CHAPTER 3.9.8.

TRANSMISSIBLE GASTROENTERITIS

SUMMARY

Transmissible gastroenteritis (TGE) is an enteric disease of pigs caused by TGE virus (TGEV), a member of the Coronaviridae. Since 1984, a distinct respiratory variant (porcine respiratory coronavirus or PRCV) has spread throughout many parts of the world. This virus is probably a deletion mutant of TGEV. PRCV does not appear to be an important primary pathogen, but it contributes to the porcine respiratory disease complex and it has greatly complicated the diagnosis of TGE, particularly by serological means.

Laboratory diagnosis is made by demonstrating the presence of virus, viral antigens or viral nucleic acid in material from suspected cases, or by demonstrating virus-specific humoral antibodies.

Identification of the agent: *Virus may be identified by virus isolation in tissue culture, electron microscopy, various immunodiagnostic assays, and more recently by specific detection of viral RNA. The most commonly employed rapid assays are probably the immunodiagnostic ones, particularly enzyme-linked immunosorbent assays (ELISAs) on faeces and fluorescent antibody tests on cryostat sections of intestine. Another enteric disease, porcine epidemic diarrhoea, is caused by a serologically distinct coronavirus that nevertheless has an identical appearance under the electron microscope. Diagnostically, immune electron microscopy circumvents this problem.*

Serological tests: *The most widely used methods are virus neutralisation tests and ELISAs. Only in the latter case is differentiation from PRCV possible, as TGEV and PRCV antibodies show complete cross-neutralisation.*

Requirements for vaccines and diagnostic biologicals: *There are no commercial biological products available internationally. However, several countries practise vaccination, and in the United States of America, licences have been issued authorising the production and distribution of monovalent and combined vaccines.*

A. INTRODUCTION

Transmissible gastroenteritis (TGE) is an enteric disease of pigs caused by TGE virus (TGEV), a member of the *Coronaviridae*. Since 1984, a distinct respiratory variant (porcine respiratory coronavirus or PRCV) has spread throughout many parts of the world and is now found in most countries where surveys for it have been conducted, one exception being Oceania. Occurrences of TGE have become more sporadic. The disease is still reported on an occasional basis from parts of Europe, North America and Asia. TGEV multiplies in and damages the enterocytes lining the small intestine, producing villous atrophy and enteritis. Diarrhoea and vomiting occur in pigs of all ages; mortality is highest in neonates. Extra-intestinal sites of virus multiplication include the respiratory tract and mammary tissues (Kemeny *et al*., 1975), but the virus is most readily isolated from the intestinal tract and from faeces. By contrast, PRCV is most readily isolated from the upper respiratory tract, the trachea, tonsils or the lungs, and little enteric multiplication of virus occurs (Cox *et al*., 1990; O'Toole *et al*., 1989; Pensaert *et al*., 1986) although PRCV can be detected by nested reverse-transcription polymerase chain reaction (RT-PCR) in nasal swabs and faeces of PRCV-infected swine (Costantini *et al*., 2004). PRCV is probably a deletion mutant of TGEV (Rasschaert *et al*., 1990) as confirmed by recent data comparing the complete 30 Kb genome sequences of TGEV and PRCV strains (Zhang *et al*., 2007).

As TGE is a contagious disease that can occur as explosive epizootics, rapid diagnostic methods for its confirmation are particularly important. The disease can also take the form of a low-level endemic problem of post-weaning diarrhoea, which is more difficult to diagnose. The occurrence of TGEV in PRCV-immune herds also

leads to milder and sporadic clinical cases of TGEV, further complicating TGEV diagnosis in such scenarios (Kim *et al*., 2000b).

Possible wild and domestic animal reservoirs for TGEV have been suggested. Wild and domestic carnivores (foxes, dogs, possibly mink) and cats seroconvert to TGEV and are suggested as potential subclinical carriers of TGEV, serving as reservoirs between seasonal (winter) epidemics. However only virus excreted by serially TGEV-infected dogs has been confirmed as infectious for pigs (Saif & Sestak, 2006). Based on genetic and antigenic similarities, it has been proposed that TGEV, PRCV, feline and canine coronaviruses represent host-range mutants of an ancestral coronavirus. Wild birds (*Sturnus vulgaris*) and flies (*Musca domestica*) have been proposed as mechanical vectors for TGEV, excreting virus for 32–72 hours, respectively (Saif & Sestak, 2006).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Virus may be identified by virus isolation in tissue culture (Dulac *et al*., 1977), immunofluorescence, reversed passive hemagglutination, enzyme-linked immunosorbent assays (ELISAs), radioimmunoassay (RIA), hybridisation with DNA probes, electron microscopy, and, more recently, by specific detection of viral RNA (Enjuanes & Van der Zeijst, 1995; Kim *et al*., 2000a; Paton *et al*., 1997; Saif & Sestak, 2006; Sirinarumitr *et al*., 1996; Woods, 1997). Molecular techniques such as RT-PCR and nested RT-PCR developed in the past few years have increased the sensitivity and specificity of detection and differentiation of TGEV and PRCV directly from field samples (Costantini *et al*., 2004; Kim *et al*., 2000a; 2000b; Paton *et al*., 1997). An alternative diagnostic method that has been recommended for laboratories lacking facilities for specialised tests is the oral dosing of susceptible TGEV/PRCV seronegative piglets with suspect intestinal contents. However, laboratory tests are still required to confirm susceptibility of the pigs prior to inoculation and to show that any illness induced in these animals is due to TGE. The most commonly employed rapid assays are probably the immunodiagnostic ones, particularly enzyme-linked immunosorbent assays (ELISAs) on faeces (Bernard *et al*., 1986; Lanza *et al*., 1995; Van Nieuwstadt *et al*., 1988b), fluorescent antibody tests (FAT) on cryostat sections of intestine (Pensaert *et al*., 1968) and immunohistochemistry (IHC) on formalin-fixed, paraffin sections (Shoup *et al*., 1996). Detection of virus by reversed passive hemagglutination has also been described (Asagi *et al*., 1986). Another enteric disease, porcine epidemic diarrhoea (PED), is caused by a serologically distinct coronavirus that nevertheless has an identical appearance under the electron microscope. Diagnostically, immune electron microscopy circumvents this problem (Saif *et al*., 1977; Van Nieuwstadt *et al*., 1988a) as does the application of PED virus-specific detection assays (Kim *et al*., 2001).

1.1. Virus isolation in tissue culture

Apart from the inoculation of live piglets (Dulac *et al*., 1977), this is the most definitive method of diagnosis. However, for routine use it is slow and laborious. TGEV does not grow well in cell culture, making this technique impractical as a routine diagnostic procedure. Moreover isolation of TGEV from pigs in PRCV seropositive herds is also problematic and often requires placement of TGEV/PRCV seronegative pigs in the suspect herd to serve as sentinels, followed by collection of samples from the sentinel pigs for TGEV isolation or detection (Costantini *et al*., 2004; Kim *et al*., 2000b). PRCV can be isolated in tissue culture using similar cell types and techniques as for TGEV, but using nasal cells or fluids and tracheal, tonsil or lung tissues or homogenates as the optimal specimens (Costantini *et al*., 2004; Pensaert *et al*., 1986).

For TGEV, isolation is usually attempted ante-mortem from faeces or post-mortem from the small intestine. Loops of affected small intestine, ligated at each end to retain the contents, or mucosal impression smears of the small intestinal luminal surface are the preferred specimens. As the virus is heat labile, all samples should be fresh or chilled.

Sample material is homogenised in cell culture medium or phosphate buffered saline (PBS), pH 7.2, containing antibiotics, e.g. penicillin (1000 U/ml), dihydrostreptomycin (1000 μg/ml), and mycostatin (20 U/ml), to produce a 10% suspension. This is allowed to stand out of direct sunlight for 30 minutes at room temperature. The suspension is then sonicated and clarified by low-speed centrifugation. The supernatant fluid may be mixed with an equal volume of heat-inactivated bovine serum in order to reduce the cytotoxic effect of the material and it is then used to inoculate susceptible tissue cultures, such as 3- to 4-day-old primary or secondary pig kidney monolayers. Other low passage porcine

cultures (such as thyroid or testis) and some cell lines (Honda *et al*., 1990; McClurkin & Norman, 1966) may also be used for primary virus isolation. After incubation at 37°C for 1 hour, the cell sheets are overlaid with a medium, such as Earle's yeast lactalbumin (EYL) balanced salt solution, containing sodium bicarbonate and antibiotics, e.g. penicillin (100 U/ml), dihydrostreptomycin (100 μg/ml), mycostatin (20 U/ml), and 1% fetal calf serum. Incorporation of trypsin into the culture medium may enhance primary viral recovery (Bohl, 1979; Honda *et al*., 1990). Uninoculated control cultures are established concurrently and all cultures are incubated at 37°C.

Viral cytopathic effect (CPE) may be observed after 3–7 days, characterised by cells rounding, enlarging, forming syncytia and detaching into the medium. Plaque formation is sometimes more reliable and easier to recognise. A suitable plaquing overlay is 1.6% noble agar in 2 × minimal essential medium with 1% $NaCO_3$, antibiotics (as above), 0.7% neutral red and 1% DEAE (diethylaminoethyl) (100 μg/ml). Wild-type TGEV does not grow readily in tissue culture, so several subpassages may be necessary before these distinctive changes become apparent. Cytopathic isolates must be confirmed as TGEV by immunostaining or by *in-vitro* neutralisation tests using appropriate TGEV-specific antisera (Bohl, 1979). If suitable monoclonal antibodies (MAbs) are available they can be used to distinguish between TGEV and PRCV by immunostaining methods (Garwes *et al*., 1988; Simkins *et al*., 1992). Differentiation of TGEV from PRCV can also be accomplished by TGEV-specific cDNA probes (Bae *et al*., 1991) or by discriminatory RT-PCR or nested RT-PCR (Costantini *et al*., 2004; Kim *et al*., 2000a; 2000b; Paton *et al*., 1997).

1.2. Fluorescent antibody test for viral antigens

The fluorescent antibody test is a rapid, sensitive and specific means of identifying TGE viral antigens in cryostat sections of intestine. A freshly dead pig is required, and the ideal animal should be under 4 weeks of age (preferably less than 1 week) and just starting to show clinical signs of the disease (that is, within 24–28 hours of infection). Within 30 minutes of death, 2 cm lengths from four different regions of the posterior part of the small intestine should be removed. Lengths of 5–10 mm are cut from these for snap freezing with solid CO_2. Correct orientation of the material is important to ensure that subsequent cutting by cryostat yields true transverse sections. Sections are cut 6 μm thick, mounted on cover-slips, air-dried and fixed in acetone. An alternative and faster procedure is to excise and longitudinally cut open a piece of the distal small intestine, gently wash the mucosal surface with PBS and prepare impression smears of the luminal intestinal surface on ethanol-cleaned microscope slides followed by air drying and acetone fixation (Bohl, 1979). The slides are then processed and stained like the cryostat sections as follows. Fixed positive and negative control sections or smears are stored at -20°C for staining in parallel. After washing with Tris buffer, pH 8.7, or PBS, the sections are stained with a diluted solution of fluorescein isothiocyanate (FITC)-conjugated TGEV antibody, and placed in a humid incubator at 37°C for 30 minutes. Any unbound stain is removed by washing in Tris buffer. If desired, the sections are counterstained with a 10^{-5} dilution of Evans blue in Tris buffer and mounted in glycerol.

Stained sections or smears should be examined by ultraviolet light microscopy as soon as possible. The quality of the staining is assessed by reference to the controls. An accurate interpretation depends on the preservation of the villous architecture, the epithelial cells of which are examined for intracytoplasmic fluorescence.

A peroxidase–antiperoxidase IHC method for the demonstration of TGEV has been developed for detection of TGEV and PRCV in both frozen and formalin-fixed, paraffin-embedded tissues (Jean *et al*., 1987; Shoup *et al*., 1996). The IHC applied to formalin-fixed tissues is advantageous because it can be done prospectively or retrospectively on the same formalin-fixed tissues used for histopathology and the fixed tissues or slides can be more readily shipped as they are stable and they do not contain live virus (Shoup *et al*., 1996).

1.3. Enzyme-linked immunosorbent assay detection of faecal virus antigens

A double antibody-sandwich system may be used, for instance with a capture MAb and a polyclonal enzyme-linked detector antibody (Lanza *et al*., 1995; Sestak *et al*., 1996). This test is based on capture of the viral antigen from the faecal sample by three MAb, two specific for the S protein (site A and D) and one for the nucleoprotein N (Lanza *et al*., 1995; Sestak *et al*., 1996). A negative coating is used as control for the specificity of the test, consisting of antibodies purified from the ascitic fluid of mice inoculated

with SP2/0 myeloma cells that do not recognise TGEV. MAbs are applied to 96-well microplates in a bicarbonate buffer, pH 9.6, and incubated overnight at 37°C. All samples are tested in duplicate wells, one containing positive coating (TGEV MAbs) and one containing the negative coating. Faecal samples are diluted in cell culture medium (1/10), vortexed and centrifuged at low speed (2000 *g*) for 15 minutes. Then the supernatant is decanted into sterile tubes and tested or stored frozen. Plates are washed twice with washing buffer (PBS containing 0.05% Tween 20) before adding the prepared faecal samples. The plates are incubated overnight at 37°C. After washing four times, a biotinylated polyclonal anti-TGEV serum is added in PBS buffer containing 0.05% Tween 20. The plates are incubated at 37°C for 1 hour. The plates are washed four times before adding a horseradish peroxidase-labelled streptavidin conjugate and incubated at 37°C for 1 hour. The plates are washed six times before adding the enzyme substrate, which is ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) with 0.03% H_2O_2 in 0.1 M citrate buffer, pH 4.2. The reaction is stopped after 30 minutes at room temperature by the addition of 5% sodium dodecyl sulphate and the absorbance determined in an ELISA reader at 405 nm. TGEV negative and positive faecal samples are included on each plate.

1.4. Nucleic acid recognition methods

In-situ hybridisation (ISH) and RT-PCR methods have been described for the direct detection of TGEV in clinical samples, with differentiation from PRCV (Kim *et al*., 2000a; Paton *et al*., 1997; Sirinarumitr *et al*., 1996). A second round of nested PCR may significantly enhance the sensitivity (Costantini *et al*., 2004; Kim *et al*., 2000a; 2000b; Paton *et al*., 1998). Differentiation between TGE viruses may be achieved by analysing PCR products with restriction endonuclease enzymes (Woods, 1997) or by sequencing (Costantini *et al*., 2004; Kim *et al*., 2000b; McGoldrick *et al*., 1999; Paton & Lowings, 1997; Zhang *et al*., 2007). Duplex RT-PCR for the combined detection of TGEV and porcine epidemic diarrhoea virus has been described (Kim *et al*., 2001).

2. Serological tests

Serology may be diagnostic if a rising titre of antibody can be demonstrated. In addition, a single seropositive result has diagnostic value if collected from a population previously known to be seronegative. As the possibility of acquiring carrier virus status among pigs can be reduced by accepting only seronegative animals, serological testing is also a common precondition for importation.

Following infection with TGEV or PRCV, viral antibodies can be detected in serum from 6 or 7 days post-infection, and such antibodies persist at least for many months. Although PRCV and TGEV antibodies show complete neutralisation of either virus, there are differences in the specificities of some of the non-neutralising antibodies (Callebaut et al., 1988; Enjuanes & Van der Zeijst, 1995; Garwes et al., 1988; Saif & Sestak, 2006; Simkins et al., 1992), as PRCV lacks certain epitopes present on the TGEV. However, virus neutralisation (VN) is not a practical method to differentiate PRCV from TGEV infection. MAbs to such regions can be incorporated into competitive ELISAs to detect serum antibody that is entirely TGEV specific. While such tests are reliable in that they do not produce false-positive results with PRCV antisera, false negatives may occur because of a reduced sensitivity compared with neutralisation tests, and because of strain variation among TGE viruses, such that a single TGEV-specific MAb may not recognise all strains (Brown & Paton, 1991; Simkins et al., 1992). The problem of insensitivity can be reduced by using the tests on a group or herd basis. These MAb-based ELISAs are the method of choice for differentiating PRCV from TGEV to qualify animals for export.

In addition, using such tests for differential diagnosis less than 3 weeks after exposure to PRCV produced inconsistent and unreliable results (Sestak et al., 1999b). More accurate results were also achieved by testing paired serum samples (acute and convalescent) in the assays and by using the recombinant spike (S) protein of TGEV as the coating antigen in place of TGEV-infected, fixed swine testicular cells (Sestak et al., 1999b).

2.1. Transmissible gastroenteritis virus/porcine respiratory coronavirus tests

These tests detect antibody to both TGEV and PRCV, and include VN tests, indirect ELISAs (Hohdatsu *et al*., 1987; Huang *et al*., 1988; Liu *et al*., 2001; McGoldrick *et al*., 1999; Rukhadze *et al*., 1989) and competitive ELISAs based on TGEV/PRCV group-specific MAbs (Paton *et al*., 1991).

VN tests can be performed with a variety of cell types and viral strains. Commonly used cell lines include swine testes (McClurkin & Norman, 1966) or primary or continuous porcine kidney cells. Such tests have been very widely used for many years and are commonly regarded as standards against

which to assess new assays. A plaque reduction VN assay using swine testes cell monolayers in six-well plastic plates and the atttenuated Purdue strain of TGEV is commonly used (Bohl, 1979). A modification of the method of Witte (Witte, 1971) described below, uses flat-bottomed tissue-culture grade microtitre plates, a cell line of A72 cells derived from a dog rectal tumour, and a field strain of virus adapted to grow in such cells: 100 $TCID_{50}$ (50% tissue culture infective dose) of virus is incubated with heat-inactivated test sera, and neutralisation is indicated by absence of CPE after further incubation with A72 cells in Leibovitz 15 medium (Sigma, United Kingdom) with added antibiotics, 10% fetal calf serum and 1% L-glutamine. The total volume of reagents in all wells should be 150 µl.

2.1.1. Virus neutralisation: test procedure

i) Sera are inactivated for 30 minutes in a water bath at 56°C.

ii) Doubling dilutions of test sera are made in cell culture medium beginning with undiluted serum (this gives a neutralisation stage dilution of 1/2 when mixed with an equal volume of virus). The dilutions are prepared in a 96-well flat-bottomed cell-culture grade microtitre plate using, optimally, three wells per dilution and 25 µl volumes per well. Positive and negative control sera are also included in the test. No standard sera are available, but internal positive standards should be prepared and titrated in an appropriate range.

iii) 25 µl TGEV stock is added to each well at a dilution in culture medium calculated to provide 100 TCID50 per well. Virus should be added to two out of the three wells containing serum at each dilution. The third well serves as a serum-only control and should receive 25 µl per well of culture medium instead of virus.

iv) The residual virus is back titrated in four tenfold steps using 25 µl per well and at least four wells per dilution; 25 µl of culture medium is added to each of the back-titration wells to compensate for the absence of a test serum.

v) The plates are agitated briefly and then incubated for 1 hour in a 5% CO2 atmosphere at 37°C.

vi) 100 µl of, for example, A72 cell suspension at 2 × 105 cells per ml is added to each well.

vii) The plates are incubated for 3–7 days in a 5% CO2 atmosphere at 37°C; the test can be performed successfully, if the plates are incubated without CO2.

viii) The plates are read microscopically for CPE. The test is validated by checking the back titration of virus (which should give a value of 100 TCID50 with a permissible range of 50–200 TCID50) and the control sera. The standard positive serum should give a value within 0.3 log10 units either side of its predetermined mean. Readings of each test serum dilution should be made with reference to the appropriate serum-only control to distinguish viral CPE from serum-induced cytotoxicity or contamination.

ix) The test serum results are determined by the Spearman–Kärber method as the dilution of serum that neutralised the virus in 50% of wells.

x) A negative serum should give no neutralisation at the lowest dilution tested (i.e. undiluted serum, equivalent to a dilution of 1/2 at the neutralisation stage).

2.2. Transmissible gastroenteritis virus-specific tests to differentiate TGEV- from PRCV-infected pigs

TGEV-specific tests are blocking or competition ELISAs that use an MAb that recognises TGEV but not PRCV (Brown & Paton, 1991; Callebaut *et al*., 1989; Sestak *et al*., 1999b; Simkins *et al*., 1992; Van Nieuwstadt & Boonstra, 1991) and are the tests of choice for qualifying animals for export. Test sera from pigs previously infected with a strain of TGEV recognised by the MAb will contain antibodies of the same specificity that can compete with it for binding to TGEV antigen-coated ELISA plates. Pigs infected with PRCV that does not contain the TGEV unique epitope will not produce antibodies to this epitope; hence, PRCV antibodies will not compete with or block binding of the TGEV-specific MAb (Brown & Paton, 1991; Callebaut *et al*., 1989; Sestak *et al*., 1999b; Simkins *et al*., 1992; Van Nieuwstadt & Boonstra, 1991). ELISA antigens may be prepared from cell lysates of kidney cell lines that were either inoculated with tissue-culture-adapted strains of TGEV, or uninfected. Alternatively TGEV-infected or uninfected swine testes cells fixed in 80% acetone have been used as an antigen source, or antigens may be prepared from recombinant S (rec-S) protein harvested in soluble form from an insect (Sf9) cell

line infected with a recombinant baculovirus expressing a TGEV S protein containing the four major antigenic sites (Sestak *et al*., 1999b; Simkins *et al*., 1992). Positive and negative antigens are coated to alternate rows of microtitre plates using bicarbonate buffer, pH 9.6. Diluted test sera, including known TGEV positive and known TGEV/PRCV negative controls, as well as known PRCV positive (negative in this test, positive in VN test) are added to appropriate wells and incubated overnight before further addition of diluted MAb to all wells. Bound MAb is detected by a peroxidase-conjugated anti-mouse antibody that induces a colour reaction in the presence of an appropriate substrate. The colour changes are measured using spectrophotometer, and for each test sample the net result is the difference in absorbance between the positive and negative antigen wells, expressed as a percentage of the result obtained with the negative control serum. The negative–positive cut-off value for the test must be determined by previous testing of known negative and positive populations. There are several commercial kits available that are TGEV specific.

Haemagglutination-based tests described to date (Labadie *et al*., 1977; Noda *et al*., 1987; Shimizu & Shimizu, 1977) were validated before the appearance of PRCV. However, they may be TGEV specific as TGEV, but not PRCV, is haemagglutinating (Schultze *et al*., 1996).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Vaccination against TGE is carried out in several countries.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

Information on experimental work or field trials of TGEV vaccines licensed for use in the United States of America (USA) has been reviewed, including possible limitations in their field efficacy and concepts related to the design of optimal TGEV vaccines (Saif, 1993; Saif & Jackwood, 1990; Saif & Sestak, 2006). Several manufacturers are licensed to produce TGEV vaccines in the USA: the vaccines include modified live and inactivated vaccines. The modified live vaccines are used for oral administration to pregnant sows (to induce passive immunity) or have also been licensed for oral administration to nursing or weaned pigs (to induce active immunity). Inactivated TGEV vaccines are licensed for parenteral inoculation of pregnant sows by the intramuscular route or for intraperitoneal administration to nursing or weaned pigs. In general, these vaccines induced marginal passive protection against TGEV challenge of nursing piglets when evaluated under controlled experimental conditions or in the field in TGEV/PRCV herds. Although they fail to adequately protect against epizootic TGE, data suggest that these vaccines may provide some efficacy against enzootic TGEV by stimulating an anamnestic antibody response to TGEV in serum and milk (Saif & Jackwood, 1990; Saif & Sestak, 2006).

The main reason proposed for TGEV vaccine failures was their inability to stimulate high levels of secretory IgA (SIgA) antibodies in milk analogous to the SIgA antibody responses found in the milk of sows naturally infected with TGEV (Saif & Jackwood, 1990; Saif & Sestak, 2006). Furthermore, these vaccines did not adequately protect the seronegative sow against TGE, such that illness in the sow often resulted in anorexia, agalatica and failure to passively protect her piglets. Thus the modified live vaccines may fail to replicate to the extent required to induce protective immunity in the intestine, or if given to seronegative neonatal animals, concerns exist regarding their possible reversion to virulence. Killed vaccines given parenterally do not induce SIgA antibodies; cell-mediated immune responses are often poor and the duration of immunity may be short-lived. Although use of PRCV strains as vaccine candidates for TGE has been proposed, experimental studies regarding their efficacy against TGEV have shown a lack of efficacy (Paton & Brown, 1990) or only partial cross-protection (Bernard *et al*., 1989; Cox *et al*., 1993; Van Cott *et al*., 1994). However, the widespread prevalence of PRCV infections in the swine population in Europe appears to have dramatically reduced the incidence of epizootic TGE in Europe (Pensaert *et al*., 1986). Newer recombinant DNA strategies for the development of TGEV vaccines include the possible use of an S protein subunit vaccine (contingent upon the development of mucosal delivery systems and adjuvants) (Park *et al*., 1998; Sestak *et al*., 1999a; Shoup *et al*., 1997) or the use of live recombinant viral or bacterial vectors that express TGEV genes important for the induction of immunity (Enjuanes *et al*., 2001; Saif, 1993; Saif & Sestak, 2006; Smerdou *et al*., 1996; Torres *et al*., 1996; Yount *et al*., 2000).

There are a number of general requirements (e.g. produced in a licensed facility, label rules, tracking capability, etc.) that apply to all biological products including vaccines. A set of regulations exist (called standard requirements, or SRs) that describe testing to be done on the vaccine and parent materials. Detailed information

on SRs for vaccines in the USA are contained in the Code of Federal Regulations (CFR) Title 9, Volume 1, Part 113 (abbreviated below as 9 CFR, 113) (United States Department of Agriculture [USDA], 1995). The general European Pharmacopoeia monograph and EMEA (European Medicines Agency) guidelines are applicable to TGEV vaccines, even though no vaccines are currently used in the European Union.

1. Seed management

1.1. Characteristics of the seed

The seed virus must be tested for purity and identity. The purity includes freedom from bacteria and fungi (9 CFR 113:27), mycoplasmas (9 CFR 113:28), and extraneous viruses (9 CFR 113:55) (USDA, 1995). The demonstration of identity is usually accomplished by VN or FAT. Genetically engineered vaccines or naturally selected vaccines with claims of antigen-coding gene deletion/inactivation are required to provide evidence (genotypic and/or phenotypic) of that identity.

1.2. Method of culture

Culture must be carried out on proven uncontaminated (approved) cells, and the number of cell culture passages is limited (usually to five). It is not required that the species of origin of the cell line be that of the target species.

1.3. Validation as a vaccine

Vaccine validation takes two forms. The master seed is considered to be immunogenic if a vaccine made at the highest passage, and according to the outline of production, is shown to be protective. The lowest antigenic level (modified live virus titre or inactivated antigen mass) shown to be protective becomes the baseline for all future serials (lots) of the product. In the case of live products, factors for titration variation and the death curve over time would be added. These trial vaccines should be tested for purity, safety, and efficacy by the manufacturer. Protection must be shown against the natural disease with the virulent challenge virus. Virulent challenge virus is defined as the dose that causes disease in $\geq$95% of the susceptible controls. Three prelicense serials must subsequently be made and tested by the manufacturer and by the licensing authority, for potency, sterility and safety.

2. Method of manufacture

This is proprietary information for each manufacturer and hence not available.

3. In-process control

This is largely proprietary. Some in-process controls refer directly to production (e.g. O_2 concentration in the fermenter). Another category, however, includes tests similar to the final container potency test. For all vaccines, the simpler the final batch or container potency test, the more likely it is that it may be used as a monitoring/blending test: for example, virus titration on sub-batches may be used to predict final blended batch titre. Ingredients of animal origin must be sterilised or shown to be free from contamination.

4. Batch control

Batches must be blended to the final specifications and bottling specifications (e.g. fermentation runs may be pooled, or one run may be split and pooled with each of three others, etc.). In some countries, bulk and process control define the product and are the subject of intense regulation and scrutiny. The emphasis in the USA is on the final product. Batch control techniques must be detailed in the outline of production and must be meaningful, trackable, and the manufacturer must discard product that fails to meet specifications. If a batch is to be exported to another country for bottling or blending, then it is subjected to all the testing as though it were final product.

4.1. Sterility

All products must be tested for sterility. The manufacturer may also run sterility tests on batches for monitoring. Tests are similar to those described in Section C.1.1.

4.2. Safety

Safety tests are done before the licence is granted, and then on the final container (Sections C.5.1 and C.5.2).

4.3. Potency

Potency would normally only be done if the potency test were a simple test (e.g. ELISA) to confirm the blending calculations before bottling.

4.4. Duration of immunity

Duration of immunity is tested in the prelicence (efficacy) serial test, not the batch control. New products are required to support label claims for revaccination schedules with efficacy trials (challenge) at the specified time after vaccination.

4.5. Stability

Stability is established before the licence is granted. Usually accelerated ageing (37°C) is used to estimate the lifetime so that the products do not have to be kept at storage temperature (4°C) for the real-time period. This will be confirmed with real-time data later. The manufacturer is not required to do stability testing. Manufacturers are required to state the amount of antigenic material that will be in their product throughout the shelf life. Samples of product are selected (usually live) and tested within 30 days of expiration to see if, for example, the titre is at the level stated in the manufacturer's outline. Stability is also affected by moisture. Moisture left in a desiccated product can shorten its life, so this has to be tested in the final product or in-process.

4.6. Preservatives

There are restrictions on the maximum amounts of antibiotics that can be in a product. Restrictions on some vaccine components are related to their safety and to whether the stated withdrawal period is long enough for the component to have cleared before the animal is slaughtered. Preservatives used are proprietary.

4.7. Precautions (hazards)

Any risks to vaccinates need to be clearly stated on the label. This usually applies to pregnancy warnings for abortogenic live viruses, and the general anaphylaxis warning, but may also attempt to warn the user about soreness or swelling at the injection site, or transient fever or inappetence in some cases. No unusual label precautions apply to the TGE vaccines currently licensed.

5. Tests on the final product

5.1. Safety

Usually this will be a mouse and/or a guinea-pig or swine safety test (9 CFR 113:33, and Witte, 1971). Sterility tests are also carried out on the final product.

5.2. Potency

There is no single test for release potency. Whatever test is used must be correlated to protection in the host animal (the efficacy tests). The potency of live TGEV vaccines can be evaluated by *in-vitro* titration of the viral infectious dose in cell culture (Saif, 1993). This titre must be correlated with the minimum viral titre required to induce protective immunity against experimental challenge, and also against natural challenge under field conditions. The potency of killed vaccines is evaluated by vaccination and challenge tests using different doses of the vaccine. Titres of neutralising antibodies induced by inoculation of laboratory animals with the vaccine may be accepted if there is an established correlation with development of protective immunity.

Particular viral antigens associated with the induction of neutralising antibodies and protection against challenge can be quantified in killed vaccines using specific MAbs in ELISA, such as neutralising MAbs to the S protein of TGEV (Saif, 1993).

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NB: At the time of publication (2024) there were no WOAH Reference Laboratories for transmissible gastroenteritis (please consult the WOAH Web site for the current list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2008.