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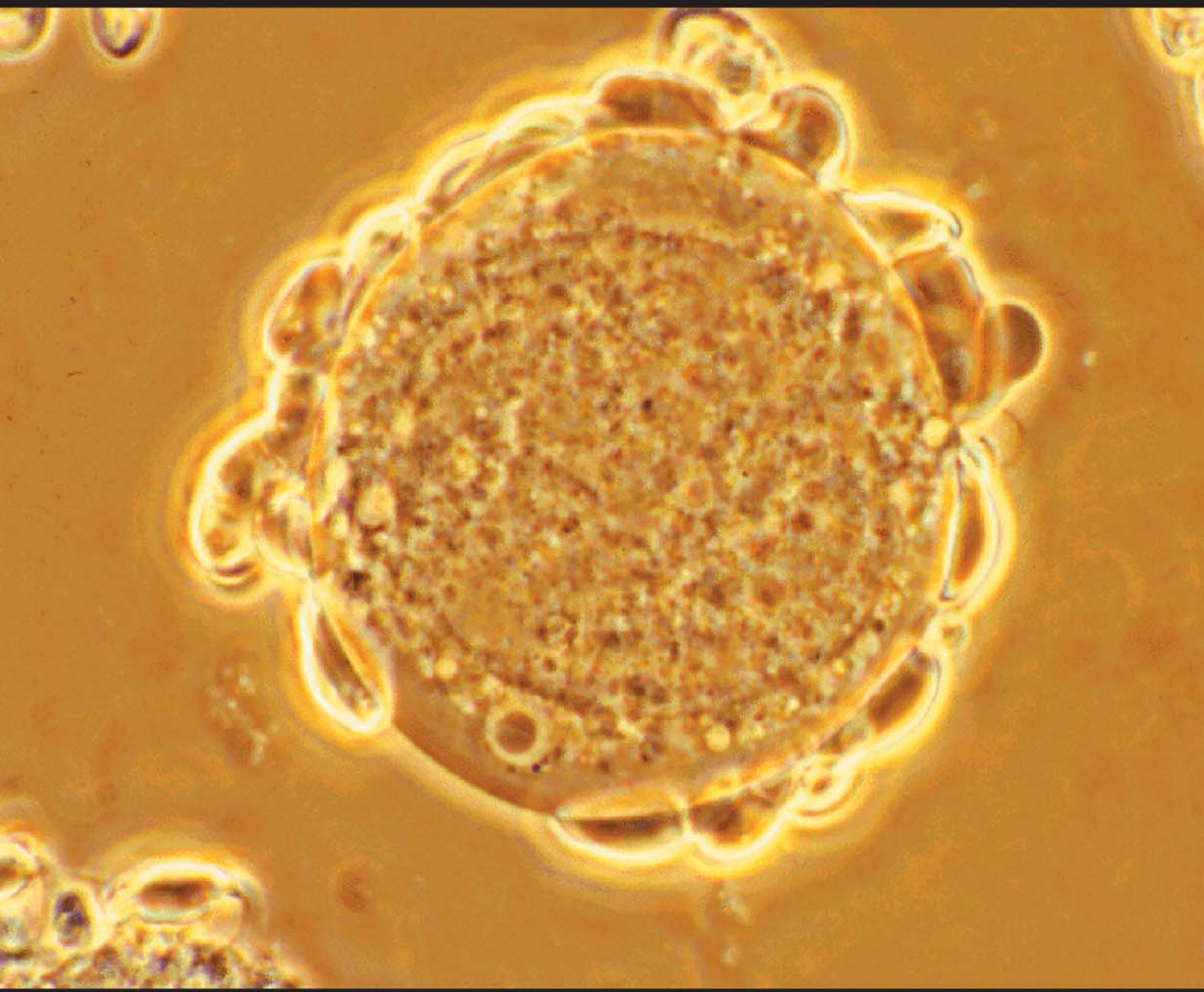

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Emerging Virus and TSE Issues for Bovine-Derived Raw Materials Used in the Manufacture of Biological Products

BY MERRIBETH J. MORIN

The use of animal products, such as bovine serum, in the manufacture of biologics is a common practice. The United States' Code of Federal Regulations, part nine (9CFR), dictates mandatory testing for viruses.[1] In the last few years, we have learned that certain viruses undetected in industry standard tests, like the 9CFR assay, can cause significant contamination of bovine products. Last year in Europe, new guidelines from the Committee for Veterinary Medicinal Products (CVMP), and draft guidelines from the Committee for Proprietary Medicinal Products (CPMP), were published to address testing requirements for bovine serum. The use of these European guidelines for bovine serum testing broadens the ability to detect viral contamination.

Even with the broader tests of the CVMP and CPMP, there are additional concerns for contamination by bovine viruses that are not detected by standard tests. In Europe, there is particular concern over *Bovine polyomavirus* (BPyV), which is a frequent contaminant of fetal bovine serum, and is potentially a zoonotic virus.[2,3,4] Similarly, the circoviruses have caused concern because they are ubiquitous in products such as porcine trypsin, and are highly resistant to inactivating treatments like irradiation.[5] Circoviruses have been identified in other species, including cattle, and raise new issues for the safety of bovine serum. An awareness of the vulnerability of animal products to vector-transmitted viruses was prompted by several incidences of catastrophic fermenter failure associated with the bunyavirus, *Cache Valley virus*. The virus entered the fermenters via serum of US origin, and was not detected by conventional virus tests. New systems have been developed to detect bunyaviruses in raw materials.

The increasing awareness of bovine viruses by European regulatory authorities is paralleled by concerns over bovine spongiform encephalopathy (BSE). European regulatory authorities now require certification of bovine products used in the manufacture of licensed products. A commission directive, issued in September 1999, states that the manufacture of any medicinal product must comply with the *Note for Guidance on minimizing the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products* and its updates.[6,7] All European marketing authorizations must now demonstrate compliance with this directive.

Testing of bovine serum according to 9CFR

The 9CFR test is a standard test performed for bovine serum and other animal-derived products. The test requires that monolayers of Vero cells, and a cell type of the same species of origin as the raw material, are prepared and maintained in media containing the ingredient under test. The cell cultures are maintained for at least 21 days and observed for any cytopathic effect. The cells are subcultured at least twice, and at least seven days after the last subculture, endpoint assays are conducted to detect viruses.

The 9CFR test is specific for the size of the monolayers to be used throughout the assay. All monolayers have an area of at least 75 cm^2, except for the last subculture, where minimum area requirements are at least 6 cm^2 each for the cytopathogenic, hemadsorbing, and immunofluorescence tests.[8,9]

The endpoint assays include both general and specific tests for viruses. The general test includes observations for any cytopathogenic and hemadsorbing agents. A change in the cytopathology of the cells, or the observation of red blood cells adhering to the cultures, indicates the presence of an adventitious agent, and would deem the raw material unsuitable for use. For the specific test, the fluorescent antibody technique is employed to test for a number of viruses. The list of viruses in the current 9CFR guidelines for bovine serum testing includes *Bovine viral diarrhea virus* (BVDV), *Bluetongue virus* (BTV), *Bovine adenovirus* (BAV), *Bovine parvovirus* (BPV), *Bovine respiratory syncytial virus* (BRSV), *Reovirus*, and *Rabies*

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virus. [Although *Parainfluenza virus type 3* (PI3) and *Infectious bovine rhinotracheitis virus* (IBRV) can be detected in the general test, they can be included, although not required, in the specific test using immunofluorescence.] Any observation of specific fluorescence will deem the material unsuitable for the preparation of biological material.

European guidelines for virus testing of bovine serum

EMEA/CVMP/743/00 - Final

The Committee for Veterinary Medicinal Products (CVMP) of the European Agency for the Evaluation of Medicinal Products (EMEA) published a guideline in January 2002 regarding the testing of bovine serum.[10] This guideline applies to bovine serum used for production of vaccines for all species of target animals. While reduction of bovine serum use is encouraged, it is understood that serum is still an essential ingredient for many cell cultures. The guideline describes the tests that need to be performed before and after inactivation of serum lots. It does not prescribe who should perform the testing; however, it is the manufacturer who is responsible for ensuring that the testing is done. Although the guideline strongly recommends that the manufacturer conduct the testing, the serum supplier or a contract laboratory can perform it.

The adventitious virus testing should include both general and specific tests on the serum prior to inactivation. These tests must be capable of detecting BAV, BVDV, Parvovirus, BRSV, Reovirus, PI3, IBRV, and viruses such as BTV that are exotic to Europe. The tests can utilize detection methods such as PCR and RT-PCR. If these detection methods are utilized, they must be validated and have a sensitivity and specificity equivalent to that in published reports. Further, the applicant will need to demonstrate whether any nucleic acid detected in these tests arose from infectious virus.

In addition to the above virus tests, a test is included specifically to detect BVDV prior to inactivation of the serum. Cells that are sensitive to pestiviruses are utilized in the test, and are subcultured four times in media containing the serum under test. At the end of the culture period, the cells are tested for BVDV using an immunofluorescence test. Immunofluorescence is an effective detection method, as not all strains of BVDV are cytopathic. Alternatively, a PCR-based technique can be used for BVDV detection. However, it must be validated and a procedure should be in place to determine whether any nucleic acid arose from infectious particles. If any infectious BVDV is detected, the amount present must be at a level that has been shown to be sufficiently inactivated by the validated inactivation method. Further, if detected prior to inactivation treatment, the serum must be tested for infectious BVDV again after inactivation.

The guideline also prescribes tests for the detection of BVDV antibodies in serum. A specific procedure is not described, but it should incorporate validated techniques. Antibodies should not be detected in the serum, or if antibodies are present, they must be at a level that does not interfere with the ability to detect BVDV. The validation of the antibody detection procedure should include a limit of detection test.

In addition to the antibody detection test, comparative titration tests should also be performed. These tests are performed by culturing cells sensitive to pestiviruses for three subcultures in test serum. After passaging, the cells are set up to determine the titer of a reference BVDV stock. The titer generated is compared to the reference titer of BVDV generated from cells passaged in control serum. The passaging of the cells in the test serum, prior to performing the titration, ensures that the cells are under the optimal conditions to detect BVDV.

The antibody and comparative titration tests expand the ability to detect viruses, as these tests can determine if the serum has any inhibitors of virus detection. If BVDV is detected in the serum prior to inactivation treatment, these tests should be conducted on the serum prior to inactivation. If BVDV is not detected prior to inactivation, these tests can be performed after inactivation treatment.

The guideline also addresses the inactivation of bovine serum. It states that it is "absolutely necessary" to inactivate bovine serum, as vaccine production can be contaminated by viruses in bovine serum. The inactivation procedure must be validated and must demonstrate consistency and efficiency. The viral clearance validation of the inactivation treatment should include viruses representative of different virus families with varying degrees of resistance to inactivation. The study should be conducted using viruses such as BVDV, IBRV, BAV, and *Porcine parvovirus*, and a test for pestivirus inactivation must also be included. The guideline recommends gamma irradiation for inactivation treatment, as this treatment has been shown to be effective while retaining the biological properties of bovine serum.

EMEA/CPMP/BWP/1793/01 - Draft

The Committee for Proprietary Medicinal Products (CPMP) of the EMEA has drafted a guideline for the testing of bovine serum.[11] This draft guideline applies to serum used in the manufacture of vaccines and biological products intended for human use. As with the CVMP guideline, reduction of bovine serum use is encouraged with the knowledge that serum is still an essential ingredient for many cell cultures. The guideline is currently in draft, but will probably come into operation in early 2003.

The draft guideline describes virus tests that need to be performed before and after inactivation of serum lots; but as with the CVMP guideline, it does not dictate who should perform the testing. The manufacturer is responsible for ensuring that the testing is done, but serum suppliers, or contract laboratories, can perform the testing. Of primary importance is the traceability of the serum from origin to final container.

The draft guideline prescribes general and specific tests for viral contaminants in bovine serum batches prior to inactivation (but after sterile filtration). The test should utilize at least two dif-

ferent cell lines, of which at least one is of bovine origin. The cell lines should be suitable for virus detection in both general and specific tests. General tests are undertaken to detect cytopathic viruses and hemadsorbing viruses (e.g.: IBRV and PI3, respectively). Specific tests are performed to detect BTV, BAV, BPV, BPyV, BRSV, BVDV, Reo3, and Rabies virus; noting that the list of viruses may need to be expanded, depending on any current epidemiological considerations. The list of viruses for specific tests may be shortened if the general tests will detect any of the listed viruses. The detection of a viral contaminant, except for BVDV, will render the serum unsuitable for use in the production of biologicals intended for human use.

As with the CVMP guideline, the draft CPMP guideline includes specific recommendations for BVDV. As the presence of BVDV in serum cannot be entirely prevented, a test to detect and quantify BVDV is required before inactivation treatment. If it is detected, the test is repeated after inactivation. A serum batch can only be used if no infectious BVDV is detected after inactivation.

As anti-BVDV antibodies could be present in batches of pooled serum, there is concern about potential inhibition of BVDV detection. The draft CPMP guideline recommends testing for anti-BVDV antibodies using validated techniques. When determining the amount of BVDV detected, the amount of antibodies should be taken into account. As with the CVMP guideline, a comparative titration test should also be performed to determine if the serum has any inhibitory effect on BVDV replication. Cells sensitive to BVDV are cultured in media containing the test serum, and then used to determine the titer of a reference stock of BVDV. This test is performed after the serum has been inactivated

The draft CPMP guideline for bovine serum strongly recommends virus inactivation treatment. The method of inactivation must validated and shown to be effective. If non-inactivated serum is to be used, there must be justification for its use.

Additional viruses of concern in raw materials of animal origin

The 9CFR test for bovine viruses, and the tests prescribed by the CVMP and CPMP, are able to detect a broad range of viruses. But there are additional viruses of concern that could contaminate bovine-derived raw materials. These include, but are not limited to, BPyV, bunyaviruses, and circoviruses. Testing for BPyV is in the draft CPMP guideline for bovine serum testing, but not in the current 9CFR or CVMP testing requirements.

BPyV is a member of the family, *Polyomaviridae*. Members of this virus family are oncogenic, as they can transform cells in culture (e.g.: SV40) and cause tumors in mice (e.g.: mouse polyomavirus). As BPyV is widespread in cattle, it is a potential contaminant of bovine serum. In one published study, BPyV was found as a contaminant in 70% of the commercial fetal bovine serum batches tested.[2] Another study produced similar results, with 66% of the tested serum batches giving positive results for BPyV.[3] In the latter study, commercial animal vaccines were also tested for BPyV with two of the 14 vaccines testing positive. BPyV is also of concern, as there is evidence that it may be zoonotic.[4] Antibodies were found in people who have regular exposure to cattle, such as farmers, abattoir workers, and veterinarians; thereby providing evidence that the virus may cross the species barrier. The current effective and reliable methods for screening bovine serum for BPyV are PCR-based, however, and an infectivity test is also useful to determine whether signals might have resulted from infectious particles.

Bunyaviruses, such as *Cache Valley virus,* are transmitted within species and across the species barrier by arthropod vectors, making the prevention of transmission difficult. There is also evidence that once a host is infected, the

virus can be transmitted across the placenta to infect the fetus.[12] This transmission of infection raises the concern for contamination of fetal bovine serum batches. There have been incidences of fermenter crashes that ultimately were determined to be caused by a bunyavirus. In at least two cases, different lots of serum had been used, and although 9CFR tests had been performed on the serum, the virus escaped detection. PCR is a more sensitive test for bunyaviruses than infectivity, and can be utilized to test bovine serum. Additionally, testing sub-lots of bovine serum can help prevent the introduction of bunyaviruses into production.

Circoviruses can be found worldwide and across a number of animal species, including humans. *Porcine circoviruses* (PCV) are contaminants of porcine materials, including trypsin. There are two biotypes of PCV: type 1 and type 2. PCV-1 is generally considered non-pathogenic, whereas PCV-2 is associated with post-weaning multisystemic wasting syndrome (PMWS), a serious disease of young pigs. Circoviruses have also been found in cattle, which raises the concern of circovirus contamination in bovine products.[13]

Of particular concern is that circoviruses are of the smallest and most resistant of the known mammalian viruses. The diameter of circovirus particles is approximately 17nm, which enables it to pass through many nanofilters. Further, gamma irradiation was shown to be ineffective for the removal of circovirus infectivity.[5] Only a tenfold reduction of infectious titer was observed when PCV-2 spiked serum was irradiated. PCR technology is currently an effective method to detect circovirus contamination, although an infectivity assay will be useful to determine if any particles detected by PCR are infectious.

There is also continuing concern for raw material contamination with transmissible spongiform encephalopathies (TSE). In Europe, manufacturers must now demonstrate that their products were manufactured according to the Note for Guidance on minimizing the risk of transmitting animal spongiform encephalopathy agents via medicinal products (commission directive 1999/82/EC).[6] All new European market applications after 01 July 2000 must have complied with the directive, whereas products already approved must have complied by 01 March 2001. Manufacturers may provide all of the required information in their applications, or provide a TSE certificate of suitability (COS) for the raw material within their applications. A COS for bovine products is obtained from the European Directorate for the Quality of Medicines (EDQM).

When performing a risk analysis for TSE, the most important factor to consider is the source of the material. The material must be sourced from countries that have compulsory notification and laboratory verification of suspected bovine spongiform encephalopathy (BSE) cases. Further, the country of origin must have a ban on importation of animals from countries with a high incidence of BSE, or from countries where feed contains meat or bone meal with a high BSE incidence.

The organ from which the material was derived must also be considered when performing a TSE risk analysis. Organs and fluids from the animal are grouped into four different categories of risk. The highest risk group is category I, which includes tissues such as the brain and spinal cord. The lowest risk group, category IV, includes tissues and fluids such as blood, heart, kidney, milk, serum, cartilage, and others. Tissues and fluids in category IV are considered to have no detectable infectivity.

Summary

As raw materials of animal origin, such as bovine serum, can be a source of adventitious viruses, testing of these materials is required. Although the overall testing strategies are similar, there are some differences between the current US and European regulations for bovine serum testing. Determining the appropriate testing for raw materials is a dynamic process since regulations are often updated. There are new regulations to test for adventitious agents that are of continual concern, as well as emerging viruses that will require additional testing considerations. Plans for marketing biological products require an awareness of new and updated regulations.

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