PART IV

Quality assurance and quality control

Quality assurance and good manufacturing practice

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In recent years, quality assurance (QA) and good manufacturing practice (GMP) have become increasingly important in the pharmaceutical industry. They are of particular importance in the manufacture of veterinary vaccines since such products have the following specific characteristics:

- The active ingredients are almost always produced by the manufacturer (and not as is generally the case by another industry, as for example with chemicals).
- Vaccine production usually requires cultivation steps, including growth of the appropriate organism and the use of substances of animal origin, which makes it easy to introduce a contaminant and to amplify low levels of contamination.
- As the end product is not usually subjected to a final sterilization step, prior to final formulation its constituents should be particularly well protected against contamination and cross-contamination.

Manufacture requires the handling of live organisms which are sometimes pathogenic for humans and/or animals. The release of these agents, with the possibility of contamination/cross-contamination, has to be regarded as a serious danger and, depending on the organism involved, the workers and the environment, together with all the materials, should be well protected. Moreover, the level of risk is further exacerbated by the large number of animal species and potential pathogenic agents. The variety of products manufactured is very great but the volume of manufacture is sometimes quite low, so manufacturing operations based on the sharing of equipment and facilities is common. In addition, other activities such as diagnosis and research are frequently linked to manufacture and this may result in opportunities for cross-contamination.

Vaccine manufacture is a complex activity, with risks, which is carried out in a complicated environment. Particular aspects of the work are important in relation to potential problems of contamination, for example contamination of the product, cross-contamination, possible amplification of contamination organisms and contamination of workers and the environment.

These factors, together with the inherent variability of biological agents and materials and the relative inefficiency of quality control tests in providing adequate reassurance for final products, means that the roles of the QA system and GMP are of the utmost importance. Not only should the requirements of general current GMP for medicinal products be applied, but also the specific requirements of particular products.

The need to maintain control over all aspects of GMP cannot be overemphasized.

In this chapter an overview of QA and GMP, with special attention to some of the particular requirements of vaccine manufacture, will be given. It has to be emphasized that responsible persons in vaccine manufacturing must have a good

knowledge of the requirements of QA and GMP, and those responsible for research and development have to appreciate the significance of QA.

The European Union's (EU) guidelines on GMPs for medicinal products (EC, 1992a) dedicates an entire annex to immunological veterinary medicinal products (IVMPs).

QUALITY ASSURANCE

Quality

The International Organization for Standardization (ISO) defines quality as: "the totality of features and characteristics of a product or service that bears on its ability to satisfy stated or implied needs" (ISO, 1994).

For medicinal products, the main components of quality, as defined by ISO, are:

- *safety;*
- *efficacy* to produce the expected effect;
- *quality,* in the narrow sense of analytical and manufacturing quality.

These are particularly important but, among others, the following should be added:

- simplicity of use (administration, storage, etc.);
- compatibility with immunoprophylaxis, if required;
- the overall cost of the product, which should be reasonable.

A medicinal product (as well as any other type of product) can only meet the required standard of quality if it has been properly designed and manufactured. This is ensured through the application of a QA system.

ISO gives the following definition of QA: "All those planned actions and systemifications necessary to provide adequate confidence that a product or service will satisfy given requirements for quality" (ISO, 1994) and the EU guidelines add the following:

> Quality assurance is a wide-ranging concept which covers all matters which individually or collectively influence the quality of a product. It is the total sum of the organized arrangements made with the object of ensuring that medicinal products are of the quality required for their intended use. (EC, 1992a.)

QA is a comprehensive system, designed, documented, implemented and furnished with personnel, equipment and other resources so as to provide assurance that products will be consistently of the required quality. The system therefore involves obtaining high quality at every level, from design to manufacture, product servicing and follow-up.

Figure 12 shows the relations between the different parts of QA.

Quality assurance for design

QA should ensure the avoidance of any design defect. The possibility of defects should always be borne in mind because they may affect all batches and their occurrence is virtually inescapable. Defects can be particularly costly, for example incomplete inactivation conditions as a result of a design defect in the manufacturing process may lead to disease in vaccinated animals.

New methods and rules enable the implementation of QA procedures into the design of manufacturing processes related to the production of vaccines.

Design reviews. These are defined by ISO as: "a formal documented, comprehensive and systematic examination of a design to meet these requirements and to identify problems and propose solutions" (ISO, 1994).

Good laboratory practice. Good laboratory practice (GLP) refers to laboratory organization and the conditions under which trials are planned, carried out and reported (OECD, 1981; EC, 1987, 1990b and 1992b).

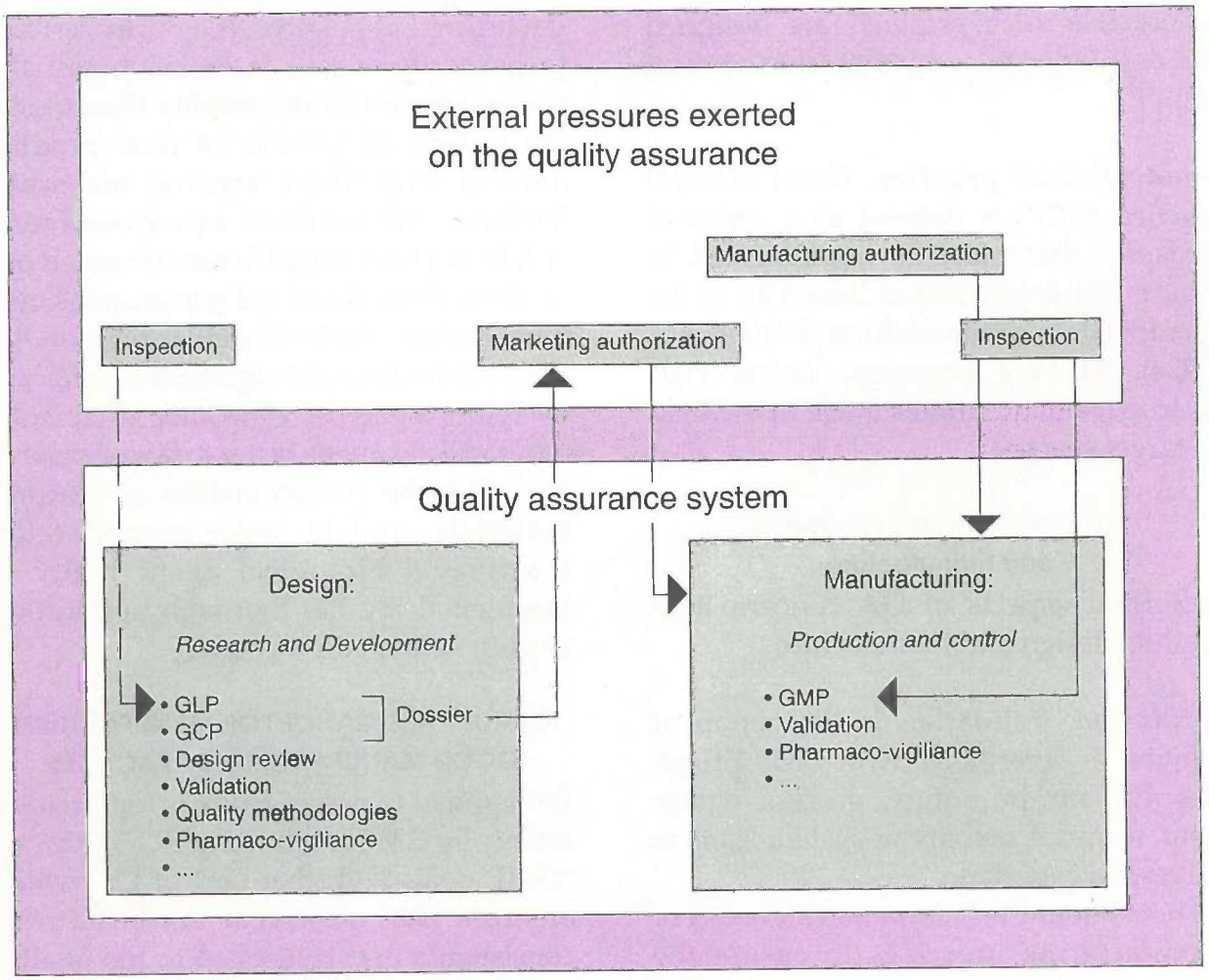

FIGURE 12
The quality assurance system

GLP also applies to the non-clinical trials designed to assess the properties and safety of veterinary medicinal products so that the quality and integrity of future trial results can be guaranteed.

The EU guidelines (EC, 1992b) indicate that the system of QA appropriate for the manufacture of medicinal products should ensure that such products are designed and developed in a way that takes account of GLP.

Good clinical practice. Good clinical practice (GCP) is defined as a series of measures that must be implemented to ensure the quality and authenticity of the scientific data obtained through trials (EC, 1992c). For GCP purposes, clinical trials mean systematic studies made in the field on target species.

Quality assurance for design and manufacture

Important aspects of QA concern both product design and manufacture.

Validation. Validation is the action of proving in accordance with GMP principles that any procedure, process, equipment, material, activity or system leads to the results expected.

There should be complete validation of manufacturing processes to ensure the continuous conformity of vaccine batches to required standards (EC, 1990a).

Production methods. Methods that ensure product quality and standards for the management of QA work (ISO, 1987a and 1987b) should be in operation.

Pharmaco-vigilance. Procedures to monitor the use of products in accordance with good standards of pharmaco-vigilance should be established. This will allow data on rare and unexpected effects to be recorded and analysed.

It must be emphasized that in both the design and manufacture of products, the prevention of defects is a better policy than relying on the results of certain in-process tests or tests of the final product (Soulebot, 1992).

With regard to control tests, it is best that they be carried out as far upstream in the process as possible. If a purity test has to be performed, it is easier to test the starting materials thoroughly than to test the dozens of batches of final product derived from those starting materials. Similarly, the results of inactivation tests will be of greater significance if carried out on the unformulated antigen suspension – whereas the results of similar tests on the final product may be significantly affected by the presence of adjuvants, excipients, etc. It will always be better to take measures to avoid the contamination of starting materials, and to make sure that the inactivated biological agent really is inactivated, by the thorough application of duly validated processes.

QUALITY ASSURANCE FOR MANUFACTURE: GOOD MANUFACTURING PRACTICES

QA applied to manufacture is represented mainly by GMPs, defined in EC (1992a) as: "GMP deals with that part of QA which ensures that products are produced consistently and controlled to the quality standards appropriate to their intended use".

Principles

QA (and GMP) should ensure the avoidance of any manufacturing or product defect. In order to achieve this purpose, the following general principles and aims should be adhered to:

- The holder of a manufacturing licence must manufacture medicinal products so as to ensure that they are fit for their intended use, comply with the requirements of the marketing authorization

and do not place animals at risk owing to inadequate safety, quality or efficacy.

- The attainment of these objectives is the responsibility of senior management and requires the participation and commitment of staff in many different departments and at all levels within the company. This also applies to the company's suppliers and distributors.
- To achieve these quality objectives consistently, there must be a comprehensively designed and properly implemented QA system that incorporates GMP and quality control. The system should be fully documented and its effectiveness monitored.
- All parts of the QA system should be adequately resourced with competent personnel and suitable and sufficient premises, equipment and facilities.

The purposes of QA

The QA system intended for the control of the manufacture of medicinal products should ensure the following:

- Medicinal products are designed and developed in a way that takes account of the requirements of GMP and GLP.
- Production and control operations are clearly specified and GMPs adopted.
- Managerial responsibilities are clearly defined.
- Arrangements are made for the manufacture, supply and use of the appropriate raw materials and packaging materials.
- All necessary controls on intermediate products, and any other in-process controls and validations, are carried out.
- The final product is correctly processed and checked, according to the necessary defined procedures.
- Medicinal products are not sold or supplied before an appropriately qualified person has certified that each production batch has been produced and controlled in accordance with the requirements of the marketing authorization and any other regulations relevant to the production, control and release of medicinal products.
- Satisfactory arrangements are made to ensure, as far as possible, that the medicinal products are stored, distributed and handled in such as way as to maintain their quality throughout their shelf-life.
- There is a procedure for in-house inspection and quality audit which regularly appraises the effectiveness and applicability of the QA system.

GMP for medicinal products

GMP is that part of QA that ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the marketing authorization or product specification.

GMP is concerned with both production and quality control. The following are the basic requirements of GMP:

- All manufacturing processes are clearly defined, systematically reviewed and shown to be capable of consistently manufacturing medicinal products of the required quality and complying with their specifications.
- Critical steps in the manufacturing process and significant changes to that process are validated.
- All the necessary facilities for GMP are provided, including: appropriately qualified and trained personnel; adequate premises and space; suitable equipment and services; correct materials, containers and labels; approved procedures and instructions; and suitable storage and transport.
- Instructions and procedures are written in clear and unambiguous language, specifically applicable to the facilities provided.

- Operators are trained to carry out procedures correctly.
- Records are made (manually and/or by recording instruments) during manufacture to demonstrate that all the steps required by the defined procedures and instructions are taken and that the quantity and quality of the product was as expected. Any significant deviations should be fully recorded and investigated.
- Records of manufacture and distribution, which enable the complete history of a batch to be traced, are retained in a comprehensible and accessible form.
- The method of distribution of products minimizes any risk to their quality.
- A system is available to recall any batch of product from sale or supply.
- Complaints about marketed products are examined, the causes of quality defects investigated and appropriate measures taken in respect of defective products so as to prevent reoccurrence.
- Rigorous standards of hygiene and cleanliness are applied.

Quality control

Quality control (QC) is that part of GMP that is concerned with the taking of samples during production, the specifications related to the product and the tests to be applied. It is also concerned with the organization, documentation and release procedures to ensure that the necessary and relevant tests are actually carried out and that materials are not released for use, or products released for sale or supply, until their quality has been judged to be satisfactory.

The following are the basic requirements of QC:

- Adequate facilities, trained personnel and approved procedures are available for sampling. Raw materials, packaging materials, intermediate, bulk and final products should be inspected and tested and, where appropriate, environmental conditions should be monitored for GMP purposes.
- Samples of starting materials, packaging materials, intermediate products, bulk products and final products are taken by qualified personnel and by methods approved by the requirements of QC.
- Test methods are validated.
- Records are made (manually and/or by recording instruments) that demonstrate that all the required sampling, inspection and testing procedures have been carried out. Any deviations should be fully recorded and investigated.
- The final products contain only active ingredients that comply with the qualitative and quantitative composition specified in the marketing authorization, are of the purity required and are enclosed within their proper containers and correctly labelled.
- Records are made of the results of inspection and the testing of materials, intermediate, bulk and final products is correctly assessed against specifications. Production assessment includes a review and evaluation of the relevant production documentation and an assessment of any deviations from specified procedures.
- No batch of product is released for sale or supply prior to certification by an appropriately qualified person in accordance with the requirement of the marketing authorization.
- Sufficient samples of starting materials and products are retained to permit future examination of the product if necessary. Products should be retained in their final packs unless exceptionally large packs are produced.

GOOD MANUFACTURING PRACTICES

GMPs are concerned with such issues as:

- personnel training, organization, safety and hygiene;
- premises and equipment;
- documentation;
- production;
- quality control;
- contract manufacture and analysis;
- complaints and products recall;
- in-house inspections.

Personnel training, organization, safety and hygiene

The establishment and maintenance of a satisfactory QA system and the correct manufacture of medicinal products rely on people. There must be sufficient qualified personnel to carry out all the tasks that are the responsibility of the manufacturer. Individual responsibilites should be clearly understood by the individuals and recorded. All personnel should be aware of the GMP principles that affect them and should receive initial and continuing training, including hygiene instructions, relevant to their needs. Staff should be well motivated.

The following are some of the important aspects relating to personnel in vaccine manufacturing establishments:

- Key personnel are the head of production, the head of quality control (who must be independent from each other) and the qualified person(s) who must ensure that each batch has been produced and checked in accordance with regulations and the marketing authorization.
- Basic and appropriate training should be given, according to training programmes.
- Personnel should be protected against possible infection, particularly in the case of microorganisms known to cause disease in humans that are either handled directly or used in work with experimental animals. Where appropriate, personnel should be immunized.
- Adequate measures should be taken to prevent microorganisms being carried outside the plant by personnel.
- The risk of contamination or cross-contamination of vaccines by personnel is particularly important. Prevention should be achieved by a set of measures and procedures (the use of protective clothing, etc.).
- Detailed hygiene programmes should be established.

Premises and equipment

Premises and equipment must be located, designed, constructed, adapted and maintained to suit the operations to be carried out. Their layout and design must aim to minimize the risk of errors and permit effective cleaning and maintenance in order to avoid cross-contamination, the build-up of dust or dirt and any other adverse effect on the quality of products.

Premises. Premises should be designed in such a way as to control the risk to both the product and the environment. This can be achieved by the use of containment, clean, clean/contained, contained and controlled areas.

Live biological agents should be handled in *contained areas*. The level of containment will depend on the pathogenicity of the microorganism.

Inactivated microorganisms and non-infected cells isolated from multicellular organisms should be handled in *clean areas*.

Open-circuit operations involving products or components that are not subsequently sterilized, should be carried out within a laminar airflow workstation (grade A) in a grade B area (see Table 14).

Other operations where live biological agents are handled (quality control, research, diagnosis) should be appropriately contained and separated if production

operations are carried out in the same building or in buildings in close proximity to those used for production.

Containment premises should be easily disinfected and have the following characteristics:

- an absence of direct venting to the outside;
- ventilation with air held at negative pressure – air should be extracted through high-efficiency particle absorption (HEPA) filters;
- a system for the collection and disinfection of liquid effluents – solid waste should be disinfected, sterilized or incinerated as appropriate;
- a changing room designed and used as an airlock, equipped with appropriate showering facilities;
- an airlock system for use when equipment is being transferred;
- in many instances, a barrier-type double-door autoclave.

With the exception of blending and subsequent filling operations, only one biological agent should be handled at a time within each area and production areas should be designed to permit disinfection between production runs with different organisms, using validated methods.

The characteristics of *clean areas* are well known and described in the GMP literature. Clean areas have a ventilation system with air under positive pressure (see Table 14).

Clean/contained areas have the characteristics of both clean and contained areas, but have a ventilation system with air at negative pressure. The outside section of the changing room is under positive pressure, the inside section is under a negative pressure that is higher than that of the work area. There is therefore an air pressure barrier. This type of area is used when it is necessary to protect the product and the environment against the handling of live organisms during certain production processes, for example the inoculation or harvest of roller bottle cultures during virus multiplication steps.

TABLE 14

Air classification system for the manufacture of sterile products

Grade	Maximum permitted number of particles per m^3 equal to or above:		Maximum permitted number of viable microorganism per m^3	Possible final filter efficiency[1] (%)
	0.5/μm	5/μm		
A laminar airflow workstation	3 500	None	Less than 1[2]	99.997
B	3 500	None	5[2]	99.995
C	350 000	2 000	100	99.950
D	3 500 000	20 000	500	95.000

Notes:

Laminar airflow systems should provide a homogeneous air speed of 0.30 m per second for vertical flow and 0.45 m per second for horizontal flow.

In order to reach air grades B, C and D, the number of air changes should generally be higher than 20 per hour in a room with a good airflow pattern and appropriate HEPA filters.

The guidance given for the maximum permitted number of particles corresponds approximately to the United States Federal Standard 209 C as follows: Class 100 (grades A and B), Class 10 000 (grade C) and Class 100 000 (grade D).

It is accepted that it may not always be possible to demonstrate conformity with particulate standards at the point of fill when filling is in progress owing to the generation of particles or droplets from the product itself.

The air pressure differentials between rooms of successively lower risk should be at least 1.5 mm water gauge.

[1] Given as an indication, percentage determined by BS 3928.

[2] The low values involved here are only reliable when a large number of air samples are taken.

Box 1
Definitions

Clean area
An area with defined environmental control of particulate and microbial contamination, constructed and used in such a way as to reduce the introduction, generation and retention of contaminants within the area.

Clean/contained area
An area constructed and operated in such a manner that will achieve the aims of both a clean area and a contained area at the same time.

Contained area
An area constructed and operated in such a manner (and equipped with appropriate air handling and filtration) so as to prevent contamination of the external environment by biological agents from within the area.

Containment
The action of confining a biological agent or other entity within a defined space.

Controlled area
An area constructed and operated in such a manner that some attempt is made to control the introduction of potential contamination (an air supply approximating to grade D [Table 13] may be appropriate) and the consequences of accidental release of living organisms. The level of control exercised should reflect the nature of the organism employed in the process. At a minimum, the area should be maintained at a pressure negative to the immediate external environment and should allow for the efficient removal of small quantities of airborne contaminants.

Primary containment
A system of containment that prevents the escape of a biological agent into the immediate working environment. It involves the use of closed containers and biological safety procedures along with secure operating procedures.

Secondary containment
A system of containment that prevents the escape of a biological agent into the external environment or into other working areas. It involves the use of rooms with specially designed air handling, airlocks and/or sterilizers for the exit of materials and secure operating procedures. In many cases it may add to the effectiveness of primary containment.

Animal houses should be separated from other production premises.

Documentation relating to the premises should be readily available.

Equipment. The equipment used should be designed and constructed to meet the particular requirements for the manufacture of each product.

Before being put into operation it should be assessed and validated as suitable for the process. Subsequently every piece of equipment should be regularly maintained and validated.

Where appropriate the equipment should ensure the satisfactory primary containment of the microorganism involved in the process and should be designed and constructed to allow easy and effective decontamination and/or sterilization.

Separate incubators should be used for infected and non-infected containers, and also for different organisms or cells in general.

Careful consideration should be given to the procedures and equipment aimed at avoiding environmental contamination (wastes, effluents, etc.).

Documentation

Good documentation constitutes an essential part of the QA system. Clearly written documentation prevents errors arising from spoken communication and permits the tracing of a batch's history. Specifications, manufacturing formulae and instructions, procedures and records must be free from errors and available in writing. The legibility of documents is of paramount importance.

Important aspects of documentation include:

- Specifications, manufacturing formulae, processing and packaging instructions, procedures and records are of paramount importance.
- It is particularly important that the data generated by the monitoring of various aspects of GMP (equipment, product, premises, etc.) are rigorously assessed and that informed decisions are made and recorded.

Production

Production operations must follow clearly defined and validated procedures in accordance with the relevant manufacturing and marketing licences. Careful attention must be paid to the constant monitoring of production and to in-process controls.

Starting materials. The properties required of starting materials should be clearly defined in written specifications. This is particularly important for substances of animal origin when the geographical origin and the animal species from which the materials are derived should be included. Special attention should be paid to the supplier's QA system.

Where possible, heat is the preferred method for sterilizing starting materials.

Media. Media should preferably be sterilized *in situ* or in line. Heat is the preferred method.

Seed lot and cell bank systems. In order to prevent the undesirable drift of properties that can ensue from multiple generations (i.e. many serial growth passages), a seed lot or cell bank system should be used. Seeds and/or cells should all be adequately characterized, tested, stored, etc.

Operating principles. Accidental spillages, especially of live organisms, must be dealt with quickly and safely.

The formation of droplets and the production of aerosols or foams should be avoided. Centrifugation and blending should be carried out in appropriate contained or clean/contained areas or in a closed system when it is necessary to prevent the transfer of organisms.

A closed system or laminar airflow cabinet should be used for operations such as the transfer of sterile media.

Equipment, the external surfaces of containers, etc. must be disinfected before transfer from a contained area and liquid and solid waste must be sterilized or disinfected.

Quality control

QC is concerned with sampling, specifications and testing, as well as with the organizational, documentation and release procedures that ensure the necessary and relevant tests have been carried out and that materials are not released for use, or products released for sale or supply, until their quality has been judged satisfactory. QC is not confined to laboratory operations, but must be involved in all decisions that may affect the quality of the product. The independence of the QC section from

the production group is considered fundamental to the satisfactory operation of QC.

Important aspects of QC include:

- Control laboratories should apply good quality-control laboratory practice.
- In-process controls play an especially important role. Important controls that cannot be carried out on the final product should be performed at an appropriate stage of production.

Contract manufacture and analysis

Manufacture under contract and analysis must be properly defined, agreed and controlled to avoid the misunderstandings that could result in a product or service of unsatisfactory quality. There must be a written contract between the contractor and the customer which clearly establishes the duties and liabilities of each party. The contract must clearly state the procedures used by the qualified person when releasing each batch of product for sale.

Complaints and product recall

All complaints and other information concerning potentially defective products must be reviewed carefully according to written procedures. A system should be in place to recall from the market, in a prompt and effective manner, all products known to be, or suspected of being, defective.

In-house inspections

In-house inspections should be conducted for the purpose of monitoring the implementation of and compliance with GMP principles and to propose corrective measures where necessary.

They should be conducted in an impartial and independent manner, following a pre-arranged programme, and should be recorded. Statements of the actions subsequently taken should also be recorded.

Most countries have their own GMP guidelines or regulations which are compulsory. Other countries are covered by the World Health Organization guidelines (WHO, 1992) which should be followed. The United States Code of Federal Regulations (CFR) (USDA, 1994), as well as EU requirements (EC, 1991a and 1991b), will also be very helpful.

There is now a move to apply the same regulations across a group of different countries, as for example in the Member States of the EU.

MAINTENANCE OF THE QUALITY ASSURANCE SYSTEM: INSPECTIONS

The integrity of the QA system, after its implementation, can only be maintained (and the introduction of undesirable small changes in the operation resisted) by the application of both internal and external pressures or checks.

Internal pressure is represented by verification and validation from in-house inspections, design reviews and the continuous attention of top management. External pressures are those exerted by competition and customers. The latter exercise direct pressure by placing orders or not. Indirect pressure is exerted by legal authorities, through official licensing (manufacturing and marketing licences) and official inspections (see Figure 12, p. 299). Such inspections are very important. Their degree of implementation differs considerably from one country to another and between GMP and GLP and an appropriate and uniform system of inspection is highly desirable.

CONCLUSIONS

When applied to vaccines, the QA system assures users that the necessary standards of quality in relation to products have been maintained (i.e. products have been suitably designed and produced and properly inspected at a reasonable cost). The QA system should also allow the manufacturer and the national control authorities to reduce costly and time-

consuming control tests that offer only a relative degree of reliability.

Such developments are to some extent new in regard to the concepts and practice of vaccine manufacture. Confidence in the product and the manufacturing process is best achieved when standards and methods of working are properly challenged by an appropriate and efficient system of QC.

The requirements of an efficient QA system should facilitate batch release and permit easy, free and fair trading and/or the exchange of vaccines for use in different regions.

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The principles of good laboratory practices, including safety in vaccine production and quality control

C.A. Mebus

The general principles of good laboratory practice (GLP) and quality control (QC) in veterinary vaccine production apply to both live and inactivated viral and bacterial vaccines. The first part of this chapter will address the general principles of good laboratory practices associated with the production of a biological product and the second part will be more specific on requirements for quality assurance.

GENERAL PRINCIPLES OF GOOD LABORATORY PRACTICE

A biological product must be pure, safe, potent and efficacious. To produce such a product there must be a proper facility that has been inspected and approved as appropriate for the manufacture of safe biological products. Close attention must be given to all the parameters associated with production of biological products, i.e. to ensure a good-quality product production must be carried out in accordance with good laboratory practices (GLPs). GLPs are a set of rules, operating procedures and practices that describe how laboratory procedures, tests and studies are planned, performed, monitored, recorded and reported, to ensure the quality and integrity of the data generated by a laboratory. These procedures and practices should be detailed and written in a clear manner, so that someone unfamiliar with them could perform the work, for example the operation and/or calibration of an instrument should be described, the speed at which to run the centrifuge should be given and the way in which to handle record charts should be described. These procedures and practices are generally written by the manufacturer and approved by the regulatory authority.

MANAGEMENT

Laboratory management is responsible for providing appropriate facilities, qualified personnel and good equipment, reagents and materials and for maintaining personnel records and ensuring that written and approved standard operating procedures, protocols and schedules are established and documented for all aspects of production.

There should be a document that specifies the authority, responsibility and interrelation of all laboratory personnel (management and staff) involved in the production of biological products. The laboratory organization should include a person responsible for ensuring that GLPs are in place, a technical manager who has the overall responsibility for production and a quality control manager who is responsible for all aspects affecting product quality, including maintenance, calibration, validation, monitoring of equipment and instruments and testing of the final product. The quality control manager should report directly to senior management.

THE FACILITY

Plans of the property showing the location of all buildings and blueprints on which

are listed all the activities carried out in these buildings should be developed and made available. To minimize opportunistic cross-contamination, specific buildings should be dedicated to the production of particular biologicals and there should be no diagnostic and/or research activities in such buildings.

Buildings should be designed and constructed to prevent cross-contamination during the process of production. Halls, rooms and production areas should be arranged to provide maximum biological security and minimal contamination of the production areas. Thus, the areas with the greatest potential for contamination of the product should be the furthest removed from the production area. Offices, eating areas, dressing rooms, toilet facilities and warehouse areas should be located so that they are accessible without passage through the production areas. Personnel and materials moving to a biological production area should not pass through the production area designated for another product.

The air supply to a production area should preferably be high-efficency particle absorption- (HEPA-) filtered and come directly from outside the building. Air pressure gradients within the building should be designed to minimize the potential for cross-contamination and exhaust air from a production area should not be recirculated to another production or building area.

In production areas, the floors, walls, ceilings and doors should be made of materials that will prevent cross-contamination and that can be easily cleaned and disinfected.

Water should be free of pollution and impurities and there must be an adequate supply and distribution of hot and cold water.

The sanitary system must have the necessary traps and vents and a disposal method that minimizes pollution. All maintenance and repairs should be documented.

The area around the building should be well-drained, clean and free of clutter. Animal facilities should be located away from production buildings and should be properly drained and ventilated and kept in a sanitary condition. An effort should be made to minimize the fly and rodent populations in the surrounding area and to keep the production areas free of these pests.

PERSONNEL

Each person working in biological production should have a personal file that includes a curriculum vitae, documentation of education and training and a detailed job description including to whom the person reports and details of those whom the person supervises.

Before working with a particular organism or agent, management must evaluate the potential for human infection and, if needed, implement immunization and/or other necessary precautions. Each area of the facility should have defined and documented operating procedures.

Production personnel must be competent in microbiological and good laboratory techniques through education and/or training. Before entering a biological production area, personnel should either change their clothes for clean laboratory clothing or cover their street clothes with appropriate laboratory garments. Hair covering, face masks, gowns and shoe covers should be used in production areas. Eating, smoking or any unsanitary practice should be prohibited in a production area.

To maintain a high level of competence, staff should receive periodic training in laboratory techniques and quality control procedures. Staff should be given opportunities for training in specific practices or of observing them in other institutions.

STERILIZATION

There should be written standard operating procedures for the washing and sterilization of all containers, instruments and equipment parts that will be in contact with the product. A recording device should be used with each sterilization cycle to ensure that the proper time, temperature and/or concentration of the sterilizing agent have been achieved. Items subjected to sterilization procedures should be labelled and dated.

Sterilization can be accomplished using live steam at a temperature of at least 120°C for not less than 30 minutes or dry heat at a temperature of at least 160°C for not less than one hour. If an instrument risks damage from either of these treatments, it can, instead, be boiled for not less than 15 minutes or subjected to chemical sterilization, for example ethylene oxide or formaldehyde would be acceptable if found to be effective.

LABELLING

Labels for identification should be placed on all ingredients, components of a biological product, biologicals in any stage of preparation and completed biological products. The label should include the date of preparation and the initials of the preparer.

SEED ORGANISMS

Responsibility for the storage of organisms to be used as master seeds should be assigned to a particular individual. All vials of seed material should be labelled and stored in a secure location and the record for all seeds should contain a documented history, test results and an accurate inventory. Protocols for the testing procedures used should also be on file.

The microorganism seeds used to produce biological products should be free of extraneous agents including viral, bacterial, mycotic and chalymydial organisms. This is best accomplished using a master seed principle. The master seed is a microbiological agent at a specific passage level from which all other seed passages are then made. When developing a vaccine, the master seed and the passage level (i.e. the number of seed passages, which is usually five) that will be used for production must have been shown to be pure, safe, potent and efficacious. The production seed is the specific passage level used to produce the antigen in the product and the working seed is a passage level that lies between the levels of the master seed and the production seed. Working and production seeds have to be shown to be free of any extraneous agent.

In viral vaccine production, the same benefits can be obtained by using a master cell principle for those cell lines used to produce viral antigen. A master cell is a supply of cells at a specific passage level from which working cells and production cells originate for the production of a biological. Master cells will have been shown to be free of extraneous agents and to be non-oncogenic, while working and production cells must be shown to be free of any extraneous agent.

STERILITY

To maximize the probability of the final product being free of extraneous agents, only certified (tested) working and production seeds and cells should be used. Media, if they cannot be heat-sterilized, should be filtered and tested for freedom from any extraneous agent before use. There should be protocols for filtration and for the testing of material for sterility.

The following are examples of ways to maintain sterility:

- Materials that need to be kept free of contamination should only be worked with in biological cabinets or in isolation rooms by people who

are properly dressed, to minimize the possibility of contamination.
- Work in cabinets or isolation rooms should be properly planned.
- Work with non-infected material should be done first, followed by work with infected material.
- After working with infected material, the area should be decontaminated and allowed to remain idle for sufficient time to allow dilution by air changes to minimize cross-contamination.
- The potential for bacterial contamination in a production area can be evaluated by incubating bacteriological media that have been exposed to the air in the area for 10 to 20 minutes.

OUTLINE OF PRODUCTION

Each biological product should have a detailed outline of production containing a protocol and guidelines. Where applicable, the outline of production should be in such detail that production could be carried out without prior experience.

An outline of production is made up of several sections.

Composition of the product

This section includes the source and passage history of the organism(s) and, if applicable, the relative proportions of organisms in the product.

Cultures

This section comprises:
- protocols and schedules (or frequencies) for identifying the organism(s) and frequency of identification;
- a protocol for determining the purity of culture(s) and, if applicable, the virulence of the organism(s) as well as the range of passage levels or subcultures to be used in production;
- the composition of the media to be used for seed and production cultures; sources of media ingredients, eggs, cell culture or tissues used; protocols for production of the media; and the methods used to determine the growth-promoting qualities of the media and their freedom from contaminants.

The protocols for production of media should include the formula, source and quality of ingredients; instructions on the storage and handling of ingredients; the quality of water required; equipment; the quality of glassware; procedures for product formulation and testing; the conditions for storage and handling of formulated media; and the product expiry date.

The protocol for testing the sterility and growth-promoting qualities of media should include preparation and testing of QC media, the source and care of QC cultures and media performance testing. Records must be made concurrently with the performance of successive steps in the production and testing of each lot of medium.

The record for each lot of medium should contain the name of the supplier, the lot number, the date of purchase, the date the seal was broken for each medium ingredient; the pH and osmolarity of the medium; the date the medium was prepared; and the initials of the preparer.

The outline of production should also include:
- a description of the containers used to grow organism(s) and instructions on how they are to be sterilized;
- storage conditions for seed cultures;
- the protocol for preparing inoculum;
- the technique for inoculation together with protocols for the preparation of production media and the titre and volume of the inoculum for each size and type of culture container;
- the duration and conditions of incubation;
- a description of the characteristics of growth and the characteristics of contamination;

- a description of the method of attenuation, if any, before the organism is used for production.

Harvest

This section comprises:

- the minimum and maximum time allowed between inoculation and harvesting and the characteristics of the culture at harvest;
- the protocol for the preparation and handling of cultures for harvesting;
- the protocol for harvesting;
- criteria for acceptable harvested material and the procedure for determining acceptability;
- instructions for the handling of discarded material not used in production;
- any additional pertinent information.

Preparation of the product

A detailed description should be given of every step from the harvest of the antigenic material to the completion of the product in the final container, emphasizing the following:

- the method of inactivation, attenuation or detoxification, if applicable;
- the composition of the preservative, adjuvant or stabilizer, the stage of production and the method of addition. (The proportions used should be stated in such a manner that the final concentration of the added component can be calculated.);
- the protocol for the method and the degree of concentration;
- if the product is standardized to a specific concentration of antigen, the procedures used to achieve this concentration and the calculations made in doing so should be given;
- serials: i) the method of assembly of units to make a serial (illustrate by example); ii) the volume of an average serial; iii) the volume of a maximum serial; and iv) other pertinent information;
- the volume of fill for each size of vial;
- a description of the method and technique for filling and sealing the final container;
- the protocol for lyophilization including procedures for determining the moisture content;
- the amount of antigenic material per dose or doses in the final container and how this is determined;
- conditions for storage of the finished product.

Permitted antibiotics and amounts per millilitre of biological product in the United States are:

- Amphotericin B 2.4 micrograms
- Nystatin (Mycostatin) 30.0 units
- Tetracyclines 30.0 units
- Penicillin 30.0 units
- Streptomycin 30.0 micrograms
- Polymyxin B 30.0 micrograms
- Neomycin 30.0 micrograms
- Gentamicin 30.0 micrograms

Permitted combinations of the above include:

- penicillin and streptomycin;
- either amphotericin B or nystatin with any of the other antibiotics alone or with a combination of penicillin and streptomycin or polymyxin B and neomycin.

Testing

A description should be given of how samples of the final product are collected, stored and tested. Protocols should be provided for the determination of purity, safety, potency, moisture content and any other test performed on the product. Each test protocol should include the minimum requirement for a satisfactory test.

Post-preparatory steps

This section should include:

- the form and size of the final container in which the product is to be distributed;

- a description of the conditions for storage.

Finally, the outline of production should include bibliographical references and any relevant appendixes.

LABELLING

All biological products should be properly labelled and packaged before leaving the production establishment. The final container label should include the following information: the name of the product; the name and address of the producer; the recommended storage temperature; the number of doses in the vial; the use, dosage and route of administration for each animal species for which the biological product is recommended; the expiry date; the serial number; and warnings or cautions, if applicable.

The expiry date is based on the earliest date of harvest and the date of the last satisfactory potency test. If applicable, the date of lyophilization should be given. A stability record should be established by testing each serial for potency at release and at the approximate expiry date.

The following are examples of warnings or cautions for products containing live or dangerous organisms: "Burn this container and all unused contents"; (for multidose vials) "Use entire contents when first opened"; "Do not vaccinate within [state number] days of slaughter"; and "Do not vaccinate pregnant animals".

The following information should be provided on the label, carton label or an enclosed leaflet: full instructions on the use of the product; if applicable, schedules of use; names of preservatives; and any restrictions on use of the product.

Containers of diluents to be used for reconstituting biological products should include on the label: the words "sterile diluent"; the name of the biological product with which it is packaged; its recoverable quantity in cubic centimetres or millilitres; the serial number; and the name and address of the producer. All necessary warnings as covered by the label for biological products should be placed on the diluent vial to cover its use and handling after reconstitution.

STORAGE

Completed biological products should be stored at 2° to 7°C, unless a different temperature has been shown to give better stability.

RECORDS

Each biological production facility should keep detailed records of all the activities carried out within the establishment. These should include a daily log of production area use. Records should be made concurrently with the performance of successive steps in the production of each lot and should contain the date and time of all critical steps, the identity and quantity of all ingredients added or removed and sufficient detail to give a clear understanding of each step in the preparation of the product. The charts and temperature records made during preparation of ingredients, sterilization of equipment or manufacture of a product are part of the record for the lot being produced. For each lot there should also be detailed records of the tests performed on ingredients, seeds, the product during manufacture and the completed serials or subserials of the product.

The facility should have a record of the location of all biologicals being prepared and the quantities held in storage and distribution channels.

QUALITY ASSURANCE

The protocols and testing procedures that were mentioned earlier can be regarded as contributing to the QC and quality assurance (QA) of biological products in the

process of manufacture. These procedures are of considerable value to the manufacturer in indicating the efficiency of the process, thus, it is of major importance that the performance of these critical factors should be documented in appropriate records. The QA monitoring of production and testing of a product should be performed by personnel not associated with the production process. The personnel responsible for QC and QA should report directly to senior management.

The requirements and procedures for testing the quality of a product should conform to the standard requirements established by the country or region in which the product will be used. In addition to tests performed by the manufacturer, product quality should be verified by a national or regional control authority's reference laboratory. Examples of standard requirements can be found in the United States Code of Federal Regulations (CFR) (USDA, 1994) and the European Pharmacopoeia (European Pharmacopoeia Commission, 1985). New test methods developed by the manufacturer should be approved by the national or regional control authority's laboratory before being used to evaluate a product.

When possible, test procedures should include a standard reference reagent, such as a virus, bacterial culture or antigen, for direct comparison with the product. Similarly a standard test reagent, sucha as a serum, antitoxin, fluorescent antibody conjugate, toxin, virus, bacterial culture or antigen, should be used to assure the correct sensitivity of the test. These reference preparations and reagents should be made available by a national or regional reference laboratory. Protocols for calibration, validation, monitoring and maintenance schedules for instruments and equipment should be based on published recommendations such as those contained in the College of American Pathologists. Instruments should be monitored frequently enough to ensure that they operate within tolerance limits 95 percent of the time.

CONCLUSIONS

The quality of a biological product is dependent on close attention to all factors directly or indirectly involved in production. These start with the design and operation of the facility, include the performance of the process and lead eventually to the testing and use of the product. The producer of a biological product must always remember that it is the manufacturer, not the user, who is responsible for ensuring that the product is pure, safe, potent and efficacious.

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The role of reference and regional laboratories

A.I. Donaldson and V. Astudillo

International organizations such as the Food and Agriculture Organization of the United Nations (FAO), the Pan American Health Organization of the World Health Organization (PAHO/WHO), the Office internationale des Epizooties (OIE) and the Commission of the European Community (CEC) have designated a series of laboratories worldwide as reference laboratories, collaborating centres and regional laboratories. These laboratories are viewed as an essential part of the organizations' respective missions to promote animal health and prevent the spread of disease through international trade in animals and animal products. The lists of FAO and OIE reference laboratories and FAO Collaborating Centres are given in the FAO/OIE/WHO (1995).

The largest network of reference laboratories (RLs) is for foot-and-mouth disease (FMD) – reflecting the international importance of the disease and the requirement for technical support so that its control and eradication can be on a regional basis. Under the aegis of FAO, OIE and CEC, different networks link a world reference laboratory, RLs, a community coordination institute and national laboratories and serve as a model for the role of reference and regional laboratories. In this chapter the background to these designated RLs and descriptions of their activities are provided.

WORLD REFERENCE LABORATORY FOR FMD

The Institute for Animal Health, Pirbright, United Kingdom (formerly the Animal Virus Research Institute) was designated by FAO in 1958 as the World Reference Laboratory (WRL) for FMD. The terms under which the WRL agreed to operate are:

- to perform tests for the presence of FMD virus on specimens sent by Member Governments of FAO and the European Commission for the Control of FMD;
- to identify the virus if present and/or the strain and antigenic properties of the isolated virus(es) if deemed necessary;
- to send all relevant information regarding the results of such tests to the government(s) requesting the test(s), with duplicate copies sent to the Animal Production and Health Division of FAO in Rome.

At the 1959 Session of the European Commission for the Control of FMD it was agreed that the WRL would be responsible for examining strains of virus found in Europe that were suspected of being types other than O, A and C, i.e. exotic strains. Additional responsibilities for the WRL were the subtyping of strains of the virus, maintenance of a reference collection of all confirmed subtypes and corresponding antisera and the supply of such antisera to other laboratories on request.

The 28th General Session of OIE in May 1960 recognized the Institute for Animal Health, Pirbright, as the WRL for FMD and authorized it to handle all seven serotypes of FMD virus. Thus the WRL became uniquely sanctioned by both OIE

and FAO to manipulate all seven serotypes of FMD virus.

Between 1958 and 1993 the WRL processed 18 200 samples from more than 110 different countries. It has also played a leading role in developing and standardizing methods for improved FMD diagnosis and virus strain characterization (Ferris and Donaldson, 1992).

REGIONAL REFERENCE LABORATORIES FOR FMD

Five regional RLs for FMD are recognized. These are:

- the Pan American Foot and Mouth Disease Center (PAHO/WHO), Rio de Janeiro, Brazil, which is designated by FAO and OIE as the regional RL for South and Central America;
- the Botswana Vaccine Institute (BVI), Gaborone, Botswana, is the OIE-designated regional RL for Southern Africa;
- the FMD Centre, Pakchong, Thailand is the FAO- and OIE-designated regional RL for FMD for Southeast Asia and Oceania;
- the Institute for Animal Health, Pirbright, United Kingdom, is the community RL for Member States of the European Union;
- the Central Veterinary Institute (CDI-DLO), Lelystad, the Netherlands, is the community coordinating institute for FMD vaccine for the European Union.

The expected functions and activities of OIE-designated RLs for FMD were reviewed by the OIE FMD and other Epizootics Commission in 1989 and adopted by the International Committee of OIE at the General Session in May 1989. They are as follows:

- The RL should act as the reference laboratory for the countries in the region and should collect and compile epidemiological data from the region with the collaboration of the national veterinary services.
- All RLs must operate under high disease-security conditions as recommended in the FAO guidelines on minimum standards (FAO, 1993). The high security systems should be regularly inspected by the relevant authorities (national and regional).
- Samples for FMD diagnosis should be received from the national veterinary services in the region.
- If a country wishes the WRL to perform the diagnosis then samples should be sent simultaneously to both the RL and the WRL.
- If a virus isolate is suspected of belonging to a serotype previously exotic to the region, samples should be sent to the WRL for confirmation and storage.
- The RL should be equipped and skilled to provide an initial diagnosis (serotyping) rapidly.
- The RL should be equipped and skilled for the determination of the serological responses of animals in terms of the serotypes of FMD virus in the region.
- The RL should use a sensitive and specific test. For this purpose it should maintain a stock of regional serotypes of FMD virus, inactivated antigens of exotic types and the appropriate immune sera.
- The RL should be equipped to propagate any FMD isolates in animal hosts and cell culture systems.
- In the case of an uncertain diagnosis, the RL should send a sample of the virus from the primary case to the WRL for confirmation and further characterization. Ideally, an aliquot of field material should be sent but, if this is not possible, animal passage material obtained from the original host species, or low cell culture passage material is acceptable. The history of animal or cell culture passage material should be provided.

- Further virus characterization should be carried out by the most up-to-date techniques, but only as a second priority.
- The RL should participate in collaborative studies with the WRL and other RLs.
- The RL should provide regular training courses in FMD diagnosis, epidemiology and disease control and should organize collaborative studies with national laboratories for the standardization of tests.
- On request, the RL should assist national laboratories by supplying reagents as required.
- The RL should perform tests and provide advice about vaccines for prophylaxis and emergency control. The definitive test is the cattle challenge test. Advice can be given, based on the results of indirect tests provided a correlation has been established between the indirect test and the cattle challenge test.
- Results and epidemiological data should be compiled and presented regularly to OIE, FAO and the WRL.
- All requests for the supply of FMD virus from any other laboratory, including the WRL, should be made through official channels, i.e. the central veterinary authority of a country must make an official approach in writing to the proposed supplier of the virus on behalf of the requesting laboratory.
- The supply of an FMD virus which is exotic to the country of the requesting laboratory should only be undertaken according to OIE and FAO procedures.

FAO expects its regional reference centres to operate along very similar lines.

The Pan American Foot-and-Mouth Disease Center

The Pan American Foot-and-Mouth Disease Center (PAFMDC) was established in 1951 as part of a technical cooperation project of the Organization of American States. Planning of the centre, which offers an international service, was done by the Pan American Sanitary Bureau, in collaboration with the InterAmerican Institute of Agricultural Sciences. Operation of the centre is the responsibility of the bureau.

The centre was built on a site and in buildings donated, together with utilities and a proportion of the labour costs, by the Brazilian Government. Major financial support was provided by the Organization of American States, and additional collaboration in specific parts of the technical and training phases of the centre's programme was received from FAO and the United States Department of Agriculture (USDA). During its formative years the work of the centre was devoted to: the training of national laboratory and field staff responsible for the control of FMD in the Americas; diagnostic services, including identification of the virus; field consultations in control and prevention techniques; and research on the nature of FMD virus and allied viruses.

National FMD control and eradication programmes were first established in South America in the early 1960s and consisted of a network of vesicular disease diagnostic laboratories, vaccine control units and field offices, the latter being responsible for disease monitoring and surveillance and the implementation of disease control procedures. PAFMDC was pivotal to these activities through the technical and advisory services it provided, particularly in the characterization of vesicular virus diseases, the production, standardization and supply of reagents to other diagnostic laboratories in the region and the training of personnel.

A continental vesicular diseases surveillance system (CVDSS), developed by PAFMDC, began operating in 1977. This linked the centre with field offices and

laboratories throughout the region. Information on vesicular disease outbreaks in individual countries is sent to the centre where it is collated and analysed to create continental disease status information which is then passed on to participating countries weekly, monthly and/or annually. The WRL, FAO and OIE also regularly receive these data.

Through this system the development of emergency disease situations, such as outbreaks in normally disease-free areas or a sudden increase in outbreaks in endemic areas, can be identified at an early stage and neighbouring countries alerted. PAFMDC's regular monitoring and characterization of virus strains circulating in the field is an important part of the CVDSS since it can identify antigenic changes and the need to alter the spectrum of strains included in vaccines. The possibility of emerging epizootics is particularly likely where there is a decrease in vaccination coverage or increased animal movement owing to trade. The links in this network are shown in Figure 13.

Significant antigenic and immunogenic differences are identified between field isolates and vaccine strains. If there are also epidemiological data indicating an increased disease incidence among vaccinated animals, PAFMDC will recommend to the relevant national authority or authorities that the strains of virus in commercially produced vaccines be changed. This may be effected by adding the new strain to the existing formulation, by substituting one of the strains or by producing a monovalent vaccine to be used together with the commercial vaccine.

Experience has demonstrated, however, that it is not advisable to substitute any strain in a vaccine when the field isolates can be covered with existing vaccine strains following intensified vaccination and revaccination. An example of this was the prediction of a type A epidemic in the Rio Plata basin region three months before its occurrence which allowed the forewarning of national authorities. The alert began in 1987 when PAFMDC established that a strain of virus, later identified as A-81 Argentina/87, present in the Rio Plata region was immunogenically different from the strain being used at that time in vaccines. PAFMDC wrote to the head of the national diagnostic laboratory in Argentina, giving details of the antigenic and immunogenic properties of the field isolate and pointing out the fact that revaccination would be necessary to achieve an acceptable level of protection and that if such measures were not immediately adopted an epidemic could be expected.

The Botswana Vaccine Institute

In 1977, Botswana suffered three outbreaks of FMD, almost simultaneously, caused by SAT 1 and SAT 2 viruses. In the Makgadikgadi area, where an outbreak of SAT 2 occurred, cattle were vaccinated with an imported vaccine but protection was poor. As imports were the only source of vaccine, the Botswana Government decided to build its own vaccine institute (in association with the company IFFA-Mérieux) but, since it was estimated that it would take at least three years to design and construct a permanent vaccine production plant and the need for vaccine was urgent, it was decided to tackle the problem in two phases as follows (Falconer, Mannathoko and Guinet, 1982):

- *Phase I*, an emergency phase, designed to produce limited amounts of vaccine while maintaining maximum disease security;
- *Phase 2*, establishment of the permanent institute, designed initially to produce 21 million monovalent doses of vaccine per year.

It was decided to use a mobile virus laboratory in order to enable work on FMD

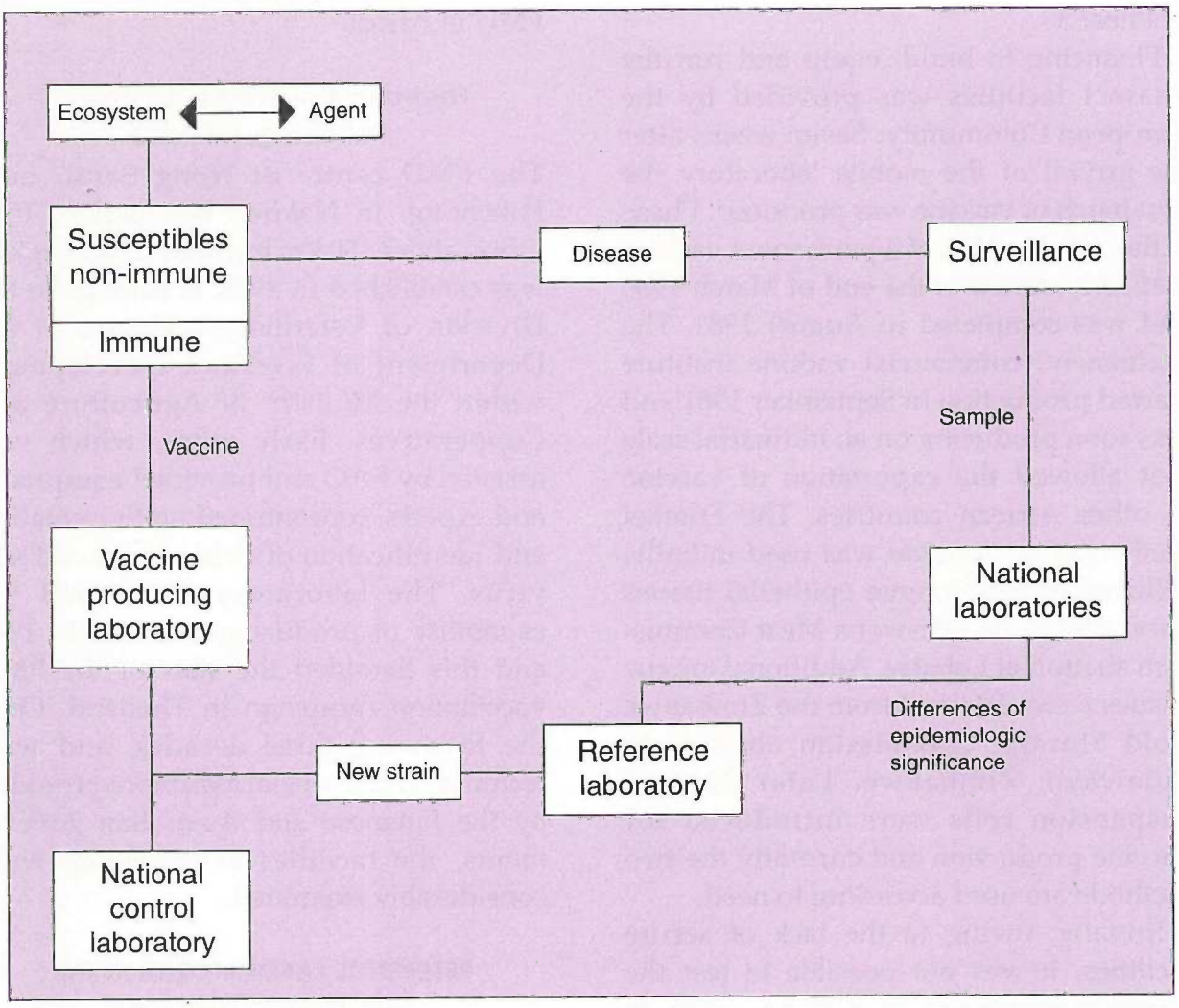

FIGURE 13
Reference system activation flow

viruses to be undertaken as quickly as possible. In August 1978, the laboratory was flown from France to Botswana in a Hercules aircraft and installed on a site in Gaborone. To provide facilities enabling work to be done outside the module's viral zone area, a large prefabricated building was erected, encompassing all the activities of Phase 1.

Financing to build, equip and run the Phase I facilities was provided by the European Community. Seven weeks after the arrival of the mobile laboratory the first batch of vaccine was produced. Phase 2, the construction of a permanent vaccine institute, started at the end of March 1980 and was completed in August 1981. The permanent, commercial vaccine institute started production in September 1981 and was soon producing on an industrial scale that allowed the exportation of vaccine to other African countries. The Frenkel method of production was used initially, utilizing bovine tongue epithelial tissues harvested at the Botswana Meat Commission abattoir at Lobatse. Additional tongue tissues were obtained from the Zimbabwe Cold Storage Commission abattoir in Bulawayo, Zimbabwe. Later BHK-21 suspension cells were introduced for vaccine production and currently the two methods are used according to need.

Initially, owing to the lack of secure facilities, it was not possible to test the vaccines produced at BVI on cattle in Gaborone and a small field laboratory at Motopi, northwestern Botswana, was used instead. Since the end of 1979, one batch out of every four produced has been subjected to a potency test in cattle and these tests are now carried out in high containment facilities at BVI, Gaborone.

One of the main responsibilities of BVI, in addition to the production of FMD vaccines specifically suited to the epidemiological situation of the countries in the region, is to study the different strains of FMD virus present in southern Africa and other parts of Africa. At the practical level, this has resulted in an increasing demand from neighbouring countries for the rapid diagnosis of suspected disease (Guinet *et al.*, 1982).

In 1985, during the 53rd General Session of OIE, BVI was designated as an RL for FMD in Africa.

Foot-and-Mouth Disease Centre, Pakchong, Thailand

The FMD centre at Nong Sarai, near Pakchong, in Nakhon Ratchasima Province, about 150 km northwest of Bangkok was established in 1958. It belongs to the Division of Veterinary Biologics of the Department of Livestock Development within the Ministry of Agriculture and Cooperatives. Early work, which was assisted by FAO and provided equipment and experts, concentrated on the isolation and identification of field strains of FMD virus. The laboratory developed the capability of producing vaccines in 1960 and this heralded the start of the FMD vaccination campaign in Thailand. Over the following three decades, and with technical and financial assistance provided by the Japanese and Australian governments, the facilities at Pakchong were considerably expanded.

REFERENCE LABORATORIES IN THE EUROPEAN COMMUNITY

As part of its policy for harmonizing the control of FMD in the European Community, as provided for in Directive 85/511/EEC, CEC decided that it would designate a community RL for FMD and a community coordinating institute for FMD vaccine.

Community Reference Laboratory for FMD

The Institute for Animal Health, Pirbright, was designated as the community RL for FMD by CEC in September 1989. Under

Articles 1 and 2 of a five-year contractual agreement, which came into effect on 1 April 1990, the agreed functions and duties are:

- To ensure liaison among the laboratories of the Member States with regard to the standards and methods of diagnosis of FMD and differential diagnosis, where necessary, in each Member State specifically by: i) receiving field samples from Member States and certain third countries with a view to determining their identity; ii) typing and full-strain characterization of FMD virus from the samples referred to in i) and communicating the results of such investigations without delay to the Commission and the Member State concerned; iii) building up and maintaining an up-to-date collection of FMD virus strains; iv) building up and maintaining a collection of specific sera against FMD virus strains;
- to support the functions of national laboratories, in particular by: i) storing and supplying to national laboratories cell lines for use in diagnosis, together with virus and/or inactivated antigens, standardized sera and other reference agents; ii) organizing and operating periodic comparative trials on FMD diagnosis at Community level and the transmission of the results of such trials to the Commission and the Member States;
- to provide information and carry out further training, in particular by: i) gathering data and information on the methods of diagnosis and differential diagnosis used and distributing such information to the Commission and the Member States; ii) making and implementing the necessary arrangements for the further training of experts in laboratory diagnosis with a view to harmonizing diagnostic techniques; iii) organizing an annual meeting where representatives of the national laboratories may review diagnostic techniques and the progress of coordination.

Under Article 3 of the contract it was agreed that:

- The RL shall operate according to recognized conditions of strict disease security as indicated in FAO, 1985.
- The RL shall formulate and recommend the disease security measures to be taken by the national laboratories in matters of FMD diagnosis, in accordance with the minimum standards referred to in the previous paragraph.

The community coordinating institute for FMD vaccine

The Central Veterinary Institute (CDI-DLO) at Lelystad, the Netherlands, was appointed as the community coordinating institute (CCI) for FMD vaccine by CEC in December 1992 and commenced its activities as a department within the CDI-DLO on 1 January 1993 under a renewable annual contract.

The functions and duties of the CCI are:

- to standardize the methods of control of FMD vaccines by national laboratories prior to authorization of vaccines by the competent authority of the Member State.
- to coordinate the control of FMD vaccine by national laboratories in each Member State specifically by: i) occasionally, or on request, receiving representative samples of batches of FMD vaccine intended for use in the Community including that produced in third countries (for use in the Community, in Community-supported vaccination campaigns or in animals intended for importation into the Community) and testing such vaccines for innocuity and potency; ii) carrying out comparative studies to ensure that innocuity and potency testing in each

Member State is of uniform methodology; iii) testing, by means of cross-immunity assays in live cattle, the efficacy of existing vaccines against important new field strains of FMD virus and communicating the results of such assays without delay to the Commission and the Member States; iv) gathering data and information on control procedures and vaccine tests and periodically transmitting such information to the Commission and Member States;

- to coordinate training and research among the various national laboratories by: i) making and implementing the necessary arrangements for the further training of experts in vaccine verification and testing with a view to harmonizing such techniques; ii) organizing an annual meeting where representatives of the national laboratories may review vaccine control and testing techniques and the progress of coordination;
- to operate according to recognized conditions of strict disease security as indicated in FAO, 1985;
- to formulate and recommend the disease security measures to be taken by the national laboratories in accordance with the standards already defined.

REFERENCE LABORATORIES AND THE QUALITY OF FMD VACCINES

The following activities of RLs have a bearing on the quality of FMD vaccines:

- *Antigenic suitability:* the antigenic characteristics of contemporary field strains of geographical relevance to the region must be constantly monitored by the RL so that advice can be given about the suitability and selection of strains already present or to be incorporated in vaccines. Suitable tests for this purpose include: virus neutralization; plaque reduction; mouse protection tests; and the liquid phase blocking enzyme-linked immunosorbent assay (ELISA) using bovine sera from a serum bank of vaccinated and revaccinated cattle. These procedures provide results from which an indirect prediction can be made of the protection likely to be conferred by selected vaccine strains against a field strain or strains and are especially appropriate in emergency situations. In the case of routine prophylactic vaccination campaigns, where speed is not so critical, the ultimate test is that of the cattle challenge. However, this has the disadvantages of high cost, greater disease security risk and the fact that it raises animal welfare considerations.
- *Independent testing of vaccines:* the RL should carry out independent and impartial quality testing of vaccines for use in different countries of the region, taking into consideration the prevailing epidemiological situations, to ensure that vaccines are suitable, potent and safe. To achieve these aims the RL should define the decision-making rules for the approval of vaccines and be capable of undertaking control assays including: vaccine production process control (antigen characterization and titration); antigenic payload (146S content) measurement (Barteling and Meloen, 1974); establishing the stability of the final emulsion; toxicity testing to ensure there are no adverse reactions in animals; and potency control tests in cattle. However, the latter are likely to be progressively replaced in the future by serological tests that demonstrate a good correlation between *in vitro* and *in vivo* results.
- *Innocuity testing:* these are essential to ensure that vaccines have been fully inactivated and are safe and sterile.

Recommended tests of innocuity include the inoculation of BHK-21 monolayer cultures (Anderson, Capstick and Mowat, 1970) and / or susceptible cattle.

- RLs should maintain stocks of reference virus strains and their antisera for supply to vaccine producers in the region.

MONITORING AND FINANCIAL SUPPORT OF REGIONAL LABORATORIES

The designation of a regional laboratory as an RL carries with it the obligation to undertake the duties and activities listed at the start of the chapter. This should be fully explained by international organizations and appreciated by candidate laboratories before they accept the designation. Furthermore, there must be a regular monitoring procedure to ensure that designated RLs are fulfilling their duties and satisfying the demands and expectations of the countries in the region.

Monitoring should be done on an annual basis through meetings of the relevant authorities of the countries of the region, by the provision of reports from the laboratories of their activities and through visits by groups of experts from international organizations (i.e. OIE, FAO, EU, PAHO / WHO).

Generally, the financial support of RLs is provided mainly by the host government and in part through specific programmes and projects. It is advisable that laboratories seeking to become designated or already performing reference work should accurately estimate the cost of these activities and request complementary funding from their usual sources or search for other sources to fulfil their needs. These other sources may include research contracts with central or local governmental agencies, international organizations and livestock associations.

Alternatively, a candidate RL may be able to persuade an international organization to meet the majority of its costs under a contractual arrangement. The advantage of the latter is that both parties should be fully aware in advance of their commitments and so both financial and technical programmes can be more effectively planned.

Note. While this chapter has been primarily concerned with describing the functions and activities of the five regional RLs involved in the control of FMD, it should be noted that, in support of FAO's Global Rinderpest Eradication Programme (GREP), there are now three regional laboratories that promote the control of the disease. Two of these are located in Africa (Senegal and Kenya) and the Pirbright Laboratory of the United Kingdom's Institute of Animal Health has recently been appointed as the FAO WRL for rinderpest. In addition, the Pan African Veterinary Vaccine Centre (PANVAC), Debre Zeit, Addis Ababa, Ethiopia, supports GREP by providing laboratory services to ensure that only vaccines of the appropriate quality and standard are used in the control campaigns currently under way in African countries.

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Description and documentation of production and quality control of veterinary vaccines

S. Ullah and G. Blocks

The products produced by veterinary biological or pharmaceutical manufacturers ultimately affect human lives, so it is necessary that companies not only produce a good-quality product that is safe, pure and efficacious but prove adherence to the regulations and guidelines that ensure every step within the manufacturing process has been carefully planned, monitored, traced and accounted for. By ensuring compliance to regulations and guidelines through these methods, companies demonstrate their ability to control the manufacturing process.

Adherence to all requirements concerning the product and processes is evidenced through documentation, so it is apparent that the documentation system is one of the most critical and complex areas of compliance with current good manufacturing practices (GMPs).

Note: It may seem as though the information in this chapter is relevant only to the functioning of large, highly developed, multinational companies. It should be noted, however, that the emphasis on the stringent control of processes, the need for testing and the development of accurate and reliable documentation, demonstrates admirably the important principles involved in establishing effective systems for quality control and quality assurance in any biological production organization – no matter how small. The lessons of this chapter are therefore strongly recommended to all those involved in vaccine production including those in small production organizations such as government veterinary laboratories in developing countries.

The initial focus of this chapter will be the general principles and premises behind GMP and the establishment of baseline criteria for developing effective documentation systems within production and quality control.

Owing to the brevity of this chapter relative to the subject matter, methodologies should not be considered comprehensive but, rather, should provide key information that producers can utilize to initiate a successful documentation system.

GENERAL PRINCIPLES

In establishing manufacturing operations, many companies equate regulations with current good manufacturing practices – indeed, it is common within the pharmaceuticals and biologicals industry to use the terms interchangeably. While GMPs are incorporated into the regulations – and in some countries are regarded as the law – clarification of what is meant by GMP should be made.

Regulations are written parameters and methodologies established by a country's government that ensure (when adhered to) that the consumer will receive a safe, pure and efficacious product of high quality. Regulations are essentially the law and non-compliance can result in censure, fines or closure of businesses. Deliberate and wilful disregard for the law can in some instances result in fines or criminal charges being levied. (Countries may use titles incorporating the word regulations, guidelines, rules, etc. when describing regulations, for example the European

Union's (EU) GMP guideline is called a guideline but is enforced as regulations throughout the EU.)

Governments must oversee all aspects of industry, including such diverse companies as meat processing facilities, medicated feed plants, biotechnology, pharmaceutical and biological manufacturing, so regulations tend to be broad and parameters vague. This is deliberate on the part of governments to allow for latitude in the way companies can produce products while still adhering to the spirit of the law.

It is the interpretation of the regulations that is difficult for many companies to decipher, institute and apply. Indeed, incorrect interpretation is the most common cause for a company to be prosecuted for non-compliance. By stating what companies must do, yet not specifying exactly how they must do it, governments have allowed for the industrial growth of manufacturing while ingeniously providing a barometer against which to measure a company's true understanding of the pharmaceutical business and all applicable laws.

GMPs are the current guidelines practised by companies that have successfully interpreted, assimilated and applied the regulations and any additional addenda to these regulations. Because regulations from countries may vary in stringency, acceptable process methodologies and requirements, it should be noted that there is more than one method of achieving successful compliance and numerous options and methods for practising GMP exist.

The common barometer for measuring a company's regulatory success is contingent on one principle: the establishment, through documentation, of accountability, traceability, standardization, reliability and control of all operational elements that have an impact on the product. Successful application of this concept should ensure a comprehensive controlled manufacturing operation and accelerate a company's global marketability by requiring minimal adjustments to another country's compliance criteria.

Traceability tracks every item and every step in a process from its source (i.e. from an individual or a substance) as a raw material, to the provider of the source substance/equipment (the vendor or supplier), through manipulations at the manufacturing facility (the processes) and as it is sent to market (distribution). This information, in conjunction with appropriate assessment measures, provides the assurance that every aspect of the process has been evaluated, monitored and controlled.

Reliability (as evidenced through validation and other documentation) assures that equipment, processes and systems will consistently perform at high standard levels that ensure a safe, pure and efficacious product.

Standardization and reliability of procedures, testing methodologies and results are achieved when companies employ one set of standards and procedures to perform specific functions. By establishing uniform guidelines and providing written instructions that allow for the consistent performance of procedures there is a significant decrease in the number of variables that could adversely affect the product.

By tracking and accounting for every component and variable in the process, a documented history of a given batch or serial on a given day is provided and corroborated through batch records, bench records, logbooks and other supporting information.

Processes and corresponding documentation that can account for every phase of manufacturing that requires testing (and can anticipate and plan the establishment of contingency procedures for undesired variables such as manufacturing discre-

pancies, unplanned deviations, investigations and rejected batches) are indicative of controlled systems.

It is necessary that regulatory agencies, customers, the public at large and the manufacturing company be assured that every action has been taken to guarantee that the best possible product is manufactured. Without documentation to prove that the above measures were consistently met, the best possible product is vulnerable to negative conjecture, claims, lawsuits and postulation owing to a lack of proof to the contrary.

It can therefore be surmised that every company that enters into the highly regulated arena of industrial veterinary biological or pharmaceutical production has the responsibility for:

- adherence to all applicable government regulations;
- application of current GMPs on a daily basis;
- meeting the contractual and possible regulatory requirements of customers;
- producing every product under controlled conditions designed to ensure that the safety, purity, quality and efficacy of the product are consistently optimal;
- continually monitoring every process to ensure standardization and reliability of procedures, testing methodologies and results;
- providing evidence of compliance to the above through concurrent and thorough documentation.

In order to establish and maintain a documentation system that will address these concerns adequately, management must have a thorough understanding of the integrative aspect of the processes and systems within the industry.

DEFINITIONS

Accessibility. As it applies to documentation accessibility means the use of a location that allows for the retrievability of controlled documentation within a reasonable amount of time (usually 48 hours), yet restricts and controls the access of unauthorized persons to the area.

Accountability. Accountability allows for each process and every component or individual involved in the processes to be evaluated, analysed and continually gauged for negative or positive effects on the system and the resultant product. Once analysed this information can be used to improve the control of a given process or system.

Auxiliary documents. Forms, diagrams, charts, logs, schematics and attachments which help to document, track and facilitate the steps in a process may be included in the batch record or referenced and kept elsewhere.

Batch record. A working copy of a master batch record, the batch record is essentially the written diary of a specific batch. Batch records trace the documented manipulations of the components and raw materials once the production process begins. For guidelines concerning batch records see Developing the infrastructure for a documentation control system (p. 349).

Bench record. This term refers to laboratory records and can apply to the raw data or the documentation that has been transcribed from the raw data. The difference between batch records and bench records is that batch records give a history of a specific product or process on a given day while bench records document the results of laboratory testing. Because bench records are essentially a compilation of testing data, the information may not be in chronological order and the data presented must be correlated to protocols or pharmacopoeias to get the complete

story. Well-written batch records tell a comprehensive story that incorporates the integration of many manufacturing processes. The production of biologicals involves numerous laboratory manipulations, so considerable skill must be used to convert the resulting bench record data into a batch record.

Cleaning feasibility studies. Tests are carried out to determine the effect of various cleansers (and methods of cleaning) on surfaces of different porosity, location and environmental conditions (e.g. the effects of heat, cold and light). Studies also help to determine the virucidal or bactericidal and fungicidal qualities of various disinfectants and the effectiveness of various solutions.

Control documents. Control documents are regulated documents that contain proprietary or confidential information concerning a company's processes, policies, ideas and methods. These documents are assigned unique identifying numbers which provide traceability and accountability for the records by allowing companies to establish and monitor who gets them and what version or revision of the document is the most current.

Controlled systems. The corresponding documentation of the integration of systems, processes and procedures, which can account for every phase of manufacturing and testing, becomes the control system. Controlled systems are challenged and validated prior to use to determine weaknesses. This allows potential problems to be anticipated and planned for in advance by providing for deviations, discrepancies, investigations and revisions.

Critical control point. A significant point in a process at which a pivotal decision is made, a critical control point affects (either directly or indirectly) the safety, purity and efficacy of the product.

Deviations. A planned or unplanned variation in a process that departs from the established steps of that process is a deviation. Planned deviations are tested and justified prior to implementation and should not affect the safety, purity, potency and efficacy of the product. Unplanned deviations are departures from the norm and should not affect the safety, purity and efficacy of the product. Companies often use the term "unplanned deviations" to describe manufacturing discrepancies.

Discrepancies. Unplanned variations in the process or procedure that could adversely affect the safety, purity, potency and efficacy of the product are referred to as discrepancies. It should be noted that, while deviations may or may not require investigation, discrepancies should always be investigated and the investigation should extend to other batches where the problem may have occurred. If it is decided after investigation to release a product affected by either unplanned deviations or discrepancies, a written scientific justification for that decision should be supplied with the product.

HEPA filters. High-efficiency particle adsorption (HEPA) filters are used to filter the air and to reduce the number of particles sized greater than 0.5 microns that enter a controlled environment. HEPA filters are used in conjunction with a manufacturing facility's air-conditioning system.

Investigation. A comprehensive and in-depth examination of the evidence elicited from reviewing the steps in a process, the investigation should be an objective, controlled exercise that focuses on root causes and provides information that can

effectively resolve discrepancies within a process or system.

Justification. An objective, scientific rationale (based on the data and situation) that supports the proposed decision is referred to as a justification.

Logbooks. Collation of data which effectively track certain activities in a process, logbooks are essentially the diary of a process and should present information in chronological order.

Material transfer forms. A form that accompanies raw material, equipment, components or product from one location to another, the material transfer form identifies the item(s), the origin and destination of those item(s) and who is responsible for the movement. Completed material transfer forms are usually retained in a readily accessible, centralized area.

Procedures. Step-by-step instructions that delineate how to perform a specific function in a process, procedures should be concise, to the point and written at a level that the persons with the requisite educational/experience background can understand. Procedures do not have to include every step in the process but significant steps must be a part of the procedure.

Process. A combination of a series of procedures that, when performed in a specified sequence, accomplishes a significant result within a system is referred to as a process.

Regulated functions. Operations that have written parameters and methodologies specified by the government or other regulatory entity, regulated functions have a direct or indirect impact on the product to the extent that non-compliance could adversely affect its safety, purity and efficacy. The requirements of regulated functions are inferred and outlined by laws (regulations).

*Stability programme.*The stability programme is a programme designed to monitor and determine the effects of environmental or physical factors on a finished product over a period of time.

PRODUCTION PROCESSES

The manufacturing operation is the engine that drives a company. Essentially, all other departments exist as support systems to augment, support and justify the results generated by the production department.

It is a common fallacy that production's main function is simply to produce, while concerns for quality are the responsibility of support systems such as quality assurance, regulatory affairs and quality control. In reality, the largest burden of proof of regulatory compliance falls on the shoulders of production owing to the impact of integrative functions and processes between production and other departments.

To understand the concept of integration, the direct processes of production need to be defined and a description of the processes from other systems that impact on production needs to be given.

Direct processes of production

In order to address effectively the application of processes to production documentation systems it is important to focus on system components and the importance of systems integration.

All operations are composed of systems which can be broken down into a series of related processes. These processes require interaction between departments at various stages of their implementation and, because it is important to demonstrate control of an operation by documentation that shows the history and control of each

process, it is necessary to decide on the key places where integration may occur – and to have records that demonstrate and support that decision.

The following production processes integrate with processes in other departments or systems although their key functions are the responsibility of production.

Receipt of raw material and components. Production receives raw material, such as the ingredients for the formulation of media, reagents and starting materials, and components, such as vials, ampoules and stoppers. These materials are received from another department (material management) through the generation of a bill of materials document.

The bill of materials lists the ingredients and components to be used in a certain process. This document is a part of the batch record and identifies items by stock or equipment identification control numbers.

Every movement of starter materials should be traceable, so documentation such as material transfer forms which identify the department transferring the material, the destination of material, the date of transfer, the reason for transfer and the person documenting this information, should be maintained.

Formulation or manipulation of starter materials. Materials used in production must usually be manipulated in some way prior to their use in the making of product. Manipulations can be divided into several functions.

Equipment such as fill line tubing, needle heads, fluid pumps, filter housings, surgical instrumentation (forceps, scissors, etc.), laboratory glassware, starter and final containers, must be cleaned and sterilized prior to use. The log records, sterilization charts and oven charts can be part of the initial batch record if the equipment will only be used in the production of one batch but, if the equipment is to be used for several products, such records should be stored separately and filed by "run" number and date of processing. In this instance, it will be important to reference which run date or number and which equipment was used on each batch record. This will help to facilitate finding the information during batch record review, investigations or an audit.

(Alternatively, when using the same equipment for several products, copies of the charts and other records can be incorporated into each product's batch record, as long as this is acceptable to government or contract regulations. When this method is used, the information describing where the original documentation is located should be referenced on every copy.)

Media must be prepared, filled and, whenever possible, sterilized.

Starter materials must be calculated and weighed and the amounts used and returned to storage should be documented (usually on the bill of materials).

The designation and identification of all components must be facilitated (e.g. the designation of fill tubing should be sent to the tissue culture production section and documentation for specific pathogen-free [SPF] eggs should go to the egg culturing section). This information is usually a part of the sterilization equipment run records and is supported by the page(s) of the batch record that require documentation of the specific equipment, components and ingredients used.

Information should be augmented by departmental logbooks which identify when material was manipulated (e.g. autoclaved, weighed and formulated) and on what piece of equipment and who performed the manipulations.

(Because of the correlation of batch

record information with logbooks and equipment, cleaning and media preparation records, it is critical that the correct information, such as equipment identification numbers, actual dates of preparation and dates transferred, be checked and verified for accuracy.

Examples of the types of discrepancy that can arise are given in The application of quality assurance to production and quality control systems (p. 354).

Preparation of the operation environment. Production is responsible for the cleaning, disinfecting and, when necessary, the fumigation of rooms used in production. In addition, the monitoring of air differentials, laminar airflow cabinets, clean rooms and HVAC systems is the responsibility of production. These activities will require the retention of logbooks and the logging of dates of cleaning and types of cleansers / disinfectants used.

If cleansers require mixing or dilution prior to use this is usually described in a type of batch record or logsheet. Schedules that demonstrate the adequate rotation of cleansers (to decrease the likelihood of microbiological resistivity) as well as cleaning feasibility studies for various cleansers must be available.

Monitoring of operation environments. Using various plating techniques and air sampling equipment, the production department continually monitors the microbial flora and air particulate concentration for every room where production takes place. The results may be documented in the batch record or the information and where to find it can be referenced in the batch record while the actual data are stored separately.

Initial operations (production run). The preparation of cell systems (i.e. the opening of eggs, grinding of tissue, etc.), inoculation, media changes, harvesting, filtering, the addition of antimicrobial agents and incubations are all examples of initial production processes. The documentation required for these processes will consist of an initial batch record, a bulk product batch record or a defined section of a comprehensive batch record (when the material is to be used to make a single final product).

Intermediate operations. Intermediate operations include storage and corresponding environmental monitoring (i.e. monitoring of freezer temperatures), sampling (archive or reserve samples for bulk solution traceability) and thawing.

When the preferred method of producing batches involves the formulation of serials (the use of initial batch solutions to make more than one type of product) the initial batch record (bulk solution batch record) is linked to the target product final batch record and the intermediate or bulk solution is referenced in the final batch record.

The batch record for the bulk solution is kept as a separate record and references every product that the bulk solution was used in. The batch record system a company decides to use should be defined in the product licence and must be well thought out and coordinated, see Developing the infrastructure for a documentation system (p. 349).

Final operations (formulation and filling). The final stages of processing involve such processes as blending, the addition of other formulating ingredients such as stabilizers, adjuvant, preservatives, etc., filling, volume checks, lyophilization (if applicable), sealing, inspection, packaging and labelling. The records generated at these stages are the final batch record and should include the records for packaging and labelling.

Packaging and labelling. This stage involves the retrieval of product from an environmentally controlled storage area, the manipulation of that product in order to package and label it and the return of the product to quarantine storage pending release.

The movement of controlled product that by virtue of its composition needs a certain temperature environment requires the documented monitoring of temperature and of the time that the product is outside the controlled environment. The documented monitoring of the controlled environment (e.g. freezer or refrigerator) requires an established procedure and frequency.

In addition, parameters of the temperature at which the product can be stored should be specified and the temperature at which immediate action must be taken (the critical action level, which is based on a critical control point) should also be recorded.

At least three types of logs are indicated by the above information:

- the log or printout used to demonstrate periodic monitoring of the temperature of the controlled environment;
- the log that documents the movement of product into and out of the controlled environment;
- the documentation within the batch record of the times of the actual packaging and labelling operations. (This information is correlated to the log information that documents the time the product was taken out of and returned to the controlled environment.)

In addition, the time out of storage is correlated to the product outline and labelling information to ensure that the time it took to process the batch did not exceed that stipulated and therefore that the safety, purity, or efficacy of the product are not jeopardized.

THE INTEGRATION OF SYSTEMS AND DEPARTMENTS WITH PRODUCTION

The complex process of documenting production processes requires that companies be aware of the relationship of other departments to the actual manufacturing operations. In order to facilitate this awareness, background information is required concerning each system that has to be included in the process. Because each system is usually the purview of a specific department, the information has to be structured to indicate the department, provide some background information concerning the department's primary focus and describe briefly how production integrates with that particular department or system.

It should be noted that the information provided here is cursory and does not include the systems integration between every department but outlines the foci of documentation on the systems of manufacturing (production) and quality control and the impact of those systems that should integrate with them.

Regulatory affairs

In order to market a biological or drug product, specific information must be submitted to the regulatory agencies that govern the industry within the country concerned.

The information submitted should outline the specifics of the manufacturing process and will usually include:

- building location and design (blueprints and legends);
- equipment and services (utilities) specifications (validation data);
- a list of the types of products a facility expects to produce;
- the procedures by which the product will be produced (including manufacturing, testing and monitoring methodologies);
- research and development data

(including master seed origin, raw materials, etc.).

The regulatory affairs department works closely with the government on a continual basis and serves as a liaison between the company and the regulatory agencies. Functions usually associated with regulatory affairs are: submission of new drug information; interpretation of licences and regulatory contracts; submission of revised product data; interpretation and application of company policies versus government regulations; the writing and facilitating of official responses to government audits; and the review and determination of information to put on product inserts, master labels and packaging information.

The establishment of criteria for all areas of the business that have to do with administrative aspects of regulatory compliance is also the responsibility of regulatory affairs.

The regulatory affairs department is also responsible for determining the adequacy of processes, procedures, master batch records and testing criteria as they pertain to changes in submissions or government expectations, as well as for facilitation and expedition of market withdrawals, recalls, etc. (In some companies, some or all of these duties may be under the purview of the quality assurance unit.)

Integration of production with regulatory affairs. The documentation generated and submitted by regulatory affairs is utilized to create master batch records, standard operating procedures, quality control protocols and other documents. These documents must be reviewed and compared whenever there are revisions or amendments to ensure that any changes to processes, procedures, raw materials, building or equipment specifications, etc. do not contradict the original submitted documents.

It should be recognized that all regulatory documents are in a state of flux. This means that as regulations, procedures, raw materials or equipment change or are clarified, the documentation within each system will need to be reviewed, evaluated and possibly revised to reflect and support the change.

Production should establish a mechanism for continually notifying regulatory affairs of any changes to processes, procedures, equipment or raw materials. New submissions should be made and government approval obtained prior to the initiation of new processes.

In addition, before writing or revising procedures, changing significant steps in the master batch record, approving manufacturing deviations or otherwise altering existing techniques, the procedures and proposed changes should be compared with the actual product outline or product licence submission. Changes that go outside the parameters established in the product outline may require an amendment to the licence (and scientific justification) prior to the change.

Regulatory affairs should apprise production of all changes and amendments and of new interpretations of procedures. It is also recommended that prior to submissions of research and development data, production is apprised and actively participates in the development of the procedures and processes that are to be a part of the product licence.

Documentation generated by regulatory affairs and continually utilized by production includes: the product outline, information concerning labelling operations, changes to the outline and facility blueprints and legends.

Research and development

Research and development (R&D) has considerable impact on the everyday operations of production. It is important to recognize the function of R&D and to

incorporate that function with the actual production process.

R&D is responsible for developing ideas into products, for example, beginning with an isolated viral strain, R&D works to develop seed that is potent, yet has minimal adverse reactive characteristics. Using feasibility studies in conjunction with seed manipulations (passages) an optimum seed preparation is developed (the master seed) and R&D must then work to build up a master seed bank that will sustain production of the product for a specified period.

The integration of R&D with production. R&D is responsible for testing and ensuring the feasibility of product use and must develop the processes used to manufacture the product. Consideration must therefore be given to the following factors:

- feasibility of the potential product as a viable vaccine that will incorporate sufficient viral activity with minimal adverse reactivity;
- reproducibility of experimental bench activity in the "real world" (i.e. in the manufacturing process);
- testing and evaluation of excipient and other raw material in conjunction with the biological component (i.e. testing for compatibility and stability);
- testing and evaluation of the packaging (composition of vials and stoppers, labelling requirements, etc.) to establish baseline criteria for compatibility and stability testing data;
- evaluation of the effects of heat, cold, light, agitation and other environmental variables on the stability of the biological component;
- development of the manufacturing and test methodologies that will be used in the actual processes.

It should be recognized that, although R&D is responsible for developing the methods of producing and testing products, the real proving ground for the success and feasibility of R&D manipulations takes place in production.

Many ideas work well in R&D laboratories where creativity, quick adjustments and manipulations using state-of-the-art equipment may be the norm and can occur throughout the evaluation process. Current GMP requirements are applied rather loosely (if at all) during the initial stages of research to allow for all avenues to be explored and such latitude is not usually allowed during actual manufacturing. Owing to the complex, stringent control mechanisms and to the recognition that production equipment is often idiosyncratic after years of use and wear, such variables as process or ingredient adjustments can only be made after serious consideration and must undergo thorough, prescribed and controlled testing. Production using methods that still need adjustments to the actual process is usually restricted or totally prohibited.

The testing methodologies developed in R&D must be reproducible on a large scale and should incorporate and reflect the methodologies that the quality control unit will actually utilize. New methodologies that cannot be referenced to manuals or pharmacopoeias usually require the approval of government testing agencies prior to use in actual production.

For successful conversion of a production process from a laboratory to a manufacturing environment the following guidelines should be utilized:

- Apprise production of the progress of products that have been approved. Visit the production site and find out the actual methodologies and equipment that are being used.
- It is far easier to assimilate a new product that uses existing methodologies than it is to create or revise a process, every effort should therefore be made to simulate the conditions,

equipment, processes and procedures that production uses. This should minimize the number of alterations a production process requires when it is transferred to the manufacturing environment.

- The validation of processes must be performed on the actual equipment that will be producing the product. This is especially critical for formulation and lyophilization processes where idiosyncrasies of equipment, utility requirements and even the availability of specific raw ingredients could compromise all of the hard work of regulatory affairs and R&D.
- It is important to recognize the importance of validation and to ensure that process validation is complete before writing final versions of the standard operating procedures (SOPs) for that process. The information gathered during the testing and challenging of the process will help to determine the critical control points and the types of SOPs that will need to be written. In addition, SOPs that compensate for the weaknesses of a process can be developed after those weaknesses have been determined during validation.
- Idiosyncrasies of equipment and processes are not only the characteristics of different manufacturers; the wear and tear from normal production use can change such diverse critical aspects as door seal integrity (and therefore the vacuum), sensor or thermocouple capabilities (for lyophilizers) or fill lines or needles for filling machines. Companies should be aware that reproducing the results with similar equipment from the same manufacturer using the same ingredients may not be acceptable for validation or product submissions – only the actual equipment used, with the actual procedures followed, can be used for such purposes.
- R&D should be aware of the manufacturing and regulatory restrictions of production. It is important to incorporate the GMP requirements of manufacturing operations in the training of the staff of support systems that are integrated with the production department.
- R&D should be apprised of the process validation requirements in enough detail to enable informed decisions to be made about developing processes or manipulations.
- The performance of pilot batches should be facilitated by R&D but production personnel should perform all the manufacturing manipulations.

Incorporating these guidelines should help to ensure that the success of the R&D department's efforts is not delayed during actual production.

The documentation that is generated by the R&D programme includes the results and evaluation data of feasibility studies, initial stability data, bench records and pilot batch data.

Quality control

In many companies, the functions of quality control (QC) and quality assurance (QA) are combined and the unit that provides testing is also the unit that determines the appropriateness of a batch for release. In addition, regulatory guidelines do not always provide an accurate distinction between the two quality units; however, owing to the types of responsibilities given to each unit, a distinction is inferred and, whenever possible, should be defined.

QC is essentially the support system for production which performs the sampling and testing of raw materials, bulk, intermediate and final product. QC also helps to facilitate or augment any analytical laboratory testing such as sterility testing of formulation vessels, validation of tests

(as required for the production processes) and monitoring of the stability programme. Because most guidelines specify that the evaluation and analysis of all manufacturing processes must take place prior to the release of the product and that such an evaluation must be performed by an independent unit not directly answerable to or influenced by production, it will be readily apparent that the QC unit (which is used to augment and test elements of the production process) should not be responsible for reviewing data performed and generated within its own department. It could not, therefore, fulfil the requirements of an independent unit.

QC utilizes controlled, standardized methodologies to test ingredients at various stages of the production process. Information, such as formulas for calculations and test procedures, is usually defined in pharmacopoeias.

If possible, the QA unit should be considered a separate department from QC.

Integration of production with QC. Most QC interactions with production involve testing at various stages of the production process and it is very important that there is continuing communication between production and QC. Changes in production caused by scale up or changes of equipment, mixing methods, ingredients, etc. can affect the application of antibiotics, pH requirements etc. during the production process and therefore have an impact on the success of QC testing. Production should apprise QC of any changes in the manufacturing process that could have ramifications on the testing of the product.

The documentation generated by QC consists of the testing records (also referred to as bench records, QC protocols, etc.); for additional information concerning QC see The role of quality control (p. 347).

Materials management

The initial integration of records which signifies the beginning of a production run involves the scheduling, receipt, storage and transfer of materials. The documentation of these actions has a significant impact on the records generated by production.

Materials management may also be referred to as distribution, logistics or production management and is responsible for the receipt, storage, inventory and distribution of raw materials as well as the scheduling of production runs and the distribution of the final product. Owing to GMP requirements, materials management is also responsible for the traceability and accountability of all raw material and components as well as for the maintenance of adequate inventory levels.

These responsibilities require the tracking and reconciliation of all materials used in the production process, together with a continual monitoring of the inventory to ensure the rotation of stock and that the oldest product in stock is utilized first. Materials management should also work in conjunction with QC to ensure that incoming shipments of raw material are adequately sampled and tested prior to their release to production.

The records maintained by materials management include: inventory cards; manufacturer, vendor and supplier lists; production schedules and logistical data; environmental monitoring logbooks for the recording of storage temperatures; material transfer forms; customer return documentation; and distribution records.

Integration of materials management with production. Production should work closely with the materials management team to ensure that production deadlines, back order issues and other logistical information is adequately recorded and passed on. Providing accurate information

concerning raw material usage, the amount of material scrapped (rejected) and re-processed, the tracking of material and the manipulations performed with material, is vital to establishing an efficient and controlled materials management process.

Production should establish processes that allow for the traceability and accountability of product usage and manipulations. This generally entails having records of when material was used, the process and/or equipment it was used with, the amount of material used, the amount (if any) returned to inventory, the lot numbers used, the date and the signature of the person performing the task(s).

Material transfer forms should be filled out accurately and should accompany any material that is to be handled in several departments.

Allowances should be made in production schedules for maintenance, environmental monitoring, R&D and validation test runs. In planning production runs, production must also consider the requirements of in-process testing and sampling of raw materials and any assays or tests that may be required prior to use of the raw material. Failure to comply with these considerations may lead to failure of the product at the final QC stage.

Examples of the documentation generated by materials management and utilized by production are: material transfer forms, bill of material data, vendor/supplier information and schedules.

Quality assurance

A successful QA programme involves the routine interaction of the QA unit with production (and with all other regulated departments) which allows the QA department to appraise operations continually and to ensure that there is communication and implementation among all integrating departments. Owing to the dynamics of manufacturing products, the timely communication of changes and amendments is extremely important to the production process.

The QA function involves the interpretation of government regulations as they apply to everyday operations and should encompass the monitoring of all regulated departments. The data generated from monitoring is collated and evaluated to determine whether patterns or trends exist. Trends are usually key indicators that a system or process may need revalidation, adjustment or possible SOP revisions.

The functions that may be incorporated under the title of QA are:

- *document control* which involves the creation of the master batch record and coordination and issuance of the production batch record and SOPs;
- *line inspection teams* which inspect line clearances and examples of the product while it is being produced (e.g. check the fill volumes and labels);
- *labelling control* which controls and tracks the dispensing of labels, inserts and cartons that contain prescriptive information;
- an *investigatory and auditing unit* which facilitates investigations of manufacturing deviations and discrepancies, monitors all regulated processes, advises on projects that require a knowledgeable view of integration of systems, performs pharmaco-vigilance and evaluates trends of adverse reactions and their subsequent impact on processes and products;
- a *GMP trainer* who facilitates the training of personnel in regulations and the practical applications of GMP – the GMP trainer may also fulfil another function within QA;
- a *validation team* which facilitates the testing and challenging of equipment and processes to ensure reliability and determine the contingencies or support

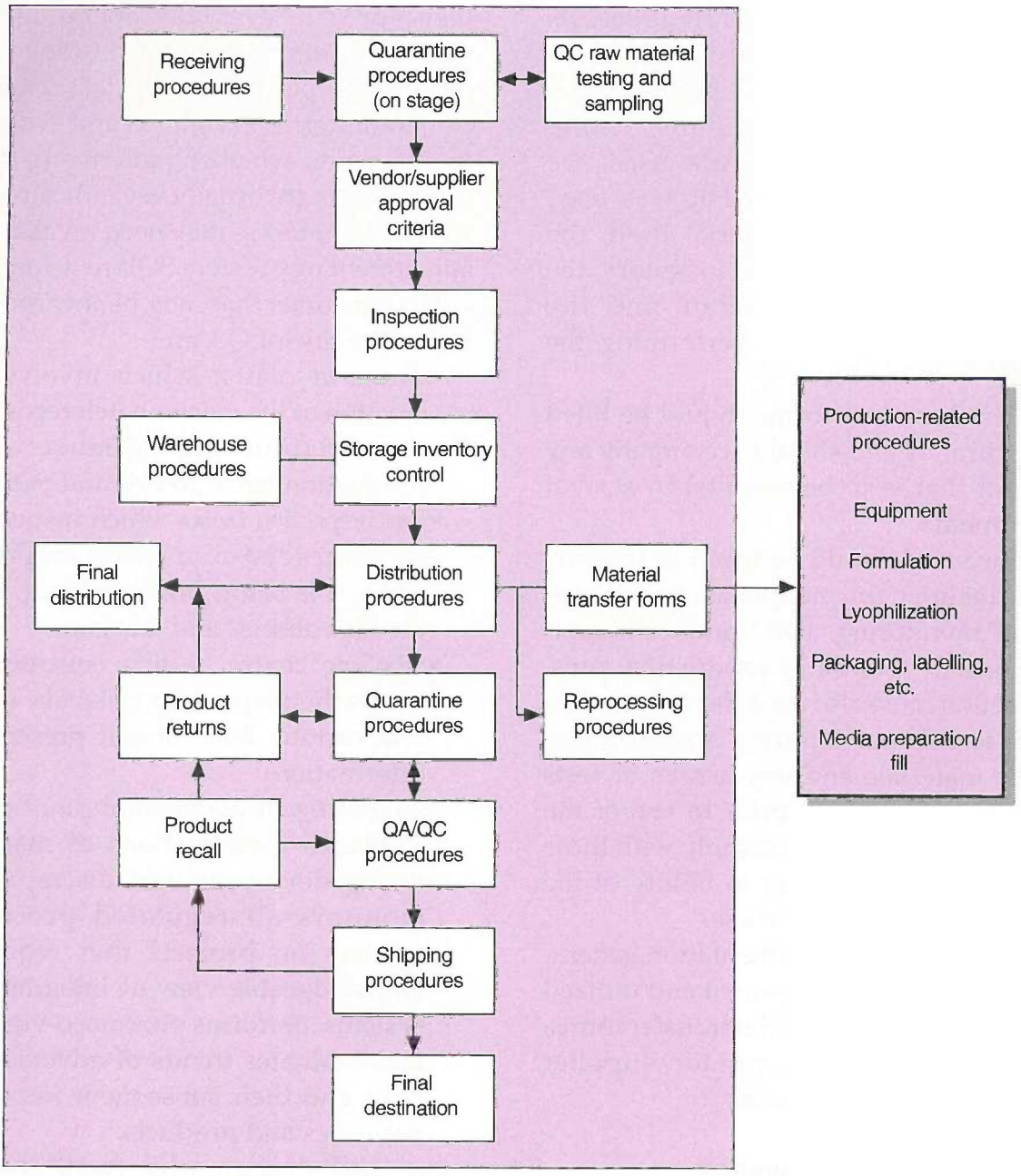

FIGURE 14
Flowchart showing production method, procedures and processes involved in material management

processes that are needed to augment a system – this includes data that will be incorporated into SOPs, tolerance parameters and maintenance programmes.

The types of documentation generated by QA include: audit reports,trend analysis data, annual product reviews, validations, evaluation and feasibility studies and release data.

The primary role of QA is to monitor continually and evaluate the application of GMP to processes and systems by utilizing audits, trend analysis, studies, etc. All regulated departments and departments that support regulated functions integrate with the QA unit and, in addition, QA performs self-assessments and evaluations.

Integration of production with QA. Companies that are proactive are discovering the advantages to having a supportive QA unit. When implemented correctly, QA works in conjunction with production to troubleshoot, investigate and solve manufacturing problems and issues. Realistic goals and empathetic attitudes are required of both departments to ensure that evaluations and corrective actions address the control of processes and systems. The following are guidelines for successful interactions between QA and production:

- Production should consult QA before implementating any revisions to processes, equipment or procedures. Owing to the comprehensive vantage point of QA, the impact of production decisions on all departments can be considered and anticipated. This will help to minimize the corrections, reworkings and other discrepancies that occur when all the ramifications have not been considered.
- QA should ensure that the present working conditions, standards and practices are considered when addressing corrective action criteria. The implementation of quality need not be expensive or time-consuming. The onus is on QA to learn the variables and factors within production and to tailor a solution that incorporates the present operation conditions with a feasible interpretation of the regulations.
- The key to successful interaction is open communication. It is beneficial for both departments to participate in departmental GMP training that provides insights into the function, responsibilities, purpose and limitations of the respective departments.

Engineering and maintenance

Together with QC, engineering and maintenance works daily with the installed processes to ensure the reliability, functionality and control of all equipment, services and processes.

The engineering and maintenance department serves as the technical support system for the facility, all equipment and services (utilities) and production. Changes or modifications of the building, services, equipment or engineering programmes should require the involvement of engineering, production, QA and regulatory affairs prior to implementation. Significant changes could affect the integrity of the manufacturing process so it is important to justify and validate all changes.

Integration of engineering and maintenance with production. Production should work closely with the engineering department to establish preventive maintenance and calibration schedules, discuss forthcoming upgrades of the facility and/or equipment and establish guidelines for the facilitation of validation protocols.

The types of documentation generated by production in conjunction with engi-

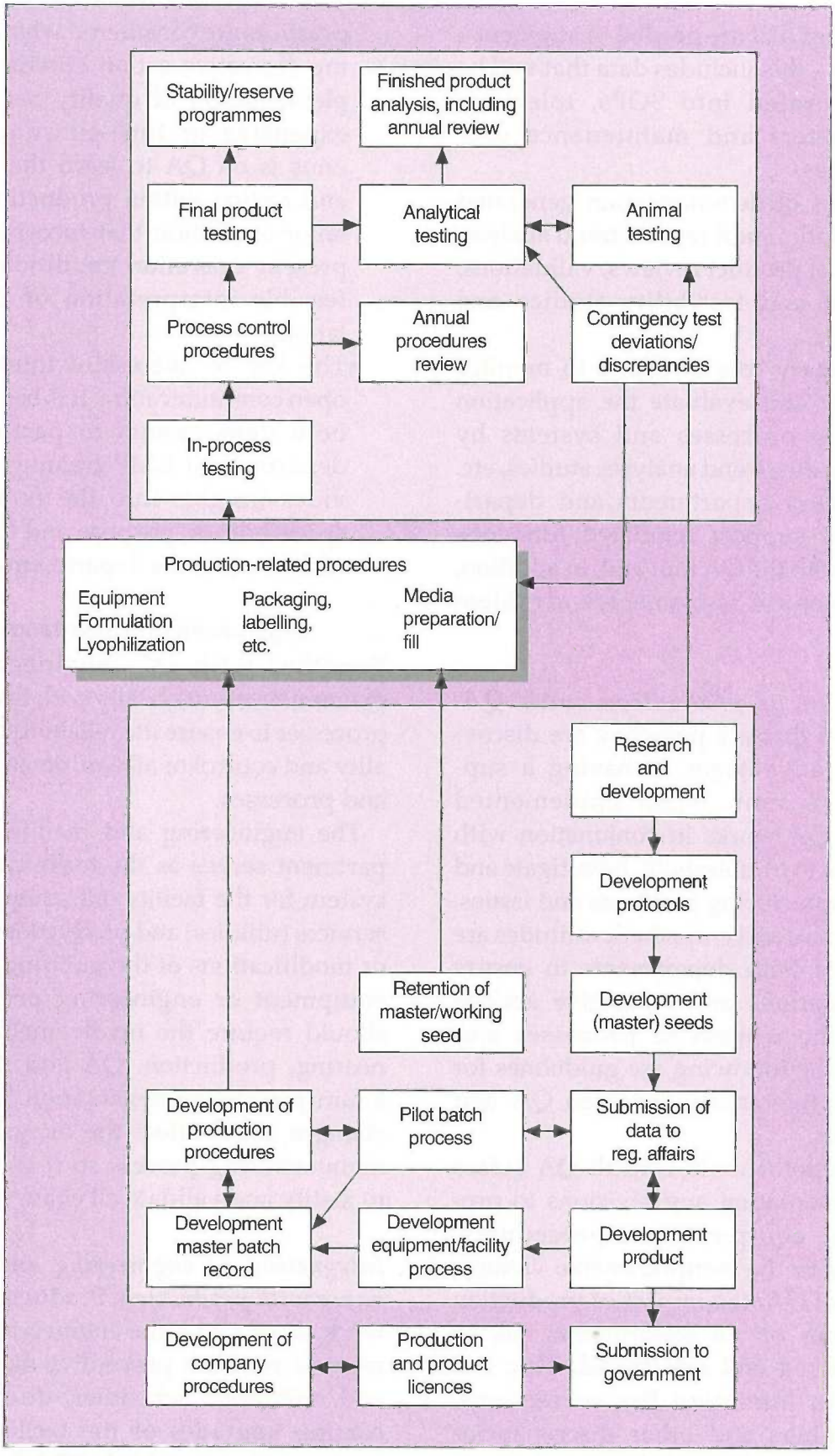

FIGURE 15
Flowchart showing production method, procedures and processes for product development and quality control

neering and maintenance are: equipment history files, maintenance and calibration schedules, manufacturer or supplier information, validation records (if not retained by a separate validation team), filter change records and equipment repair work orders.

Documentation control

Owing to the complexity of pharmaceutical and biological processes, companies have a tendency to departmentalize tasks when designing the documentation system, which results in a fragmented system that must be continually monitored, amended, revised and augmented. To remedy this situation, it is necessary to understand the flow of operations prior to establishing or revising a documentation system. Documentation exists and is mandatory for every regulated system within the manufacturing facility; therefore consideration of the facility, product, processes and systems should be paramount.

Documentation control essentially involves the dissemination of master batch records, SOPs and other forms that serve to corroborate production processes. Charts delineating regulated systems, the flow of operations and the key components of documentation are given in Figures 14, 15 and 16.

Integration of production with the documentation control system. The master batch record and SOPs are often perceived as the main components of the production documentation system, leading to the emphasis usually being placed on developing these formats instead of developing a comprehensive system.

A common error which occurs within the industry (and which creates considerable confusion and discrepancies) is to allow different departments to develop their own SOPs, including procedure formats, control numbering systems and methodologies, independently of an overall company plan. This often results in a myriad of formats, numbering systems, logs and procedural steps being developed even when all departments are performing the same or similar tasks. Because streamlining procedures reduces the amount of paperwork, contributes to the standardization of operations and increases manufacturing productivity, production should write multifunctional SOPs wherever possible (see Developing the infrastructure for a documentation control system on p. 349).

Another key area in documentation control is the process for revising existing documents. Revision of existing documents generally requires a review of the product outlines to ensure that new information or proposed procedures are not in conflict with the original submissions. Product outlines are regulated documents containing proprietary information. This means that they should be managed under a controlled system that designates specific numbers for each document and tracks the whereabouts of all copies of the product outlines including the current version.

Steps should be taken to ensure that the revising and amending of existing documents occur in a controlled manner and that the personnel in all integrative systems are aware of the impending changes and are prepared to modify their records accordingly. This is generally performed through a change control procedure. Production should also ensure that all changes that occur are reflected in the SOPs and that new copies are distributed to each department and obsolete copies are retrieved and destroyed. It is largely through the coordination efforts of the documentation control department that records are adequately maintained.

Companies that do not have the resources for a separate documentation

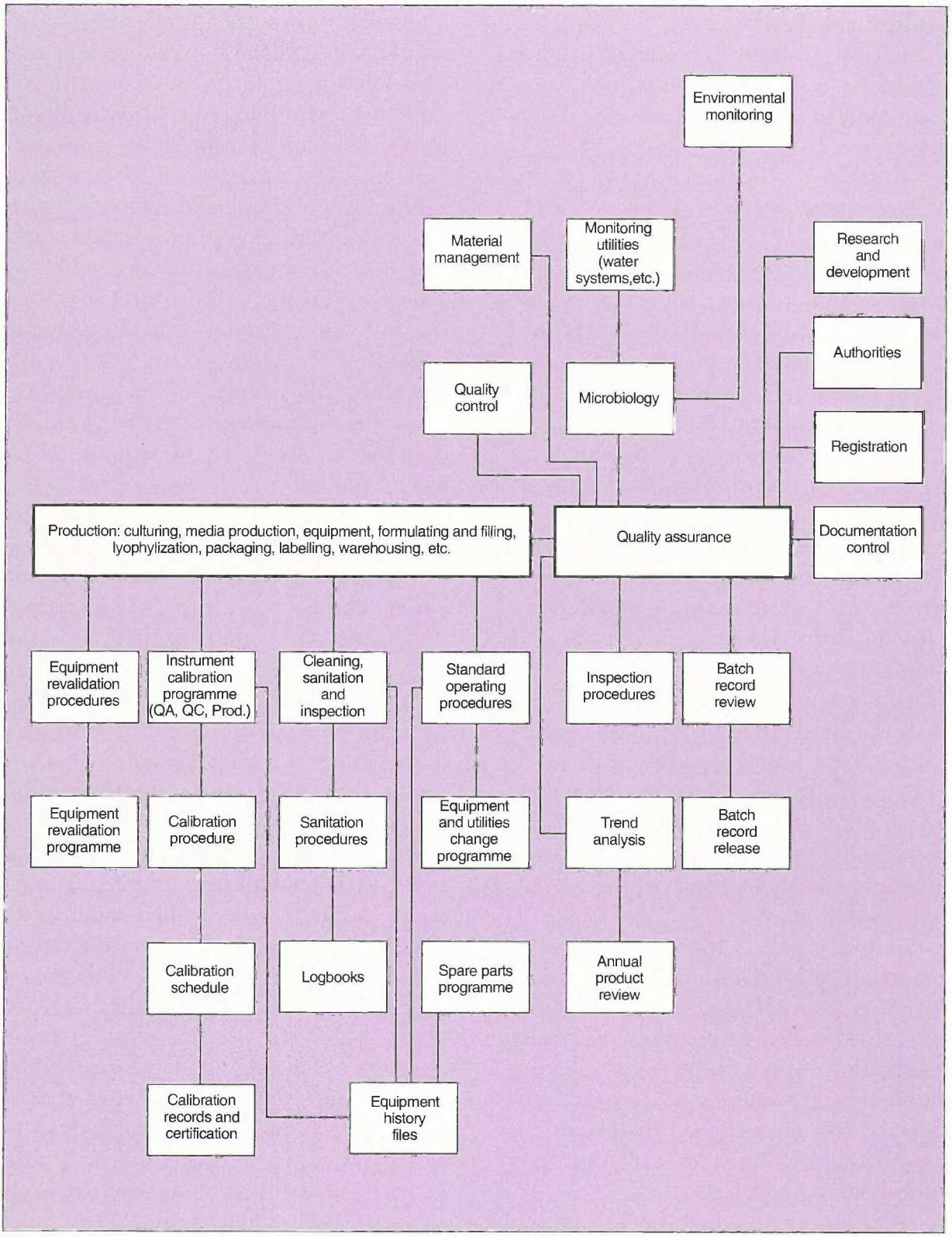

FIGURE 16
Flowchart showing quality assurance-related procedures

control unit (which is usually a component of the QA department) may integrate these functions with another department.

Although it is beneficial to assimilate the documentation control concept throughout the company, dividing document control responsibilities among various departments is not recommended. A centralized, secure area for the retention of batch records, production outlines, protocols, etc. should be utilized. Access should be limited to authorized personnel only and careful monitoring and logging of the batch records removed from the area should be performed.

The types of documentation generated and maintained by the documentation control department include: the master batch record log, the master SOP tracking log, the batch record issuance log, the form tracking log, the master company signature list and the master control records (batch records, SOPs).

Purchasing

Although purchasing is usually not considered a regulated department, the purchasing department does make decisions that can have a critical impact on production and ramifications on regulation.

Specific ingredients and processes are defined in the product outline, so it is very important that the company's purchasing department is aware of the production criteria prior to contracting with vendors. Owing to the technical nature of this information, it is recommended that the personnel involved be experienced in the processes, ingredients and component composition and that an evaluation of vendors' manufacturing and QC processes, as well as pricing factors, be considered before selecting a supplier. Purchasing personnel should also be made aware of all pertinent regulatory requirements that could affect the criteria for ordering certain products, services or equipment.

Integration of purchasing with production. In procuring equipment, products and services for a regulated industry, purchasing personnel need to be aware of the regulatory impact of their decisions. All too often, purchasing departments obtain deals for the company, only to have the money and effort saved nullified by the costs of regulatory redress.

Staff from purchasing, QA, production and engineering and maintenance should confer whenever major equipment purchases or changes in existing services need to be made. It is often required that company personnel audit the site and evaluate the suitability of a specific supplier. Purchasing personnel should undergo auditor's training or be accompanied by the company's in-house auditor whenever a new vendor is to be assessed.

Ideally, the persons responsible for procuring goods and services for production should undergo GMP and advanced technical training. Personnel should have an understanding of the ramifications of choosing certain kinds of flooring, duct work, irradiation services, etc.

The types of documentation that are generated by purchasing and that have an impact on production are: purchase orders, evaluation of vendors (vendor/supplier approval programmes), documentation of the continual monitoring of vendors and the establishment (if feasible) of a vendor/supplier certification programme.

Packaging and labelling

Information is constantly being revised, updated and clarified, so it is critical that there is an open dialogue among regulatory affairs, production and labelling control. The instructions provided on labels, inserts and cartons must reflect the same instructions submitted to the government and procedures for the facilitation of this dialogue should be written and followed.

Packaging and labelling are the production processes that are usually initiated after product testing is complete and the results of all tests have been found acceptable. The operations involved are: removal of the final product from quarantine storage, clearing away evidence of other labelling operations from the production lines, visual inspection of the vials, continual monitoring of the packaging and labelling lines and the actual process of packaging and labelling.

Key aspects of this process are the receipt, sampling, inspection, storage and dispensing of labels, inserts and cartons (these are usually functions of a division of the QA unit in conjunction with regulatory affairs).

Integration of production with packaging and labelling. On receipt of labels from labelling control, it is imperative that the production department, and all support systems, participate in the control and monitoring of the labelling operation. The procedures to be defined and followed should include: proofing or review of labels; disposal of obsolete old labels; and effective retrieval, reconciliation and destruction of defective labels. The personnel in packaging are in the position of inspecting the product and labels for extended periods of time, so it is beneficial for them to be aware of information concerning specific product parameters (such as temperature or light exposure restrictions, label and cap coloration).

In addition, each department that integrates with another assumes some of the responsibility for evaluation and criticism at the point of integration. This includes not only the labelling, but also the review of line status signage (identification of the type of product and batch number to be labelled and the phase in the process that is being performed – start-up line clearance, tear-down or changing of the line information), the expiry date, reconciliation records, etc.

It is important that key activities (such as line clearances to ensure that all labels from previous runs are removed and that no evidence of the previous run exists) are performed, verified and documented.

It is also of key importance that labels be accounted for and that a limit for label reconciliation discrepancies (how many can be unaccounted for without requiring an investigation) be established and followed. There should be investigations of all discrepancies that surpass the discrepancy threshold.

Microbiology

When a company can afford the implementation of a separate unit to perform environmental monitoring (in this instance, environmental monitoring refers to particulate and environmental flora sampling), the testing and plating of personnel and production areas and the testing for pyrogenicity, cleaning validation studies, etc., it is beneficial to have a separate microbiology department.

A testing department separate from QC allows routine production tests to be performed by one department (QC) while the other department monitors auxiliary factors that have an impact on the production process.

The microbiology department is usually concerned with the monitoring and culturing of production process components such as water systems, the evaluation of gown sterilization techniques, the testing of sterility and pyrogenicity, the manufacture and testing of various culture media (used to culture bacteria, mycological and parasitic organisms) and the evaluation of microbiological testing results.

Integration of production with the microbiology department. The microbiology department provides periodic monitoring

of all production environments (including the environmental hoods used in QC), establishes criteria for the periodic testing and culturing of clean room and laboratory environments, tests filters (including HEPA filters), undertakes tests of personnel gowning techniques and subsequently cultures all samples taken. The department must schedule the testing and evaluation of test data in such a way that a minimal time elapses between materials/environments being sampled, cultured and evaluated and materials being used. This reduces the likelihood that sampled material could be used in other products or processes prior to the results of the initial culturing being available.

Where the resources of a company do not provide for a separate microbiology department, these responsibilities may be divided between QC and production.

The types of documentation generated within this microbiology include: culturing protocols, bench record information, data and evaluation of personnel gowning and room environmental results. These types of data are considered auxiliary and are usually retained within the microbiology department. Specific information, such as the results of settling plates used during a production run, can be referenced in the batch record.

THE ROLE OF QUALITY CONTROL

The QC unit is generally responsible for all of the sampling and testing of ingredients, components, reagents and raw or starting materials. The test phases include initial sampling and testing of raw materials, in-process testing, the testing of intermediate bulk materials, animal testing and final product testing.

QC must also test for anomalies, perform periodic reassays of reagents and participate in testing involved in investigations and manufacturing discrepancies. The general instructions for tests are outlined either in a country's regulatory guidelines or in pharmacopoeias; but, in cases where more than one test methodology is listed, QC must ensure that the procedures used are the same as the procedures written in the product outline.

QC should also ensure that all raw materials and components are identical to the list provided to the government in the outline. When changes in either procedures or ingredients are indicated, regulatory affairs should be notified prior to implementation.

QC data are usually presented in the form of bench records which provide the results from tests and should include the formulae used and the type of test performed, as well as referencing the established test methodology. The procedures utilized should also be referenced along with all other pertinent data (the age of the animals being tested, product outline parameters, etc.).

Other types of documentation generated by QC are: raw material assays, QC sampling logs, stability programme data, certificates of analysis, equipment QC, laboratory instrumentation calibration programme, logbooks and scheduling of the animal-handling facility and the supporting documentation.

As with production, the QC unit involves specific interactions and communications with other departments. The departments that integrate with QC are: R&D, regulatory affairs, materials management, engineering and maintenance and QA.

R&D and regulatory affairs

The test methodologies invented or designed by R&D must be incorporated into a format that can be utilized by a controlled laboratory. The latitude allowed within R&D is usually not permissible within QC. Most countries have established specific methodologies for the

testing, assaying and evaluation of test manipulations. In addition, companies that export must also incorporate or augment their country's testing requirements with the explicit methodologies mandated by their customer.

The stability programme (which may be initiated by R&D but is maintained by QC) is a mechanism that monitors the effects of such elements as time, preservatives, heat, light, vial/stopper composition and temperature on the product.

Initially, R&D may perform an abbreviated version of this programme to determine the selection of vials, stoppers, raw materials, etc. on the viability of the product. This information is critical in creating an expiry date, storage temperatures and product ingredients. The QC unit utilizes this information to establish the parameters for monitoring the product.

The ongoing product/component interaction should be monitored constantly to determine if certain factors such as container or stopper composition, preservatives, heat and light react adversely with the product over time. This is the main premise for establishing a stability programme. Since the data produced could affect the product's expiry date, ingredients or components, QC should continually update the regulatory affairs department and alert it to any data that arise on the questionable stability status of a product.

Materials management

One of the responsibilities of the QC unit is to ensure the quality of all components used to make a product, so QC must perform in-process sampling and testing of raw materials and components. This involves establishing sampling programmes that specify the amount of each product to be sampled and the acceptable quality level (AQL) that must be achieved on inspection.

The AQL for vials, for example, would require establishing the type of glass, the colour, size and shape of each vial. Another element could be whether the imperfections identified are classified as critical, major or minor, and how many of each kind of imperfection could be allowed within the vial and within each sample size.

QC personnel usually have a designated space within the materials management area to sample each shipment. Using good lighting and such tools as callipers, tape measures and spatulas various items are sampled and the results are documented.

Suppliers often furnish certificates of analysis which may describe the tests done by the supplier and the results. It is suggested that certificates from other companies be augmented with parallel sampling and testing until a degree of confidence can be developed. This includes the periodic assay or testing of various raw materials (including reagents) to determine testing characteristics and to provide baseline data from which to establish supplier approval criteria.

Companies should remember to establish a programme for the reassay of reagents and ingredients that are retained for a period of one year or more.

Engineering and maintenance

One of the responsibilities of QC is to monitor the water systems of the company (if this is not performed by a separate microbiology unit), so it is important that QC be apprised of any impending changes to service systems or existing drains and of any remodelling of the facility. In addition, engineering and maintenance must perform or assist in the calibration and validation of the various instruments in the QC laboratory.

The majority of instruments in the laboratory will require a schedule of monthly, quarterly, biannual or annual

calibration. Reagents and controls generally have built-in QC measures that require the testing or challenging of the control prior to use.

Quality assurance

The QA unit is responsible for the review of all QC data prior to release of the final product. QA monitors QC to ensure adherence to all applicable standards and test methodologies.

DEVELOPING THE INFRASTRUCTURE FOR A DOCUMENTATION CONTROL SYSTEM

Storage

Documents should be stored in a secure location with limited access. In order to facilitate documentation reviews such as annual product reviews, trend analyses, SOP revisions, evaluations of master batch records, lyophilization cycles, etc., it is essential to maintain various types of documentation in centralized locations.

The centralization of processes and documents allows the number of system variables and personnel involved to be more effectively controlled. A system that is accessible to several departments is vulnerable to the vagaries of departmental interpretation.

The following are recommendations for the storage of records and other documents:

- Provision should be made to ensure the storage of controlled records for a specified period of time.
- Records should be stored in a safe, secure area and efforts should be made to minimize the damage that could be caused by environmental factors such as humidity and water.
- Records should be stored in an area where they are easily retrievable and should either be located at the site where the activities described took place or should be readily accessible to authorized inspecting personnel.
- Procedures should be established for the storage and retention of records and should include the methods of storage used and the steps necessary to retrieve information.
- Procedures should exist for the revising, correcting, tracking and destruction of all controlled documentation.
- In order to facilitate storage requirements, a log should be retained delineating the numbers of controlled documents, who has copies of these documents, when they were distributed and when obsolete copies were returned.
- A procedure should exist for the archiving of records. Consideration should be given to storage periods, the types of records that should be retained, how records should be packaged for storage and who has authorization to store and retrieve archived information.

Development of master documents

Master documents are the original permanent copies of documents from which authorized working copies are made. The purpose of master documents is to have an official copy that contains the original information of the process and, when combined with copies of all of the retired (obsolete) master documents, they chronicle the information necessary for the formulation of a specific product, procedure or process.

There should be only one set of master documents. Appropriate identification of this set can be made by virtue of the original signatures, by its being printed on controlled, specially designed or coloured paper (possibly paper with a watermark or of a special restricted colour or company letterhead paper) or by its being stamped with an authorized red stamp. The key is to have some identifying characteristic that makes it possible to distinguish a master

from a copy. Accessibility to the identifying characteristic (coloured or letterhead paper, for example) should be restricted.

Among the master documents are the SOPs, which are step-by-step instructions used to perform a specific function in a process. Procedures should be clear, concise and to the point. Text should be written in language that persons with the requisite education and experience can understand.

For a procedure to be effectively described there are key elements that must be present in the document format. The following information may be written as a cover sheet or in the form of a header/footer on the first page of the document:

- *SOP title:* a descriptive title of the function to be performed;
- *procedure number:* a sequential control number assigned to the procedure;
- *departmental category:* the department to which the procedure applies;
- *page number:* each page must be numbered;
- *effective date:* the date the procedure is to be officially used is typed in here. This date must be after the date of the final signature;
- *approval signatures:* appear in the blocked section and should be handwritten;
- *written by:* the signature of the person who wrote the SOP.

Packaging/labelling. Because information is constantly being revised, updated and clarified, it is critical that an open dialogue exists among regulatory affairs, production and labelling control. The instructions provided on labels, inserts and cartons must reflect the same instructions submitted to the government. Procedures for the facilitation of this dialogue should be written and followed.

The persons responsible for reviewing and approving an SOP should have a background commensurate with the subject matter they are reviewing. It is the responsibility of all signees to have a working knowledge of the subject matter and to evaluate the document in the light of their area of expertise.

The approval of production SOPs usually involves the departmental supervisor or head, the production manager, regulatory affairs and QA. (In companies where QA functions as the regulatory liaison, it may not be necessary to have the signature of the head of regulatory affairs.)

The following information should also be contained in the SOP:

- *objective:* explaining the main purpose of the procedure and defining whether the purpose is to outline the method of operation, maintenance, etc.;
- *scope:* the exact limitations of the procedure, which define the targeted elements. The procedure may be directed towards a function, a piece of equipment, a certain process, etc.;
- *responsibility:* direct responsibility should be assigned to an individual title or group, to ensure that the procedure is enforced;
- *procedure:* step-by-step directions written in wording that the person performing the task can understand;
- *review and retention:* a statement regarding the date for regular review (review of procedures should take place at least once a year) – the process for revising documents and making them obsolete may be written on each cover sheet if desired.

SOPs should pertain to specific equipment or processes. When SOPs describe the functions of a larger process, it is suggested that the last step or a reference section direct readers to the next SOP. (If a company does not have an SOP coordinator or a documentation control unit, leading references to the next SOP should be avoided.)

Any numerical values written in the SOP should have tolerance parameters whenever possible. Parameters should be realistic and, wherever possible, be broad enough to allow moderate process fluctuations. Numerical values that indicate the specific actions to be performed must be linked to documents that allow the verification of that action.

It is important to remember that the master batch record will contain abbreviated information from SOPs that specifies the significant steps. For example, a record specifying that a certain step must be verified must provide a logbook, a batch record or some other document that, when filled out, indicates that the verification took place. Information that has incorporated numerical fluctuation (i.e. tolerance) is represented as in the following example: the temperature 37°C ± 5°C means the allowable temperature range for that product is 32°C to 42°C. This means that a product found within that range is acceptable for use.

In devising tolerance ranges for processes, the data to justify ranges should be provided within the product outline.

Documents that specify time parameters must be linked to documents that allow verification of that information, for example: "Agitate the liquid for five minutes ± one minute".

There should be a logsheet or batch record providing the following information: the times the agitation began and ended; who it was performed by, with the date; and who it was verified by, with the date.

All SOPs should follow the parameters and specifications of the product outline.

All reviews of revised SOPs should incorporate a correlation of the current version of the document (if it is a revision) and the product outline.

The routing of SOPs and SOP revisions should be documented on an SOP master log.

Development of historical records

Historical records are the working batch records. The master batch record is a blank format that has been designed with specific instructions to produce a specific product of a specific size, potency, etc. The batch record is essentially the diary of all of the processes and procedures performed on a given day to produce a specific product. In a manual system, this will be the document that actual performance information is recorded on.

Batch record. A batch record is basically an abbreviated form of a process SOP that, when filled out, becomes the diary for a specific lot of a product made on a particular day.

Batch records are derived from master batch records and are authorized copies of the master document.

The following information pertains to master batch and working batch records:

- The formulation information and the equations used for scale up should be written under the guidance of regulatory affairs, R&D and the department(s) involved in scale up.
- All equations and significant steps pertaining to the production of a batch should be described.
- Master documents are always printed on official company paper. (Working batch records are copies of the master.)
- The standard type of documentation to be found in a batch record includes: the name of the product; the product registration or drug code number and the lot number of the initial product (or the raw material lot numbers)
- A bill of materials page should describe the materials and ingredients to be used and the quantity requested, with space provided in which the quantities used and returned to inventory (i.e. stores) can be documented. An example of a bill of materials record is as follows:

Product code	Material	Raw material (No.)	Expiry date	Quantity required	Quantity used	Quantity returned	Operator's initials	Date
6215400	6mm West 4880 grey stoppers, siliconized	540STPRS	6 July 1998	7 500				
7348221	20-cc type II clear flint glass vials	321GLSCC	6 July 1998	7 500				

- Original auxiliary records or copies, with a note of where the originals can be referenced, should be included in the batch record.
- All documentation that reflects the significant steps in a process such as inoculation, incubation, harvesting, centrifuging, mixing, filling, packaging and labelling should be included in the batch record.
- References and documentation should be made describing what containers and pieces of equipment were used, when the equipment was sterilized and who performed the critical steps.
- Data such as inoculation and candling results should be included in the batch record.
- Lyophilization steps should be described step by step.
- The formulae used to perform calculations for specific gravity, multiplicity of infection and fill volume criteria should be an integral part of the batch record.
- Monitoring fill volume, line clearances and other processes that are exclusive to the operation of a specific batch should be included in the batch record.
- The specific logs used to monitor filling, sealing and labelling phases of the process should be included in the batch record.
- A monitoring record should supply the following information: product name, code number, issue date, potency, etc.; any specific instructions on the monitoring procedure (e.g. "A representative vial for each nozzle should be checked at 30-minute intervals as per SOP 001.11.111"); the filling machine name; the equipment identification number; the fill room number; the manometer reading; who performed the operation, with date; the number of fill needles used; the acceptable fill range (e.g. 5.0 ml to 5.3 ml); the target range (e.g. 5.2 ml); the time started; and the time ended. A specimen monitoring record is shown in the box on the next page.

The record would then indicate an immediate readjustment of the fill volume and would probably have start and finish times of 8:40 am and 3:10 pm, respectively.

Records such as refrigeration monitoring records and the making of media or cleaning solutions are usually indicative and apply to processes that will be used for several products. These preparation records are usually written as process batch records and are retained in specific areas of the department that performed the work. The product batch record may reference the media preparation or disinfection preparation batch records but

Vial number	Time	Amount *(ml)*	Vial number	Time	Amount *(ml)*
1	8:00 am	5.25 ml	1	2:30 pm	5.13ml
2		5:20 ml	2		5.22ml
3		5:12 ml	3		5.21 ml
4		5.20 ml	4		5.18 ml
1	8:30 am	5:12 ml	1	3:00 pm	5:11ml
2		5:20 ml	2		5:26 ml
3		5:32 ml[1]	3		5:23 ml
4		5:29 ml	4		5:08 ml[2]

[1] Fill volume out of range, fill operator called and instructed to adjust needle number 3.
[2] Low fill, fill operator called and instructed to adjust needle number 4.

copies of these types of documents need not be included with the batch record.

- Batch records should contain a documentation list stating the documents that are to be found in the batch record, the results of the batch record review and whether the record is acceptable for release based on the review.
- A completed batch record usually has copies of the QC records including the certificate of analysis and the results of the testing that was performed for final product testing.

Maintenance and retention of auxiliary records

Records that support the production processes or provide documented proof of certain activities, such as lyophilization and autoclave charts, validation data, feasibility studies and stability data must be regularly maintained, monitored, updated and retained. Lyophilization, autoclave and oven charts should be maintained in the batch record when the load includes only those items that are traceable to a specific product. For example, autoclave records for stoppers or lyophilization records should be maintained in the batch record but autoclave records for media or tubing, which could be traceable to other products, may be copied with the original referenced document or the records may be kept separate and the batch number of the autoclave run referenced in the batch records.

Examples of other records that are auxiliary and may be stored separately (and referenced by a control number in the batch record) are: the results of microbiological monitoring; validation information; QC records that provide data for more than one product; preventive maintenance records for equipment; calibration records; sanitation and disinfection records; and inventory records.

Whether this practice is acceptable is contingent upon the regulations of each country's government and the requirements of importing customers (when applicable).

Control and tracking documentation

Control documents are regulated documents that contain proprietary or confidential information concerning a company's processes, policies, procedures or ideas. These types of documents are assigned unique identifying numbers which provide traceability and accountability by allowing the company to establish and monitor who gets the documents and what version or revision of the document is most current, as well as by coordinating the replacement of obsolete documents. Examples of controlled documents are product outlines, SOPs, batch records and labels.

Assigning control numbers involves the systematic application of a sequence of numbers to documents in order to track information and the corresponding recipients. Examples of tracking documents are logbooks, routing forms, perpetual inventory control cards and material transfer forms.

The information written in the batch record is correlated to the documentation in logbooks. Logbook documentation should complement the data in the batch record, so the following points should be considered:

- Documentation should be made as soon as work has been completed whenever possible. For instance, verifications should be signed by a witness immediately after the worker completes the procedure. When information is signed a day or two later, the accuracy of the information can no longer be assured, especially as it is already apparent that the personnel involved have a questionable memory demonstrated by their inability to sign the record on time.
- The same personnel who sign the "Performed by" and "Verified by" sections of the batch record should also sign the appropriate sections of the logbooks.
- Information in logbooks should be in chronological order and, wherever possible, books should be bound as opposed to loose-leaf binders.
- Significant information in either logbooks or batch records should be handwritten and not typed, stamped or computer printed.
- Master logs should describe the master documents on file and what revision number or version number is current.

Change control procedure

This procedure provides a mechanism whereby the revision or amendment of regulated documents is effectively monitored, evaluated and controlled. It involves establishing the critical control points, the authorization personnel and the criteria for making a decision for approval or rejection of a proposed change.

The change control procedure should require that several departments review proposed revisions to any procedure, equipment or processes. These include changes in temperature ranges, incubation times, ingredients, filling and manipulation of the product, testing and storage.

The procedure should be designed to eliminate the possibility of a process conflicting with the product outline and should provide for the justification of clerical as well as scientifically sound changes.

Change control procedures are considered critical and should take into account information provided by the evaluation of pertinent documents, the expertise of persons reviewing the change and the justification written to support the change. Discrepancies in any of these criteria could result in the invalidation of the change (which could jeopardize all the products made subsequently to the change).

An example of a change control procedure form is given in Figure 17.

THE APPLICATION OF QUALITY ASSURANCE TO PRODUCTION AND QUALITY CONTROL SYSTEMS

The success of a company's implementation of GMP can generally be evaluated by a review of the documentation within or surrounding the production process. From the focal point of a batch record, which is a diary of a particular batch of product, an auditor can evaluate SOPs, the product outline, QC data, distribution, purchasing and inventory records. In addition, the proficiency of regulatory affairs, labelling, engineering and mainte-

Company name _____ Attachment I of SOP _____
Form control _____ Effective date _____
Address _____ Supersedes _____

Section 1 – Responsibility: author/initiator of change
Document title _____ Date initiated _____
Proposed document revision number _____
Document number _____ Draft initiated by _____

Indicate change(s) (x):
( ) Revise SOP
( ) Convert policy to procedure
( ) Revise production batch record
( ) Convert procedure to policy
( ) Change validation parameters
( ) _____

Indicate type of change(s) (x):
( ) Clerical corrections
( ) Make amendment to SOP*
( ) Addition of step(s)
( ) Make attachment to production batch record

( ) Deletion of step(s)
( ) Revalidation
( ) Clarification of existing information
( ) Other (explain)

* Must be justified by department head

Section 2 – Responsibility: department supervisor or designate
Evaluate the information in Section 1 in conjunction with the document submitted.
Determine if change is feasible and warranted.

( ) Approve as is
( ) Approve as corrected
( ) Revised draft required prior to approval (if checked return to author)

Reviewed/approved by _____
Department supervisor or designate
Date ___ / ___ / ____
☞ Route approved document to education trainer

Section 3 – Responsibility: Education training
Education training review the revised document and determine if training is necessary.

( ) Training indicated
( ) Original training is sufficient

Reviewed/approved by _____
Education trainer or designate
Date ___ / ___ / ____
☞ Route to quality assurance

Section 4 – Responsibility: QA manager or designate
QA review proposed change(s). Indicate below justification(s) (x):

( ) Directed by regulation
( ) Provides clarification to document
( ) Directed by in-house requirements
( ) Reflect to actual practice
( ) Other (document) _____

( ) Not a significant step
( ) Does not affect safety, purity, potency of product
( ) Correction action
( ) New process/equipment

( ) Approved as is
( ) Revised draft required prior to approval
( ) Approved as corrected

Reviewed/approved by _____
Quality system manager or designate
Date ___ / ___ / ____
☞ Route to QA

Section 5 – Responsibility: QA
( ) Revalidation of process required
( ) Validation not applicable
( ) Validation of step required

Document _____
( ) Approved as is
( ) Approved as corrected
( ) Revised draft

Reviewed/approved by _____ Date ___ / ___ / ____
QA director or regulatory affairs

FIGURE 17
Sample documentation change control form

nance, documentation control and QA can be evaluated by using the batch record.

If a company fails to understand the need for integration of systems (and the corresponding documentation), an audit such as this wil be damaging for the company and revealing for the auditor.

It is important to remember that one of the primary reasons for the proliferation of documentation is to provide information to regulatory agencies and other auditing bodies. Since the lack of appropriate documentation is a key indicator of inadequacies within a system, and poor or incorrect documentation may be indicative of questionable or inappropriate operations, documentation review is the key focus of any audit.

By reviewing the batch record in conjunction with supporting documentation, auditors are able to make a relatively accurate evaluation of the manufacturing processes and systems.

The methodology for performing a systems audit via the batch record is as follows (the name of the department usually responsible is indicated in brackets):

- The auditor asks to review specific batch records and, because the information must be in accordance with the product submissions and licences, also asks for the product outline.
- If a specific licence has stipulations to meet contractual agreements (for countries that export), licence amendments, addenda and contracts may also be requested.
- If the batch record does not provide step-by-step documentation of the process but instead resembles a bench record, SOPs specific to that product and supporting functions will be requested.
- Beginning with the bill of materials, the auditor will ensure that the materials used reflect the specifications submitted to the government or agreed to in the contract. This encompasses the dilutions of ingredients and reagents, special treatments of media, the type of packaging containers, stipulations that specific manufacturers or vendors must be used, the amounts dispensed of each ingredient, lot numbers used, etc.
- If there are discrepancies in the information listed above, auditors will usually ask to see the change control procedures (regulatory affairs and/or documentation control), the annual review of procedures documentation (regulatory affairs and/or QA), certificates of analysis, which evaluate incoming raw materials (QC), documentation of the process of scale up or converting R&D batches into production batches (regulatory affairs and R&D), the supplier approval process (QA and purchasing) and the procedure for developing a master batch record (documentation control). If lot numbers are not used in a controlled, sequential manner, or if it appears that more of a lot number was documented as having been used than was received by the company, inventory records as well as procedures will be scrutinized (inventory control or materials management).
- Any ingredient that indicates the involvement of outside vendors or suppliers (such as the need for irradiation, HVAC filter changing and cleaning services, suppliers of media, eggs and tissue) will involve the evaluation of the purchasing department and should include the QA unit. Whether a company implements audits of outside contractors and includes the participation of the QA unit prior to the purchase of major equipment or supplies is a direct reflection of the company's understanding of GMP requirements. Lack of QA involvement

in the decisions of building a facility or purchasing equipment and supplies inevitably leads to GMP infractions which must be remedied or justified later.

- Both the ingredients and the formulation steps of the batch record are reviewed against the product outline and appropriate procedures (regulatory affairs, production, QC).
- The parameters for inoculation, harvesting and incubation will be compared with the product outline and contract requirements. Auditors will request logbooks to ensure adequate maintenance and disinfection of equipment and compare the dates, names, locations and equipment in the batch record with the dates, names, locations and equipment in the logbooks' lists of equipment (production, QA).
- The actual usage of ingredients will be compared with the amounts dispensed and remaining, as well as with the chronological order of lot numbers. Key points will be whether there is a system in place to ensure that the oldest ingredients are used first; a tracking system to ensure that all raw materials are reconciled; traceability of where and when ingredients were used; and an established action level threshold beyond which the company will investigate inventory discrepancies (materials management, production).
- Testing methodologies will be compared with pertinent pharmacopoeias, licence submissions and sampling plans. Evaluation methods and data will be reviewed (QC, microbiology).
- All numerical values of temperatures, incubation periods, mixing times, etc. will be reviewed and correlated to the equipment-use logs which indicate the dates of operation, the cleaning and disinfection of the various areas, environmental monitoring data (e.g. manometer readings, settling plate or air particulate sampling) and the personnel who have performed significant steps.
- The methods companies use to handle returned product, complaints and product recalls, as well as the review of equipment specifications, history and validation records, utility systems and building design are also common areas of assessment.
- Autoclave and Lyophilization charts and logs give definitive information concerning the functions of a system that encompasses equipment maintenance, biological indicator monitoring, product management, calibration, disinfection and cleaning, validation, product outlines, manufacturing discrepancy and deviation processes, quarantine/holding and rejection/disposal information. The documentation commonly reviewed at this juncture includes: equipment history files; preventive maintenance and calibration programmes; validation and revalidation data; cleanser feasibility studies (for effectiveness of disinfectants and cleansers); utility system data, especially water systems; lubrication programmes; and load configurations (production, engineering and maintenance, microbiology, regulatory affairs, QA).

The information given here is by no means comprehensive, but it should convey the relevancy and importance of documentation. As this information is reviewed, it becomes apparent why an adequate and comprehensive documentation system is necessary.

The complexity of the documentation and integration of the production processes require the use of resources that can provide an overview of the systems inherent in the manufacturing of veterinary vaccines. It is recommended that in the

development of systems for a new facility, the requirements of documentation should be given paramount importance. Companies that have an interest in retroactively addressing documentation issues or that wish to improve an existing system are encouraged to assess their manufacturing systems. A successful systems review will reveal the documentation inadequacies in existing processes while providing the initial springboard for addressing and developing new documentation requirements.

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Assessment of potency in bacterial vaccines

P.A. Knight

Regular performance of potency tests on every production lot is essential for the maintenance of efficacy in most bacterial vaccines. Failure to perform such tests has often resulted in a gradual decline in potency and vaccines from previously untested sources rarely perform satisfactorily when tested. Potency tests are prescribed in most bacterial vaccine monographs and where these have been consistently applied the incidence of failure in the field has been very low. It is, however, important to recognize that mere conformity to the requirements of a pharmacopoeia does not guarantee that a new formulation will necessarily prove efficacious in the field. It is always essential to demonstrate that a representative batch of any new formulation elicits the required level of protection or of protective antibody in the target species.

This chapter is concerned with the principles on which valid potency tests should be based, in cases where pharmacopoeial requirements are inapplicable or unavailable and considers the potency tests laid down in three major compendia, viz. the European Pharmacopoeia, which is mandatory throughout the European Union (EU), Chapter Nine of the United States Code of Federal Regulations (USDA, 1994), which is mandatory in North America, and the British Pharmacopoeia (British Pharmacopoeia Commission, 1985), which is harmonized with the European Pharmacopoeia but contains some unique monographs and is widely available in many developing countries. In the British Pharmacopoeia all the veterinary monographs are contained in a single volume, so it may be more convenient to use than the European Pharmacopoeia where they are scattered through several volumes.

Although the requirements contained in these documents are not binding to veterinary vaccine producers outside the EU, the United States or the United Kingdom, they are based on extensive experience of the production and control of effective bacterial vaccines and the methods that they prescribe have been well tried. Their adoption in other countries can, therefore, avoid the lengthy and expensive process of test development and validation. In the case of new vaccines, for which no pharmacopoeia requirement is relevant, validation of a potency test method and, where appropriate, a reference preparation should be integrated into the process of vaccine development.

KILLED VACCINES

The majority of bacterial vaccines are killed. They may contain whole inactivated cultures, suspensions of killed organisms, exoantigens from culture supernatants or extracted somatic antigens. The need for potency tests of killed vaccines has sometimes been questioned, particularly for those in which the protective antigens can be identified and measured, on the grounds that if the right quantity of antigen is present, the potency will always be satisfactory. This is not so. Potency tests are essential to the consistent production of efficacious killed vaccines because the potency of the final product is affected by a wide range of variables which cannot be completely controlled. Although the

impact of these variables can be defined in a few instances, in most cases it is impossible to predict from the density of a culture, the concentration of a toxoid or its antigenic profile, the potency of an adjuvanted final vaccine prepared from it. This is because the expression of protective antigens is affected by the quality of the growth medium and the time and conditions of culture growth. The immunogenic quality and quantity of protective antigens are also affected by small differences in conditions of detoxification. The immunogenicity of the protective antigens in the final vaccine is affected to varying extents by adjuvants and other antigens which are either deliberately added to form multivalent products or are present as impurities in the final formulation.

Although a few monovalent, unadjuvanted vaccines prepared from highly purified antigens that do not require inactivation are issued for human use without any potency test, none are as yet available for veterinary applications. For all inactivated veterinary vaccines it remains impossible to predict the potency of a batch of vaccine on the basis of antigen content, culture density or previous experience.

The foregoing points emphasize the importance of maintaining rigorous standardization of every aspect of the manufacturing process and the need to control accurately the quantity of protective antigen in a dose of vaccine whenever it can be measured.

An ideal potency test should meet two requirements. It should: measure a response that is relevant to the efficacy of the vaccine; and provide assurance that the size of that response is sufficient to guarantee efficacy.

Measurement of the immune response

The method of measurement or titration of the response is critical to the relevance of the test that assesses the efficacy of the vaccine. Most potency tests on veterinary bacterial vaccines rely on the use of a challenge with live organisms to measure immunity in the test animals.

Challenge methods have the advantage that they are perceived to demonstrate efficacy directly. This is undoubtably true when the route of administration is analogous to the route of infection in the field or when the pathology of the laboratory infection is similar to that of the natural disease. However, it is a far less reliable guide if, for example, an intraperitoneal challenge is used to determine the potency of a vaccine directed against an enteric or respiratory disease. Thus, vaccines based on adhesin antigens of *Escherichia coli*, which are highly effective against colibacillosis in piglets, completely fail to protect mice against intraperitoneal challenge with live *E. coli* in the laboratory. Similarly, the relevance of intraperitoneal challenge of laboratory animals to the efficacy of vaccines directed against swine erysipelas or the pneumonic forms of *Pasteurella haemolytica* is questionable. Nevertheless, live challenges are the most relevant indicator of potency for the majority of inactivated bacterial vaccines.

Challenge tests, however, do have considerable disadvantages. They are inefficient, using large numbers of animals to obtain limited information. The procedures to which the animals are submitted are so severe that in the EU such tests can only be tolerated in the absence of a satisfactory alternative and many laboratories are active in the development of methods to replace them. In addition, the use of challenge methods precludes the determination of potency for more than one component in a multicomponent vaccine with a single group of animals. For these reasons there are considerable attractions in the use of non-challenge methods of evaluating the immune re-

sponse, provided that such methods can be shown to reflect the efficacy of the vaccines concerned with sufficient accuracy.

The best-established examples of non-challenge methods are found among the clostridia, where the substantial role played by lethal toxins in the pathogenesis of the relevant diseases allows the potency of many vaccines to be determined in terms of the antitoxic response elicited by the vaccine in rabbits and titrated *in vivo* or *in vitro*. For many years such tests have been instrumental in maintaining high levels of efficacy in a range of clostridial vaccines used in Europe, Australasia and North America, despite the fact that in almost every case antitoxic immunity provides only a part of the total protection offered by the vaccine.

As the antigens responsible for protection against bacterial diseases have been identified, vaccines based on these antigens have been developed. The routine potency tests of such vaccines have been based on levels of antibody directed against the protective antigens. Although some authorities might describe such tests as immunogenicity rather than potency tests, they may furnish as good an index of efficacy as a challenge test, provided that the antibodies measured are specific to the molecular species responsible for protection. Ideally the antibodies measured should be shown to neutralize the biological action of the protective antigen. Thus, for example, antibodies directed against adhesins should be shown to block adhesion of bacteria to target cells, and those directed against toxins should be shown to prevent their toxic effects.

Unfortunately, apart from the clostridia, serological techniques such as enzyme-linked immunosorbent assay (ELISA), immunoprecipitation or agglutination have measured antibodies directed against preparations of the protective antigen(s) (which are often only partially purified) without making reference to their biological activity.

The value of such techniques as indicators of potency depends on their specificity for the protective antigen(s). Use of monoclonal antibodies (MAbs) directed against the protective antigen in capture or competition ELISAs provides a useful means of attaining specificity when such MAbs are available. Alternatively, specificity may be attained by exhaustive purification of the protective antigen(s).

Purification of large protein antigens is not always easy, even from the native state, and may be impossible for preparations derived from formalin-inactivated cultures. The choice of serological technique is not critical but ELISA offers a wider range of strategies for the attainment of specificity and many methods based on the use of MAbs are only practicable with ELISA. Detailed descriptions of a variety of methods for the serological evaluation of antitoxins are included in the World Health Organization (WHO) manual for potency testing of diphtheria, tetanus and pertussis vaccines (WHO). However, for those laboratories that lack facilities for reading ELISA tests, a more limited range of strategies for the attainment of specificity is still possible. Although tests based on the agglutination of bacterial cells have been widely used in the past, they are often dominated by immunoglobulin M (IgM) responses to O antigens and the number of antigens presented by whole organisms is so large as to make the attainment of high specificity for protective ones extremely difficult. Some of these difficulties may be overcome by the use of agglutination inhibition and single radial diffusion techniques.

Whatever method of response evaluation is chosen, it is important to establish that the selected method, when applied to sera from vaccinated animals, reflects their resistance to challenge and that results

from the complete test reflect the relative efficacy of different vaccines in the target species. It is also important that all serological data are related to a laboratory reference preparation which should preferably consist of a pool of typical test sera. This is essential in order to ensure that the results obtained on different occasions or even different plates are validly comparable.

Potency test formats

The other element of the test is the elicitation of an immune response in animals. This may be observed in laboratory animals or in animals of the target species. While the latter possess obvious advantages in terms of the ease with which test results can be translated into evidence of field efficacy, the greater ease of standardization, management and containment of challenge infections, together with the better prospects of using statistically adequate numbers, have favoured the use of laboratory animals for most tests.

An ideal potency test should provide a quantitative estimate of the potency of the vaccine, which would be the same if the test were performed at any laboratory and which would exactly reflect the efficacy of the vaccine in the target species. As well as estimating the potency of the vaccine an ideal potency test would also indicate a level of confidence that the true potency exceeded minimum requirements. Because tests that rely on an absolute level of response are affected by the varying levels of sensitivity of the animals used, the potency of a vaccine in an ideal test should be determined in comparison with a standard or reference preparation.

The classical six-point assay is designed to provide that comparison. In such a test the responses of groups of animals receiving three dose levels of the vaccine under test are compared by challenge, titration or serological methods with the responses of similar groups given dilutions of a standard vaccine.

Standard and reference preparations. Standard vaccines may be supplied by national control authorities such as the United States Department of Agriculture (USDA) or international bodies such as the European Pharmacopoeia Commission or WHO. If no appropriate national or international standard is available, and particularly if the vaccine to be tested is a new formulation, a batch of vaccine of proven efficacy should be adopted as a local reference preparation.

A standard or reference vaccine should be qualitatively identical to the test vaccine. Strong interactions frequently occur between the components of multivalent vaccines. When these occur it is often better to adopt a separate multivalent reference preparation than attempt comparison with a single component reference. Use of such reference preparations may facilitate potency testing of several vaccine components in a single group of animals, resulting in considerable cost savings. If a reference preparation is intended for use over a period of more than three years it should be lyophilized and then, preferably, tested for efficacy in the target species. If this is impracticable, the lyophilized material should be shown to be identical in potency to the liquid vaccine that has been proved to be efficacious. Particular care needs to be taken when lyophilizing vaccines that contain aluminium adjuvants as these often suffer recrystallization, resulting in a substantial loss of potency when the vaccine consists entirely of adsorbed soluble antigens. The effect is largely mitigated when whole bacterial cells are present.

Challenge tests. When the immune response of animals is evaluated by challenge, the proportion of animals surviving in each treatment group is used to estimate

the 50 percent protective or effective dose (ED_{50}) values for test and standard vaccines. The method of calculation of ED_{50} values is not specified but, if access to a computer is available, the preferred method is probit analysis which makes the most efficient use of the available data. The quantitative estimation of potency by six-point assays assumes that the dose-response curves of the test and standard vaccines are linear and parallel and it is usual to verify that estimates of potency are valid in this respect. (A computer program for probit analysis is available from WHO, Biologicals Division, Geneva 27, Switzerland. This estimates the relative potency of the test vaccine, together with its confidence limits, and verifies the statistical validity of the estimate.)

In the absence of a computer, ED_{50} values can be calculated by the methods of Reed and Muench (1938), provided that the available data satisfy the requirements of that method (i.e. test groups show 100 percent and 0 percent effects), or by use of tables of precalculated data such as those of Toothill, Robinson and Adams (1969). An estimate of the potency of the vaccine under test relative to a standard – the relative potency (RP) – can then be calculated as the quotient of the ED_{50} value estimated for a standard preparation divided by the ED_{50} estimated for the vaccine under test. These methods, however, are less effective than probit analysis and do not test the statistical validity of the data or estimate confidence limits.

Quantitative response tests. The six-point assay described above is applied to quantal data (i.e. proportions of survivors). The same principle can also be applied to quantitative (continuous) data such as scores or titres. The scores or titres are used to estimate the potency of the test vaccine relative to the standard by parallel line analysis. It is usual to transform both the dose levels and the response data to logarithms at the beginning of the analysis unless they are already in a logarithmic form such as serial dilution numbers. Assays based on quantitative data usually require smaller numbers of animals than quantal assays and are therefore preferable. A computer program for parallel line analysis, similar to the one referred to above for probit analysis, is also available from WHO.

Although six-point assays provide the best estimate of the potency of a vaccine, they are rarely used in veterinary applications because of the large numbers of animals that they require. The tests for *Pasteurella multocida* and swine erysipelas vaccines in the North American and European requirements need 120 and 96 mice per test, respectively.

Absolute response level tests. The majority of the tests prescribed by pharmacopoeias do not make use of a reference preparation but base the criterion of acceptance on the elicitation of a defined level of response by a fixed dose of vaccine. This approach requires a high degree of standardization of the laboratory animals which may vary from strain to strain depending on their diet and other conditions of husbandry. The effects of these variables (which can be very large) are difficult to isolate and may influence the observed potency value of the vaccine under test. This can lead to the rejection of good vaccine or the acceptance of bad. A partial solution to this problem can be found in the occasional use of a reference vaccine to verify that it elicits a typical response in the hands of the testing laboratory. A suitable reference vaccine with target ranges for six clostridial vaccines is available from the Central Veterinary Laboratory, Weybridge, Surrey, United Kingdom. For other types of vaccine, the only means of establishing that the animals in a laboratory are producing

typical results is to re-test material previously tested by a reputable laboratory, elsewhere.

Single point comparative tests. A third approach has been adopted by WHO to overcome the cost problem of six-point assays for human vaccines against diphtheria and tetanus while retaining the use of a standard vaccine (WHO). These tests are designed to provide assurance that a dose of the vaccine under test is significantly superior to a defined dose of a reference vaccine. The advantage of this method is that it offers a direct comparison of the vaccine under test with an established reference preparation but requires only one-third of the number of animals needed to determine the RP.

In the form prescribed by WHO the test offers statistical confidence that the potency of the vaccine is superior to that of the reference dose. The difficulty with this approach is that it predicates that routine batches of vaccine will be substantially better than the minimum required to protect the target species and this may not always be so. Such superiority should be sought whenever possible since definitive challenge tests in the target species are almost invariably performed shortly after vaccination is complete and make no allowance for declining immunity in the period preceding a recall vaccination.

If a comparative test is to be set up, the following procedure is recommended:

i) Establish the dose of reference vaccine (D) that is just enough to achieve minimal protection in the target species after one or two doses. If a minimum requirement has already been defined, D is the dose of reference material that exactly meets that requirement.

ii) Establish the dose of reference vaccine that, using a one- or two-dose schedule, elicits a minimal measurable response in the chosen laboratory species (d). For a challenge test the response should be approximately 10 percent protection; for an antibody response test, at least 80 percent of the animals should respond, but the titres should be low.

iii) Calculate the laboratory animal test dose (t) as the dose recommended for the target species (T): $t = T.d/D$.

iv) Inoculate groups of laboratory animals with doses d and t of the reference and test vaccines respectively. The number of animals required is discretionary; if D is much lower than T few animals will be needed; if D is only slightly greater than T very large numbers will be required. There is therefore a strong incentive to produce highly potent vaccine.

v) For both groups, follow the same vaccination and challenge or serum sampling schedule as was used for ii) above.

vi) If responses were evaluated by challenge, determine whether the response to the test vaccine (P) was significantly greater ($P < 0.5$) than those to the reference preparation by Chi-square test (Fisher's contingency tables are the most convenient way of doing this). If responses were evaluated as titres or numerical scores, they should be transformed to logarithms unless they are already in logarithmic form (end-point tube numbers from titrations involving serial dilutions and some scores are already log transformed). Using the transformed data, determine whether the responses to the test vaccine are significantly greater than those to the reference using Student's "t" test or Welch's variation if the group variances are unequal.

vii) The test vaccine is satisfactory if it

elicits a significantly greater response than the reference preparation. Alternative criteria adopted for some veterinary vaccines have included requirements that the response to the test vaccine should be simply greater or even not significantly inferior to the reference preparation. Such reduced criteria do not automatically provide assurance of adequate potency but rely on the use of minimum numbers of animals to provide confidence.

WHO does not permit the use of single-dose tests until data from six-point assays have confirmed that the assumptions of linearity and parallelism of dose-response curves, which underlie all biological tests and assays, are justified. Since evidence of linearity and parallelism is not required, even from the six-point assays prescribed for veterinary vaccines, it appears inappropriate to require it for veterinary applications of the single-point comparison described above.

Pharmacopoeial tests. The tests prescribed in the European and British pharmacopoeias and in the United States Code of Federal Regulations are summarized in Table 15.

Clostridial vaccines

Clostridial vaccines may be prepared from toxoids, whole anacultures (bacterin-toxoids) and, very occasionally, bacterins alone. With the exceptions of *Clostridium haemolyticum* and *Cl. chauvoei*, which are tested by challenge of guinea pigs with live bacteria, both the European Pharmacopoeia and the United States CFR prescribe antitoxin response tests for all the major clostridial vaccines, whether bacterial cells are included or not. The form that the tests take and the criteria of acceptance imposed by the two compendia are summarized in Table 16.

Live challenge tests for clostridia. Potency tests involving challenge of guinea pigs with live organisms are prescribed for *Cl. chauvoei* vaccines by the United States CFR

TABLE 15

Potency tests applied to inactivated bacterial vaccines

Causative organism	European and British pharmacopoeias	United States CFR	Reference used?
Clostridium novyi	Antitoxin titre	Antitoxin titre	No
Cl. perfringens A	Not included	Not included	No
Cl. perfringens B, C, D	Antitoxin titre	Antitoxin titre	No
Cl. septicum	Antitoxin titre	Not included	No
Cl. tetani	Antitoxin titre	Antibody titre	No
		or toxin challenge	Yes
Cl. haemolyticum	Not included	Challenge	No
Cl. botulinum C, D	Toxin challenge	Toxin challenge	No
Cl. chauvoei	Live challenge	Live challenge	No
Cl. sordellii	Not included	Antitoxin titre	No
Leptospira interrogans hardjo	Not included	Not specified	No
L. i. pomona	Not included	Live challenge	No
L. i. canicola	Live challenge	Live challenge	No
L. i. grippotyphosa	Not included	Live Challenge	No
L. i. icterohaemorrhagiae	Live challenge	Live challenge	No
Escherichia coli	Absolute response	Not included	Yes
Pasteurella multocida	Not included	Live challenge	Yes
Pasteurella haemolytica	Not included	Not included	No
Erysipelothrix rhusiopathiae	Live challenge	Live challenge	Yes
Brucella abortus	Live challenge	Not included	No
Mycoplasma	Not included	Not included	No

TABLE 16
Compendium requirements for clostridial vaccines

Species	European requirements			United States requirements		
	Test animal	Test type	Criterion	Test animal	Test type	Criterion
Cl. perfringens C	10 rabbits	AT resp.	10 Bu/ml	8 rabbits	AT resp.	10 Bu/ml
Cl. perfringens D	10 rabbits	AT resp.	5 Eu/ml	8 rabbits	AT resp.	2 Eu/ml
Cl. perfringens B	10 rabbits	AT resp.	10 B+5E u/ml	No United States requirement		
Cl. botulinum C, D	20 mice	Tox. chall.	> 80% live			
Cl. botulinum C	5 mink	Tox. chall.	> 80% live			
Cl. novyi B	10 rabbits	AT resp.	3.5 u/ml	8 rabbits	AT resp.	0.5 u/ml
Cl. septicum	10 rabbits	AT resp.	2.5 u/ml	No United States requirement		
Cl. sordellii	No European requirement			8 rabbits	AT resp.	1 u/ml
Cl. tetani poly	10 rabbits	AT resp.	2.5 u/ml			
Cl. tetani mono	10 guinea pigs	AT resp.	7.5 u/ml	10 guinea pigs	Ab resp. (ELISA)	2 Au/ml
Cl. tetani horse	10 guinea pigs	AT resp.	30 u/ml			
Cl. chauvoei	10 guinea pigs	Live chall.	10/10 live	8 guinea pigs	Live chall.	> 80% live
Cl. haemolyticum	No European requirement			8 guinea pigs	Live chall.	> 80% live

Notes: AT resp. = antitoxin response; Tox. chall. = toxin challenge; Live chall. = live challenge

and the European Pharmacopoeia and for *Cl. haemolyticum* vaccines by the United States CFR alone. The United States method for both involves a lower vaccine dose but permits a proportion of deaths among the vaccinates in a two-stage test in which vaccines that protect seven-eighths of the animals are accepted, those protecting less than five-eighths are rejected while intermediate results invoke a second test.

Spore preparations are available from the United States Plant and Animal Protection Service for both *Cl. chauvoei* and *Cl. haemolyticum* but their routine use is unlikely to be practicable for laboratories outside the United States. Challenge preparations can be prepared by culture of *Cl. chauvoei* in Buddle's medium containing 0.5 percent glucose until the first signs of gas appear. A satisfactory culture should contain at least 1 000 50 percent lethal doses (LD_{50}) per millilitre when injected intramuscularly in broth containing 2.5 percent calcium chloride. Should it prove impossible to achieve this level of virulence, a more virulent culture may be obtained by inoculating Buddle's medium with blood from a guinea pig at the point of death from *Cl. chauvoei* infection. If -70°C refrigeration, solid carbon dioxide (CO_2) or, better, liquid nitrogen is available, it is preferable to divide the culture into 2-ml aliquots and freeze at -70°C or lower. The lethality of the culture can then be determined before it is used. Culture stored at -70°C or in solid CO_2 will retain its full lethality for several weeks. If stored under liquid nitrogen it is stable for up to a year. It is advisable to dilute the fresh or stored culture to contain 100 LD_{50} doses in each challenge dose.

The United States type of test, by setting the final pass criterion at close to 75 percent protection, makes the outcome less susceptible to distortion by the death of a single non-responsive guinea pig. Despite this relaxation the overall stringency of the United States and European tests are very similar.

Challenge with *Cl. chauvoei* subjects guinea pigs to gangrenous lesions in the leg followed by invasion of the ventral body wall. The need for a less traumatic

alternative has been widely recognized. An alternative test, based on the measurement of antibody responses in vaccinated animals by an ELISA method (based on surface antigens) has been approved in Australia. Such tests do not reflect all of the protective antigens present in the bacteria and the supernatant components of anaculture vaccines, but they have been shown to correlate well with the performance of similarly formulated vaccines in challenge tests.

It has been found that relatively crude sonicates and sodium dodecyl sulphate (SDS) extracts of bacterial cells can be used as antigens to sensitize polyvinyl chloride (PVC) or polystyrene plates. Before an ELISA method can be accepted for any particular vaccine, it is essential that a correlation be established between the serum titre corresponding to a marginal pass and the results of the associated challenge test.

No challenge test is prescribed for *Cl. haemolyticum* in Europe but some manufacturers have adopted tests based on the anti-beta-haemolysin response of rabbits which are performed in parallel with the tests for *Cl. perfringens, Cl. novyi, Cl. septicum* and *Cl. tetani*. This permits the potency of *Cl. haemolyticum* components in multivalent vaccines to be assessed in the same group of rabbits as the other components. Beta-haemolysin is the most prominent toxin produced by *Cl. haemolyticum*. Although there is no direct proof of its importance as a protective antigen there is evidence that most of the protection provided by *Cl. haemolyticum* vaccines is derived from the supernatant, and vaccines controlled and formulated on the basis of beta-haemolysin content have proved consistently effective in the field. Beta-haemolysin toxins, toxoids and antitoxins can be titrated according to the principles discussed below for the lethal toxins of other clostridia but with the use of *in vitro* indicators such as haemolysis of sheep erythrocytes or coagulation of ovolecithin.

Antitoxin response tests for clostridia. The great majority of clostridial vaccines are tested for potency by inoculation into rabbits and subsequent measurement of their antitoxin titres. This allows the costly immune response phase of the potency test to be shared among all the components of a multivalent formulation. European and United States requirements are broadly parallel – where they exist – the main differences being that larger vaccinating doses, higher response criteria and larger numbers of animals are used in Europe than in the United States.

The vaccination doses used by both authorities are large and are far beyond the upper asymptote of the dose-response relationship for most of the components. It is therefore dangerous to rely on potency test results for the adjustment of the antigen content of vaccines. With the exception of the USDA test for tetanus, the titres of pooled rabbit sera are determined by toxin neutralization (TN) tests. In TN tests the volume of a test toxin solution required to neutralize a defined quantity (e.g. 1 unit) of standard antitoxin is first determined. This is the L+ dose. The volume of each test antiserum required to neutralize the lethal effects of an L+ dose of the same toxin is the reciprocal of the serum titre expressed in units per millilitre.

In principle, the serum titre is equal to the number of units of standard antitoxin required to neutralize the test dose of toxin, divided by the volume of test serum required to produce the same effect on the same occasion. Although the toxin neutralization tests prescribed by the European and United States requirements are described in different terms both are based on the same principles – the United States method determines only the minimum acceptable titre, while the European

method provides a quantitative estimate of titre. It is recommended that manufacturers should determine titres quantitatively so that adverse trends in potency can be recognized and remedied before the point of batch failure is reached.

Before testing an unknown serum it is necessary to select a suitable test dose of toxin. This is the smallest quantity of toxin which, when mixed with a defined dose of standard antitoxin (e.g. 1 unit or 0.1 units) produces an end-point effect when injected into an animal or exposed to an *in vitro* indicator. This quantity is described as L+ for 1 unit or L+/10 for 0.1 units, if the indicator is death.

Similarly, it is possible to define test doses for paralytic end points (Lp and Lp/10), for intradermal reactions (Lr and Lr/10) and for haemolytic end points (Lh and Lh/10). It is preferable to select an end point as close to neutrality as possible, such as 50 percent effect for an L+, an Lp or an Lr in the animal tests and 30 percent haemolysis in haemolytic tests.

Although toxin neutralization tests may be performed at either high or low concentrations it is important that the concentration of toxin in the test mixture and the dose of toxin administered to the indicator (the test dose) should be kept constant. This is because the test antisera (produced by two-dose immunization of rabbits) are far less avid than standard preparations which are prepared from hyperimmunized horses. In consequence, the apparent titres of test antisera are reduced when the concentration of reactants in the test mixture is low. In practice, the test dose needs to be large enough to contain at least ten indicating doses of toxin and small enough to be neutralized by 0.1 ml of a serum of the lowest acceptable titre, for example the minimum requirement for *Cl. septicum* in the European Pharmacopoeia is 2.5 units per millilitre. The highest possible test dose would therefore be 0.25 units equivalent (L+/4). In order to test down to 2.0 units per millilitre a level of L+/5 has been chosen which is the lowest level that can be supported by a good toxin which should contain ten lethal doses in one L+/5 dose.

Outline of test method. (The following description assumes that mixture volumes containing four test doses will be appropriate but other volumes are equally valid.) Having determined the test dose of toxin, select, on the basis of previous assays, an estimate of the titre of the test serum and calculate the volume required (v) to neutralize four doses of toxin and the volume of serum (m) that would be required if the true potency of the serum was of the lowest acceptable titre. Prepare a series of mixtures containing four times the test dose of toxin and one of a logarithmically graded series of volumes of the test antiserum, centred on n and including m at its upper end.

Prepare a parallel series of five mixtures, each containing the same amount of toxin and 120, 110, 100, 90 and 80 percent of the standard antitoxin dose against which the toxin test dose was determined. Make up the total volume of each mixture to four times the volume to be administered to the animal or other indicator system. Allow the mixtures to react for 30 to 60 minutes at 20° to 25°C before inoculating them into the animal or *in vitro* indicator. Volumes of 0.2 or 0.5 ml of mixtures containing *Cl. perfringens, Cl. septicum* and *Cl. sordellii* toxins are injected intravenously into mice, whereas mixtures containing *Cl. novyi* toxin are injected subcutaneously over the base of the tail and mixtures containing *Cl. tetani* are inoculated subcutaneously over the lumbar region of the spine.

The inoculated animals are observed for 48 or 72 hours and deaths are recorded, except for animals injected with *Cl. tetani*, which are observed for four days. An end point of minimal tetanic paralysis is used

in many countries. In mice injected as described above, the end point is manifested as failure to retract either hind leg. The test is valid if a coherent end point is obtained in the standard range but, if the end point for the standard is found to be more than 10 percent above or below the expected value, the estimate of titre for the test serum should be adjusted proportionately.

Alternative indicators. Although the standard pharmacopoeial tests for clostridial antitoxins use death or paralysis of mice as indicators of excess toxicity, many of the toxins have other biological activities that are also suitable indicator effects for toxin neutralization tests. Table 16 shows some of the alternative indicators that have been shown to be valid indicators for clostridial toxins.

The use of alternative end points retains the toxin neutralization principle and represents a minimal change from the official lethal test method. It can result in considerable savings without prejudice to the reliability of the test provided that the alternative activity is a property of the same molecular species as the lethal toxin. There is evidence that the cytopathic activity of *Cl. perfringens* C filtrates for EBTR cells is not caused by the beta toxin and this gives cause to question the identity of the dermo-necrotic moiety of *Cl. septicum* and the cytopathic moiety of *Cl. novyi* B with the corresponding alpha toxins. In all other cases there are no reasons to doubt this assumption.

Serological tests based on assays of antibodies without reference to the toxin neutralization (TN) principle have been proposed for many of the clostridia. Most of the methods proposed have used ELISA methodology, but tests based on passive haemagglutination (PHA) and single radial diffusion (SRD) have also been proposed. ELISA methods offer the best prospects of consistent correlation with the titres obtained from TN tests. PHA and SRD require less-sophisticated equipment and reagents than ELISA but PHA at least is excessively sensitive to IgM antibodies which do not generally neutralize toxins.

In at least one instance (the United States test for tetanus) an ELISA method has been adopted as definitive. The value of such tests is primarily dependent on the purity of the antigen used and, for tetanus at least, there is evidence that simple ELISAs based on highly purified antigen can produce results that are almost identical to those

TABLE 17

Non-lethal indicators of clostridial toxins

	Indicating activities	Comments
Cl. perfringens alpha	Dermo-necrotic	Not used for test
	Haemolysin	Reflects lethal test
	Phospholipase C	Reflects lethal test
Cl. perfringens beta	Dermo-necrotic	Reflects lethal test[1]
	Cytopathic	Not identical to lethal toxin
Cl. perfringens epsilon	Dermo-necrotic	Reflects lethal test[1]
	Cytopathic	Reflects lethal test[1]
Cl. novyi alpha	Dermo-necrotic	Not used for test
	Cytopathic	Reflects lethal test[2]
Cl. septicum alpha	Dermo-necrotic	Reflects lethal test[2]
	Cytopathic	Reflects lethal test

[1] Lethal and alternative activity neutralized by the same monoclonal antibody.
[2] Correlation between lethal test and alternative incomplete.

obtained by TN over the range of titres expected from potency tests.

More elaborate strategies such as toxin binding immunoassay (TOBI), developed for the accurate measurement of low titres, and ELISAs based on antigen capture by non-neutralizing monoclonal antibodies, developed to overcome the poor correlation problems between TN and serological tests for diphtheria antitoxin, offer only small gains in tetanus potency tests.

The accuracy with which the results of serological tests reflect those of TN tests for other clostridial toxins has not been critically evaluated.

Clostridial potency tests based on toxin challenge. Toxin challenge tests are performed on *Cl. botulinum* vaccines only and their use for other clostridia is not recommended. The tests prescribed by the United States and European authorities both require the animals to be challenged intraperitoneally 21 days after a single dose of vaccine but differ in certain respects. In the United States, five mink receive a full recommended dose. These, with three controls, are challenged with 10 000 LD_{50} of toxin and all controls must die while 80 percent of vaccinates survive for seven days. In Europe, 20 mice receive 0.2 ml of one in eight dilution of vaccine. These, with ten controls, are challenged with 25 paralytic doses of toxin. All controls must show specific botulinum paralysis and 80 percent of vaccinates must show no signs of botulinum paralysis for seven days.

(In some countries special permission is required before undertaking tests in which animals are subjected to intoxication with *Cl. botulinum.*)

Test reagents. Although some authorities provide standard toxin materials, it is important to recognize that the definitive standard for the test is always the standard antitoxin. Standard antitoxins may be laboratory or national preparations that have been calibrated directly or indirectly against the relevant international standard. International standards are provided by WHO through the Statens Seruminstitut, 80 Armager Boulevard, Copenhagen, Denmark, for *Cl. botulinum, Cl. novyi, Cl. septicum, Cl. perfringens alpha, Cl. sordellii* and *Cl. tetani* antitoxins. *Cl perfringens beta* and *epsilon* antitoxin standards are distributed by the WHO/FAO Laboratory for Biological Standards at the Central Veterinary Laboratory, United Kingdom.

These preparations are not intended for use as working laboratory standards but should be used to calibrate national standard preparations or, in countries where these have not been produced, laboratory working standards.

Laboratory working standards are best prepared from a large pool of sera typical of the samples intended for test. The pool is carefully calibrated against the relevant national or international standard by the exact method laid down in an authoritative compendium such as the European Pharmacopoeia, preferably with the collaboration of the national control laboratory. The use of a laboratory reference preparation of the same species as the test sera is, of course, unavoidable for ELISA tests but is also highly desirable for other serological tests and TN tests using alternative indicators, where differences in avidity between standard and test preparations can lead to misleading results.

Although neither European nor United States requirements invoke a standard vaccine, it is useful to verify that the animals in a testing laboratory are of similar sensitivity to those used when the requirements were formulated. For this purpose a reference preparation, for which "normal" responses have been established by collaborative assay, has been prepared by the Central Veterinary Laboratory.

In many cases it will be necessary for laboratories to prepare their own test toxins. Test toxins can be prepared from

production cultures by harvesting them a few hours earlier than for vaccine production. The toxic supernatant may be separated from the cells by centrifugation or filtration. (*Cl. perfringens epsilon* toxin is present as a prototoxin and the filtrate should be incubated for one hour with 2 percent pancreatic homogenate before stopping the reaction with soybean trypsin inhibitor.) The filtrate is concentrated and partially purified by precipitation with ammonium sulphate. Further purification is not recommended since it is likely to result in reduction of the crucial ratio of toxicity to L+. The resulting precipitate may be dried in a vacuum over phosphorous pentoxide and ground to a homogeneous powder from which weighed quantities can be dissolved to prepare the test toxin solution. When weighing dried toxin it is important that the whole contents of the bulk container are thoroughly mixed on each occasion since the surface layer often deteriorates. It is advisable to prepare sufficient toxin solution to last for several weeks and to stabilize it with 40 percent glycerol. The concentration of toxin should be high enough to ensure that not more than 5 mg of glycerol is injected in the test mixture.

If equipment for freeze-drying is available it is preferable to redissolve the toxin and dialyse it against buffered saline, then add an excipient such as 5 percent hydrolised gelatine to stabilize it before dispensing the resulting solution as accurately as possible into ampoules and then freeze-drying.

The minimum lethal dose, L+ and protein nitrogen content of the solution should be measured before freeze-drying and only solutions that contain a sufficient number of lethal doses per L+ dose should be dried. The number of L+ and indicating doses per container should be determined as accurately as possible before using the toxin.

The number of LD_{50} or minimum lethal dose (MLD) per L+ dose or the equivalent ratio of non-lethal indicating doses is critical to the suitability of a toxin. Each test dose (the quantity of toxin contained in the volume of toxin-antitoxin mixture injected into an animal or exposed to an *in vitro* indicator) should contain at least ten indicating doses. Suggested minimum levels for specific toxicity are included in each assay method in the British Pharmacopoeia. Limits for purity of test toxins are similarly included but these are less critical.

Leptospira vaccines

Little is known of the identity of the antigens responsible for the protective effects of *Leptospira* vaccines. There is therefore no rational basis for serological potency tests and the only methods recommended depend on challenge. All but one of the five serovars of *L. interrogans* used in vaccine production are lethal for hamsters (the single exception is *L. interrogans hardjo*, which infects but does not kill). Some serovars can also be tested in guinea pigs but, since hamsters are more robust and suitable for all serovars, it is convenient to use them in every case.

Both European and United States requirements specify inoculation of hamsters with a small fraction of the dose intended for the target species, followed at an interval of about 16 days by a challenge of between 10 and 10 000 LD_{50} of a virulent strain, which should preferably be a local isolate. The animals are observed for 14 days post challenge. European and United States requirements for four serovars are summarized in Table 18.

Leptospira cultures do not easily survive freeze-drying and most laboratories therefore maintain them in liquid culture. It is frequently necessary to passage the strain through hamsters to regain virulence before growing the challenge culture on Korthof's medium. (*L. interrogans* serovars

are dangerous to humans. All manipulations should be carried out in safety cabinets and protective clothing – gloves, goggles and masks – should be worn during inoculation of animals. Infected animals and their excreta should be decontaminated before disposal.)

The European and United States requirements differ in the vaccination dose as a proportion of the target species dose, the number of animals used and the pass criteria which make provision for a repeat test. In these respects the United States requirement is slightly more stringent but less likely to fail to produce good vaccine than the European.

No potency test is prescribed for serovar *hardjo* but a renal infection end point can be substituted for the lethal challenge in a test based on comparison with a reference vaccine. Renal infection is determined 14 days post challenge by aseptically removing hamster kidneys and crushing them in a syringe. The crushed tissue is then inoculated into Korthof's medium and incubated for 14 days before examination for viable leptospires.

The crushing of kidneys is likely to generate spurts of infected fluid. This procedure should therefore be performed in a glove box. An alternative potency test based on the elicitation of agglutinins in guinea pigs has also been proposed for *hardjo*. Although production of agglutinins has been shown to correlate with efficacy in calves it is unlikely that the IgM antibodies measured in the test are important to the protection of calves.

Escherichia coli vaccines

Vaccines against bovine, ovine and porcine colibacillosis may depend on at least three distinct protective mechanisms; somatic antigens, adhesin antigens and enterotoxin. It is unlikely that somatic antigens alone will furnish adequate protection. No requirements for *E. coli* vaccines have been issued by USDA and no international standards or reference preparations exist, but monographs covering porcine and bovine vaccines recently completed for the European Pharmacopoeia provide a suitable basis for a review of potency tests for these vaccines.

Both monographs describe a test under potency which is not intended to be routinely performed for batch release but is prescribed in order to establish that one or more lots of the vaccine are effective against each of the strains or immunotypes against which protection is claimed. A vaccine lot shown to be satisfactory in this test may then be adopted as a reference preparation in the batch potency test (see Standard and reference preparations, p. 362). In the potency test, pregnant females of the target species are vaccinated and the resistance of their progeny to oral

TABLE 18

Comparison of European and United States requirements for leptospiral vaccines

Serovar	Vaccinating dose		Number of doses		Survivors required for pass		
	US	Europe	US	Europe	US test 1	US test 1 + repeat	Europe
Icterohaemorrhagiae	1/80	1/40	14-18	15-20	> 7/10	> 14/20	> 7/10
Canicola	1/80	1/40	14-18	15-20	> 7/10	> 14/20	> 7/10
Pomona	1/800	NI	14-18	NI	> 7/10	> 14/20	NI

challenge with heterologous strains bearing each of the claimed antigen types is compared with those of unvaccinated controls. The monograph requires 15 controls and 15 piglets from vaccinated gilts or sows, or five controls and ten calves or lambs from vaccinated dams, to be challenged.

The criteria governing the outcome of the test are too complex to be summarized in detail here but they correspond to protection indices ranging from 0.55 to 0.67 for pig vaccines and 0.875 for vaccines intended for ruminants. For manufacturers operating in countries not bound by the European Pharmacopoeia a protective index of 0.7 might be taken to represent an adequate level of protection. (The protective index is calculated by subtracting the percentage mortality of vaccinated animals from that of control animals and dividing the result by the percentage mortality of the control animals.)

The prescribed batch potency test may be performed in rabbits, guinea pigs or mice. Rats can also be considered for some of the adhesin antigens. The test depends on serological determination of the response to protective antigens after one or two doses of vaccine and requires that the mean response shall not be significantly ($P < 0.05$) inferior to the response elicited by a reference vaccine of proven efficacy under the same conditions.

Good assay practice dictates that the reference vaccine is tested in parallel with the test vaccine on every occasion. If the mean titre from the test vaccine is less than the reference the difference should be tested for significance by Student's "t" test. To satisfy the European Pharmacopoeia, the mean of the log titres elicited by the test vaccine must be shown to be not significantly inferior to those elicited by the reference vaccine.

Although this criterion avoids the release of grossly inferior vaccine it offers no assurance that the vaccine is as good as the reference and a requirement that the test vaccine is significantly superior to the reference would be preferable if such a standard could be consistently maintained. A test based on serological responses in the target species is also permitted for pig vaccines but, since no acceptance criterion is included, it is difficult to see how this could be applied.

Any valid serological method may be used in the batch potency test but ELISA methods are favoured by the European Pharmacopoeia. Although methods based on bacterial agglutination or PHA have been used, the achievement of consistent results of appropriate specificity has often proved difficult. However, the use of ELISA methodology does not, in itself, guarantee specificity or reproducibility and test antigens need to be purified with great care. The use of monoclonal antibodies in competition ELISAs offers a short cut to specificity for some antigens. Monoclonal antibodies directed at many of the protective antigens are available from the Central Veterinary Laboratory, United Kingdom. Although these recognize only one of several epitopes expressed by the test antigen, this deficiency is less serious than the intrusion of accessory antigens in partially purified preparations.

Use of the batch control test prescribed by the European Pharmacopoeia is dependent on the establishment of a statistically significant correlation between results of the batch control test and the full potency test described in the previous paragraph. While it would theoretically be possible to establish a relationship between the outcomes of full and batch control tests over a range of batches of vaccine, such a study would be prohibitively expensive. It is therefore more practicable to establish a correlation between the proportion of progeny protected against challenge and the colostrum titre of their dams as

measured by the serological method developed for the batch control test. If a direct ELISA is used, a change of conjugate and laboratory reference preparation will be required before it can be applied to the target species.

Pasteurella vaccines

Vaccines against mammalian and avian strains of *Pasteurella multocida* and mammalian strains of *P. haemolytica* are considered here. No European requirements for any *Pasteurella* vaccines exist but the United States CFRs contain requirements for vaccines against both avian and mammalian strains of *P. multocida*.

The mammalian vaccine requirements are applicable to each of the immunotypes present in the vaccine. Three fivefold dilutions of the test vaccine and three identical dilutions of a standard vaccine are inoculated intraperitoneally into groups of 20 mice. The dose volume prescribed is one-twentieth of the lowest dose volume prescribed for the target species but, for laboratories not constrained by the requirements, a fixed dose of 0.5 ml may be more convenient. The standard may be provided by USDA or may be an in-house reference preparation. The initial dose is repeated after 14 days, the animals challenged intraperitoneally with 100 to 10 000 LD_{50} of a virulent culture ten to 12 days later and the proportion dying in each of the treatment groups is recorded for ten days after challenge.

The test is valid if the proportion of survivors in at least two of the groups that received standard vaccine lies between 0 and 100 percent and if the 50 percent end-point dilution for the standard lies between the highest and lowest dilution. There are no requirements for the dose response regressions to be linear or parallel. Instead the relative potency (RP) is determined as the reciprocal of the 50 percent end-point dilution of the test vaccine, divided by the equivalent figure for the reference. The method of calculation of 50 percent end-point dilution is not specified but it would be appropriate to determine the 50 percent effective dose (ED_{50}) by a standard LD_{50} calculation such as probit analysis or Reed and Muench (1938). If the protection afforded by the test preparation is so complete (>80 percent survivors at the lowest dose) that a 50 percent end point cannot be determined, while a valid end point is obtained for the reference preparation, the vaccine is satisfactory.

The acceptance criterion requires that the RP is 0.5 or more. If this criterion is not met, two further tests may be performed and a (geometric) mean value for the RP determined. There are complex provisions for the exclusion of very low initial estimates if repeat tests are satisfactory.

Although the form of the data produced in this test is suitable for full statistical evaluation by a method such as parallel line probit analysis, the actual data may not satisfy the strict validity requirements of such assays very often and the less rigorous approach is therefore justified. Although simplified, single-dose level tests have never been used for *Pasteurella* vaccines, it is probable that their use could offer assurance of potency with smaller numbers of animals than the prescribed six-point assay.

Most strains of *P. haemolytica* are far less virulent for mice than *P. multocida* but the virulence of most strains of *P. haemolytica* can be enhanced sufficiently to allow a challenge of 100 LD_{50} by inclusion of either 5 percent yeast or 5 percent hog gastric mucin in the challenge suspension. The choice of enhancing agent can only be determined by trial and error – some strains respond to yeast and others to mucin, in an apparently random fashion, and a few strains respond to neither.

No requirements for killed *P. haemolytica* vaccines exist in either the United States

CFR or the European Pharmacopoeia, but the USDA test for *P. multocida* bacterins could be applied to *P. haemolytica* with the use of virulence enhancement. However, the relevance of the mouse test to protection is questionable, particularly for the pneumonic forms and, in view of the large number of strains included in many vaccines, it may be more realistic to accept a serological test of consistency without any particular claims to the measurement of efficacy.

Serological tests currently being developed by some manufacturers to detect antibodies directed against leukotoxins and other virulence factors offer a prospect of replacing the challenge tests with serological alternatives that are in fact relevant to protection, in the near future.

The United States CFR includes requirements for avian vaccines prepared from strains of *P. multocida* types 1, 3 and 4. In each case 21 birds of the target species are vaccinated with the recommended dose and revaccinated three weeks later. After 14 days of observation, 20 of the vaccinated birds and ten unvaccinated controls are challenged with a virulent culture of an appropriate specified strain: X-73 for type 1, P-105 for type 3 and P-1662 for type 4. The numbers of birds dying during the 14 days following challenge are recorded. At least eight of the ten control birds must die for a test to be valid. If six or fewer of the 20 vaccinates die, the vaccine passes. If nine or more die the vaccine fails. If seven, eight or nine die the test is repeated and if the total number of vaccinates dying in both tests is 15 or fewer out of 40, the vaccine passes.

Erysipelas vaccines

Both United States and European requirements include potency tests in which the responses of three groups of mice receiving graded doses of the vaccine under test are compared with those of mice receiving three similar doses of a standard preparation. However, the European test has several advantages. The standard used is the WHO International Standard for swine erysipelas vaccine or a laboratory preparation that has been calibrated against it. This allows vaccines to be standardized on an international basis. The United States test is similar in structure to that for mammalian strains of *P. multocida* described above and does not yield a truly quantitative estimate of potency. The European test, from similar numbers of animals but using statistical methods such as parallel line probit analysis, can provide relatively precise estimates of potency.

Three groups of 16 mice are vaccinated subcutaneously with 0.5 ml of three graded dilutions of the test vaccine on a fivefold range (dilutions of 1/5, 1/25 and 1/125 are suggested). Similarly, three further groups of mice, which have been randomly allocated from the same population, are vaccinated with three similarly graded doses of the standard preparation (0.2, 1 and 5 units are suggested). Ten mice from the same population are retained as unvaccinated controls. After 21 days all the mice are challenged intraperitoneally with enough of a virulent culture to kill the unvaccinated controls in three days. The proportion of mice surviving challenge by eight days in each treatment group is counted and the proportions used to calculate the potency of the test vaccine relative to the standard by a suitable statistical method such as parallel line probit analysis. No formal requirements of parallelism or linearity are required, but normal statistical requirements of validity are usually easy to satisfy. The vaccine passes if the estimate of potency exceeds 50 units per dose for swine.

Although the above test is statistically more satisfactory than many potency assays there is some evidence that it does not reflect the ability of vaccines to protect

swine against percutaneous challenge very accurately. The United States version of the test is very similar in principle and therefore unlikely to be more reflective of efficacy in swine.

Brucella vaccines

No requirements for inactivated *Brucella abortus* vaccines are included in the United States CFR, but a monograph in the British Pharmacopoeia specifies a challenge potency test in guinea pigs.

Inject 12 or more guinea pigs intramuscularly with one-tenth of the cattle dose. (If the vaccine is formulated as a water-in-oil emulsion do not attempt to dilute it.) Set aside six or more unvaccinated controls from the same population. After 40 days, inoculate the vaccinated guinea pigs and six unvaccinated controls intramuscularly with a suspension containing 5 000 organisms of a suitable CO_2-dependent strain of *B. abortus* (strain 544 grown in trypticase soy broth containing 5 percent equine serum may be suitable). The virulence of the suspension should be sufficient to produce a 50 percent infective dose (ID_{50}) in guinea pigs with fewer than 100 organisms. Thirty-five days after challenge weigh and kill the vaccinated and control guinea pigs and aseptically remove the spleen from each animal. Weigh each spleen and homogenize the tissue in casamino acids solution. Inoculate a volume of homogenized suspension corresponding to 0.05 g of spleen into a suitable medium and incubate at 37°C for four days.

The vaccine is satisfactory if the following criteria are satisfied:

- viable *B. abortus* organisms of the challenge strain are present in all the spleen suspensions from control animals but in not more than 25 percent of those from vaccinates;
- The mean weight of spleens in the vaccinated animals expressed as a percentage of total body weight is not more than 0.3 percent;
- the geometric mean serum agglutination titre of the vaccinated animals 35 days after challenge is not more than 600.

Care should be taken to avoid accidental self-inoculation with oil-emulsion vaccines. The process of extraction and homogenization of spleens infected with the virulent challenge culture is potentially hazardous and these manipulations should be performed in safety cabinets with barriers to contain sudden spurts of infectious material, as well as the aerosols generated during homogenization.

Although the above test is lengthy and costly to perform, it is the best available index of potency for a vaccine against which serological responses are notoriously unreliable.

Parallel test methods may be applied to other inactivated *Brucella* vaccines.

LIVE VACCINES

A minority of bacterial vaccines consist of suspensions of live organisms whose virulence has been reduced to permit their safe administration to the target species.

Routine potency tests in laboratory animals are not generally appropriate for such preparations. Potency is better guaranteed by the careful evaluation of the protective efficacy of vaccine prepared from a master seed strain in the target species and careful control of the numbers of bacteria in serials produced from it. The United States CFR uses a set formula in the majority of its requirements for the control of potency of live bacterial vaccines. This entails the adoption of a strict seed lot system in which no more than five subcultures are permitted between the final product and the master seed lot. This is supported by the performance of a challenge test in the target species every three years to show that a pilot batch, containing

a carefully determined number of organisms derived from the master seed, is able to protect the target species.

If the number of organisms in the pilot batch is insufficient to attain the required level of protection a further pilot batch containing more organisms may be prepared and tested. If a master seed continues in use for more than three years, new pilot batches must be prepared and tested at three-year intervals. Further batches prepared from the same master seed can then be released on evidence that the viable count is high enough to ensure that twice the number of organisms shown to be protective in the original potency test will be present until the expiry date.

Bacterial counts on the pilot batch should be performed in five replicates using dilutions that ensure that between 30 and 300 colonies appear on each plate. Counts for the release of serials should be performed in duplicate. The test methods prescribed for five vaccine classes in the United States CFR are summarized in Table 19.

Some living bacterial vaccines, however, do not multiply in the host but consist rather of a mass of preformed antigens, which may incorporate adjuvants and are more similar to killed preparations.

Live *Pasteurella* vaccines

There are no European or British requirements for live *Pasteurella* vaccines. The tests in the United States CFR are based on the formula described above. The main test parameters and criteria of acceptance for the three vaccine classes specified in the requirements are summarized in Table 18. In contrast to tests on killed vaccines, vaccinates and controls must be housed separately.

Pilot batches prepared from new seed lots of *P. multocida* for bovines are used to vaccinate calves that are challenged by a

Table 19

Potency tests for live bacterial vaccines in the United States

	Pasteurella multocida		*P. haemolytica*	Anthrax	Erysipelas
	Bovine	Avian	Bovine		
Animals used					
Vaccinated	10 calves	20 birds	10 calves	30 guinea pigs	20 swine
Control	5 calves	10 birds	5 calves	12 guinea pigs	10 swine
Days to challenge	14 to 21	14 or more	14 to 21	14 to 15	14 to 21
Challenge strain	Virulent pneumonic	Virulent *P. multocida*	Virulent pneumonic	Virulent for guinea pig	Virulent strain
Route of challenge	Respiratory	Intramuscular	Respiratory	Subcutaneous	Not stated
Evaluation	Symptom scores 4 to 10 days post challenge	Survival for 14 days post challenge	Symptom scores 4 to 7 days post challenge	Survival for 10 days post challenge	Symptoms and pyrexia over 7 days post challenge
Acceptance criteria	Vaccinate score significantly better than control ($P < 0.05$)	> 7 controls die, > 13 vaccinates survive	Vaccinate score significantly better than control	> 9 controls die, > 26 vaccinates survive	> 79% controls affected > 89% vaccinates clear

respiratory route, such as aerosol or tracheal instillation, with a virulent culture preferably of a local isolate of *P. multocida*. Resistance to challenge is evaluated by a numerical scoring system. The score should take account of the extent and severity of lung lesions and whether the animal dies. It may also take account of post challenge symptoms, but the importance assigned to these observations should be much less than that assigned to mortality and lung pathology. Surviving calves are killed painlessly after a fixed interval within the range of four to ten days post challenge. The extent and severity of lung lesions are assessed in these animals and in any animals that may have died and a numeric score is assigned to each animal. The mean scores for the vaccinate and control groups should be compared statistically using Student's "t" test. For the pilot vaccine to satisfy the requirements of the test, the mean score for the controls must be significantly greater than for the vaccinates.

New seed lots of *P. haemolytica* type 1 for bovines are tested by a similar procedure. The interval from vaccination to challenge by the respiratory route with a pneumonic strain of *P. haemolytica* is the same but the symptoms among the control animals are not expected to include pyrexia and surviving calves should be killed earlier – between four and seven days after challenge.

Live *P. multocida* vaccines for birds are tested by similar methods. If more than one species of bird is indicated on the label, the efficacy of a pilot batch prepared from each new master seed lot must be demonstrated in each of them. The potency test conforms to the formula described above and the main test characteristics and criteria are summarized in Table 19. The challenge culture should be of a virulent avian strain, preferably of local origin. If the pilot batch fails to meet this requirement, another batch containing more bacteria may be prepared and tested for potency (and safety).

Bacillus anthracis vaccine

Potency tests for living anthrax vaccines based on challenge are included in both United States and European requirements. However, the United States CFR relies on a spore count for the release of final vaccine and only requires a potency test when new master seed lots are introduced, whereas the European Pharmacopoeia requires a potency test on every lot of final vaccine. The difference may perhaps be explained by the inclusion of adjuvants in some European vaccines. It follows that the United States test is suitable for the control of monovalent vaccines that contain no adjuvant whereas the European method is more suitable for vaccines containing other antigens, adjuvants or other components capable of modifying the immune response elicited by a given number of spores.

The United States CFR requires that new master seed lots are tested for potency by a method based on the formula that has already been discussed. The test differs from other formula-based tests in that large guinea pigs each weighing 400 to 500 g are used instead of animals of the target species. Since the test is carried out in this species the challenge strain does not have to be virulent for sheep. The Pasteur vaccine strain is therefore recommended. Although the requirement specifies that the challenge dose should contain 4 500 LD_{50} doses, it allows two out of 12 survivors among the controls and is therefore less severe than the European requirement.

Although the number of spores in each batch of vaccine prepared from the master seed is determined from the number present in the pilot batch there is an overriding requirement that the number of spores should not in any case be less than 2 million per dose. The number of

spores is verified by direct count on every batch of final vaccine.

The European requirements make provision for vaccine strains of three levels of virulence. Mild strains, such as the Sterne strain, which are harmless to guinea pigs and mice are tested for potency in guinea pigs. Intermediate strains which are lethal for guinea pigs but harmless for rabbits are tested in rabbits and less attenuated strains which kill a proportion of rabbits must be tested in the target species. The following method relates to vaccines prepared from the Sterne strain, but the numbers prescribed are the same for each test species. The potency test is performed on every lot of final bulk vaccine or on each filling lot.

Ten guinea pigs are inoculated subcutaneously with the minimum dose prescribed on the label for sheep and observed for 21 days. If more than two of the guinea pigs die from non-specific causes, the test is repeated. After 21 days, the vaccinates are challenged with 100 LD_{50} of a strain of *Bacillus anthracis* that is virulent for the test species used and three unvaccinated controls are challenged with 10 LD_{50} of the same preparation. For guinea pigs the Pasteur vaccine strain is sufficiently virulent and less hazardous to operators and the environment than fully virulent strains.

The test is valid if all the control animals die within ten days. The vaccine passes the test if all of the vaccinates survive for ten days. If one vaccinate dies the test may be repeated but all the vaccinates must survive in the repeat test.

A bacterial count is performed on every batch to confirm that the count stated on the label is valid.

Live swine erysipelas vaccine

Potency requirements for live swine erysipelas vaccines are included in the United States CFR. The outline of the requirements conforms to the formula already applied to anthrax and live *Pasteurella* vaccines.

Potency test variables are summarized in Table 19. Since body temperature is an important index of protection, the body temperature of the vaccinates and of ten unvaccinated controls are measured daily for 14 to 21 days to establish a baseline. All 30 swine are then challenged with a virulent culture of *Erysipelothrix rhusiopathiae*. A suitable culture is available from the United States Animal and Plant Inspection Service. The animals are observed for seven days post challenge during which time at least 80 percent of the controls must register a temperature greater than 105.6°F (40.9°C) on two consecutive days and also show typical signs of infection such as hyperaemia of ears and abdomen, moribundness, skin lesions, depression, arthritis or death, in a valid test. If at least 90 percent of the vaccinates remain free of the above signs, with body temperatures remaining below 104.6°F (40.2°C), the master seed lot is satisfactory.

Live *Brucella abortus* (strain 19) vaccine

No test of efficacy is required for strain 19 vaccine released in the United States where potency is assured by viable count determinations alone with minimum requirements of 3×10^9 and 3×10^{10} live organisms per dose for reduced and standard dose formulations, respectively. These are supported by requirements that no more than 5 percent of the colonies obtained when the vaccine is plated on potato agar should be frankly rough and no more than 15 percent, in total, deviant from typical smooth colony morphology.

It is suggested that any laboratory producing strain 19 vaccine outside the United States should verify the efficacy of its product by the European Pharmacopoeia method described below whenever a new master seed is adopted and in

any case at intervals of not less than three years.

The requirements for strain 19 vaccine in the European Pharmacopoeia include a potency test in which 12 guinea pigs are vaccinated intramuscularly with one-fifteenth of the calf dose of the test vaccine in 1 ml. After an interval of not less than 40 days, the vaccinates, together with six unvaccinated controls, are challenged with a virulent CO_2-dependent strain of *B. abortus*, such as strain 544. (1 LD_{50} of the challenge suspension should contain no more than 100 organisms.) After a further 35 days, the animals are weighed and killed. The spleen of each animal is weighed and then emulsified and a volume of suspension equivalent to 0.05 g of spleen is inoculated into a suitable medium such as trypticase-soy, containing 2 to 5 percent equine serum. The inoculated medium is incubated for four days at 37°C. The test is valid if the spleens of all the control animals are infected. The potency of the vaccine is satisfactory if 75 percent or more of the spleens from the vaccinated animals remain uninfected.

This potency test is supported by a viable count requirement of 4×10^{10} viable organisms per calf dose. The European requirement is thus far more stringent than the United States one in this case.

Live *Salmonella dublin* and *Salmonella cholerae suis* vaccines

Monographs for live vaccines against both *Salmonella dublin* and *S. cholerae suis* in the British Pharmacopoeia do not include any potency test and rely entirely on viable count. These monographs have been written for a single specific strain in each case and the required viable count of 2.5×10^9 organisms per dose would not necessarily be appropriate for other strains. Laboratories preparing vaccines from other strains would be advised to adopt the formula used by the United States CFR and maintain a strict seed lot system supported by efficacy tests on pilot batches produced from each new master seed to validate release on the basis of bacterial count alone.

Other live bacterial vaccines

It is recommended that the formula applied by the United States CFR should be adopted as a guiding principle in the evaluation of potency in live vaccines for which no compendial requirement exists.

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Potency control of modified live viral vaccines

C. Terpstra[1]

Since Pasteur's pioneering work on the attenuation of rabies virus by the serial passaging of "street" virus in rabbit brains and use of the "fixed" virus in animals and humans (1885), modified live viral (MLV) vaccines have been developed against a vast number of viral diseases of humans and animals.

The development of most viral vaccines, however, did not begin until the introduction and large-scale use of tissue culture techniques at the end of the 1940s when antibiotics became available. Until recently, MLV vaccines were obtained in much the same way as in Pasteur's time, i.e. by multiple passages in cell cultures, embryonated eggs or in another animal species to which the virus is not naturally adapted. After a certain number of passages the virus is tested for vaccine efficacy and safety. Using this rather empirical approach, a number of highly efficacious vaccines have been developed. For example the cell culture-adapted Kabete O strain against rinderpest received over 90 passages in bovine kidney cells (Plowright and Ferris, 1962) and the so-called Chinese strain (C strain) of swine fever virus over 800 passages in rabbits (Lin and Lee, 1981). Both vaccines are highly immunogenic and safe. A single vaccination with the Plowright vaccine protects for over ten years against a lethal challenge with virulent rinderpest virus (Plowright, 1984) and pigs vaccinated once with the C strain were fully protected against challenge with the virulent Brescia strain of swine fever virus five to six years later (Terpstra, unpublished results).

Attenuation usually reflects the selection of certain variants already present in a heterogenous population of wild-type virus, a single-step mutation or a multistep mutation. This process of alteration can hardly be influenced and its results are largely unpredictable. A more accelerated approach, although with an equally unpredictable outcome, is physical or chemical mutagenesis. Examples of the first category are the temperature-sensitive mutants used in some vaccines against virus-associated respiratory disease. A promising example of chemical mutagenesis is the serially mutagenized MV P12 variant of Rift Valley fever virus (Morill *et al.*, 1987; Hubbard, Baskerville and Stephenson, 1991).

Conventional vaccines are to a certain extent developed by "trial and error" procedures, although modern biotechnology has created tools for the well-defined engineering of vaccines. The design of such vaccines, however, requires knowledge of the nature of immunogens, the mechanisms underlying virulence and immunity and the mapping of non-essential virulence genes. Examples of MLV "biotech" vaccines are deletion mutant vaccines and vector vaccines. The latter category uses vaccinia or other poxviruses, herpesviruses and perhaps in the future also adeno- or other viruses as carriers and expression

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systems of genes from heterologous microorganisms that code for immunizing proteins.

Without the systematic use of vaccines against, for example, foot-and-mouth disease (FMD), rinderpest and swine fever it would have been impossible to control these diseases, let alone eradicate some of them from certain regions of the world. Indeed, vaccination has become one of the most cost-effective forms of veterinary health care.

Vaccines, whether developed conventionally or constructed by biotechnological means, must be evaluated for efficacy and safety before being released to the field.

The production of MLV vaccines should be based on a seed lot system. A stock of master seed virus is produced and all batches of vaccine are prepared from this within a limited number of passages. The master seed virus is subjected to extensive testing to ensure that it is immunogenic, sterile and free from extraneous viruses, as well as from pathogenic and, where applicable, oncogenic effects. A seed lot system offers the advantage that efficacy tests and, if necessary, safety tests on each batch of vaccine can be limited. In general, the minimum requirements for safety seem to be better defined and more severe than those for efficacy. The primary and ultimate goal of a vaccine, however, is to be efficacious.

This chapter describes various aspects of efficacy, *in vivo* and *in vitro* assays for determining the potency of vaccines and criteria for assessing the endurance of immunity and the value of field trials.

ASPECTS OF EFFICACY

Vaccine efficacy can be defined as the degree to which a vaccine induces a protective immunity in the target host. In most viral diseases a natural infection induces a strong and long-lasting protective immunity against reinfection and, particularly, stimulates immunological memory. Ideally an MLV vaccine simulates the natural infection without causing disease. However, there is no such thing as a perfect vaccine. A vaccine that is completely safe cannot usually induce as strong an immunity as the natural infection.

In descending order of value, the following levels of protection can be distinguished in vaccinated animals:

i) prevention of the initiation of a primary infection;
ii) reduction of virus replication at the site of entry;
iii) prevention or reduction of virus spread to other critical tissues such as blood;
iv) prevention of the virus shedding into the environment;
v) prevention or reduction of transmission of the virus to contact animals;
vi) prevention of the development of clinical disease;
vii) prevention or reduction of economic losses.

In addition: the offspring of vaccinated animals should be protected against intrauterine or post-natal infection; and immunity after one vaccination should be long-lasting, preferably throughout the economic life span of the animal.

Many vaccines offer clinical protection against disease (level vi) above) but do not (completely) prevent virus multiplication and shedding on exposure of the animal. The protection level a vaccine must primarily provide differs according to the disease and also depends on the aim of vaccination and whether an eradication policy is to be pursued or not.

The following examples illustrate these points:

- Because porcine parvovirus is only harmful to foetuses before the seventieth day of gestation, an efficacious

vaccine must prevent the transmission of parvovirus from the sow to foetuses during that period.

- Persistent infections with bovine viral diarrhoea (BVD) virus, owing to transplacental transmission, play a key role in the pathogenesis, clinical manifestation and epizootiology of BVD. Thus, an efficacious BVD vaccine must primarily prevent viraemia and, in consequence, must ensure that no congenitally BVD-infected calves are born.
- Preventing viraemia after exposure to field virus or decreasing its level below the threshold required to infect the invertebrate host must also be the primary protective requirements of all vaccines against vector transmitted diseases such as Rift Valley fever, African horse sickness and bluetongue.
- Vaccination of pigs against Aujeszky's disease (AD) has been practised widely for decades. At first, the aim was to produce high antibody titres in sows because piglets, which are the most susceptible group, had to be protected; later when clinical AD occurred in finishing herds the prime goal was to protect the fattening pig from developing severe growth retardation when infected with field virus. For these purposes, vaccines offering protection level vii) sufficed. The change of policy in some countries to a combined vaccination and eradication programme for AD not only required the use of serological "marker" vaccines but also the selection of vaccines that prevent the establishment of latency after infection (Kimman, 1992) and reduce the average number of secondary cases per infectious individual (i.e. the basic reproduction ratio R_0) below unity (De Jong and Kimman, 1994).

In general, vaccination campaigns launched to eradicate a disease, whether in combination with serological methods or otherwise, must utilize vaccines that are able to break the animal-to-animal transmission of field virus. Vaccination must, therefore, prevent or at least reduce virus shedding to the extent that the transmission of virus to contacts is impeded (levels iv) and v)). Examples of vaccines that have proved to be effective in arresting the transmission of field virus after exposure of the vaccinated host are the cell culture-attenuated Kabete O strain of rinderpest virus (Plowright, 1984) and the Chinese strain of swine fever virus (Terpstra and Wensvoort, 1988). Annual vaccination of cattle in the JP15 Rinderpest Campaign with the Plowright vaccine strain was successful in eradicating the disease in most East and West African countries (Lépissier and MacFarlane, 1966). In the Netherlands, classical swine fever has been eradicated from enzootic areas (where there is intensive pig farming) by systematic vaccination with C strain virus in combination with a "stamping out" policy (Terpstra, 1991).

BIOASSAYS TO ASSESS POTENCY

The potency of MLV vaccines can be determined *in vivo*, either in the target host or in a suitable laboratory animal model, or *in vitro*, the latter two options being derived parameters. Determination of potency *in vivo* may be based on assaying the 50 percent protective dose (PD_{50}), a titration of the virus or the serological response induced in either the target species or a laboratory animal model. To reduce the use of laboratory animals, efforts should be made to replace *in vivo* titration of vaccine virus – usually by intracerebral inoculation of baby mice – by titration in a suitable cell culture system.

PD_{50} assay on master seed virus

Production of MLV vaccines should be based on a seed lot system and, as regards

efficacy, the relation between virus content of the vaccine dose and the resulting protection in the target species should be determined at least once in the history of a master seed virus. The dose-response relationship may be determined on a sample of the master seed or on a representative derivative.

For a quantitative dose-response analysis, groups of target animals are given graded doses of vaccine virus and challenged thereafter with a standard dose of virulent virus, preferably administered via a natural route. The virus dosages used for vaccination should be calculated on the basis of the geometric mean titre of at least five replicate virus titrations in a suitable cell culture system or in embryonated eggs.

To obtain meaningful results, 100 percent of the unvaccinated controls should respond to the challenge. For this reason it is common practice to use an overdose of challenge virus, usually 10^3 to 10^5 50 percent infective doses (ID_{50}). The aim of the dose-response analysis is to estimate the median protective dose (PD_{50}), the 95 percent protective dose (PD_{95}) or other aspects of the dose-response curve. The PD_{50} is the vaccine virus dose that protects 50 percent of the vaccinated animals. Using an infinite number of doses (and therefore animals), the dose-response relationship would follow approximately a symmetrical sigmoid curve. This empirical fact often leads to the so-called logistic regression or probit analysis as a satisfactory statistical method for the estimation of the PD_{50} value. For ethical as well as cost reasons, the number of target animals available for a PD_{50} experiment will be limited. It is important therefore to design the experiment in such a way that an optimal estimate of the dose-response curve is obtained.

The following aspects should be considered in designing a dose-response experiment:

- At least two doses should result in a partial response (i.e. neither 0 percent or 100 percent of the animals receiving these doses should respond to the challenge) otherwise an invalid estimate of the dose-response curve is obtained.
- Doses providing less than 50 percent and more than 50 percent protection are essential for estimating an accurate PD_{50}.
- If the requirement is to estimate the PD_{95} – the dose providing 95 percent protection which is to be aimed at in the field – care should be taken to assure that some groups receive doses high enough to ensure complete protection, otherwise the curve has to be extrapolated in the upper region, which on the basis of a sigmoid curve leads to large statistical uncertainties.
- Doses providing 0 percent protection are less critical and not strictly necessary.
- Care should be taken that the majority of the responses are not located in the extreme lower or extreme upper part of the curve as this would not be an efficient way of using the available animals.

The natural quantitative criteria to be satisfied are:

- the mean squared error of the PD_{95} should be minimal;
- the mean squared error of the PD_{50} should be minimal;
- the number of times that an unreliable estimate is found (i.e. all doses used in the experiment are smaller than that of the estimated PD_{95}) should be minimal.

The dilution range to be covered may be assessed in a pilot experiment using tenfold dilutions and one or two animals per group. The optimal design of the ultimate experiment depends on a priori knowledge of the curve and on the specific quantitative criteria aimed for. Computer simulations can be carried out, using a varying number

of doses and varying numbers of animals per dose to see how well the different designs perform with regard to the quantitative criteria mentioned above. The simulations are based on the parameters of the curve as estimated in the pilot experiment. In general, it is advised to use five or six different doses and 3.2-fold (0.5 log) dilutions as this provides a compromise among different objectives and makes it possible to test the model. A useful review of the literature on the subject can be found in Chapter 8 of Morgan (1992). The best-fitting curve of a dose-response experiment can be determined by computer, using a probit or, easier to use, logit analysis. If a logistic regression model is chosen, the dose providing "100p" percent effective protection (PD_{100p}) can be calculated according to the following formula:

$$PD_{100p} = e^{a - \frac{\left[\ln\left(\frac{1}{p} - 1\right)\right]}{b}}$$

in which "a" stands for the natural logarithm of the dilution giving 50 percent protection, "b" for the slope of the curve and "p" for the protected fraction.

Parameter "a" (PD_{50}) determines the place of the curve, whereas parameter "b" determines its shape. The higher the value of "a" the higher the PD_{50} and the less potent the vaccine, while the higher the value of "b" the steeper the curve. Figure 18 illustrates a dose response function with "a" = -3.117 and "b" = 1.511. Standard deviations of "a" and "b" can also be calculated from one dose-response curve.

The above formula allows the calculation of the dose for each protection level wanted. For example the dose providing 95 percent protection in the PD_{50} experiment represented by the curve in Figure 18 equals:

$$e^{-3.117 - \frac{\left[\ln\left(\frac{1}{0.95} - 1\right)\right]}{1.511}} \qquad e^{-3.1}$$

$$= e^{-3.117 + 1.9486} = e^{-1.168} = 0.31 \qquad e^{-3.1}$$

In this example one PD_{50} ($e^{-3.117}$) equals 0.044.

The unit to be used in expressing the PD_{50} value will depend on the most reproducible titration system available for a particular pathogen, for example plaque-forming units, tissue culture infective units and egg infective units.

In principle, a dose-response curve can be determined for any of the vaccine efficacy levels i) to vii) discussed on p. 382. For reasons of easy and quantifiable measurement, physical signs of disease such as fever, viraemia or viral excretion are the parameters chosen most often in allocating individual animals to "protected" or "susceptible" categories after challenge.

The PD_{50} and the relationship between protection and vaccine virus dose obtained in a dose-response analysis are only valid for the conditions applied in that particular test. The outcome therefore may not be extrapolated to another master seed virus, another animal breed with a different susceptibility for the pathogen, a different target species or a different vaccination or challenge route. A dose response analysis is performed only occasionally, for example when the manufacturer changes to another virus strain or when a new master seed has been produced.

The protection observed in a properly performed vaccination challenge experiment in the target species should be regarded as the sole primary parameter of immunity. Any other parameter having an established correlation with the primary parameter and used to measure immunity should be regarded as secondary. Examples of secondary parameters are: protection obtained in non-target (laboratory) animals, *in vitro* infectivity titre of vaccine virus and level of antibody provoked in the target species.

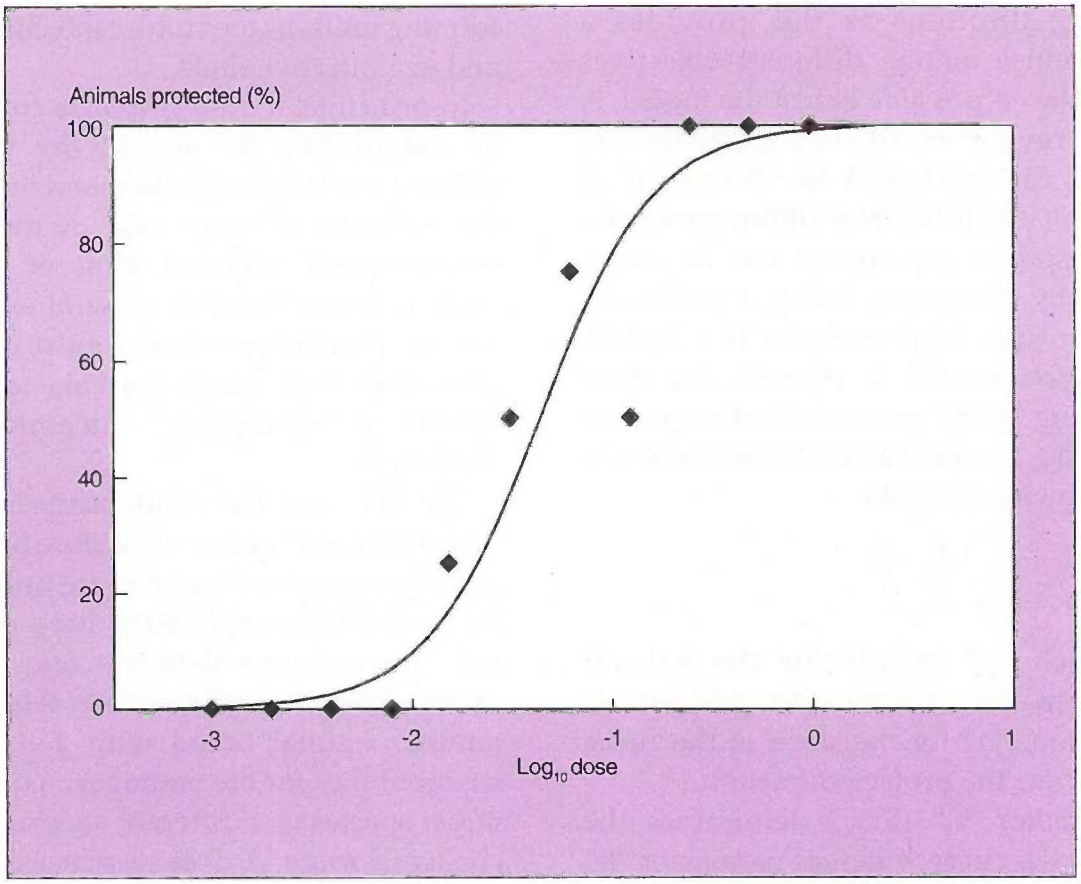

FIGURE 18
Dose-response relation using 11 groups of four animals (a = 3.117 and b = 1.511)

Calculated versus observed PD_{50}. By definition 1 PD_{50} protects 50 percent of the animals vaccinated. It follows that a vaccine containing a calculated 100 PD_{50} per dose protects 50 percent ($p = 0.5$) of "n" animals when used at a dilution of 1:100 ("n" = large number). When testing a random sample of, say, ten animals vaccinated with a 1:100 dilution, the probabilities of finding nought, one, two, etc. up to ten animals protected are binomially distributed (Table 20).

The probability of each outcome "z" can be calculated according to the formula:

$$\text{probability} = \binom{n}{z} \times p^z \times (1-p)^{n-z}$$

in which: "n" = sample size (10 in the example) and "p" = protected fraction (0.5 in the example).

The probability that fewer than five animals are protected is the sum of the probabilities of finding nought, one, two, three and four protected animals, which equals 0.37695 (Table 20). The probability, therefore, that the batch fails to pass the test is 37.7 percent, despite the fact that one dose contains 1 PD_{50} in the dilution tested. A vaccine producer therefore has to incorporate more than the calculated 100 PD_{50} per dose to increase the probability that the PD_{50} as observed by the controlling authority meets the requirements.

In order to obtain, say, 90 percent confidence that the batch will pass the potency test the sum of probabilities to find fewer than four animals protected should be ≤0.1. By using the above formula in a spreadsheet model, a value of 0.1 for this sum of probabilities is obtained when "p" = 0.6458. This means that the manufacturer has to safeguard a protected fraction of 0.65 per dose instead of 0.5. The dose providing 65 percent protection depends on the shape of the dose-response curve and can be calculated with the logit formula. In the example of the dose-response curve shown in Figure 18, the PD_{65} = 0.0667, which is 1.52 times the 0.044 required for 1 PD_{50}. The producer should therefore incorporate at least 152 PD_{50} per dose in order to be 90 percent certain that the vaccine batch will pass the potency test requirements.

Potency tests on vaccine batches

Unlike inactivated vaccines, which require an *in vivo* potency test on each batch, the PD_{50} of an MLV vaccine is assessed only

TABLE 20

Calculation of the probability distribution of the number of protected animals in a random sample of ten out of a population vaccinated with 1 PD_{50} per animal

Number protected in random sample (z)	Possibilities	Probability
0	1	0.000977
1	10	0.009766
2	45	0.043945
3	120	0.117188
4	210	0.205078
5	252	0.246094
6	210	0.205078
7	120	0.117188
8	45	0.043945
9	10	0.009766
10	1	0.000977
Σ	**1 024**	**1.000002**

once on the master seed virus or a representative vaccine batch of the virus seed. The potency of subsequent batches prepared from the master seed can be based on the relation between virus dose and protection as observed in the dose-response curve. A titration of the vaccine virus, therefore, according to the method used in determining the dosage titre in the dose-response analysis, enables the calculation of the required infectivity per dose, thus ensuring a certain degree of protection.

Subject to the regulations of the appropriate national authority, a limited *in vivo* test may be required for each batch or may be omitted as a routine control if the final product, after reconstitution, contains per dose not less than the quantity of virus that conferred an accepted degree of protection in a potency test on the master seed. Whatever the method, a general rule is that a protection test is required for each vaccination route recommended by the manufacturer and for each strain incorporated in the vaccine when different virus strains are combined.

Tests in the target species

A commonly used procedure for estimating the potency of a vaccine batch *in vivo* involves two groups of target animals, one being vaccinated and the other serving as a control. After a certain immunization period the animals of both groups are challenged with a highly virulent virus strain by an appropriate route.

The degree of protection of the vaccinated animals is measured by comparing their reactions with those of the controls. The number of animals used, their age, the interval between vaccination and challenge, the challenge dose and the route differ from vaccine to vaccine, and so do the criteria for an acceptable degree of protection such as signs of disease, height and duration of virus excretion and growth retardation. Table 21 lists some specifications from the published monographs of the European Pharmacopoeia for potency tests of batches of MLV vaccines for farm animals.

An alternative procedure for estimating potency in the target species is based on determing the antibody titre in the serum of vaccinated animals. The response of usually neutralizing or haemagglutination-inhibiting antibody is measured according to a standard method, using positive and negative reference preparations as controls. The reliability of serological methods depends on an established correlation between humoral antibody and protection. It goes without saying that the procedure is less satisfactory as the vaccine-induced protection is more dependent on mucosal or cellular immunity.

Tests in laboratory animals

Whereas potency testing in laboratory animals is common practice in human vaccine production, it is the exception in veterinary vaccines. The use of laboratory animals, however, may be indicated when the costs of testing in the target species are prohibitive, when non-immune target animals are difficult to obtain or when alternative methods for quantifying the vaccine virus are lacking. Examples of each category are equine vaccines, vaccines against arthropod-transmitted diseases, which may be widespread in an animal population, and the C strain of swine fever virus.

Potency estimations of vaccines against vector-borne diseases may be made in mice and potency estimations of the C strain by titrating the vaccine virus in rabbits. For example 1 PD_{50} of the C strain virus for pigs corresponds to approximately 4 ID_{50} for rabbits (Bironi and Leunen, 1988). The potency of rabies vaccines for domesticated animals and wildlife is also estimated in mice, which are analogous to human rabies

TABLE 21

Specifications from the European Pharmacopoeia for potency tests of MLV vaccines for farm animals

Specifications	IBR fowlpox	AD	CSF	AIB	Vaccine against AIE		IBD	NCD	MD
E.P. monograph No.	696	745	65	442	588	587	450	589	649
Year of publication	1990	1991	1983	1985	1988	1988	1989	1994	1989
Animal species	Cattle	Pigs	Pigs	Chickens	Chickens	Chickens	Chickens	Chickens	Chickens
Age/weight	2-3 mo.	15-35 kg	6 wks	min.	3 wks	min.	min.	1day	n.sp.
No. vaccinated animals	5	≥5	2 x 5	≥20	≥20	≥20	≥20	≥30	≥20
No. control animals	2	5	2	10	20	20	10	30	20
Challenge at days after vaccination	21	21	14	21	28	14	14-21	9	21
Challenge route	i.n.	i.n.	i.m.	i.tr.	i.c.	i.o.	i.m.	n.sp.	i.d./i.f.
Observation period (days)	21	7	14	7	21	10	10	70	21
Criterion	Virus excretion	Av. daily weight gain	100 PD_{50}/ dose	≤20 % no virus excretion	≥80% no signs of disease	≥90% no severe bursal lesions	≥90% no signs of disease	≥80% reduction of spec. lesions	≥90% no signs of disease

AIE = avian infectious encephalomyelitis;
AD = Aujeszky's disease;
AIB = avian infectious bronchitis;
CSF = classical swine fever;
IBD = infectious bursal disease (Gumboro);
IBR = infectious bovine rhinotracheitis;
MD = Marek's disease;
NCD = Newcastle disease;
i.c. = intracerebral;
i.d. = intradermal;
i.f. = intrafollicular;
i.o. = intraocular;
i.m. = intramuscular;
i.n. = intranasal;
n.sp. = not specified;
i.tr. = intratracheal;
min. = minimum age as specified for vaccination;
mo. = months.

vaccines (WHO, 1984). A requisite for all these tests is that a good correlation has been obtained between the response measured in laboratory animals and protection in the target species.

In vitro tests

An *in vitro* prediction of efficacy can be based on titrating the vaccine virus in a suitable cell culture system or in embryonated eggs. The relationship is partly explained by the fact that virus titre and the level of immunity induced in the target animal are both functions of virus replication. In this light, it is not surprising that the relationship between the 50 percent tissue culture infective dose ($TCID_{50}$) or the 50 percent median egg infective dose (EID_{50}), on the one hand, and PD_{50}, on the other, often approaches unity. For example, for cattle 1 PD_{50} of the cell culture-adapted Kabete O strain of rinderpest virus, at any passage level between 34 and 90, was virtually identical with 1 $TCID_{50}$ (Plowright and Ferris, 1962). Similarly, in pigs 1 PD_{50} of the cell culture-adapted C strain of swine fever virus equals 1.6 $TCID_{50}$ (Terpstra, Woortmeyer and Barteling, 1990) and 1 PD_{50} of the Turkey herpes virus strain of Marek's disease virus corresponds with four plaque-forming units (PFU) (Witter and Burmester, 1979). On the other hand, the PD_{50} of the cell-associated CVI 988 strain of Marek's disease virus was found to be about 40 PFU (De Boer *et al.*, 1981).

It is common practice, however, to use a wide safety margin to compensate for inaccuracy in titration, loss of titre during storage, inexact dosage, etc. For cell culture-adapted rinderpest and swine fever vaccines $10^{2.5}$ $TCID_{50}$ has been the norm. Each final lot of vaccine ready for use in the field should undergo a titration to verify that the infectivity contained in each dose is as expected. A standard reference virus preparation should be titrated in parallel to ensure that the *in vitro* test system employed is of uniform sensitivity.

With respect to infectivity, the European Pharmacopoeia generally requires that one dose of reconstituted vaccine contains not less than the expected amount of virus according to the minimum virus titre stated on the label or in the leaflet, and that this amount satisfies the criterion for potency acceptance of the product.

The United States Code of Federal Regulations (CFR) for animals and animal products (1993) generally requires that batches of MLV vaccines contain, at any time within the expiration period, a virus titre $10^{0.7}$ greater than the sample of master seed virus that provided an accepted level of protection in an animal potency test assessed by challenge.

The minimum requirements of the quantity of virus per dose are specified for the following vaccines:

- $10^{2.5}$ $TCID_{50}$: bovine para-influenza, infectious bovine rhinotracheitis, bovine virus diarrhoea, Aujeszky's disease, Venezuelan equine encephalitis;
- $10^{2.0}$ EID_{50}: fowl pox, avian infectious bronchitis;
- $10^{2.5}$ EID_{50}: avian infectious encephalomyelitis;
- $10^{2.5}$ EID_{50} or $10^{2.5}$ $TCID_{50}$: avian laryngotracheitis;
- $10^{5.5}$ EID_{50}: Newcastle disease;
- $10^{2.0}$ PFU or $10^{2.0}$ ID_{50}: infectious bursal disease, infectious tenosynovitis;
- $10^{3.0}$ PFU: Marek's disease.

Similar to the methods based on serology, the *in vitro* titration tests are not potency assays in the strict sense, since they merely ensure an adequate viability of the vaccine batch at a level similar to products that have been shown to be effective.

DURATION OF PROTECTION

In the previous sections, the efficacy of a MLV vaccine was determined on the basis

of a vaccination challenge experiment in the target animal (i.e. a primary parameter) or on the basis of deduced (secondary) parameters. The results of a potency test are usually read two to three weeks after vaccination when immunity may be expected to be at its peak.

In principle, the endurance of protection claimed by the producer should be based on the successful outcome of a vaccination challenge experiment in the target animal made after a defined period of time. Important requisites for accepting the results are that the vaccine used in the duration experiment was of minimum potency and that a predetermined percentage of the vaccinates (usually 80 to 90 percent) passed the requirements of the challenge test.

The actual duration of immunity can be expected to be greater, since protection does not end at the time of challenge. For reasons of economy, the lack of facilities to accommodate experimental animals for prolonged periods of time or reductions in the usage of experimental animals, it may be justifiable to extend the anticipated duration of immunity by extrapolating from additional data obtained in the vaccination challenge experiment, for example the path of the antibody curve. Published data on the duration of immunity with a parent strain may also be extrapolated to an otherwise identical daughter strain of a higher passage level or a daughter strain adapted to grow on a different substrate. This is legitimate provided that the immunogenic relationship between primary and/or secondary parameters and protection remains unchanged in comparison with that of the parent strain, and that the frequency, interval and application route are also the same.

In addition to the use of a vaccine in a primary target species, a secondary related species (e.g. cattle/sheep; horse/donkey) or an unrelated species may also be involved. Age and interval of vaccination, antigen dose, PD_{50}, immune response and persistence of protection may all vary among species. As a consequence, the extrapolation of data concerning the duration of immunity in the primary species to a secondary species is not justified without a successful vaccination challenge experiment in the secondary species. However, when a positive correlation can be shown between protection – or the value of a secondary parameter such as serology – in the primary and the secondary target species, data obtained in the former species may be used to evaluate the effect of the vaccine in the latter species.

THE VALUE OF FIELD TRIALS

Veterinary vaccines are not only developed to control or eradicate a contagious disease, they should above all satisfy the farmer, who has the need to apply preventive measures against a certain agent for reasons of economy. The vaccine therefore should be efficacious not only in laboratory tests but also under field conditions.

Field trials are, however, accompanied by a number of difficulties:

- they require large numbers of animals, are difficult to monitor, are laborious and consequently expensive;
- as opposed to laboratory tests, in which only seronegative animals are used, animals vaccinated in field trials may possess, either actively or passively, acquired antibodies;
- the prevalence of the agent and, thus, the immune status may vary from farm to farm;
- other infections present on the farm may interfere with the development of immunity or may mask the clinical effects of vaccination;
- the effect of vaccination on the spread of field virus cannot be measured unless a marker vaccine with a com-

panion serological test is used to distinguish vaccine-induced antibodies from antibodies against field virus;
- the use of controls is hampered, because farmers are reluctant to accept that groups of animals should remain unprotected, and becomes virtually impossible (even with separate housing) when the vaccine virus spreads;
- a standardized challenge with a virulent strain of field virus is not acceptable.

In view of the above, field trials are often not undertaken. The omission, however, raises the question of whether it is justifiable to extrapolate from the results of laboratory tests to performance of the vaccine in the field.

The following are the conditions for a well-designed efficacy trial in the field as formulated by Pijpers and Verheijden (1991):
- a representative group of animals that remains unvaccinated or is given another vaccine must be included as a concurrent control;
- randomization, the process by which all animals are equally likely to be assigned to either the vaccination or the control group, is necessary to avoid bias;
- codification (i.e. the basis of a "blind" trial) is always desirable and is essential if subjective parameters such as the severity of clinical signs are used for evaluation;
- an adequate statistical analysis of the results is of paramount importance.

Furthermore, for a meaningful evaluation of the vaccine under field conditions it is essential that:
- the vaccine contains the minimum titre of potency per dose as specified by the producer;
- a natural challenge occurs during the observation period, preferably with a virulent virus strain.

As suggested by van Oirschot (1992), a workable compromise between assessing vaccine efficacy in the laboratory and in the field could be to vaccinate animals under field conditions, keep them with unvaccinated controls and, if they do not become infected, to subject both groups to a challenge infection in the laboratory. It should be emphasized that little value can be attached to field trials, which in essence do not comply with the requirements above.

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Potency assessment of inactivated viral vaccines

T.R. Doel

A broad division exists between the methodologies employed to evaluate the potencies of live and inactivated viral vaccines. With live vaccines, it is common practice to assess a preparation by measuring the number of infectious particles in an *in vitro* assay, where a correlation has been established previously based on the minimum titre required to give an effective vaccine. Live vaccines may occasionally be potency tested in the target species particularly where the cost of the animals is relatively low, as with poultry.

In contrast, potency testing of inactivated vaccines is in the main done in the target species or an animal model. One reason for this is the often complex nature of formulations where potency is not caused solely by the antigen content but also the influence of the adjuvants which are invariably used in the final product. This is perhaps best illustrated by foot-and-mouth disease (FMD) vaccines where the list of regularly used adjuvants includes aluminium hydroxide gel and saponin (the efficacy of the latter may vary significantly with the source and purity), simple and complex emulsions with mineral oil and avridine.

Clearly, potency testing of inactivated vaccines can be very costly, particularly if the host is a large animal and cannot be recovered for further use. A good example is the United Kingdom potency test for FMD vaccines in which 26 cattle are challenged with live virus, after 24 of them have been vaccinated, and are subsequently destroyed.[1] Alternative tests have been devised because of this and other considerations such as the unavailability of seronegative animals and high-security large animal facilities. The complete range of tests used with inactivated vaccines may be summarized as follows:

i) vaccination and live virus challenge of the target species;
ii) vaccination and serology only of the target species;
iii) vaccination and live virus challenge of a small animal model;
iv) vaccination and serology only of a small animal model;
v) estimation by physical methods of antigenic/immunogenic content prior to formulation of the final product.

These basic test procedures are listed in Table 22 in the context of a range of important diseases for which inactivated virus vaccines exist. Frequently, of course, workers may also undertake serology with categories i) and iii) to give added confidence in the interpretation of the data. In the case of categories ii), iii) and iv) it is necessary that a firm correlation should have been established between potency in the target

[1] It should be noted that in disease-free countries such as the United Kingdom it is unacceptable for FMD-seropositive cattle to be returned to the seronegative population. This is because it is difficult to exclude the possibility that a seropositive animal is persistently infected and it is necessary to convince trading partners that there is no risk of disease if they import livestock or livestock products.

TABLE 22

List A and list B diseases of animals for which approved inactivated virus vaccines exist

Disease	Target species		Model species	
	Challenge	Serology	Challenge	Serology
FMD	+ (cattle)	+	+ (mouse and guinea pig)	+
Rift Valley fever	–	–	+ (mouse)	–
Newcastle disease	+ (chicken)	–	–	–
Rabies	–	–	+ (mouse)	–
Infectious bovine rhinotracheitis	+ (cattle)	–	–	–
Eastern and western equine encephalitis	–	–	–	+ (guinea pig)
Equine influenza	–	+ (horse)	–	+ (guinea pig)
Equine herpes (EHV-1, types 1 and 2)	+ (horse)	+	+ (hamster)	+
Japanese equine encephalitis	–	–	+ (mouse)	–
Venezuelan equine encephalitis	–	+ (horse)	–	–
Avian infectious bronchitis	+ (chicken)	–	–	–
Infectious bursal disease	–	+ (chicken)	–	–
Bovine viral diarrhoea	–	+ (cattle)	–	–

Source: OIE, 1992. Other diseases/viruses for which inactivated vaccines exist include canine parvovirus, hepatitis and distemper, swine influenza, parvovirus and enterovirus encephalomyelitis (previously Teschen disease), egg drop syndrome and African horse sickness.

species and that predicted from either protection or serology of the animal model.

For the future, ethical considerations will have an increasingly important influence on the use of animals for vaccine testing and will stimulate the development of animal-free *in vitro* assays, as indicated in category v) above (Soulebot, Milward and Prevost, 1993) and more widespread acceptance of serological alternatives to live virus challenge procedures i.e. categories ii) and iv) in the list above.

STATISTICAL ANALYSIS

Closely related to the issues of cost and logistics of potency testing in target and model species is the question of the statistical significance of the test with the numbers of animals used. The variation seen with potency tests is for the most part an expression of the individual sensitivities of the test animals which, with a large population, have been shown to obey a binomial distribution (Prigge, cited by Hendriksen, 1988). In practical terms, a

quantitative assay based on a relatively small number of animals is very inaccurate. For example, the 90 percent confidence interval for the 50 percent protection dose (PD_{50}) test for FMD vaccines using three groups of five cattle lies between 45 and 220 percent of the potency (Hendriksen, 1988). Thus potency testing in small animal models or *in vitro* tests with large numbers of sera from vaccinated target species, provided a precise correlation can be made with potency in the target species, has the attractions of reduced costs while giving improved statistical significance to the data. *In vitro* tests also offer the opportunity to include a reference serum which allows inter- and intratest differences to be quantified and corrected for.

The problem of correlation between a test in the target species and a small animal model or *in vitro* assay has been debated for many years by those involved in the testing of FMD and other vaccines. Added to this there is, perhaps, an understandable preference among the users of vaccines for direct potency data from the target species despite the inherent statistical limitations. A key issue in the establishment of a correlation is that the conditions and materials being used in the two tests are clearly defined and recognized. Although this may seem obvious, there has been a tendency, with experimental FMD vaccines at least, to make the assumption that the neutralizing antibody correlates as well with protection as that induced by the conventional inactivated viral vaccines.

That this assumption is not always valid is demonstrated by work done with synthetic peptide vaccines which induced very high levels of neutralizing antibodies in cattle but gave considerably lower levels of protection than would have been predicted (DiMarchi *et al.*, 1986).

Another issue to consider here is the level of correlation desired by a national regulatory authority and what can be achieved realistically. Thus, protective immunity will, at the very least, depend on a complex and variable interaction among antibodies (which themselves have different affinities, isotypes and antigenic specificities) and phagocytic cells involved in the clearance of antigen-antibody complexes. This may be contrasted with the extreme simplicity of an *in vitro* serological assay which measures, at best, total antibody presence and may not discriminate among the more important antibodies in relation to protection. Thus, the mouse protection test for FMD vaccine sera gives the best correlation with protection in cattle, whereas the enzyme-linked immunosorbent assay (ELISA) correlates less well but has the considerable advantages of ease of use, lower cost, reproducibility, minimal use of animals (antibody reagents), etc. Progress towards replacing live virus challenge in the target species with *in vitro* assays will be greatly assisted by a general recognition of the likely deficiencies in the correlations made and a full discussion among all the interested parties.

There are two basic types of potency test. The first type is the single-dose or qualitative test in which animals are given a single dose of vaccine followed by challenge and/or the taking of blood samples. The results are amenable to simple statistics but often the only information they provide is percentage protection. Highly potent vaccines may be overlooked because more than 100 percent protection cannot be measured. While this approach ensures that the vaccines released for sale will have a minimum potency, the test results are less informative in terms of comparisons between different batches or formulations unless a good correlation between potency and a serological parameter has been established.

An alternative, but more costly, approach is to use a quantitative test in which

TABLE 23

Specimen potency data for the calculation of PD_{50} values

Dilution factor (log_{10})	Number of animals	Number protected	Proportion protected	Cumulative			Cumulative protection (%)
				Protected	Not protected	Total	
Neat (0.000)	10	10	1	23	0	23	100
3 (1.5228)	10	7	0.7	13	3	16	81
9 (1.0457)	10	4	0.4	6	9	15	40
27 (2.5686)	10	2	0.2	2	17	19	11
81 (2.0917)	10	0	0	0	27	27	0

a dilution series of the vaccine is prepared and each dilution tested in a group of animals. For proper analysis, a minimum of three dilutions is used and, if possible, these should be arranged so that the highest and lowest vaccine concentrations confer 100 and 0 percent protection respectively. This allows calculation of the dose of vaccine that protects, or is effective on, 50 percent of the population (PD_{50} or ED_{50}) and, usually, a confidence interval which gives information on the inherent variation intrinsic to the method.

There are many methods of calculating PD_{50} values including Reed-Muench, variants on Kärber, logit and probit analysis (Fontaine *et al.*, 1985) and even more opinions as to which is the most suitable method. Kärber procedures are probably the most widely used and are generally replacing Reed-Muench which, although simpler to use, was rejected by the British statistician, D.J. Finney, who considered Spearman-Kärber to be equal or superior at all times (Finney, 1971). It is clear that Reed-Muench should not be used if the 50 percent end point lies outside the dose titration series (Henderson, 1985). On the other hand, if the extreme dilutions of the titration series show 0 and 100 percent responses, and this could often be the case with assays in cell culture or small animals, then Reed-Muench is acceptable if simplicity of calculation is an important consideration. For this reason, both procedures are given below (Astudillo and Wanderley, 1976) based on a hypothetical set of protection data (Table 23), although they may equally be applied to the calculation of 50 percent end points for other assays.

Reed-Muench

Ideally, the value of the PD_{50} should fall approximately in the middle of the dose titration which should be a geometric series composed of equally sized groups of animals. No attempt should be made to calculate a PD_{50} value when the 50 percent end point lies outside the dose titration series (Henderson, 1985).

Initially, the column at the far right, which gives percentage cumulative protection, is scanned for the two values which bracket the percentage protection required and the corresponding log dilution factors are noted. Of course, this is 50 percent protection for the 50 percent lethal dose (LD_{50}) calculation but 25 and 75 percent are required for the estimation of the standard error.

The general Reed-Muench equation is given by:

$$Log_{10}PD_{A\%} =$$

$$\frac{\text{Log Inf} + A\% - \%\text{Protection at Log Inf}}{\%\text{Protection Log Sup} - \%\text{Protection Log Inf}}$$

$$\times (\text{Log Sup} - \text{Log Inf})$$

Where A% is the percentage protection required, i.e. 50, 25, etc; log inf is $\log_{10}$ of the dilution that gives less than A% protection; log sup is $\log_{10}$ of the dilution that gives more than A% protection; %protection log sup and % protection log inf are cumulative percentage protection at the dilutions which bracket A%. Thus:

$$\text{Log}_{10}\,PD_{50} = 1.0457 + \left[\frac{50 - 40}{81 - 40}\right] \times [1.5228 - 1.0467]$$

which calculates to $\text{Log}_{10}\,PD_{50}$ of 0.8380 or an arithmetic PD_{50} of 6.89, this being the dilution factor required to give 50 percent protection.

The following equation allows an approximation to the standard error of the $\log_{10}\,PD_{50}$ and was suggested by Pizzi (cited by Finney, 1964):

$$SE_{PD50} = \sqrt{\frac{0.79 \times h \times R}{n}}$$

Where 0.79 is a constant; n is the number of animals per dose; R is the interquartile interval ($PD_{75} - PD_{25}$) calculated using the standard Reed-Muench formula as described above; h is the $\log_{10}$ interval between doses, which is as follows, in the example given:

$$\frac{\text{Log}_{10}\text{ dilution neat vaccine} - \text{Log}_{10}\text{ dilution 1 in 81 vaccine}}{\text{number of doses} - 1}$$

i.e. $$h = \frac{0 - (2.0917)}{4} = 0.4771$$

From the Pizzi equation, the SE of the PD_{50} of the above data set calculates as follows:

$$SE = \sqrt{\frac{0.79 \times 0.4771 \times 0.6541}{10}} = 0.157$$

Therefore, the 95 percent confidence limits of the $\log_{10}\,PD_{50}$ value are $\pm 1.96 \times 0.157$ or in arithmetic form the potency of the vaccine is 6.89 PD_{50} with upper and lower 95 percent confidence limits of 13.99 and 3.39, respectively.

Spearman-Kärber

The extreme dilutions of the dose titration series should show 0 and 100 percent protection for the best use of this method although, if necessary, an assumption can be made that either of these values lies beyond the end of the series. The only important effect of making such an assumption is that it will not be possible to deduce the true PD_{50}. Rather, it will be necessary to express the calculated PD_{50} as equal to or greater than the observed value.

The basic formula for Spearman-Kärber is as follows:

$$\text{Log}_{10}PD_{50} = \sum_{i=1}^{k} \frac{(p_{i+1} - p_i)\,(x_i + x_{i+1})}{2}$$

Where k is the number of doses, p_i and p_{i+1} are the proportions protected in doses i and i+1, and x_i and x_{i+1} are $\log_{10}$ of the dilution factors for doses i and i + 1.

The calculation of

$$\frac{(p_{i+1} - p_i)\,(x_i + x_{i+1})}{2}$$

is made for each dilution in the data set given above and the products summed to give the $\log_{10}\,PD_{50}$. For comparison with Reed-Muench, the same data set gave an arithmetic PD_{50} value of 7.23.

The standard error of the $\log_{10}\,PD_{50}$ is calculated from the following expression:

$$SE = d\sqrt{\sum \frac{p_i q_i}{(n-1)}}$$

Where $d = x_i - x_{i+1}$ and x_i and x_{i+1} are $\log_{10}$ of the dilution factors for doses i and i+1, respectively; p_i is the proportion protected in dose i; q_i is $1 - p_i$; and n is the number of animals per group.

Using data from Table 22, the SE calculates as follows:

$$SE = 0.4771 \times \sqrt{\frac{0.61}{9}} = 0.124$$

Therefore, the 95 percent confidence limits of the $\log_{10}$ PD_{50} value are $\pm 1.96 \times 0.124$ or in arithmetic form the potency of the vaccine is 7.23 PD_{50} with upper and lower 95 percent confidence limits of 12.64 and 4.13, respectively.

Probit analysis

A more complete approach than the Reed-Muench and Spearman-Kärber procedures is to subject the potency data to probit analysis (Finney, 1971). Essentially, the percentage protection data are transformed into probit responses by reference to tables and weighted iterative regression analysis is performed on the resulting $\log_{10}$ dose/probit response line. For many years, the Wellcome FMD production laboratories at Pirbright, United Kingdom, used sets of probit tables calculated for all possible permutations of two versions of the European Pharmacopoeia test for FMD vaccines (Pay and Parker, 1977). In the case of the experimental protocol using three groups of five cattle, there are 216 possible sets of responses of which only 88 have positive slopes. Furthermore, it is not possible to calculate the lower 95 percent confidence on more than 20 sets. It should be emphasized, however, that less sophisticated procedures such as Spearman-Kärber cannot be relied on any more readily.

While it is extremely important to recognize that the PD_{50} value and confidence limits obtained may vary with the statistical method employed – particularly where small numbers of test animals are used (Pay and Parker, 1977) – it is also probably true to say that large differences between the statistical methods are not likely to be seen, given good data in which the PD_{50} value is bracketed by the doses tested (Henderson, 1985). This can be seen here with the two calculation methods used. In the final analysis, it requires little extra effort to submit the results of a potency test to several statistical methods provided the experimental design is compatible with each.

Finally, a further option with the quantitative test is the use of a standard or reference vaccine in parallel with the product under test. Clearly this increases the cost of potency testing but provides more information with particular respect to controlling or at least recognizing and allowing for experimental variables other than individual animal variation. In this way all batches of vaccine may be compared against the baseline of the reference preparation so that trends or major fluctuations in quality can be identified. The slopes of the respective dose-response curves of the reference and test vaccines provide additional information. Thus, essentially equivalent preparations differing only in antigen content should have parallel slopes. Significant deviation from this may be indicative of problems of vaccine quality or the manner in which the potency test was performed – having made the assumption that the reference vaccine is stable and has not deteriorated with time.

POTENCY TESTING OF SOME INACTIVATED VIRAL VACCINES

The following review, which is not intended to be comprehensive, draws heavily on OIE (1992).

Foot-and-mouth disease

Numerous procedures have been developed for the testing of FMD vaccines. Some of these are direct methods involving the challenge of cattle, others are indirect methods based on correlations with

protection data. *In vitro* procedures are growing in popularity.

In the case of direct methods, probably the best known is that prescribed by the 1985 version of the European Pharmacopoeia. This specifies the use of 18- to 30-month cattle obtained from FMD-free areas that are seronegative for FMD and have never been vaccinated. Vaccine is diluted in an inert diluent to give three dose levels at not more than fivefold intervals and is used to inoculate groups of at least five cattle according to the volume and route stated on the label, using one dose level per group. Three weeks after vaccination, the vaccinated animals and a control group of two non-vaccinated cattle are challenged intradermolingually with 2 × 0.1 ml of a suspension containing 10 000 ID_{50} of cattle-adapted virus that is fully virulent and appropriate to the virus type of the vaccine under test. Animals are observed for eight days. Unprotected animals show lesions at sites other than the tongue and control animals must develop lesions on at least three feet.

Occasionally, vaccinated animals may develop a lesion on one foot despite evidence to suggest that the vaccine is potent. These "one-footers" are most probably the result of virus entry via an external abrasion rather than a consequence of viraemia and are considered by some workers as protected. High-quality FMD vaccines such as those prepared from antigens stored in emergency reserves (Doel and Pullen, 1990) not only protect against generalization, even at high dilution, but can suppress the febrile response and vesicular erosion of the tongue at the sites of injection of challenge virus.

For the vaccine to pass the European Pharmacopoeia test it must contain at least 3 PD_{50} per dose.

The most recent version of the European Pharmacopoeia Monograph on FMD vaccines (1993) describes a number of important changes in procedure. First, cattle of not less than six months of age are indicated which is more consistent with the general practice of a number of European laboratories and has considerable operational and cost advantages. It is also probably more relevant to test the vaccine in animals at or closer to the age recommended for the first vaccination (four to six months).

The second change is more controversial and indicates the use of different volumes of vaccine to vary the dosage rather than using different dilutions. While this procedure may prove to be acceptable, it must be said that it is a significant departure from well-accepted practices and it does not have a large body of data to support it.

Another direct test has been used by workers at the Institute for Animal Health in which three groups, each of eight cattle, are vaccinated with a twofold, a tenfold or a fiftyfold dilution of the field dose in adjuvant. Challenge is as above except that 10 × 0.1 ml of a suspension containing 100 000 ID_{50} of cattle-adapted virus is used. The inclusion of adjuvant rather than inert diluent gives a flatter dose-response curve and, thus, higher PD_{50} values than those for vaccines tested by the United Kingdom method. This is reflected in the United Kingdom pass-mark of 6 PD_{50} and Ahl *et al.* (1990) have demonstrated that the approximate relationship is:

$$\text{The European } PD_{50} = \sqrt{\text{of the United Kingdom } PD_{50}}$$

Several procedures have been described in which protection of cattle with a single field dose of vaccine is measured. The K index procedure described by Lucam and Fedida (cited by Pay and Parker, 1977) was widely used by a number of countries to assess their FMD vaccines. Briefly, virus is titrated intradermolingually in four vaccinated cattle and compared with the titre in

four susceptible control animals, the reduction in titre being proportional to the potency of the vaccine. To the author's knowledge this procedure is now rarely used.

A more popular test is the protection against generalization (PG) procedure in which each of 16 cattle is vaccinated and subsequently challenged, usually 21 days later, with 10 000 ID_{50} of virus (Vianna Filho *et al.*, 1993). For a vaccine to pass the test, it is usually necessary to protect at least 12 cattle. This test is perhaps most suited to South America, where it is applied extensively to the many batches of vaccine being produced for routine use in the subcontinent, because it provides the information required in an unambiguous form, i.e. the minimum percentage of animals that will be protected if the vaccine is used. With the PG method there is not the temptation to apply statistics to inadequate data, in particular the calculation of PD_{50} values from data lacking a dose-response relationship. According to Vianna Filho *et al.* (1993), 3 PD_{50} by the European Pharmacopoeia method corresponds to 78, 78 and 79 percent protection by the PG method for virus serotypes O, A and C, respectively.

Following recovery from challenge, cattle may be reused to test a vaccine of another serotype despite the fact that they may have high-quality immunity to the first serotype and may be persistently infected. The reuse of cattle is particularly appropriate to testing based only on serology, where there is no disease security risk involved in holding the animals for a significant period of time.

Other species are occasionally used for direct potency testing of FMD vaccines. For example, oil-adjuvanted vaccines for pigs may be tested in this species although the procedure is complicated by factors such as overchallenge which can occur because of the very high level of virus excretion by susceptible animals. With oil formulations, it is common practice to deliver a range of volumes of vaccine although it is possible to prepare a dilution series.

Guinea pigs have been used extensively as a model species for potency testing of FMD vaccines. They are less favoured now, however, owing partly to the occasional failure to correlate the results with potency in cattle and the development of serological tests to replace challenge work in animals. In the author's experience, guinea pigs are most useful with vaccines made from pure or semi-pure antigens such as those held in antigen banks (Doel and Pullen, 1990) where the correlation between cattle and guinea pig potency appears to be good. There is some evidence that the high levels of contaminating cellular proteins in conventional FMD vaccines may interfere in the immune response of guinea pigs to viral antigens (T.R. Doel, unpublished results). It is important to recognize another possible reason for the occasionally poor correlation between the two challenge tests, namely the respective passage histories of the cattle- and guinea pig-adapted challenge viruses and, indeed, the vaccine production strain, which may lead to significant antigenic divergence.

In addition to the ethical issues involved in live challenge work, there are two main disadvantages associated with direct challenge methods in cattle. The first of these is the risk of dissemination of FMD from the testing area which may have additional cost implications depending on the level of biosecurity required by the veterinary authorities. The second disadvantage is that it is not feasible to challenge test more than a single strain of virus at any one time whereas most commercial FMD vaccines are multivalent, containing two, three or even four strains of the virus.

For these and other reasons, many workers have examined the correlation

between direct indices of protection and serological parameters, in particular neutralizing antibody (Stellman *et al.*, 1968; Sutmöller, Gomes and Astudillo, 1984: Ahl *et al.*, 1990; Pay and Hingley, 1986 and 1992). Pay and Hingley (1986) claimed that a good correlation could be obtained between virus neutralizing antibody and vaccine potency when both were expressed in $\log_{10}$ antigen PD_{50} units. Because the procedure required three separate regression slopes, these authors refined their method to allow the conversion of a mean titre virus neutralizing antibody titre directly into a percentage protection value using a single regression slope (Pay and Hingley, 1992). In commenting on some apparent batch-to-batch variation that was found, they stressed the necessity for each laboratory to set up its own database because of variables such as the virus neutralization test (i.e. cell sensitivity, etc.) and the relationship between the serum assay virus and the vaccine virus.

Ahl *et al.* (1990) also made a valuable contribution to this subject. Using a plaque-reduction assay with a large number of cattle sera obtained from potency tests, they concluded that their approach fulfilled the requirements to replace the official PD_{50} challenge test in Germany. They also calculated the relationships among a number of different serological and protective parameters used by various FMD laboratories including United Kingdom and European PD_{50} values.

Sutmöller, Gomes and Astudillo (1984) compared the microtitre neutralization test with a passive immunity test in mice (i.e. mouse protection test or MPT) and protection data from cattle. They derived a table of expected percentages of protection (EPP) versus the reciprocal of the log serum dilution that neutralized 50 percent of 100 ID_{50} of virus and concluded that the serum neutralization test was a useful alternative for the estimation of the potency of FMD vaccines, although it tended to underestimate the number of vaccines that would be approved compared with the mouse protection test. In the latter procedure, six- to seven-day mice are injected with cattle serum and, one hour later, challenged with a dilution series of mouse-adapted virus. The results of the test are expressed as the mouse protection index (MPI), being the difference between the $\log_{10}$ virus titres obtained in the presence and absence of test serum (Gomes and Astudillo, 1975). The MPT is in regular use for the potency assessment of FMD vaccines made at the Pan American Foot-and-Mouth Disease Center and is the only procedure capable of assessing reliably the levels of protection conferred by some synthetic FMD peptide vaccines in cattle. Thus, the virus neutralizing antibody titres of peptide vaccinated cattle give little indication of the probability of protection in contrast to the MPT titres of the same sera, which show a good correlation (Mulcahy *et al.*, 1991). The relative superiority of the MPT for the serological assessment of vaccine potency is understandable. Pure *in vitro* tests such as ELISA and the neutralization test are less able to discriminate between those sera that have the affinities/isotypes/specificities of antibodies most appropriate to protection and those sera that do not (Steward *et al.*, 1991). Furthermore, these tests take no account of *in vivo* antigen-antibody clearance mechanisms, in contrast to the MPT.

Despite these limitations, ELISA in one of its many versions has been or is being evaluated by a number of FMD laboratories as an assay to measure the protective capacity of cattle sera. A liquid phase-blocking ELISA, described by Hamblin, Barnett and Hedger (1986), is used regularly by the World Reference Laboratory for FMD as an alternative to the serum neutralization test. In addition, a similar assay is being used in a large study in

South America involving laboratories in Argentina, Brazil and Uruguay and coordinated by the Pan American Foot-and-Mouth Disease Center. The titres of almost 1 000 sera from vaccine potency tests are being determined using a liquid phase-blocking ELISA, a virus neutralizing antibody test and the MPT. The correlations achieved will be of considerable interest to manufacturers and users of FMD vaccines.

Rift Valley fever

The test for inactivated Rift Valley fever vaccine is performed in two stages. Serial fivefold dilutions of vaccine are prepared and a single dose of 0.5 ml is used to inoculate ten adult mice per dilution by the intraperitoneal route (OIE, 1992). The mice are challenged two weeks later with 10^5 to 10^6 mouse LD_{50} of an appropriate challenge strain by the subcutaneous route. The challenge strain should be different from the vaccine strain. A second assay is conducted based on the results of the first but using serial twofold dilutions to bracket the expected 50 percent end point of the test. Data from the second test are plotted on probit paper and the confidence limits of the assay determined. Thus, the two-stage procedure allows a more precise end point to be determined without using very large numbers of mice.

Rabies

It has been established that the antigenic potency in mice of inactivated rabies vaccine is a reliable indicator of efficacy in the target species. One of two tests may be used (OIE, 1992). In the case of the European Pharmacopoeia test, groups of at least ten mice, aged three to four weeks, are inoculated with single decreasing doses of vaccine, whereas the National Institute of Health (NIH) test requires two doses, separated by a period of one week. A sufficient number of dilutions must be made to permit the calculation of the dilution of vaccine at which 50 percent of the mice are protected against intracerebral challenge 14 days after the last vaccination. A WHO reference vaccine is available for testing in parallel and allows the expression of the potency of the vaccine under test in terms of international units (IU).

To be valid, the PD_{50} of each set of data should lie between the extreme points of the dilution series. Statistical analysis should show dose-response lines that are linear and parallel with significant slopes and the 95 percent confidence limits should be not less than 25 percent and not more than 400 percent of the estimated potency. Finally, the titre of the challenge virus should be not less than 10 ID_{50} per 0. 03ml. The vaccine passes the test if the estimated potency is not less than 1 IU.

There has been considerable dispute surrounding the available potency tests for rabies vaccine because of their failure to distinguish satisfactorily between different vaccine products and the widely differing results observed when the same vaccine is tested by different laboratories. As a result, *in vitro* methods such as ELISA have been investigated with the aim of replacing the problematic *in vivo* methods (Hendriksen, 1988). One of the newer methods is an antibody binding test in which a vaccine dilution is mixed with an equal volume of neutralizing antiserum followed by live rabies virus. This mixture is inoculated on to a chick embryo fibroblast culture and the infectious foci detected by fluorescent antibody techniques. The potency of the vaccine dilution is thus proportional to the number of infectious foci on the cell sheet. This test has been validated in comparison with the NIH test and gave less variable and more reproducible data.

Viruses of poultry

With inactivated virus vaccines for poultry, it is completely feasible to test all prepara-

tions in the target species. The diseases of major importance include Newcastle disease, avian infectious bronchitis and infectious bursal disease (OIE, 1992).

Newcastle disease vaccines are tested by a number of methods (OIE, 1992). The European Pharmacopoeia indicates the vaccination of 20 birds at the minimum recommended age by the route and dose given by the manufacturer. After 14 to 21 days, the vaccinated birds, along with ten control birds, are each challenged with 10^5 LD_{50} of a suitable strain of Newcastle disease virus such as the Herts 33 strain. For the vaccine to pass the test, all control birds should die within six days of challenge and 90 percent of the vaccinated birds survive at least ten days with no sign of disease.

The potency test used for avian infectious bronchitis (AIB) vaccines depends on the type of protection required (OIE, 1992). To protect laying birds, 30 or more specific pathogen-free (SPF) birds are vaccinated at the earliest possible age (not later than three weeks of age). A second group of 30 control birds are included in the test and all are housed separately until four weeks before peak egg production. At this time they are housed together and individual egg production is monitored until it is regular, at which time all birds are challenged. Egg production is monitored for a further four weeks and the challenge should be sufficiently severe to ensure a loss of at least 67 percent of production in the control group during the first three weeks of the post challenge period. The group given one dose of inactivated vaccine should show a drop of production intermediate between the control group and what is normally observed with live AIB vaccines.

In fact, it is not unusual to carry out a composite potency test with live as well as inactivated preparations. Sera are collected from all birds at vaccination, four weeks later and following challenge. None of the sera from the control group should show antibodies against the virus.

If the vaccine is required to protect against respiratory disease, groups of 20 SPF birds, aged four weeks, are vaccinated as indicated by the manufacturer. Their antibody responses, as well as those of 20 control birds, are determined four weeks later. All groups are housed together and there should be no antibody response in the control group. Finally, all birds are challenged with 10^3 ID_{50} of virulent virus and killed four to seven days later. Sections of the trachea are examined for cilial motility and at least 80 percent of the control group should display complete ciliostasis whereas the tracheal cilia of a similar percentage of vaccinated birds should remain normal.

With infectious bursal disease (IBD) vaccines, an efficacy test is performed initially and once only, using a typical batch of vaccine (OIE, 1992). In this test, 20 SPF birds, near to point of lay, are vaccinated with a single dose of vaccine by the recommended route and the antibody response measured with reference to a standard antiserum over a period of four to six weeks post vaccination. In the United Kingdom, this antiserum may be obtained from the Central Veterinary Laboratory (CVL), Weybridge, United Kingdom. Eggs are collected for hatching and 25 progeny chicks challenged at four weeks of age by installation of eye drops containing 10^2 ID_{50} of a virulent strain such as CVL 52/70. Ten control birds of the same breed are also challenged. After three days, the bursa of Fabricius is removed from each bird and examined histologically or tested for the presence of virus antigen by the agar gel precipitin test. For the test to be acceptable, all control birds must show evidence of IBD infection whereas not more than three of the progeny from vaccinated hens should be affected.

For the routine vaccine potency test, 20 chicks, approximately four weeks of age, are vaccinated as above. The vaccinated birds are housed with ten control birds and the antibody responses of each bird determined with reference to a standard antiserum over a period of four to six weeks post vaccination. The mean antibody level of the vaccinated birds should not be significantly less than the level recorded in the efficacy test and the control birds should remain seronegative.

Viruses of horses

OIE (1992) describes six virus diseases of horses for which inactivated vaccines exist. These are Japanese, eastern, western and Venezuelan equine encephalomyelitis (JEE, EEE, WEE and VEE, respectively), equine influenza and equine rhinopneumonitis. The need for these vaccines relates both to the high value of bloodstock and the fact that humans are hosts for all of the equine encephalomyelitis viruses listed above, occasionally with fatal consequences.

Potency testing of both EEE and WEE is performed by inoculation of ten guinea pigs with one-half of a horse dose of vaccine on two separate occasions. The interval between the vaccinations should be 14 to 21 days and the route used with the guinea pigs should be as indicated for the horse. Two to three weeks after the second dose, sera are collected and tested by a plaque-reduction neutralization test. The EEE and WEE titres should be ≥ 1.4 and ≥ 1.32 respectively.

With JEE, a mouse protection test exists in which 30 mice, aged three to four weeks, are inoculated intraperitoneally with 0.1 ml of a tenfold dilution of vaccine in phosphate-buffered saline. The vaccine is given twice at three-day intervals. Eight days after the first inoculation the vaccinated mice, along with an equivalent uninoculated group, are challenged with graded doses of live virus and observed for 14 days. For the vaccine to pass the test, the survival rate should be more than 40 percent in the immunized group and the mortality rate in the control group should be more than 90 percent.

Inactivated VEE vaccines are tested in horses. Each of 20 susceptible animals is inoculated subcutaneously as recommended and sera taken within 21 to 28 days of vaccination. For a valid test, at least 19 of 20 horses must have haemagglutination inhibition (HI) antibody titres of at least 1 in 20 or serum neutralizing antibody titres of at least 1 in 40.

Equine influenza vaccines may be tested either in horses or in guinea pigs. For the target species, one vaccine dose is inoculated by the recommended route into each of five susceptible horses. A second dose of the same vaccine is injected after the period stated on the manufacturer's label. Blood is collected from each animal one week after the first vaccination and two weeks after the second vaccination, sera are prepared and the haemagglutinating activity (HA) determined.

For the vaccine to pass the test, the HA antibody titre of each serum taken after the second vaccination should not be less than 1 in 64 and the serology after the first vaccination must not show evidence of an anamnestic response. The latter safeguards against the use of horses that have pre-existing low levels of immunity to the disease. Such animals may be replaced and the test repeated in fresh animals. In general, it is not necessary to carry out this test with subsequent batches of vaccine prepared from the same seed lot system. Instead, ten guinea pigs are vaccinated as specified on the label and blood samples taken 21 days later. HA antibody titres are determined and should not be less than 1 in 16.

Equine rhinopneumonitis is caused by an equine herpesvirus of which there are two antigenically, biochemically and bio-

logically distinguishable types referred to as EHV-1 and EHV-4. The former is the more common cause of abortion and the latter is more commonly found with acute respiratory disease of young horses.

Vaccine potency is tested preferentially in horses and, in the case of anti-abortion vaccine, in pregnant mares. Challenge is by the intranasal route with a virulent strain of virus. In the case of vaccines containing inactivated EHV-1, the potency test in horses may be substituted by a protection test in which vaccinated hamsters are challenged with a lethal dose of hamster-adapted virus. Because EHV-4 does not adapt to hamsters, vaccines containing this subtype may be tested by demonstrating seroconversion of virus-neutralizing antibodies in the test animal.

CONCLUDING COMMENTS

It is worth reflecting on the role of vaccine potency testing in the overall scheme of vaccine production and usage. First and foremost it exists to ensure that the customer, often a regional or national veterinary authority in the context of the diseases reviewed here, receives a product of known minimum potency. Indeed, the same authorities may control the official potency test and naturally wish to see an increase in the minimum potency requirements so that future batches of vaccine purchased by them are more effective.

At the same time, the manufacturer obtains information from a potency test which may be of value in recognizing problems during production and/or allows either the future production of more potent vaccine or an increased output of the minimum potency product. The latter option is particularly attractive because of the potential for increased profitability in an area where profit margins are relatively small.

Thus, the control authority and the manufacturer are occasionally in conflict over the most appropriate passmark for a vaccine. It is clear, however, that excessive demands for more potent veterinary vaccines may persuade the manufacturer to change to more lucrative products or result in very costly vaccines. The net consequence will often be the same. The customer may not be able to maintain a programme of vaccination either because the producer no longer makes the vaccine or because the cost of the vaccine is too high. The latter is particularly relevant to developing countries where the funds available for the purchase of animal vaccines are extremely limited.

With diseases of great economic importance such as FMD the consequences of a reduction in the level of vaccination for the agricultural economy of a country may be disastrous. There is a need, therefore, for a mutual understanding between the vaccine manufacturer and the control authority. In other words, control authorities should consult fully with vaccine manufacturers so that all concerned appreciate the consequences of a requirement for higher potency vaccine.

In the same vein, the move towards replacing official challenge tests in animals with *in vitro* assays will be greatly facilitated by full and proper discussion between the interested parties at all stages of the process.

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In vitro potency testing of inactivated biologics: current situation in the European Union

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The EEC Council Directive 90/677 (EC, 1990) defines an immunological veterinary medicinal product (IVMP) as: "a veterinary medicinal product administered to animals in order to produce active or passive immunity"; and: "the quantitative particulars of an IVMP shall be expressed by mass or by international units, or by units of biological activity or by the number of germs or by specific protein content where possible, as appropriate to the product concerned". The mass, number of germs or specific protein content are not determined *in vivo* but rather *in vitro*. This definition would allow, in principle, for the replacement of potency tests performed in animals by *in vitro* tests. Such a change would not only save animal lives (which is an ethical requirement) but also save costs (animal tests are particularly costly and often of long duration) and solve some practical difficulties – certain tests can adequately be performed only in specific pathogen-free (SPF) animals, animals that are free of specific antibodies or animals that have never previously encountered the specific organism and, in many cases, such animals are not available. Both industry and official laboratories should obviously be in favour of such changes.

The directive cited above is part of a whole series of regulations, which are implemented throughout the European Union (EU), in the field of IVMPs. These regulations complement the monographs of the European Pharmacopoeia which is itself widening its field of application. The object of these regulations is to ensure a very high level of safety and efficacy but, unfortunately, they often lead to the increased use of animals in test procedures, especially target animals for routine safety tests and, for ethical, economic and practical reasons, this is undesirable.

Although in the past the assessment of biological products – particularly tests of potency of vaccines – were mainly carried out in animals, either laboratory species or domestic livestock, such tests could be expensive, time-consuming and, in the case of some products, of only limited value. The advent of modern physico-chemical, immunological or molecular biological methods of measuring with considerable precision the essential immunizing component of antigens destined for the preparation of vaccines, offers significant advantages to those involved with developing in-process quality control tests. Not only are there advantages in terms of precision and the saving of time and financial costs, there is also no need for the use of animals – a not inconsiderable factor from the viewpoint of those concerned with animal welfare.

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REGULATIONS

In the EU, three levels of rules must be considered. At the most general level, recently published Commission Directive 92/18/EEC (EC, 1992c) modifying the annex to Council Directive 81/852, indicates that potency tests may be: "based upon *in vitro* or *in vivo* methods,... (and) in exceptional circumstances, potency testing may be carried out at an intermediate stage, as late as possible in the production process". This represents a significant opportunity, allowing for instance to move the control test to a stage upstream of the addition of adjuvants, thus avoiding all the difficulties related to the need to separate the active ingredients from the adjuvants and other ingredients for testing. However, the term "in exceptional circumstances" considerably limits the scope of this opportunity.

The directive is supplemented by guidelines including "General requirements for the production and control of inactivated mammalian bacterial and viral vaccines for veterinary use" (EC, 1992a) which, however, does not give any further precision concerning potency tests and merely states: "the vaccine shall be shown to be of satisfactory potency using validated methods".

In the meantime, some countries have already adopted species-specific guidelines. In the United Kingdom, for example, guidelines are available for the production and control of avian virus vaccines, veterinary bacterial vaccines, porcine virus vaccines, canine virus vaccines, equine virus vaccines and bovine virus vaccines. These documents do not include any particular indication concerning *in vitro* or *in vivo* potency tests – it is only stated that: "each batch of vaccine shall be shown to be of satisfactory potency using an approved method" (Veterinary Medicines Directorate, 1991) – except in the avian guidelines where the use of serological tests for four specific vaccines (Newcastle disease, egg drop syndrome, infectious bronchitis and infectious bursal disease vaccines) are planned (Veterinary Medicines Directorate, 1990).

The final level of rules within the EU are the national pharmacopoeias, which include IVMP monographs (for example British Pharmacopoeia Commission, 1985; Commission Nationale de Pharmacopée, 1992). It should be noted that national pharmacopoeias must use the European Pharmacopoeia monographs where they exist, but may include monographs that are not in the European Pharmacopoeia.

THE EUROPEAN PHARMACOPOEIA

The European Pharmacopoeia extends its domain of application beyond the EU, since it concerns at least all the member countries of the Council of Europe, 19 nations in all. The Pharmacopoeia consists of monographs, general notices and analytical methods.

Monographs

The European Pharmacopoeia includes 12 monographs for killed vaccines and a general monograph for "veterinary vaccines". None of these specifies the use of *in vitro* tests. Five other monographs for killed vaccines are at the level of projects published for public appraisal (European Pharmacopoeia Commission, 1991a, 1991b, 1991c, 1991d and 1991e). None of these projects plans for *in vitro* tests, with the exception of the document concerning foot-and-mouth disease (FMD) vaccine. The chapter pertaining to in-process control of antigenicity states: "the content of antigen is determined by an *in vitro* method (e.g. 146S-particle measurement by sucrose density gradient centrifugation and ultraviolet spectrophotometry at 259 nanometres)". This test may be used for the release of vaccine but only in cases of extreme urgency. The section reads: "If the

test for potency has been carried out with satisfactory results on a representative batch of vaccine prepared from a given batch of antigen, then a batch of vaccine prepared from the same batch of antigen may, in cases of extreme urgency and subject to agreement by the national authority, be released before completion of testing if it has been shown that the batch has an antigen content not less than that of the representative batch, determined in an in-process control by an *in vitro* method."

It may seem that the European Pharmacopoeia excludes the use of *in vitro* tests and refers exclusively to challenge tests or vaccination followed by antibody titration. However, certain monographs allow that: "if the test for potency (in animals) has been carried out with satisfactory results on a representative batch of vaccine, this test may be replaced by a manufacturer as a routine test on other batches of vaccine prepared from the same seed lot by an alternative test for which satisfactory correlation of the results with those of the Pharmacopoeia method has been established by a statistical evaluation, subject to agreement by the national authority."

General Notices. The preamble to Part IV-1, General Notices (European Pharmacopoeia Commission, 1985) states that the monographs are official standards applicable within countries of the Contracting Parties (countries of the Council of Europe) and that: "statements under the headings Identification, Tests, Assay and Potency are mandatory requirements", but that: "with the agreement of the national authority, alternative methods of analysis may be used for control purposes, provided that the methods used enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official methods were used".

Analytical methods

An opening in favour of substituting *in vitro* test for *in vivo* assay seems to appear. The fifteenth fascicle (European Pharmacopoeia Commission, 1991f) introduces in its chapter on analytical methods, under heading V 2.1.10, the important paragraph concerning immunochemical methods: "These methods are employed to detect or quantify either antigens or antibodies". Of course, it is underlined that: "the results of immunochemical methods depend on the experimental conditions and the nature and quality of the reagents used. It is essential to standardize the components of an immunoassay and to use, wherever available, international reference preparations for immunoassays".

Two main types of methods are quoted as quantitative methods for determining antigens. These are methods in which a labelled antigen or a labelled antibody is used and methods in which an unlabelled antigen or antibody is used. The latter group is divided into immunoprecipitation methods such as single radial immunodiffusion (SRID) and immunoelectrophoretic methods such as crossed immunoelectrophoresis, electroimmunoassay (often referred to as rocket immunoelectrophoresis) and counter-immunoelectrophoresis.

No technical details are described. However, validation criteria and validation methods are detailed. It is stated that a quantitative immunochemical method is not valid unless:

- the antibody or antigen does not significantly discriminate between test and standard. For a labelled reactant, the corresponding reactant does not significantly discriminate between labelled and unlabelled compound;
- the method is not affected by the assay matrix, i.e. any component of the test sample or its excipients, which can vary from sample to sample and may

include high concentrations of other proteins, salts, preservatives or contaminating proteolytic activity;
- the limit of quantification is below the acceptance criteria stated in the individual monograph;
- the precision of the assay is such that the variance of the results meets the requirements stated in the individual monographs;
- the order in which the assay is performed does not give rise to systematic errors.

In order to verify these criteria, the validation design includes the following elements:
- the assay is performed in at least triplicate;
- the assay includes at least three different dilutions of the standard preparation and three dilutions of sample preparations of activity presumed to be similar to the standard preparation;
- the assay layout is randomized:
- if the test sample is presented in serum or formulated with other components, the standard is likewise prepared;
- the test includes measurement of non-specific binding of the labelled reactant;
- for displacement immunoassay maximum binding (zero displacement) is determined and dilutions cover the complete response range from values close to non-specific binding to maximum binding, preferably for both standard and test preparations.

The following is provided on the statistical calculation of results: "to analyse the results, response curves for test and standard (must be) analysed.... Significant non-parallelism indicates that the antibody or antigen discriminates between test and standard and the results are not valid. In displacement immunoassays, the values for non-specific binding and maximum displacement at high test or standard concentration must not be significantly different. Differences may indicate effects due to the matrix, either inhibition of binding or degradation of tracer".

From all that has been said about regulations in the EU, it can be concluded that *in vitro* potency testing on final products is almost never proposed but is not excluded and that *in vitro* antigen quantification methods are indicated but with no technical details. Validation criteria and methods are more detailed, but no or few data are available for statistical calculations. Reference preparations, which would be particularly useful, are not available. Thus, *in vitro* tests are essentially used for in-process controls. Methods are generally internal to the different laboratories and may be part of the industrial expertise – under such conditions, it is understandable that few methods have been standardized. The following are examples of widely used methods.

Foot-and-mouth disease. The antigenic content of FMD vaccine (Fargeaud, Fayet and Roumiantzeff, 1969) is determined by first preparing sucrose density gradients. Samples clarified by centrifugation are then loaded on the gradients, ultracentrifugation is performed and ultraviolet spectrophotometric results are plotted. The area under the peak is proportional to the concentration of 146S particles of the sample. Using a standard curve established with different dilutions of a purified viral suspension containing a known concentration of 146S particles, the concentration of 146S particles in the test sample can be calculated.

Rabies and pseudorabies. Antigenic glycoprotein content is determined using a single radial immunodiffusion test, derived from Ferguson's method (Ferguson and Schild, 1982). A concentration gradient is established for the antigen diffusing from

an external source into the gel medium containing the corresponding antibody at a comparatively low concentration. When the equilibrium between the external and the internal reactants has been established, the circular precipitation area, originating from the site of the external reactant, is directly proportional to the amount of the antigen applied and inversely proportional to the concentration of the antibody in the gel. Various dilutions of the test glycoprotein and standard glycoprotein solutions are used. The formula for the linear response can be established as $d2 = ac + b$ where: "d" is the diameter of the circular precipitation area; "a" is the slope; "c" is the dilution rate; and "b" is the original intercept.

The titre of the test suspension is expressed by the ratio of the slopes multiplied by the concentration of the standard expressed in micrograms per millilitre. For rabies, many other methods have been proposed, from enzyme-linked immunosorbent assay (ELISA) tests (Bruckner *et al.*, 1988) to antibody binding tests (Barth *et al.*, 1985) or even determination of the *in vitro* production of specific interleukin-2 (Joffret *et al.*, 1991).

Tetanus vaccine and other clostridial vaccines. Current pharmacopoeia requirements for tetanus vaccines for veterinary use specify a final product potency test based on direct challenge of mice or titration of antibodies from vaccinated guinea pigs or rabbits. The titration of antibodies is performed by a serum neutralization method against a standard toxin in mice. In all cases, these tests require the use of animals. Although variations of these standard methods have been proposed to reduce the number of animals (Knight and Roberts, 1987; Huet, Relyveld and Camps, 1990), *in vitro* tests should be considered as alternatives. One of the first objectives could be to replace the existing toxin neutralizing tests performed in mice by an *in vitro* antibody assay method. Numerous test systems have been proposed: gel diffusion, nephelometry, immunoelectrophoresis and ELISA (Calmels *et al.*, 1981). The present availability of reagents, high standards of quality and widespread use of the technique would make the ELISA test a good candidate to replace *in vivo* testing. Many other researchers have developed such a test and correlation with *in vivo* methods has often been found to be good (Cox *et al.*, 1983; Gentilli, Pini and Collotti, 1985).

Complete *in vitro* potency testing would require the direct titration of tetanus toxoid in the final product. Such methods have been proposed (Melville-Smith, 1985) and are currently in use for the identity testing of tetanus vaccines. The earliest test for direct quantification of the tetanus toxoid is Ramon's flocculation test. Although cumbersome, it has the advantage of referring to an international standard and is used to quantify the tetanus toxoid prior to vaccine formulation (i.e. 100 Lf/dose). Toxoid measurement in the final product is more easily performed by gel diffusion or ELISA. When run against a standard, a quantitative estimation can be obtained.

While tetanus vaccines have been the most widely studied, all that has been said could also be applicable to veterinary clostridial vaccines. *In vitro* antibody titration methods have already been proposed (Knight *et al.*, 1990). The main problem connected with these approaches remains the reliability of measurement on adjuvanted vaccines and is discussed in Conditions for the development of *in vitro* tests (p. 416).

Pasteurella multocida. In this example, *in vitro* tests for the determination of dermonecrotoxin content (DNT) have been made possible owing to the much better knowledge that now exists of the pathogenesis of

the disease. Research has moved on from a situation where the etiology of atrophic rhinitis (AR) was ascribed to many infectious and non-infectious causes, through the narrowing of the cause to two bacteria, *P. multocida* and *Bordetella bronchiseptica*, to the discovery and proof through challenge models of the role of the dermonecrotic toxin (DNT) of *P. multocida*. This has allowed the preparation of vaccines of progressively better-defined content. Current modern vaccines are based on purified DNT toxoid which gives an opportunity to apply several *in vitro* tests.

Toxin determination, initially tested by intradermal injection in guinea pigs can now be performed *in vitro* in embryonic bovine lung cells or Vero cells. This assay can then be used to determine the neutralizing antibody level.

An ELISA test has been designed to measure toxoid content in the active ingredient and the final vaccine. Using a sandwich technique, the test has been successfully used to titrate toxoid content in adjuvanted vaccine. As in previous examples, the critical point is the quality (purity and stability) of the standard. This test is used to standardize batch formulation and could be used as a release method provided that a reference vaccine from the same production process with the same amount of toxoid has been shown to be protective.

CONDITIONS FOR THE DEVELOPMENT OF *IN VITRO* TESTS

Most vaccines are adjuvanted, either as aluminium hydroxide-adsorbed formulations or as oil emulsions. This makes testing more difficult and is a source of variability.

It is necessary either to separate the antigen in the vaccine, taking into account the loss or denaturation of antigen, or to adapt the test to use on the finished product with possible interference from other vaccine constituents.

Two ways around this difficulty would be either to estimate the average amount in the final product and the variability of a given process without attempting to retrieve the exact amount introduced in the formulation (as seen for DNT) or to accept the in-process testing upstream of formulation provided that all steps in formulation are correctly described, validated and performed. The second approach is preferable because it allows a precise determination of the amount of antigen that will be introduced in the vaccine and thus constitutes a guarantee, provided that quality is ensured and the final steps of formulation are reproducible. This is why the possibility provided by Directive 92/18/EEC (EC, 1992c) to carry out potency tests upstream in the process, even if limited to "exceptional circumstances", is particularly important. This approach is only acceptable under the following three conditions, however:

- batch-to-batch consistency must be formerly validated;
- a quality assurance system must be implemented;
- the system must be properly maintained and regularly subjected to a suitable inspection procedure.

The first condition is obvious. It may, however, be useful to remember that: "validation is the action of proving, in accordance with the good manufacturing practice (GMP) principles, that any procedure, process, equipment, material, activity or system actually leads to the expected results" (EC, 1992b) and that: "total validation of manufacturing processes, in order to ensure continuous conformity of batches" is required for IVMPs (EC, 1990).

Concerning the second condition, it is not only necessary to validate batch-to-batch consistency, but it must also be shown that this batch-to-batch consistency

will last indefinitely – the defect detection and reaction system must be replaced by a defect prevention system. This will consist of developing an overall quality assurance system.

The International Organization for Standardization (ISO, 1991) defines quality assurance as: "all those planned and systematic actions necessary provide adequate confidence that a product or service will satisfy given requirements for quality". EU good manufacturing practice (GMP) (EC, 1992b) adds: "Quality assurance is a wide-ranging concept which covers all matters which individually or collectively influence the quality of a product. It is the total sum of the organized arrangements made with the object of ensuring that medicinal products are of the quality required for their intended use. Quality Assurance, therefore, incorporates Good Manufacturing Practice plus other factors outside the scope of this Guide."

Regarding GMP, principles and guidelines were laid down for veterinary medicinal products (VMPs) by Directive 91/412/EEC (EC, 1991) and are explained in detail in the 162 pages of EC, 1992b, in which 16 pages are dedicated to the specific requirements of IVMPs. GMP deals with that part of quality assurance that ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use. GMP refers to "personnel, premises, equipment, documentation, production, quality control, contract manufacture and analysis, complaints and product recall as well as self-inspection". Its implementation is compulsory and subject to inspection.

What would be the use of applying GMP, implementing a rigorous quality assurance system, if this did not ensure quality, especially in the final blending stages of vaccine formulation, and thus allow the limiting of final product testing? The last steps at least should be subject to a parametric release system, i.e. they should be based on documented production data rather than on control data.

The third condition concerns the quality assurance system maintenance (Soulebot, 1992). Once implemented, the quality assurance system, like any other system, can be maintained and avoid drifting only under the influence of a double set of pressures – internal and external. Internal pressure is represented by verifications, such as self-inspections, as recommended by GMP, but also the continuous attention of top management, etc. External pressures are those exerted by competition and customers. The latter exercise direct pressure (by placing orders or not) or indirect pressure through legal authorities, audits and other official inspections. Such inspections are, in all circumstances, of the greatest importance as controls alone have proved to be insufficient (nevertheless, controls should be submitted to GMP). The application of GMP is then of prime importance. Consequently, it is particularly important that an efficient inspection system be implemented for IVMPs. The new inspection system, concerning specifically quality assurance and quality control implemented by the United Kingdom (Lee, 1992) besides GMP general inspection, is a big step towards making this evolution easier by increasing the necessary confidence of all.

CONCLUSION

By following the suggestions made in this chapter, *in vivo* potency tests could be progressively replaced by *in vitro* tests. *In vivo* tests would be used only for product development, establishment and validation of correlations and exceptional verifications. A last point must be emphasized; there are no standardized *in vitro* techniques or reference preparations in the EU, in spite of the fact that many tests are used or being developed. In order to avoid

future problems, it is particularly important that, as of now, efforts be made among Europe, the United States and other countries for the harmonization of new techniques and standard preparations.

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Sterility management and testing of vaccines and raw materials for adventitious agents

G. Blocks

Sterility management is the system that combines the continuous efforts of research and development (R&D), production, quality control (QC) and quality assurance (QA) in reducing and controlling the level of microbiological contamination of products. The principal sources of microbiological contamination are personnel, air and equipment. Therefore, to maintain the sterility of a product, the "bioburden" of the premises has also to be controlled.

The risk of infection during the production, storage, sampling and testing of a batch of product has to be controlled, monitored and documented. Production has therefore to be in accordance with current good manufacturing practice (GMP) and QC has to be in accordance with both GMP and good laboratory practice (GLP).

An important factor is that in R&D, production, QC and QA, all actions, results, findings and observations should be properly documented, i.e. clear descriptions, which are dated, signed and easily retrievable should be given.

Sterility is a built-in characteristic of the production process. During R&D of a product, special attention has to be given to minimize the risk of contamination in the production process. Special consideration should also be given to the number of aseptic handlings, the production process must be designed in accordance with the premises and utilities available and, if there are incompatabilities between the designed process and the production facilities, these should be addressed before starting production. Changes in the process or in the production facilities after the start of production are less time-effective and cost-effective than establishing a well-designed process before starting production. There is also an increased risk when producing outside the boundaries of the product licence if adaptations are made after the start of production.

One essential component of a sterility management system is the assurance that the necessary samples are collected, stored, tested and interpreted in the proper manner. Trend analyses can be performed to determine consistent problem factors but corrective actions need to be immediate and cannot wait for a trend analysis timetable.

Products that have to be sampled include: starting materials, intermediate products and final product. Utilities that must be sampled are: water systems, steam systems and the compressed air system. Premises that need to be monitored are those critical working areas where the product is in direct contact with the environment, including: surfaces, air, fingertips and the laboratories that are the background for the critical working areas. For each sampling point, test requirements have to be defined, including the sampling method, the frequency of sampling, alert levels and action levels.

Sterility is defined as the absence of microbial agents in a product. Microbial agents can be bacteria, mycoplasmata, moulds, yeasts and viruses. An indication of sterility can be obtained by performing

the appropriate tests for the absence of microbial agents on a representative sample of the batch of product.

The selection criteria for tests to demonstrate sterility have to be based on the origin and properties of the product, the possible contaminants and the species for which the product is destined.

Tests of sterility may be limited in their ability to detect live and/or inactivated microbial agents and their residues. During interpretation of test results, the following points should be considered:

- the range of species of microbial agents detected;
- minimum detection levels of live microbial agents;
- minimum detection levels of inactivated microbial agents;
- minimum detection levels of sublethally damaged microbial agents;
- sampling and storing procedures;
- QC laboratory procedures;
- the activity of antimicrobial agents.

The interpretation of test results therefore has to be based on the validity of the test procedures available for the particular product. Test results have to be compared with the results of positive and negative control samples, which should be an integral part of every test that is performed.

Special consideration should be given to metabolites of killed microbial agents such as endotoxins. These can be the cause of adverse effects in the target species and can also influence the production process.

References are given at the end of this chapter for further details of guidelines and appropriate tests.

SAMPLES

Samples can be taken from different sources. The origin of the sample influences the sampling method. It is therefore necessary to have standard operating procedures (SOPs) in which each sampling method is described. The sample should be representative of the product from which it is taken. It will be obvious that, before a sample is taken, the contents of the container must be homogenous.

Furthermore, special consideration should be given to the prevention of contamination of the sample at the time of sampling. Samples should only be taken by properly trained personnel.

GENERAL RECOMMENDATIONS ON CONTROL TESTS

For non-biological starting materials and sterilized biological starting materials tests should be made for bacterial and fungal sterility.

For biological starting materials tests should be made for: bacterial and fungal sterility; the absence of *Mycoplasma* spp.; and the viral sterility of product on sensitive cell cultures including primary cells from the species of origin.

For cell substrates tests should be made for: bacterial and fungal sterility; the absence of *Mycoplasma* spp.; and the viral sterility of the product (i.e. tests for cytopathogenic viruses, haemadsorbent viruses, specific viruses of the species of origin and specific viruses of the intended species).

For bacterial seeds tests should be made for: bacterial monoculture; and the absence of *Mycoplasma* spp.

For mycoplasmal seeds tests should be made for: bacterial and fungal sterility; and mycoplasma monoculture.

For viral seeds tests should made for: bacterial and fungal sterility; and the absence of *Mycoplasma* spp. Tests should also be performed on: primary cells of the species of origin of the virus; cells that are sensitive to viruses pathogenic for the species for which the vaccine is intended; and cells that are sensitive to pestiviruses.

On the final product to be administered parenterally tests should be made for: bacterial and fungal sterility (in the case of

a live bacterial vaccine the test for bacterial monocultures should be used); and the absence of *Mycoplasma* spp.

For the final product to be administered non-parenterally tests should be made for bacterial and fungal sterility (if the test fails, a viable bacterial count may be performed – the maximum acceptance level is one non-pathogenic organism per dose – and, in the case of a live bacterial vaccine, the test for bacterial monocultures should be used); and the absence of *Mycoplasma* spp.

TESTS FOR BACTERIOLOGICAL AND FUNGAL STERILITY OF A PRODUCT

Tests of sterility are necessary for assessing samples from a product that should be bacteriologically or fungally sterile, since they give information on the presence of viable aerobic or anaerobic bacteria and of viable fungi. Tests for sterility are described in detail in various pharmacopoeias. Based on the regulations that are in force in the country of production and in the country of destination of the product, a suitable pharmacopoeia should be selected to be used as a reference for the preferred test method.

Where sterilization is the final step in production, the validation of the sterilization process for a particular product and the documentation on the actual sterilization run give the greatest assurance of sterility.

In the case of aseptically filled products, the results of tests for bacteriological and fungal sterility must be combined with the validation of the production processes, such as filling, lyophilization and capping, and the actual batch record.

The working area in which control tests are performed should be protected against contamination at the same level as the production areas. Assurance that tests are performed under suitable conditions is derived from the validation of the tests (i.e. negative control samples) and from microbiological monitoring of the critical working areas (surface swabs, air samples and fingertips) and also from the additional negative control samples included in every test. The results of such negative control samples should be that no growth is observed.

Test methods

Two test methods – membrane filtration and direct inoculation – are described in the European Pharmacopoeia. Whenever it is possible, based on the nature of the product and on the capacities of the QC laboratory, the membrane filtration technique should be used. The membrane filtration method is preferred over the direct inoculation method because of the lower sensitivity to anti-microbial agents in the product and because it involves fewer aseptic handlings.

The suitability of a test for a particular product is derived from the validation of that test and from the positive control samples included in every test. Preparations that are used as positive control samples should consist of 100 viable microorganisms (aerobes, anaerobes and fungi).

The results of the test on the positive control samples should be that early and copious growth is observed.

Interpretation of results

Test media are observed at intervals throughout the incubation period. They are examined for macroscopic evidence of microbiological growth.

The test results can be accepted if the test is validated by the appropriate controls, i.e. if the negative control samples do not show evidence of microbiological growth and if the positive control samples do show evidence of microbiological growth.

If a product fails a test that has been

shown to be valid, an investigation must be undertaken by the QA section into the causes of the contamination. Microscopic examination of the contaminating organism should be undertaken by means of Gram's-stained slides. In the case of a product that contains inactivated bacteria, special attention must be given to the possibility of failure of the inactivation process. Only when it can be shown that the probable cause of contamination occurred during sampling or testing are the results of the re-testing acceptable, otherwise the entire batch of product must be rejected.

If a product fails a re-test that is declared valid, all the previously mentioned points regarding the reasons for failing a test should be considered. A second re-test is only acceptable if the microorganism identified is without doubt different from that isolated in the first test; if it is not, the entire batch of affected product must be rejected.

If tests on negative control samples frequently (>1 percent of the samples) show microbial growth, the test should be revalidated with special attention given to possible sources of contamination (culture media, glassware, critical working area and laboratory technique).

If tests on positive control samples frequently (>1 percent of the samples) show no growth, the test should be revalidated with special attention given to the possibility of the presence of growth-inhibiting substances in the product.

If trend analysis of the results of tests of a product show a rise in positive results, while the control samples give the expected results, investigations should be made into the cause of the contamination of the product. Special attention should be given to the production process, sample taking techniques and the quality of the containers used to fill the product and for the taking of samples.

TESTS TO DEMONSTRATE FREEDOM FROM CONTAMINATION IN PRODUCTS CONTAINING LIVE BACTERIAL OR MYCOPLASMAL STRAINS

With bacterial seeds or products containing a live bacterial strain, the standard tests to demonstrate bacterial and fungal sterility cannot be used since microbial growth will always result. The same applies to sterility tests on products containing live strains of mycoplasmata. Nevertheless, it is as important to demonstrate freedom from contamination with such products as it is with products containing inactivated organisms.

The tests used for this type of product are likely to be less sensitive than those used with inactivated preparations, making it even more important to ensure that the production conditions fully meet the requirements of GMP / GLP, that all control tests are completely validated and that attention is given to the need for microbial monitoring of the production process and the associated sampling methods.

The tests to be used need to exclude as much as possible the presence of microbiological strains other than the intended one. Such tests are based on biochemical, serological or morphological properties.

Examples of tests

Plating of samples on solid agar medium. Media to be used can be those that have general growth-promoting properties, such as blood agar in Petri dishes, or specific media, which have growth-promoting properties for a selected range of microbiological organisms.

The result of this type of test must be an early and copious growth of only one type of colony, which has the macroscopic properties of the production strain. At least five colonies should be sampled individually and fixed and stained according to Gram's method. The microscopic examination of these Gram's-stained prepa-

rations should show only one type of microorganism that has the characteristics of the production strain. Where appropriate, other staining techniques can also be applied.

Serological tests. Suitable laboratory test animals are inoculated with the test sample. During the test no clinical signs of disease may be observed other than those signs that normally occur after inoculation of the tested product.

At the time of inoculation and after an interval of three weeks (usually), blood samples are taken for serological testing. The paired blood samples should only show a rise in the level of antibodies that are correlated to the microbiological strain of the product. If evidence is found of other types of antibodies, the test should be repeated. If in the repeat test the same antibodies are detected, the product must be rejected.

The test should always include at least two animals that are not inoculated. Their paired sera are used as negative controls and, if in the sera from the control animals or from the test animals an unexpected antibody increase is observed, there is a high probability that these animals were infected during the test. Such a situation does not constitute evidence of contamination of the sample.

Biochemical tests. There are several biochemical test kits available commercially. When using one of these kits, a negative control sample consisting of a "blank product" should always be included. A blank product is similar to the product under test but does not contain the essential microbiological strain. If possible it should be sterile.

If there are consistent results with the control blank at the time of validation of the test, these values can be disregarded as background "noise". The results of the test should always be in accordance with the biochemical properties of the production strain.

If unexpected results are obtained, it will be necessary to undertake a re-test and, if in the re-test similar results are obtained, the product should be rejected.

TEST FOR THE PRESENCE OF BACTERIAL ENDOTOXINS

If a batch of starting, intermediate or finished product is negative in tests for bacterial sterility (i.e. no viable bacteria are detected) it is still possible that there are residues of inactivated bacteria. Among these inactivated bacteria are bacterial endotoxins, which can give rise to adverse reactions in vaccinated animals or can cause undesirable effects during the production process.

In some cases it is not possible to remove all of these bacterial endotoxins. In such a situation a limited amount of endotoxin may be acceptable. In all other cases, the presence of bacterial endotoxins is the result of bacterial infection and the product is therefore not acceptable. Products that should give a negative result in this test but prove positive must be re-tested and, if the re-test confirms the finding of the first test, the batch of product should be rejected.

TESTS FOR THE ABSENCE OF MYCOPLASMATA IN PRODUCT

Two tests are described in the European Pharmacopoeia. The first detects the avian strains *Mycoplasma gallisepticum* and *M. synoviae* using two different media. The other test detects the non-avian strains *M. hyopneumoniae* and *M. urealyticum*. During validation of the test, it is necessary to demonstrate the sensitivity of the test system to neutralizing agents, which may be part of the tested product. If neutralizing agents are present, a suitable inhibitory substance should be used. This inhibitory

substance must not interfere with the sensitivity of the test.

Positive controls are used to show that the test system is capable of detecting small numbers of mycoplasmata. Negative controls are included to show whether contamination has occurred during the course of the test or during other laboratory manipulations.

Special attention has to be given to products that normally contain live mycoplasmal strains. By using these tests and, where appropiate, tests capable of giving serological evidence of the presence of other strains, assurance can be given that the product is not contaminated with other strains of mycoplasmata.

For the interpretation of test results together with the results of positive and negative controls, refer to Tests for bacteriological and fungal sterility of a product (p. 423).

TESTS FOR VIRAL STERILITY OF PRODUCTS

Tests should be conducted on starting materials that contain material of animal origin and on the final product. Starting materials of animal origin include the master seed virus, the production cell system and the serum and trypsin used during the production process.

Samples that contain the master seed virus or the live virus vaccine require to be inactivated in such a manner that the vaccine strain is inactivated – without affecting any contaminating viruses that are present. Inactivation can be achieved by the use of serum with a high titre of neutralizing antibodies specific to the seed virus used to prepare the vaccine. Care has to be taken in the preparation of the antiserum that the virus used is well characterized and possibly purified by cloning. If not there is the possibility that, should there be a contaminating virus or viruses present in the original master seed, they may also be present in the antiserum prepared with the latter. If possible the antiserum should have a different source and history than the master seed.

If this method of producing neutralizing antibodies is not possible, other methods of selectively removing contaminants from the seed virus may be used.

Tests can be categorized as general tests that demonstrate the presence of large groups of viruses and tests that demonstrate the presence of a specific virus or group of viruses. Tests may also be described in terms of their ability to demonstrate the presence of live viruses or to show that the sample under test can induce the production of antibodies in test animals against the specific virus or group of viruses under consideration.

Tests for viral sterility are described in detail in several pharmacopoeias and guidelines. Based on the regulations that are in force in the country of production and in the country of destination of the product, the applicable set of guidelines and tests should be selected.

The suitability of a test for any particular product will be indicated by the original validation of the test (i.e. the results from positive control samples) and also from the positive control samples included in every subsequent test. In tests that give evidence of the presence of live viral agents, the test cultures are observed at intervals during the incubation period and at its conclusion. Test cultures are observed for both macroscopic and microscopic evidence of viral growth. The test is valid and the results can be accepted if the negative control samples do not show evidence of viral growth and if the positive controls do show evidence of typical viral growth.

If a product fails a test that is declared valid, an investigation must be made by the QA section into the causes of the product failure. Identification of the virus strain isolated should be made using a

suitable method. Only when it can be shown that the probable cause of contamination occurred during sampling or testing, is a re-test acceptable, otherwise the batch of product must be rejected.

If a product fails a valid re-test, the batch of product must be rejected.

If tests of negative control samples frequently (>1 percent of the samples) show the presence of virus, the test should be revalidated with special attention given to possible contamination sources (i.e. starting materials, critical working areas and laboratory techniques).

If tests of positive control samples frequently (>1 percent of the samples) indicate the absence of virus growth, the test should be revalidated.

If trend analysis of the tests of a product show a rise in positive results, while the control samples give the expected results, investigations should be made into the cause of the contamination of the product. Special attention should be given to the production process, sample taking techniques and the quality of the containers used to fill the product and for the taking of samples.

BACTERIOLOGICAL MONITORING OF SERVICES

Only the minimum microbiological monitoring of services is described here. All other types of analyses on these systems are not included. Depending on the production site and on the regulations that are in force, an extended programme of bacteriological monitoring may be advisable.

In the case of production problems related to bacteriological sterility or the validation of a new or modified production process, an extended programme is also advisable. Such programmes may consist of a higher frequency of sampling, the sampling of additional work areas and the use of other specific culture media, etc.

Some services provide basic requirements or elements of the process and, with respect to the clean steam and compressed air systems, their end products may come in direct contact with the biologicals that are produced, up to the final stages of production.

The services that have to be monitored for microbiological contamination include the main water supply system, the purified water (PUW) system, the water for injection (WFI) system, the main steam system, the clean steam system and the compressed air system.

Services should be checked on a regular basis, preferably every week, for the number of viable microorganisms present and for the level of degradation products of microorganisms such as endotoxins.

The standards for PUW and WFI are defined in various pharmacopoeias. Condensates of clean steam and compressed air have to be in accordance with the requirements for WFI.

Tests should be made on samples taken from sampling points that are close to the point of usage in the system.

If a sample does not fulfil the requirements, another sample should be tested. If this sample also fails to fulfil the requirements, an investigation should be made into the cause of the contamination. From the results of this investigation corrective measures such as sterilization and/or maintenance of the system should be instituted.

BACTERIOLOGICAL MONITORING OF PREMISES

Effective SOPs should be written to describe the rooms that are sampled, the sampling points, the sampling techniques, the frequency of sampling and the time at which samples are taken. For air samples, the volume of air sampled should be prescribed and for surface swabs the number of square inches/centimetres of

surface to be sampled should be prescribed.

The reasons for the bacteriological monitoring of premises are:

- to check on the effectivenesss of previously validated sanitary, disinfection and sterilization procedures – samples should be collected by using surface swabs or contact agar plates and air samplers. The frequency of testing should be once a month for clean rooms and once every two months for other areas that are microbiologically monitored;
- to check on contamination levels during production.

Samples should be taken at critical working points such as: air (settle plates), surfaces (swabs or contact plates) and fingertips (contact plates). Furthermore, samples (swabs or contact plates) should be taken from the environment of critical working areas (e.g. clean rooms and QC laboratories).

The following are recommended frequency levels for testing:

- *critical working areas* (usually a laminar airflow cabinet) using settle plates should be tested continuously during production. (It is recommended that plates should be changed every two hours, depending on how fast they dry out which is mainly dependent on the composition and thickness of the medium in the plate);
- *surfaces* should be tested once per production day;
- *fingertips* should be tested once per working day for every laboratory technician.
- surfaces in *the critical working area environment* should be tested once every week.

Sampling methods should be validated and special attention should be given to the detergents, disinfectants and sterilizing agents used in the production process since residues of these can decrease the viability of microorganisms in the various samples taken for testing. Where this is the case, neutralizing agents for these residues should be used.

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