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Overview of rapid microbiological methods evaluated, validated and implemented for microbiological quality control

The risk for patients through spoiled or otherwise adulterated pharmaceuticals has been acknowledged for many centuries and led to the establishment of Good Manufacturing Practice (GMP) and pharmacopoeial guidelines. Besides chemical purity, pharmaceuticals also have to meet microbiological standards, the latter primarily depending on the administration route. Drug products which are injected directly into blood vessels or tissues or that are applied directly into eyes and ears represent a greater infection risk than products which are administered orally or onto intact healthy skin. While parenteral drug products are required to be free from any viable microorganism (USP <71>, Ph. Eur. 2.6.1), oral and topical products are not required to be sterile, but are subject to strict guidelines limiting the number and types of acceptable microorganisms (USP <61> and <62>, Ph. Eur. 2.6.12 and 2.6.13).

It is the responsibility of the pharmaceutical industry that these microbiological standards are maintained until secondary packaging of the drug product. Knowledge of the microbiological quality of the used excipients and active ingredients, microbiological monitoring of the environment in which the pharmaceuticals are produced, as well as release-testing of the final drug product contribute to maximising patient safety. Testing for microbiological quality requirements relies on traditional methods based on visual detection of a large enough population of microorganisms, either as a colony on solid nutrient medium or as turbidity in liquid nutrient medium. The duration until microbial growth can be detected visually is dictated by the generation time of the microorganisms present; whilst fast-growing microorganisms like *E. coli* can be seen within hours, visual detection of slow-growing microorganisms can take days or even weeks. Therefore, microbiological quality control often represents the bottleneck for release of drug products after manufacturing. In addition, the late detection of a microbiological quality issue complicates subsequent investigations for the root cause of the contamination. Accordingly, there is high interest throughout the pharmaceutical industry to replace traditional test methods by faster alternative methods. The encouragement by several health authorities to implement such alternative microbiological test methods, as well as official validation guidance documents for the pharmaceutical industry (USP <1223>, Ph. Eur 5.1.6,[1]) heralded a start to the transition to the use of alternative, faster test methods. In this article, several Rapid Microbiological Methods which were evaluated or validated by Novartis will be presented.

Sterility

Since 2010, a rapid microbiological method for sterility testing has been implemented as routine at several Novartis sites, allowing an incubation time of only five days (compared to 14 days which are requested by the pharmacopeial sterility test)[2]. Following acceptance by FDA CDER and FDA CBER, approvals were gained by EMA and MHRA to conduct this rapid sterility test for certain products. The method is based on the Millipore Milliflex Rapid system, which detects micro-colonies on membrane filters incubated on a nutrient medium. In contrast to the method established by the supplier which demands the use of liquid nutrient medium cassettes, Novartis decided to use solid nutrient medium for this purpose. Therefore, identification of a

solid nutrient medium which is suitable for enabling growth of stressed micro-organisms was a prerequisite for successful validation of the test method[3].

The system was chosen because it offered several advantages regarding an application as a rapid sterility test. First, it is growth-based and therefore similar to the traditional sterility test. Second, it was shown by the supplier that the requirement of sterility testing, the detection of 1 CFU following incubation, can be fulfilled. Third, the principle of membrane filtration was feasible and the system seemed adaptable to sterility testing. The principle of detection is based on ATP bioluminescence, during which ATP (derived from the growing micro-organisms) delivers energy for the conversion of Luciferin to Oxyluciferin. The reaction is catalysed by the enzyme Luciferase and leads to the emission of light photons which are captured by a highly sensitive CCD (Charge Coupled Device) camera, enabling a quicker detection of the growing microorganisms. Because the microorganisms are two-dimensionally distributed on the filter membrane, individual colonies can eventually be distinguished and enumerated. Therefore, the system is able to get information about the extent of contamination, which in general is not required for sterility testing, but might become useful in certain cases. Novartis validated the method drawing upon official guidelines (USP <1223>, Ph. Eur 5.1.6,[1]) and was able to demonstrate that it delivers robust, reliable results and performs equivalent to the compendial sterility test method in terms of robustness, ruggedness, repeatability, limit of detection, specificity and accuracy / precision. Novartis also showed that although the method is (partially) destructive since it demands extraction of the microbial ATP, a large enough fraction of microorganisms per colony survives the treatment to enable re-growth of potential contaminants for identification. In addition to this general method validation, Novartis successfully demonstrated equivalence between the alternative method and the compendial sterility test specifically for several drug products.

The rapid sterility test based on the previously described system has the limitation that test samples have to be filterable. Therefore, another system had to be taken into consideration for rapid testing of non-filterable sterile drug products. The choice of Novartis fell on the Celsis Advance System, which is also based on ATP bioluminescence for detection of growing microorganisms. In contrast to other systems based on ATP bioluminescence, the reaction is amplified by exploiting the microbial enzyme Adenylate Kinase which converts ADP to ATP, therefore intensifying the bioluminescence reaction. The system demands incubation in liquid nutrient medium, therefore an application for non-filterable drug products might be feasible. Since this method is very similar to the 'traditional' test method, the envisioned validation strategy will be similar to the one used for the previously described system. Only a small fraction of the incubated volume is used for the actual test, therefore subsequent identification of potential contaminants does not pose a difficulty with this system.

Besides non-filterable sterile drug products, an application for the microbiological examination of non-sterile drug products / excipients is currently under evaluation. Although the system is not able to quantify potential contaminants, it might be used to deliver a fast presence / absence result for microbial growth, indicating if further microbiological examination is necessary. Since this alternative microbiological examination of non-sterile drug products would demonstrate the general absence of growing microorganisms in the sample, it is foreseen to combine the TAMC (Total Aerobic Microbial Count), TYMC (Total Yeast/Moulds Count) and inspection for specified microorganisms in one single test. Besides the advantage of a faster test result (initial study data indicate approximately three days), this combination of experiments would reduce the amount of work for routine test performance significantly. Because of these strict microbiological requirements, only non-sterile drug products/excipients which rarely harbour growing microorganisms would be interesting for this application, an aspect which would be included in the product-specific validation.

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Water

Water analysis is currently performed according to the pharmacopoeial guidelines (USP <1231>) and respective monographs using mixed ester cellulose membranes incubated on R2A for five to seven days and subsequent visual enumeration.

Validation work for a rapid water analysis using the Growth Direct System by Rapid Micro Biosystems is making good progress and is envisaged to be finished early in 2011. The system counts colonies of microorganisms by detecting their autofluorescence with a CCD chip. Since this setup offers a far better resolution than the human eye, detection of much smaller colony sizes is possible, therefore allowing for a significantly reduced incubation time of approximately three days. The detection threshold for visual counting is 10^6-10^9 cells, whereas the system can enumerate colonies consisting of only around 50-100 cells[4,5]. Autofluorescence, which is detected by the CCD chip and used to enumerate the micro-colonies, is mostly derived from flavin groups, aromatic amino acids and NADH/NADPH when bound as co-factor. Since these molecules are ubiquitous in cells, all cells fluoresce yellow-green when illuminated with blue light, allowing detection of any cultivable microorganism[6-9]. Readings are performed every two to six hours, therefore the system basically offers online monitoring and can provide early warnings if relevant microbial limits are exceeded. The method is non-destructive, allowing subsequent identification of detected micro-organisms. Since only the detection system, but not the overall method, is different from the traditional method, this system can rather be considered as an automated method than an alternative method, potentially reducing the extensiveness of the validation. Because incubation time, reading intervals and final enumeration are controlled and conducted automatically by the system itself, the variability of many experimental parameters is reduced when compared to the 'traditional' test method. Since the common, non-automated work steps remain the same for the rapid method compared to the compendial method, the intrinsic variability of these parameters has to be the same for the two methods. For these reasons, Novartis evaluated that USP <16> (Automated methods for analysis) might be applicable, limiting the validation to the detection system itself. Therefore, the validation only focused on the parameters accuracy and precision, specificity and equivalence to the compendial method in routine operation (using regular water samples). Because the system has a limited loading capacity, Novartis decided that the rapid

method will be exclusively implemented for water qualities which have a direct influence on release of sterile drug products.

Bioburden

Manufacturing of parenterals also demands microbiological in-process controls (bioburden tests) in order to monitor the bioload of the drug product prior to sterile filtration or heat sterilisation. The bioburden test is performed as described in several official guidelines[10,11] using membrane filtration followed by incubation on Tryptic Soy Agar and MacConkey Agar. Potential contaminants are visually enumerated and usually identified. Regarding the requirements a rapid bioburden test would have to fulfil (membrane filtration, enumeration and possibility for subsequent identification), two different systems are currently in our focus for this application. On the one hand there is the system which is currently being validated for water testing, on the other hand there is a system which was released only quite recently, the Millipore Milliflex Quantum. This system detects microorganisms based on staining with Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE), which enters the cells through diffusion and is cleaved and retained exclusively in viable cells. The product of the cleavage, Carboxyfluorescein succinimidyl ester (CFSE), fluoresces green when excited by blue light. Like the Growth Direct System, the Milliflex Quantum has the potential to reduce the time to result by several days. It is less expensive and not as complex in built than the system intended for rapid water testing, but has the disadvantage that validation is more time-consuming since USP <16> does not apply and that it does not offer early warnings in case of exceeding results. Additionally, readings are not performed automatically by the system and the hands-on time in the lab is slightly longer due to the necessary staining step.

Conclusion

As outlined in this article, RMMs hold a lot of potential to accelerate and even improve microbiological quality control. Besides allowing drug products to reach the market and therefore the patient earlier, RMMs also allow quick and effective investigative and corrective actions in case of a microbiological quality issue. The growing interest for RMMs leads to an increase of different available systems from different suppliers, offering choice to identify the most suitable system for the intended application.

As described in this article, Novartis currently has its main focus on growth-based methods which still demand for a certain incubation time. Although inevitably being slower than for example RNA/DNA-based methods which would virtually deliver real-time results, growth-based methods offer the advantage of being closer to the 'traditional' methods making pharmacopeial requirements easier to apply. However, some of these novel approaches offer a higher sensitivity compared to growth-based methods, e.g. by detecting viable but non-culturable microorganisms which still represent the largest portion of the microbial fauna[12]. Therefore, a certain risk to more frequently exceed compulsory limits can hardly be denied. It will hence be a future challenge for the pharmaceutical industry as well as for the health authorities to define guidelines and requirements to adapt to the new possibilities that alternative approaches to microbiological quality control are about to offer.

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