Biologicals: test procedures available to assess components and products, with limitations

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Summary: Contamination with a pathogen, residual live organisms in inactivated products, and a lack of safety of live products may be considered to constitute the greatest risks associated with the use and the international movement of biological products. Although tests are conducted to monitor the risks, the limitations of such tests must be recognised and steps should always be taken to minimise and, as far as possible, avoid the risk. Tests must be suitably sensitive and specific for their function, but they must also be practical and reproducible. Studies on the sensitivity of tests provide a measure of their limitations.

The author describes points to consider for the assessment of the suitability of different test methods. Emphasis is placed on tests for parameters associated with risk, such as extraneous agent testing. Examples of such analyses are discussed. There is a need for an exchange of information and a scientific debate on such issues if international acceptance of test methods and harmonisation of requirements are to be achieved.


INTRODUCTION

The term ‘biologics’ is often used to refer to immunological products such as vaccines and antisera, but it can also be applied to any product manufactured using materials which are biological in origin, such as in vitro diagnostic kits. Many of the principles relating to the risks associated with international trade in biologicals apply to all such products.

Biological components and products are subjected to a vast range of tests, each of which is designed to check the quality, safety or efficacy of the product.

At the national level, all aspects of safety, quality and efficacy have to be considered, and risks must be considered in relation to the benefits to be derived from the use of a product. Nevertheless, the following may be considered to constitute the greatest risks associated with the use and the international movement of biological products:

- contamination with a pathogen
- residual live organisms in inactivated products
- lack of safety of live products.

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For this reason, the testing designed to provide assurance on these aspects can be considered to be the most important for international trade. This paper will therefore be restricted to consideration of these three main areas.

Although tests are performed to monitor risks, such tests have limitations, and steps must always be taken to minimise and avoid risk, as far as possible. There is always a need to ‘build in’ quality, by using good quality starting materials and production procedures designed to minimise the risks: this reduces reliance on quality testing. This has been the approach in the United Kingdom and the rest of Europe for many years, and the relevant European Commission Directives (2, 3) and guidelines (4) reflect these ideas.

**SAFETY TESTING OF LIVE VACCINES**

The dossiers supporting applications made to any Member State in the European Union (EU) for marketing authorisations for vaccines must contain information on the results obtained from tests conducted to comply with the requirements laid down in Directive 81/852/EEC, as amended (3).

For safety studies, a series of specific laboratory tests must be conducted, including tests for the safety of administration of a single dose, an overdose and repeat administration of a single dose. These tests must be performed using vaccine of the highest titre and least attenuated passage level in susceptible animals, usually of the youngest age, and in pregnant animals (if appropriate). The studies must provide data on the safety of all recommended routes of administration.

In the case of live vaccines, the following studies are required:

- possibility of the spread of the vaccinal organisms
- dissemination of the organisms in the vaccinated animal
- possibility of reversion to virulence on passage between animals
- biological properties of the vaccinal strain (e.g. neurotropism).

The possibility of recombination or genomic reassortment in the wild must also be considered.

The applicant must also address the following issues:

- safety for reproductive performance
- immunological function in the target species
- possible interactions with other products
- potential for residues in food.

These laboratory tests are designed to demonstrate the safety of the product through tests using worst-case conditions.

Laboratory tests have certain limitations, however. First, the animals are kept under ideal conditions. Second, only one breed or strain of animal is usually available for such work. It is therefore expected that field trials will be performed to complement the laboratory studies and provide supporting data.

Field studies can provide information on product use in a relatively large number of animals and different breeds or strains, under practical conditions. The effect of
intercurrent disease, different husbandry practices and the incidence of side-effects, such as post-vaccinal secondary infections (e.g. after spray vaccination), can be monitored and assessed. Unexpected effects on the environment may be observed.

The usefulness of field studies can be limited if the investigator takes insufficient care to obtain data and document the effect of vaccination. It is sometimes assumed that no complaint means no adverse reaction, but this assumption is unacceptable if no other field data are available. Data should be available from detailed follow-up studies in a proportion of vaccinates.

In addition to conducting laboratory and field trials, the applicant is required to provide an assessment of the potential harmful effects which the product may have on the environment.

For many products, if a sound data package is established to demonstrate the safety and the risks from use of the product as recommended, with studies and tests conducted as described above, the information should be applicable for consideration of the risks associated with use of the product throughout the world.

The following criteria, however, may lead to a requirement for additional safety testing, if there is a proposal to extend marketing to a region where the initial trials were not conducted:

a) There may be a different range of breeds in an area, and if it is suspected that one or more of these may be particularly susceptible to the antigen or adjuvant, it may be wise to conduct a small additional field trial.

b) There may be a particular intercurrent infection in the area which may necessitate trials in infected animals, or demonstration of compatibility with another vaccine administered in the region at the same time.

c) Where there are marked differences in husbandry between the location of the initial field tests and the proposed new market, specific testing may be necessary.

d) There may be a particular environmental risk, the extent of which may need to be assessed.

A good basic safety data package should provide sufficient data to minimise the extent to which such additional safety testing is required when extension of markets is proposed.

INACTIVATION

Regulatory authorities and manufacturers wish to be assured that, where appropriate, inactivation of products has been successful. In the context of international trade, this is particularly true when the agent to be inactivated is a more virulent strain than that which naturally occurs in the importing country, or an exotic pathogen.

The limitations in the methods available for detecting live organisms, particularly when testing the final product, are often compounded by the presence of residual inactivant. Therefore, even when tests for inactivation are conducted, it is very important to avoid problems by ensuring, as far as possible, that the inactivation process is satisfactory.
The procedures and check test methods for immunological products to be sold in the EU are described below.

After addition of the inactivant, the vessel should be inverted or shaken, or the material should be transferred to a second vessel, to ensure that all live organisms are in contact with the inactivant.

The inactivation process must have been validated. To provide a safety margin, inactivation incubation is expected to be 33% longer than the time demonstrated to be necessary for inactivation.

Validation data must be obtained from a study relevant to the inactivation process to be used during manufacture. The study can be performed on a small-scale pilot batch, providing that the test design takes into account a number of features of the production process, such as the normal titre of harvested material. Samples must be taken at intervals sufficiently beyond the point where negative samples are obtained, and a sufficient number of samples must be taken around the critical point where negative samples start to occur. Another point which should be addressed, if relevant, is whether or not residual inactivant has an inhibitory effect on the sensitivity of the system used to detect live organisms.

Confirmatory inactivation testing of each batch of product is then expected at two stages. First, the inactivated harvest should be checked immediately after the inactivation process, to ensure that the procedure has been successful. A further test is then applied to the finished product or, if an adjuvant is present which does not permit a suitable test to be performed, the test is conducted as late as possible in the production process (usually immediately before adding the adjuvant). This provides assurance, for example, that only inactivated antigens have been blended.

All these tests need to be sufficiently sensitive to detect small numbers of live organisms, and they need to be validated. In EU guidelines (4) and European Pharmacopoeia monographs (5), the basic elements of the recommended method are as follows:

- an appropriate quantity (e.g. the equivalent of 10 doses or 1.0 ml of antigen) should be tested in a suitably sensitive in vitro test system
- the test should include one passage of the material.

It is the responsibility of the manufacturer to validate the system used.

In addition to ensuring that the inactivation process is performed thoroughly, the production process must be conducted under conditions of ‘good manufacturing practice’ (GMP) (2). This should ensure complete separation of live and inactivated organisms in the factory.

With such controls over inactivation, there should be no need for concern regarding residual live organisms in international trade of inactivated biological products.

**TESTS FOR EXTRANEOUS AGENTS**

The possibility of importing a pathogen in a biological product (e.g. vaccine, diagnostic kit or reagent) is of great concern to national authorities, and this appears to be the main reason for the ban on importation of biological products in some parts of the world.
A vast number of potential test systems exist for detecting extraneous agents but – as with all other areas of testing – sensitivity and reliability are limited. As it is impracticable to test the whole batch of a substance, it may be possible to obtain a negative result from a contaminated product because the sample was not truly representative. Also, the test system used for contamination screening may not be sufficiently sensitive to detect a small number of contaminants, or the tests applied may have been inappropriate for detecting the contaminant in question, as there is a tendency to look only for organisms about which something is known.

Therefore, all steps must be taken to assess and minimise the risks of products being contaminated with extraneous agents.

Consideration of the risks from extraneous agents must be based on a consideration of the consequences of such contamination. These consequences depend on both the agent and the proposed uses of the potentially-contaminated substances. In some cases, the consequences are such that the risk of contamination must be reduced to as near zero as possible, while in other cases some degree of risk can be tolerated.

For example, in the case of highly contagious and infectious exotic notifiable diseases, such as foot and mouth disease, the consequences of contamination with the causal organisms are considerable even if the organisms have been inactivated. For pathogens such as these, it is therefore important to minimise the risk by obtaining substances from a disease-free source, rather than relying on testing of the substance or product. Testing will still be required on some occasions, but this will be performed as required, on a national or regional basis, to confirm freedom from the disease.

In the case of organisms which are less readily transmitted (e.g. bluetongue virus), a greater risk of contamination may be acceptable. For such organisms, assurance of freedom from contamination might be based on acceptance of tests for freedom from the organism, with or without an inactivation procedure applied to a potentially-contaminated substance (e.g. serum). In this case, the use of the substance must be considered (e.g. serum for production or master seed) before a decision can be taken on the acceptability of a particular proposal.

During the manufacture of biological products, contamination with extraneous agents may occur from a wide range of potential sources. Some of these are described below.

**Contamination in the factory**

Contamination can occur at any point in the production cycle, from the starting substance to the final product. This risk must be minimised through the application of GMP (2); adherence to such principles eliminates the risk of cross-contamination at the production site.

**Contaminated starting materials**

*Virus and bacterial seeds*

A seed lot system should be used for viruses and bacteria, and the seeds should be tested for purity as thoroughly as possible, to minimise the risk of contaminating each batch.

*Cell substrates*

A seed lot system should also be used for cell substrates, and the seeds should be tested as thoroughly as possible to demonstrate the absence of extraneous agents and thus minimise the risk of contaminating each batch.
The use of primary cells presents a risk of contamination with each batch of cells, and their use should be avoided wherever possible. When the use of primary cells is unavoidable, a well-tested specific pathogen-free (SPF) source should be used. For poultry vaccines, however, primary tissues such as eggs may be used and, in the case of inactivated vaccines, a healthy non-SPF source may be used for production of batches of vaccine, providing that the inactivation process has been shown to inactivate any likely contaminants.

Substances of animal origin

Substances of animal origin (e.g. serum) also present a risk of contamination with each batch, and their use should be kept to a minimum.

As far as possible, substances of animal origin should be obtained from animals of known disease status. This is usually achieved by sourcing from countries which are free from major exotic pathogens, such as foot and mouth disease virus. As indicated above, for more ubiquitous organisms or those which are less hazardous, it may be acceptable to achieve freedom from live extraneous agents through the use of a validated inactivation process and/or by testing the substance for the agent and rejecting any batches from which samples are found to be positive.

In summary, under some circumstances there may be a number of options for the stage at which tests may be conducted, but in the manufacture of most biological products testing of materials is necessary. A single one-off test may be sufficient (e.g. for seeds), or testing may be performed on a batch basis (e.g. serum or final product) or as part of a validation study.

The consequences of a particular contamination with an extraneous agent depend on the agent and the proposed use of the substance; these same factors limit the choice of test used to detect the agent.

All tests used must be suitable for the purpose. This means that tests for extraneous agents should be sufficiently specific, sensitive, reliable and reproducible.

TEST SPECIFICITY

A highly specific test may be required under some circumstances (e.g. for an identity test) but care must be taken when testing for extraneous agents, to ensure that the test is not too specific.

For example, one of the more recently developed systems for detecting microorganisms is hybridisation with DNA probes. Kwang et al. (6) studied the use of four deoxyribonucleic acid (DNA) probes derived from a cytopathic strain of bovine virus diarrhoea virus, in a dot blot assay for detection of these viruses. Two of the probes detected most of the cytopathic strains studied, but non-cytopathic strains were detected much less successfully.

Similarly, if a serological test is being conducted for the detection of antibodies or antigens where the agents can exist as one of a number of serotypes, it is necessary to ensure that a group-specific reaction will be detected. Otherwise, a test must be performed for each serotype. One example of this is the detection of antibodies to avian adenoviruses in SPF flocks.
TEST SENSITIVITY

Highly sensitive tests are required.

Staff at the Central Veterinary Laboratory in Weybridge, United Kingdom, have performed various studies on the sensitivity of different test systems for a range of avian pathogens (7, 8, 9). An approach similar to that described by Nicholas et al. (7) may be used. These authors compared the sensitivity of various test systems for detecting avian bronchitis virus in an experimentally-contaminated Newcastle disease vaccine. *In vitro* detection systems were found to be more sensitive than the use of chickens (7).

Wilcox et al. (11) concluded, from their studies of the sensitivity of serological tests, that their microneutralisation test and enzyme-linked immunosorbent assay (ELISA) were of comparable sensitivity in the detection of antibodies against infectious bronchitis, while the agar gel precipitation test was less sensitive.

Increasing numbers of papers are now being published on the sensitivity of hybridisation techniques and polymerase chain reaction for the detection of microorganisms or viruses. Noteborn et al. (10) have described a sensitive technique for detecting cells infected with chick anaemia virus, and are currently comparing the sensitivity of various detection systems.

TEST VALIDATION

Whatever method is used, it is important to ensure that the test will achieve its function in the test laboratory and that the sensitivity and specificity are reproducible. For example, Nicholas et al. (8) studied the reproducibility, as well as the sensitivity, of three serological tests for antibodies to infectious bursal disease virus. It was concluded that the ELISA test gave the best overall results.

Nicholas et al. (8) also indicated the need to test standard preparations on most occasions. In fact, in most test systems, positive and negative controls must be tested in parallel to demonstrate the validity of the assay.

A neutralising serum may be needed for testing master seed viruses. It is necessary to produce this with a pure antigen, rather than deriving it from the master seed, and to demonstrate that it does not neutralise any extraneous agents which may be present in the test substance and which the tests are designed to detect.

AGENTS OF CONCERN

It is obviously impracticable to conduct tests for all possible contaminants in all materials. In the EU, it is considered necessary to examine the risks of contamination with pathogens of the source species and the target species for the product, but it is occasionally necessary also to consider the risks for in-contact animals, humans and the environment.

INFLUENCE OF THE AUTHORITIES

In conclusion, tests must be conducted on the sources of starting materials, on the starting materials themselves, and/or on the intermediate or final product, to detect extraneous agents which are potentially hazardous. The methods of testing must be
suitably sensitive, specific, reproducible and reliable. The manufacturer must perform these tests to his/her own satisfaction, and that of the regulatory authorities of the countries where the product is to be marketed.

The European Pharmacopoeia (5) and EU guidelines (4) provide some guidance on test systems likely to be acceptable to authorities in the EU. For example, the European Pharmacopoeia sets out requirements for testing SPF flocks and detailed methods for some testing of avian vaccines for extraneous agents. EU guidelines for mammalian vaccines describe general tests which can be used for screening and will detect a range of agents. This is complemented by a table of tests for extraneous agents in mammalian vaccines, which indicates that specific tests are needed for some agents.

In the United States of America, equivalent guidance is included in the Code of Federal Regulations (1).

When proposing to sell a product in the EU, the onus is on the manufacturer to demonstrate that the most suitable testing regime for extraneous agents has been used, and that scientific and technological developments have been taken into account. It can be very difficult, therefore, for a manufacturer to comply with the requirements of different authorities world-wide, in view of the range of test methods available (the acceptability of inactivation versus testing, for example, for some agents in some substances) and the need to pay heed to guidance notes.

It is clear that there is much to be gained from international harmonisation of risk assessment and agreement on appropriate test procedures for extraneous agents. If this is to be achieved in a manner which is considered satisfactory by the authorities and manufacturers, data will be required on the merits of different options, so that conclusions can be reached on the basis of science.

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VALIDITÉ ET LIMITES DES ESSAIS DESTINÉS À ÉVALUER LES PRODUITS BIOLOGIQUES ET LEURS COMPOSANTS. - A.M.T. Lee.

Résumé : La contamination par un agent pathogène, la présence de micro-organismes vivants résiduels dans des substances inactivées et l’absence d’innocuité des produits à micro-organismes vivants constituent les principaux risques liés à l’utilisation et aux échanges internationaux de produits biologiques. Or, force est de constater que les essais effectués afin d’évaluer les risques ont des limites, et que des mesures doivent être prises pour réduire, autant que possible, ces risques au minimum. Les tests doivent être non seulement suffisamment sensibles et spécifiques, mais également pratiques et reproductibles. Des études sur la sensibilité des épreuves en ont démontré les insuffisances.

L’auteur décrit les critères permettant d’apprécier la pertinence des différentes méthodes d’essai. Il met notamment l’accent sur les tests applicables aux facteurs de risque, tels que les tests portant sur les agents extérieurs. Des exemples de ces analyses font l’objet de la discussion. Il faut promouvoir des échanges d’information et des débats scientifiques sur toutes ces questions si l’on veut aboutir à l’homologation des méthodes d’essai et à l’harmonisation des normes au plan international.
PROCEDIMIENTOS DE PRUEBA DISPONIBLES PARA LA EVALUACIÓN DE COMPONENTES Y PRODUCTOS BIOLÓGICOS, Y SUS LIMITACIONES. – A.M.T. Lee.

Resumen: La contaminación con un agente patógeno, la presencia de organismos vivos residuales en productos inactivados y la falta de seguridad de los productos vivos constituyen los mayores riesgos asociados al uso y al movimiento internacional de productos biológicos. Pese a la práctica de pruebas para controlar los riesgos, es preciso reconocer las limitaciones de las que tales pruebas adolecen, y actuar siempre en consecuencia para minimizar y, en la medida de lo posible, eliminar el riesgo. Es necesario que las pruebas sean lo bastante sensibles y adecuadas a su función, pero también prácticas y reproducibles. Los estudios sobre la sensibilidad de las pruebas proporcionan una medida de sus limitaciones.

El autor describe los aspectos que se han de tener en cuenta en la evaluación de la idoneidad de los distintos métodos de prueba. Hace especial hincapié en las pruebas para parámetros ligados al riesgo, tales como los tests de detección de agentes extraños. Se comentan algunos ejemplos de estas técnicas analíticas. Un mayor intercambio de información, así como un debate científico centrado en estas cuestiones, son condición necesaria para alcanzar un acuerdo internacional tanto sobre los métodos de prueba como sobre los requisitos que han de cumplir dichas pruebas.


REFERENCES


