inducing no abnormal clinical reaction on inoculation into rinderpest-susceptible cattle.

iii) **Efficacious**: Inducing an immunity to rinderpest in rinderpest-susceptible cattle.

2. **Method of manufacture**

Individual vaccine batches are prepared by infecting cell cultures and, after an appropriate incubation period, harvesting the overlying media into which large numbers of live virus particles have been released. To facilitate long-term storage and cold-chain distribution, this fluid is freeze-dried in the presence of a cryoprotectant consisting of 5% lactalbumin hydrolysate and 10% sucrose. Virus may be grown in primary kidney cells from bovine embryos or calves, or cells derived in a homogeneous manner by up to ten serial subcultures from either of these sources. In addition, vaccine may be manufactured in approved continuous cell lines provided the cells are known to be non-infected with bovine viral diarrhoea (BVD) virus and are maintained in a seed lot system; Vero cells have been used for this purpose. To constitute a batch, infected cultures must have been inoculated with the same seed virus and incubated and harvested together. Two harvests are permissible from the same set of cultures and may be pooled to form a bulk suspension. Written records must accompany all stages of vaccine manufacture.

3. **In-process control**

**Cells**: Primary cells, serially cultivated primary cells or continuous cell lines must have been derived from normal looking animals or embryos, and must retain a normal morphology during cultivation. They must be shown to be free of contamination with adventitious viruses, particularly BVD virus. Whatever cells are committed for vaccine production, uninfected control cultures must be maintained using the same media and incubation conditions as the rinderpest-infected cells. They must be subjected to frequent microscopic observations. After harvesting the vaccine, the control cultures should be washed to remove ox serum and re-incubated for 10 days in media containing ox serum substitutes. They are again subject to frequent microscopic observations for evidence of cytopathic change. Simultaneously a sample of the cultures should be examined for the presence of noncytopathic BVD virus using an immunofluorescence or immunoperoxidase test or RT-PCR. The serum used in the culture media must come from rinderpest-susceptible animals.

**Virus**: A virus titration must be undertaken on the seed lot using tenfold virus dilutions in a microplate or roller tube system and employing ten replicates per dilution. A similar titration must be undertaken on the final bulk. Virus should be derived from cultures maintained in roller bottles and may not be harvested more than 10 days after the date that these cultures were infected. The harvest should be clarified by low-speed centrifugation before mixing with cryoprotectant. Prior to lyophilisation it may be held for not more than 5 days at 4°C but for considerably longer if frozen at –20°C to –60°C. As adventitious viral contamination may arise during a manufacturer's manipulations or from the use of contaminated media, rabbit hyperimmune serum should be used to neutralise the rinderpest content of the bulk suspension, after which the mixture should be used to infect calf kidney or Vero cells, which are handled as described above. The final bulk must be tested for freedom from bacteria, fungi and mycoplasmas.

4. **Batch control**

a) **Identity**

The contents of one container from each filling lot must be exposed to neutralisation by rabbit rinderpest antiserum, using a varying virus/constant serum method, and inoculated into bovine kidney cells. The identity of the product is established if no rinderpest-specific CPE develop.

b) **Sterility**
Tests for sterility and freedom of contamination of biological materials may be found in Chapter 1.1.9.

c) Safety and efficacy

Using rinderpest susceptible cattle, the contents of five randomly selected vials are pooled and used to inoculate one ox with a volume equivalent to 100 cattle field doses and one ox with a volume equivalent to 1/10th of a cattle field dose. These animals are maintained in close contact with an uninoculated ox for the following 3 weeks. During this period the animals are subjected to daily temperature recording and frequent clinical inspections. At the end of the 3 weeks, the cattle are examined for rinderpest neutralising antibodies and challenged with a strain of rinderpest capable of inducing a pyrexia. The vaccine is considered safe and efficacious if it does not induce any abnormal clinical reaction, if both animals receiving vaccine are protected and if there is no evidence that the vaccine virus has been transmitted. This test is not a potency test. Each vaccine lot must also be tested for innocuity in small animals.

d) Potency

The close relationship between immunising potency and infectivity allows the latter to be used as the basis for potency estimations. Three infectivity titrations are undertaken using cells of an approved continuous line or cells grown from each of three different bovine calf or embryonic kidneys. For the first titration, the pool of vials used for the safety test may be employed. The second and third estimates are made on further pools, each of three final containers. The sensitivity of the cells used in each working session must be measured using a standard laboratory rinderpest virus preparation. The final titre is the geometric mean of the three estimates, each undertaken using tenfold dilutions and ten observations per dilution.

e) Duration of immunity

It is unnecessary to routinely establish the duration of immunity to TCRV. Reported results indicate that lifelong immunity can be expected following the successful vaccination of cattle free of all vestiges of maternal immunity.

f) Stability

TCRV is highly stable when correctly freeze-dried and will keep for long periods at either +4 or –20°C provided the product is stored under vacuum. Recent evidence indicates that the rate of degradation of lyophilised TCRV can be altered by the choice of stabiliser and by variations in the drying cycle. The most advantageous results were associated with the use of a 5% lactalbumin hydrolysate/10% sucrose stabiliser, a 72–74 hour drying cycle under reduced vacuum (100 milliTorr), initial drying for 16 hours at –30°C, and a final shelf temperature of 35°C. With high release titres, such vaccine can be used in the field for 30 days without refrigeration. Following reconstitution in either normal saline or 1M magnesium sulphate, the virus becomes much more thermolabile. The period for field distribution of reconstituted vaccine should not exceed its half-life, but as this parameter is temperature dependent and varies between 8 and 24 hours over a range from 4°C to 37°C, a common sense limit must be applied; this can be determined by National Control Authorities, but a universal period of 4 hours can be recommended.

g) Preservatives

TCRV contains lactalbumin hydrolysate and sucrose which are added as cryoprotectants; otherwise it contains no specific chemical preservative.

h) Precautions (hazards)

There are no known hazards associated with the manufacture or field use of TCRV.

REFERENCES


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**NB:** There are OIE Reference Laboratories for Rinderpest (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).