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Committee for Medicinal Products for Veterinary Use (CVMP)

# Questions and Answers on allogenic stem cell-based products for veterinary use: specific questions on sterility

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# Background

Cell-based medicinal products, including stem cell -based products are heterogeneous with regard to the origin and type of cells and to the complexity of the product.

The use of allogenic stem cell -based products in the veterinary sector is increasing and raising questions for manufacturers, authorities and users.

One of the questions under discussion concerns the sterility of the finished product (absence of bacteria, fungi and mycoplasma). As allogenic stem cell -based products are veterinary medicinal products to be administered parenterally, they should be sterile. The active substances of stem cell-based products are living cells which themselves cannot be sterilised by physical or chemical methods, and also the final product can neither be terminally sterilised nor sterilised by filtration.

Microbiological contamination can occur at various steps during the manufacturing process: from the initial sampling of the cells/tissue up to the final product when packaged into containers. A crucial step is the sourcing and collection of the stem cells as at this step it is not always possible to fully implement aseptic techniques. Further sources of microbiological contamination are raw materials. Also the *in vitro* processing of stem cells carries the risk of contamination.

Furthermore, control for the absence of microorganisms is a pivotal aspect of *in process* controls and quality evaluation of cell preparations at selected stages of the production, including the final stem cell-based product.

The presence of endotoxins in stem cell-based products is also a safety concern. Therefore, control of endotoxins of stem cell-based products is an essential element of any quality control program.

Currently no specific guidance is available for stem cell -based products for veterinary use in Europe. Guidance documents have been established for human cell-based products requesting human cell-based products to be sterile [1,2,3,4] or for general requirements on animal cell based products in the USA [5].

The EU Guide to Good Manufacturing Practice (GMP) covers in Part I basic GMP principles for the manufacture of human and veterinary medicinal products. Annex 2 to this guide covers the manufacture of human biological products including Advanced Therapy Medicinal Products (ATMP). The principle provisions laid down in that Annex are considered to be applicable also to stem cell–based products for veterinary use.

For the microbiological control of medicinal products, including veterinary biologicals, a number of methods and recommendations are established and described in the European Pharmacopoeia (e.g. 2.6.1 ‘Sterility’, 2.6.7 ‘Mycoplasmas’, 2.6.27 ‘Microbiological Control of Cellular Products’, 5.1.6 ‘Alternative methods for control of microbiological quality’, 5.1.9 ‘Guidelines for using the test for sterility’). In the general text 5.14 on ‘Gene transfer medicinal products for human use’ the Ph. Eur. advises to test genetically modified cells for specific characteristics (sterility, mycoplasma, endotoxin). Guidance on raw materials of biological origin has been recently developed by the Ph. Eur. (5.2.12 ‘Raw materials of biological origin for the production of cell-based and gene therapy medicinal products’) [6].

The United States Pharmacopeia (USP) has established a specific chapter 1046 addressing ‘Cellular and Tissue-based Products’ [7], which gives information on sterility, mycoplasma and endotoxin testing, amongst other things.

Microbiological control of stem cell –based products and control methods are a pivotal aspect of process control and quality evaluations of all cell preparations and therefore tests for the absence of microbiological agents, at selected stages of the production need to be established. In addition, a thorough testing for the absence of bacteria, fungi, mycoplasma and endotoxins should be performed at the level of the finished product at release. Classical sterility testing is addressed in various regulations worldwide, including the European Pharmacopoeia (Ph. Eur.). As the finished product shelf-life is extremely short the established methods for microbiological control have limitations and are normally not applicable.

Following a review of the scientific information relating to stem cells, a number of areas have been identified that would benefit from further consideration by relevant experts and, where appropriate, the elaboration of specific guidance in the form of a questions and answers document (Q&A).

Eight specific questions for further consideration have been identified relating to sterility aspects. These questions, together with an answer to each question, are presented below.

1. **Are there any recommendations regarding the use of other approaches or methods or further issues applicable for the sterility control of stem cell–based products *in process* and/or at the finished product? How can the absence of bacteria, fungi and mycoplasma (sterility) of the finished cell-based product be ensured?**

   Sterility of a final stem cell -based product is a crucial parameter. As stem cell -based products are veterinary medicinal products to be administered parenterally, the final product should not contain any detectable microorganism. Therefore it is crucial to develop an overall microbiological control strategy that will not only rely on finished product testing, but ensure the microbiological purity of the product by using appropriately qualified and tested starting and raw materials and applying a validated aseptic manufacturing process including appropriate *in process* and finished product controls. Tests for sterility, the absence of mycoplasmas and endotoxins must be part of the release specification.

2. **Given that it is not always possible to fully implement aseptic techniques, what measures are recommended when sourcing and collecting the stem cells?**

   The collection procedure should only be performed by experienced/well trained veterinarians using aseptic techniques. The risk of contamination and cross-contamination needs to be considered and steps should be taken to minimize this risk e. g. by appropriate training of personnel, by having written procedures for collection etc. It is recommended that the collection procedure is carried out in a secluded room with windows and doors closed and with restricted entry (to minimise air flow and as far as possible avoid air-borne contamination). The room/area should be as clean as possible with documented cleaning procedures in place. Appropriate parameters for cleanliness should be established based on the type of areas in which collection takes place (barn vs. surgical room).

   Standards for hygiene and aseptic surgery should be followed; collection procedures/requirements must be recorded. The sample should be collected into prepared, sterile and sealable containers which should only be opened immediately before the sample is put in and closed as quickly as possible afterwards. Procedures for packaging and shipping, including temperature control, should be carried out according to defined procedures. Since sourcing and collection of animal stem cells are not comparable to practices for human tissues/cells in terms of potential microbiological contamination from the animals and in terms of tissue sampling techniques, antibiotics may be used in the manufacturing process in order to prevent microbiological contamination. These antibiotics should be shown to be removed in the downstream manufacturing process.

**3. How can the sterility of raw materials be ensured as raw materials are potential sources of microbiological contamination?**

There are two types of raw materials to be considered: biological material and synthetic material. Biological material includes blood, sera, cells, tissues. Synthetic material includes media and media supplements. Vials and stoppers that are in direct contact with the product should comply with Ph. Eur. requirements.

Media and media components used in the entire production process should be tested for sterility. Use of media and media components from certified suppliers is recommended. For sera used as a supplement in cell culture media, special criteria should be met which are outlined in respective veterinary guidance documents (e.g. CVMP guideline on the requirements for the production and control of immunological veterinary medicinal products, Annex 2 - EMA/CVMP/IWP/206555/2010-Rev.1 [8]). Appropriately small volumes of media, supplements and solutions should be used in order to avoid long storage times and multiple repeated opening and closing of the containers. Quality control of each batch of incoming raw material should be ensured. The use of raw materials produced according to Ph. Eur. standards is preferred.

**4. Are there any recommendations regarding general sterility and safety in the whole manufacturing process?**

All manufacturing steps and procedures should be designed to prevent circumstances that compromise product quality or increase the risk of (cross-) contamination. The production process should be set up to minimise this risk and appropriate control measures should be included in the process. The manufacturing steps should be performed in defined manufacturing areas. Procedures, including those for cleaning and sanitation, should be well described and only qualified and trained personal should be involved in production. All equipment used must be sterile. Any re-usable equipment should be thoroughly cleaned and sterilised in an autoclave using a validated cycle.

The entire production process should be validated and all critical steps and parameters identified. Each step should be precisely documented and the specifications for acceptance criteria and product release criteria described. These specifications are the quality standards that confirm the quality of products and materials used in the production process (tests, analytical procedures, acceptance criteria).

Stem cells should be filled into sterile tubes/containers shown to be tightly closed and impermeable. The properties and configuration of the containers/vials should enable easy handling with a low possible risk of contamination during transport of the cells and by the veterinarian upon handling the cells. The product information should include a detailed description how to handle the product.

**5. When during the production process is testing for sterility critical?**

Testing for sterility at all critical steps of production is strongly recommended. Sterility testing should start upon arrival of the tissue sample at the stem cell procurement site, depending on the tissue material used. Further *in process* controls for sterility should be implemented at every critical step of the production process. During culturing of the cells additional macroscopic and microscopic evaluation of samples should be performed encompassing opacity of the culture medium and microscopic detection of bacteria. If contamination is confirmed during one of these processing steps the intermediate product should be discarded. Provided that all *in process* controls are negative for bacterial contamination, the active substance batch can be approved for preparation of the final product. Testing of sterility is also required for the final product.

It is recommended to test for sterility at the following points in the production process:

- all media and components prior to cell cultivation.
- tissue/blood after sample collection.
- culture media after 2 days in culture (first media change).
- at each cell passage.
- prior to packaging/finished product.

If antibiotics are used in the early manufacturing process their removal during the manufacturing process should be documented. Only trace amounts of antibiotics should be present in the finished product.

If during the manufacturing process the product undergoes cryopreservation, re-seeding after thawing or further manipulation (washing, culturing) the sterility testing should be repeated.

If the finished product is frozen before its use it is recommended to test the product prior to cryoconservation. The proposed shipping conditions should not compromise the quality/sterility of the product.

During cryopreservation and thawing of the intermediate or the finished product cross contamination should be avoided. The basic principle of successful cell cryopreservation and resuscitation should be followed: a slow freezing and a quick thawing process.

**6. Which suitable (rapid) microbiological methods, alternative validated testing methods may be acceptable or might be appropriate for sterility testing of stem cell -based final products?**

The Ph. Eur. sterility test is the reference (official) method (harmonised with USP and JP). Therefore compliance with this method is required. However, due to the short shelf life of stem cell-based products results from the classical test according to Ph. Eur. 2.6.1 may not be available before administration of the stem cell-based product. Rapid microbiological methods are available which can give results within a shorter time-frame, depending on the test method. Use of such methods may be acceptable, if justified. Alternative methods may be used as long as they ensure sterility of the final product, lead to the same pass/fail result and are validated. It is the responsibility of the manufacturer to demonstrate the suitability of the alternative test and to ensure comparability of results with the outcome of Ph. Eur. recommended techniques. This approach may also be acceptable for the testing of absence of mycoplasma.

To date no rapid sterility tests are available providing results within less than 24 hours as would be required for final product testing of stem cell based products: the quick tests that are currently available require a time-to-detection of up to 48-72 hours and depend on the degree/amount of contamination. Any results obtained earlier than that are preliminary and not conclusive.

The FDA has evaluated and compared rapid tests for the detection of microorganisms e.g. in liquid media: BacT/ALERT® by Biomerieux, BACTEC™ from Becton Dickinson and Rapid Milliflex from Merck Millipore. The three rapid microbial methods were evaluated for sensitivity and speed of detection of spiked microorganisms and showed equivalent sensitivity at detection of lowest spiked microorganism level.
(http://www.fda.gov/downloads/BiologicsBloodVaccines/ScienceResearch/UCM266975.pdf)

Rapid Milliflex® consistently detected spiked organisms at 1 CFU/10ml within 5 days (usually 1-3 days). Similar results were obtained at 10 and 100 CFU/10 ml [9,10].

According to Biomerieux, 98% of isolates can be detected using the BacT/ALERT® within 72 hours from samples like blood and sterile body fluids [9,11,12].

Also for BACTEC™ the range of time-to-detection in hours was claimed by Becton Dickinson to be less than 72 hours for each of the organisms listed in their Quality Control Certificate [9].

However, even these rapid tests are not quick enough to provide final results before the product would ideally be administered to the animal. In Europe, a combination of results of rapid microbiological test(s) and Gram stain for deciding on release of the product, before finalisation of the classical Ph. Eur. sterility tests, is the currently applied approach. If an alternative test method has been cross validated with a Ph. Eur. test it is generally not required that the Ph. Eur. test is run as well as the alternative test. The Ph. Eur. 5.1.6 on alternative methods for microbiological control provides further information and guidance.

However, as long as there are no tests available which are validated and certified to reliably provide a comprehensive result within less than 24 hours and therefore product release and administration is before final sterility test results are available there will be a remaining risk of contamination of the final product. The applicant should address the actions to be taken in the event that the sterility test is determined to be positive after the product is administered to the patient (e.g. notifying the treating veterinarian). Appropriate information and warning will be given in the SPC.

**7. Is PCR an acceptable alternative to sterility testing?**

PCR is a very sensitive, quick and relatively cheap method to obtain information on the presence of undesired DNA in a cell based product. However to ensure sterility it may not be considered an appropriate test method, except for mycoplasma. Due to the high specificity of the method it would be necessary to include a specific primer for every microorganism potentially present in the tissue/sample/final product. The spectrum of bacteria and fungi is huge; therefore it would be difficult to perform a comprehensive test for bacteriological contamination using this method. However, PCR can be a valuable tool to look for specific bacteria e.g. bacteria which may not usually be detectable by standard sterility tests.

To detect mycoplasma the use of PCR-based mycoplasma assays or other rapid detection assays are recommended. Testing should be conducted on both cells and supernatant. An option could be Hoechst fluorescent staining for mycoplasma and other undesired DNA in culture medium. Hoechst stains are part of a family of blue fluorescent dyes commonly used to stain DNA.

**8. Is the presence of endotoxins in stem cell-based products a safety concern and how can quality control of endotoxins in the manufacture of stem cell-based products be performed?**

The presence of endotoxins is a valid safety concern because of the high clinical implications, e.g. in horses. This can be tested at the level of the active substance and the finished product using validated Ph. Eur. tests.

# Conclusion

The sterility assurance of the finished stem-cell product is critical in light of the fact that the product may be administered prior to a final sterility result being obtained. To address the whole process of sterility assurance of stem-cell based products more detailed questions are developed and discussed.

Stem cell -based products are veterinary medicinal products to be administered parenterally. Therefore they should be sterile. Due to the nature of the stem cell - based products - living cells which

themselves cannot be sterilised, and also the final product can neither be terminally sterilised nor sterilised by filtration - sterility of the product is a crucial parameter. To establish an overall microbiological control strategy that will not only rely on finished product testing is of high importance. The microbiological purity of the product has to be ensured by using appropriately qualified and tested starting and raw materials and applying a validated aseptic manufacturing process including appropriate *in process* controls. Strict microbiological monitoring during the entire manufacturing process from the sourcing of materials until the finished product is essential. Testing for sterility, for the absence of mycoplasmas and for endotoxins has to be performed using suitable and validated methods.

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